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PhD thesis

Exploring the participation of VviNAC factors in the transcriptional regulatory network which governs grapevine maturation processes

S.S.D. AGR/07

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ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the most important fruit crops as it is widely cultivated, and the winemaking industry has a huge worldwide economic relevance.

The global warming has affected viticulture altering the maturation processes; in particular, the anticipation of the onset of the berries ripening (*veraison*) has changed the physiological characteristics of grapes and, consequently, has negatively influenced the wine quality. In this contest, uncovering the molecular mechanisms of the ripening could provide the key in maintaining high quality grapes and wine.

For all these reasons, the identification and characterization of master regulators controlling the transition from vegetative-to-mature growth are the challenging but fundamental tasks of this research project. At first, to provide insight into the transcriptional programs controlling the development of grapevine, a global gene expression atlas was generated (Fasoli *et al.*, 2012). Combining this dataset with a berry-specific one (Massonnet *et al.*, 2017), an integrated network analysis was performed (Palumbo *et al.*, 2014) and a new category of genes (*switch* genes), which are significantly up regulated during the developmental shift and inversely correlated with many genes suppressed during the mature growth phase, was identified. Moreover, many transcription factors are present among them, strongly indicating that they could represent master regulators of the developmental phase transition; between them, the plant-specific NAC (NAM/ATAF/CUC) transcription factors represent an interesting family due to their key role in plant development processes and stress responses (Jensen et al., 2014). Fourteen *VviNACs* genes were selected for functional characterization as key candidates of the major transcriptome reprogramming during grapevine development: *VviNAC01*, *VviNAC03* (D'Incà, 2017), *VviNAC08*, *VviNAC11* (D'Incà, 2017), *VviNAC13* (D'Incà, 2017), *VviNAC15*, *VviNAC17*, *VviNAC18*, *VviNAC26*, *VviNAC33* (D'Incà, 2017), *VviNAC38*, *VviNAC39*, *VviNAC60* (D'Incà, 2017) and *VviNAC61*.

Different approaches have been carried out to investigate the function of *VviNACs* gene: the transient over expressions, DAP-seq (DNA Affinity Purification and sequencing, Bartlett *et al.*, 2017) and, only for VviNAC60, the ChIP-seq (Chromatin Immunoprecipitation sequencing, Kaufmann *et al.*, 2010). All of them

are powerful techniques used to identify possible targets of the selected transcription factor regulations. VviNAC01 showed its important role in the ethylene pathways; VviNAC03 did not reveal a well-defined identity but seems to be plant growth related; VviNAC08 seems to have a possible role in the gibberellin-related and circadian mechanisms; VviNAC11 seems to be related to the control of the auxin pathways and the chlorophylls degradation; VviNAC13 revealed a probable action in the lignin and phenylpropanoid metabolic processes; VviNAC15, regulating many other TFs, highlighted its role in the regulation mechanisms orchestration; VviNAC17 appeared to be a regulator of the jasmonic acid-induced gene expression; VviNAC18 analyses reported its role in the chlorophyll degradation; VviNAC26 presented the regulation of many genes related to the sugars biosynthesis and the anthocyanin synthesis; VviNAC33 terminates the photosynthetic activity and organ vegetative growth; no data are available for VviNAC38; VviNAC39 resulted to up regulate the transport of sugar and lipids and the ubiquitin-conjugating; VviNAC60 revealed a lot of hormones related up regulated direct target genes and many transcription factors, highlighting again the important and major role of this transcription factor in the grapevine maturation processes; VviNAC61 revealed a predominant role in the regulation of the aromatic compounds biosynthesis. Concerning VviNAC60, ChIP-seq data were also obtained and one interesting gene, the *SRG1- SENESCENCE-RELATED GENE 1 OXIDOREDUCTASE* (VIT_10s0003g02400), was found. In order to define a regulation and co-regulation network between *VviNACs*, some candidate targets genes taken from the different DAP-seq datasets (*VviNAC01*, *VviNAC05*, *VviNAC08*, *VviNAC34*, *VviNAC37* and *VviNAC61*) were tested by Dual Luciferase Reporter Assays to see by which of the selected TFs were actually regulated. The obtained results showed that VviNAC01 directly repressed *VviNAC05* expression, whereas activated the *VviNAC08* one; moreover, VviNAC01 was validated as repressor of its own transcription. VviNAC03 resulted a repressor of *VviNAC05*. VviNAC11 directly up regulated *VviNAC34*, *VviNAC37* and *VviNAC61*. VviNAC13 resulted to regulate *VviNAC34* and *VviNAC37* expression. VviNAC15 activated *VviNAC34*. VviNAC17 acted as a repressor of *VviNAC05*, *VviNAC08* and *VviNAC61* expression. VviNAC18 resulted a direct activator of *VviNAC05*.

VviNAC26 positively regulated the expression of *VviNAC05* and directly down regulated the *VviNAC08*, *VviNAC34* and *VviNAC61* expression. VviNAC33 was found as a direct activator of *VviNAC05*, *VviNAC08*, *VviNAC34*, *VviNAC37* and *VviNAC61* expression. VviNAC60 showed to induced *VviNAC05*, *VviNAC34* and *VviNAC61* expression.

This PhD thesis lights up the possible roles of some *VviNACs* in the grapevine development and presents a preliminary regulatory network between this family members; further analysis must be conducted to completely elucidate this complex regulation system.

CONTENTS

OUTLINE OF THE THESIS

The main goal of this PhD project was to investigate the role of the *Vitis vinifera* NAC transcription factors in the grapevine maturation process through genomic, transcriptomic and functional assay techniques.
The attention was focused on 14 *VviNAC* genes: *VviNAC01, VviNAC03, VviNAC08, VviNAC11, VviNAC13, VviNAC15, VviNAC17, VviNAC18, VviNAC26, VviNAC33, VviNAC38, VviNAC39, VviNAC60* and *VviNAC61*.

Chapter 1 reports a general introduction to provide all the current knowledges about the maturation process in grapevine, the NACs transcription factor role in plants, the plant transcription regulatory mechanisms and the last findings concerning the plant cistrome landscape.

Chapter 2 presents the *VviNACs* genes selection criteria and their functional characterization in grapevine. Indeed, after the selection a transient over expression of the selected *VviNACs* was performed to obtain an overview of their primary effects on the grapevine transcriptome and to highlight their hypothetical target genes.

Chapter 3 describes the study of the selected VviNACs cistrome through the DAP-seq approach and the first ChIP-seq attempt in grapevine on VviNAC60, the master regulator of the berry ripening. A huge bioinformatic work has been done to interpretate the sequencing results and all of them were integrated with the transcriptomic data described in **Chapter 2.**

Chapter 4 reports the validation of the VviNAC-*VviNACs* interactions, aimed at the reconstruction of the grapevine maturation processes regulation. These results were supported by bioinformatic analyses and luciferase assay and permitted to identify a preliminary and partial regulatory network between *VviNACs*.

Chapter 5 discusses all the obtained results and suggests further analyses and technical improvements to fully elucidate the molecular mechanisms underlying the maturation process in grapevine.

1. BACKGROUND KNOWLEDGES

1.1 THE GRAPE RIPENING PROCESS

Grapevine (*Vitis vinifera* L.) is one of the most worldwide cultivated fruit crops as wine industry has a high economic impact on the economy of many developed countries (Martinez-Esteso *et al.,* 2013).
It is a woody temperate perennial plant which presents a period of active growth, from spring to fall, and a dormancy period during winter and until the budburst (Hellman, 2003); to ensure plant survival, all organs undergo an acclimation process for the low temperature toleration during this last phase. It has a biennial reproductive cycle, in fact buds formed in the first year give rise to shoots bearing fruit in the second year.

Berry ripening is a complex and irreversible developmental process affected by many endogenous and exogenous factors (Jackson, 2014; Kuhn *et al.*, 2014). It is tightly coordinated with seed development, originates in the pulp near the stylar end and then moves to the skin and it is genetically and epigenetically programmed (Castellarin *et al.*, 2011).
The berry development exhibits a double sigmoid pattern of growth (Rogiers *et al.*, 2017; **Fig. 1**) which is divided into four distinct stages: a green phase, which lasts from flowering to about 60 days after that, involves rapid growth and cell division; a lag phase, during which the growth slows and the organic acid concentration reaches the highest level; a maturation phase, in which sugars, pigments, volatile secondary metabolites and aromatic molecules are synthetized and organic acids, such as tartrate and malate, accumulate in the vacuole. Moreover, during this last described phase some first growth produced compounds decline, leading to a decrease of acidity and astringency. The third described stage starts with the *veraison* (the onset of ripening), a striking metabolic transition phase characterized by important biochemical and physiological changes, such as softening and coloring of the berries; it is also characterized by an accumulation of specific ROS (Pilati *et al.*, 2014). Berry ripening then proceeds to a fourth phase described as overripening or senescence; during this phase, cell integrity becomes compromised and the berry loses water.
Brassinosteroids, which are well known hormones involved in plant growth and

development, increase at the *veraison*, suggesting they may play a primary role in the regulation of this process (Kuhn *et al.*, 2014). After *veraison*, the levels of ABA increase in berries, playing a role in seed maturation, acquisition of dormancy and resistance to water stress deficit. ABA is also specifically involved in the fruit accumulation of sugar and phenolic compounds (Conde *et al.*, 2007). Despite grape is a non-climacteric fruit, ethylene promotes ripening through complex interactions and may influence berry acidity and the development of flavor and aroma (Fortes *et al.*, 2015; Conde *et al.*, 2007); instead, auxins delay the ripening associated processes (Kuhn *et al.*, 2014).

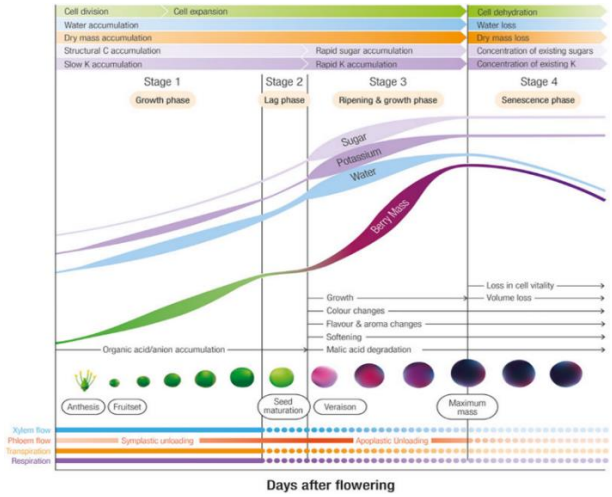

Figure 1: Double sigmoid curve of the four developmental stages of grape berries (Rogiers *et al.*, 2017).

The phenology of grapevine development, which is predominantly influenced by water and inorganic nutrients supply (Jackson, 2014), light exposure (Kuhn *et al.*, 2014) and temperature (Pearce and Coombe 2004; Jones and Davis 2000), is an important aspect that must be considered for the fruit quality and yield (Spayd *et al.*, 2002; Marais *et al.*, 2001; Haselgrove 2000).

The undergoing global warming is changing the timing of grape ripening with consequences on the harvest date and therefore negative impact on wine quality (Webb *et al.*, 2007); low temperatures are necessary to increase total soluble solid and anthocyanin content and to decrease total acidity, while high temperatures cause a reduction in berry weight, total soluble solid, anthocyanins and flavanol contents (Kuhn *et al.*, 2014).

Viticultural, enological and plant culture short-term methods, such as canopy

management (Greer *et al.*, 2010), wine chemistry, the shifting of grapevine growing areas (Hanna *et al.*, 2013; van Leeuwen *et al.*, 2013; Ollat *et al.*, 2011) and the development of new climate adapted cultivars (Ollat *etal.*,2014; Duchenne *et al.*, 2010), are not sufficient and more powerful solutions are required.

The expansion of genetic resources has provided many useful tools to understand the ripening mechanisms. In fact, a grapevine global gene expression atlas on all *Vitis vinifera* organs was created (Fasoli *et al.*, 2012), showing the transcriptional organization of the whole plant.

The atlas revealed a clear distinction between vegetative/green and mature/woody sample transcriptomes, suggesting a fundamental shift in global gene expression as the plant *switch*es from the immature to the mature developmental program (**Fig. 2**). Maturation involves the suppression of diverse metabolic processes, including photosynthesis, energy metabolism, carbohydrate metabolism, cellular component organization, and the cell cycle, which are related to vegetative growth. In contrast, only a few pathways were specifically induced, including secondary metabolic processes and responses to biotic stimuli. This evidence suggests that the transition to mature growth predominantly involves the suppression of vegetative pathways rather than the activation of mature pathways and these results indicate the existence of specific regulatory genes that promote the vegetative-to-mature transcriptomic transition.

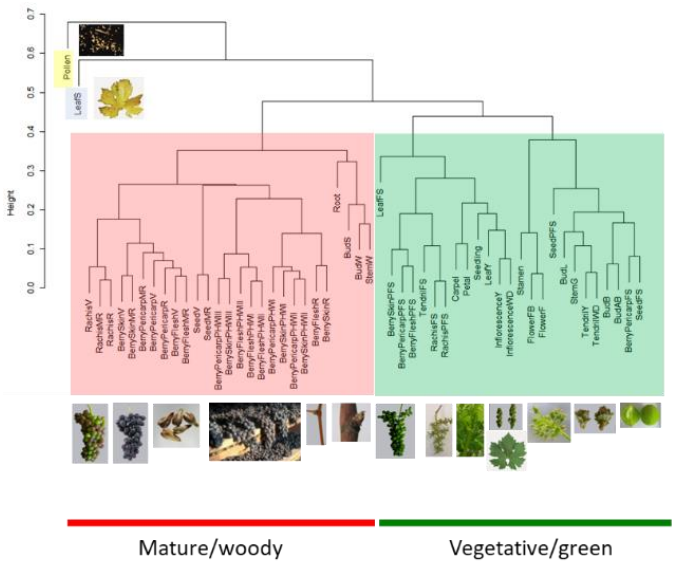

Figure 2: The grapevine global gene expression atlas (Fasoli *et al.*, 2012) showed a clear distinction between samples, which clustered predominantly in relation to temporal dynamics rather than organ identity.

The identification of the key genes involved in deep transcriptome shift that occurs in grapevine was carried out using a gene network analysis (Palumbo *et al.*, 2014) on the gene expression atlas. 1686 differentially expressed genes (DEGs) between vegetative/green and mature/woody samples were identified; among them, 1220 genes were down regulated and 466 were up regulated, suggesting that the transition mainly involves the suppression of vegetative metabolic processes rather than the activation of mature pathways. Moreover, a gene co-expression network with the DEGs was generated and a new subset of 113 genes was identified (**Fig. 3**). These genes were called *switch* genes as they were lowly expressed in the vegetative/green tissues and highly expressed in the mature/woody organs. The most represented functional categories were secondary metabolic process and transcription factors. The same approach was used to analyze the berry-specific transcriptomic dataset (Massonnet *et al.*, 2017) and other *switch* genes involved in the berry development regulation were identified: 190 for the red berry dataset and 212 for the white one. Transcription factors were found as the most significantly over expressed functional category.

By comparing the grapevine atlas and the berry datasets, a list of 131 shared *switch* genes was obtained, representing the key regulators of the global transcriptomic reprogramming necessary for the organ transition.

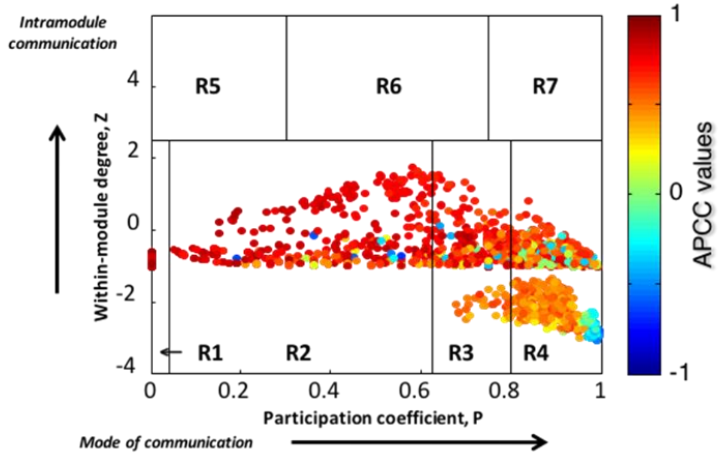

Figure 3: Co-expression network of the differentially expressed genes highlighting a small group of localized *switch* genes that were down regulated during growth phase and up regulated during the mature phase (Palumbo *et al*., 2014).

To further improve the molecular knowledges related to berry ripening and to identify with more accuracy the key genes involved in the regulation of this process, an RNA-seq transcriptomic analysis has been performed (Fasoli *et al*., 2018). The results showed that during the progress of berry development and maturation, transcripts are divided into four classes: genes expressed during pre-*veraison* (Class 1), which expression rapidly decreases during berry development; gene expressed during *veraison* (Classes 2 and 3), which expression shows a peak at *veraison* and then subsequently declines; gene expressed during later ripening (Class 4), which are expressed during late-ripening stages with an increasing expression throughout development.

Furthermore, the transcriptomic analysis of berry around *veraison* (early *veraison*, mid-*veraison* and late *veraison*), showed that the onset of berry ripening could be represented by two molecular transitions starting from 14 days before veraison (**Fig. 4**). The most represented functional gene categories of both transitions molecular biomarkers were the one related to response to hormone stimulus, cell wall metabolism, secondary metabolic processes and transcription factors; many of these biomarkers were already identified as *switch* genes in the transcriptomic datasets previously analyzed.

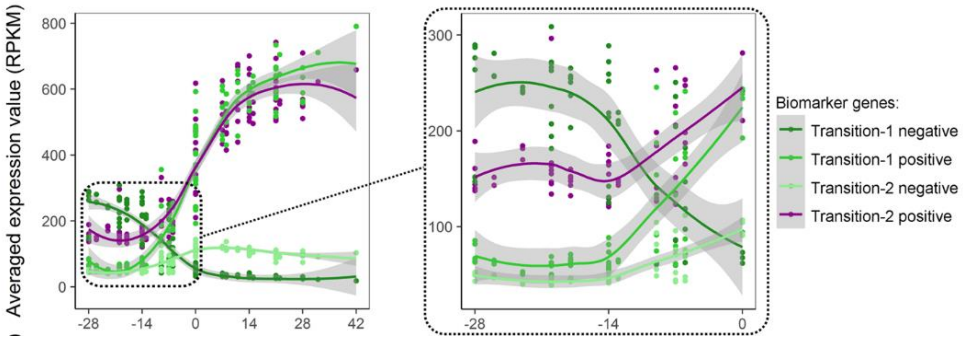

Figure 4: The averaged expression profile of transition-specific putative biomarker genes shown over the whole development (left) and during pre-veraison phase (right), (Fasoli *et al*., 2018).

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1.2 THE NAC TRANSCRIPTION FACTORS

The grapevine global expression atlas (Fasoli *et al.*, 2012) revealed a clear distinction between vegetative/green and mature/woody tissues, reflecting the existence of a deep transcriptome reprogramming occurring during organ phase transition to mature growth. An integrated network analysis (Palumbo *et al.*, 2014) identified the *switch* genes, significantly up regulated in the mature/woody tissues and inversely correlated with many genes down regulated during the developmental transition, which might represent master regulators of transcriptomic rearrangements that drives the entire plant into a maturation program. By combining the atlas dataset with the berries one (Massonnet *et al.*, 2017), *switch* genes, some present in both transcriptomes and other specific for one of them, were identified. All the lists showed several transcription factors (TFs) encoding genes as basic helix-loop-helix (bHLH), MYB, zinc finger (C2H2 and C3HC4 type) family members, some lateral organ boundary (LOB), WRKY DNA-binding and NAC domain-containing proteins (**Fig. 5**).

GENE_ID	*GENE_description*	*GENE_Name*	*Atlas*
VIT_17s0000g00430	basic helix-loop-helix (bHLH) family	*bHLH075*	
VIT_15s0046g00150	DOF affecting germination 1	*DAG1*	
VIT_06s0004g07790	Lateral organ boundaries domain 15		*
VIT_03s0091g00670	Lateral organ boundaries protein 38		
VIT_13s0158g00100	putative MADS-box Agamous-like 15a	*VviAGL15a*	
VIT_07s0031g01930	myb TKI1 (TSL-KINASE INTERACTING PROTEIN 1)		
VIT_02s0033g00380	R2R3MYB transcription factor	*VvMybA2 (C-term)*	
VIT_02s0033g00390	R2R3MYB transcription factor	*VvMybA2*	
VIT_02s0033g00450	R2R3MYB transcription factor	*VvMybA3*	
VIT_14s0108g01070	NAC domain-containing protein	*VvNAC11*	
VIT_02s0012g01040	NAC domain-containing protein	*VvNAC13*	
VIT_19s0027g00230	NAC domain-containing protein	*VvNAC33*	*
VIT_08s0007g07670	NAC domain-containing protein	*VvNAC60*	*
VIT_07s0005g01710	WRKY Transcription Factor	*VvWRKY19*	
VIT_05s0020g04730	Zinc finger (C3HC4-type ring finger)		
VIT_08s0040g01950	Zinc finger (C3HC4-type ring finger)		*
VIT_18s0001g01060	Zinc finger (C3HC4-type ring finger)		*
VIT_03s0091g00260	Zinc finger protein 4		

Figure 5: Comparison between the Atlas and the berry dataset (Massonnet *et al*., 2017). The list shows all the transcription factors among the *switch* genes.

Among them the NAC TFs, one of the largest plant-specific family, are particularly interesting as they are functionally involved in the control of late developmental processes (Olsen *et al*., 2005). Members of this family are involved in flower morphogenesis (Hendelman *et al.,* 2013) and as regulators of the fruit ripening (Zhu *et al.,* 2014). They are implicated in shoot apical meristem development (Nikovics *et al.,* 2006), in lateral root development (He *et al.,* 2005) and in cell division (Kato *et al.,* 2010); they also take part in xylem development (Endo *et al.,* 2015), embryo development (Duval *et al.,* 2002), regulation of secondary cell walls biosynthesis (Mitsuda *et al.,* 2007; Zhong *et al.,* 2006) and cell death (Niu *et al.,* 2014). Moreover, many of these TFs are associated with senescence processes such as the regulation of chlorophyll degradation in leaves (Oda-Yamamizo *et al.,* 2016) and in the regulation of anthocyanin biosynthesis (Zhou *et al.,* 2015).

The NAC TFs are also important for the tolerance to biotic stress response (Jensen *et al.,* 2010; Bu *et al.,* 2008; Fujita *et al.,* 2004; Jensen *et al.,* 2007) and abiotic stress, such as drought and salinity stress response (Mao *et al*. 2012; Nakashima *et al.,* 2012; Christianson *et al.,* 2010; Tran *et al.,* 2004; Puranik *et al.,* 2012). Furthermore, the NAC TFs undergo intensive post-transcriptional regulation that includes microRNA-mediated cleavage of genes (Khraiwesh *et al.,* 2012; Mallory *et al.,* 2004) and are also characterized by a complex post- translational regulation involving ubiquitination, dimerization, phosphorylation or proteolysis (Nakashima *et al.,* 2012; Puranik *et al.,* 2012).

The NAC acronym derives from the first three characterized proteins with the so-called NAC domain (Aida *et al.,* 1997; Souer *et al.,* 1996): NAM (no apical meristem) from petunia, *Arabidopsis* ATAF (*Arabidopsis* transcription activator factor) and CUC (cuo-shaped cotyledon). A huge number of putative *NAC* genes have been identified in many plant species such as *Arabidopsis* (117), rice (151; Nuruzzaman *et al.,* 2010), soybean (152; Le *et al.,* 2011), apple (180; Su *et al.,* 2013), banana (167; Cenci *et al.,* 2014), tomato (104; Su *et al.,* 2015) and so on.

In grapevine 74 family members have been identified (*Wang et al*., 2013), however their specific functions are poorly known.

The wide studies on model plants revealed the presence of a highly conserved N-terminus containing the NAC domain which is approximately 150-160 amino acids

in length and can be divided into five sub-domains (A-E; Ooka *et al.,* 2003; Ernst *et al.*, 2004)): subdomain A may have an important role in the formation of functional NAC dimeric proteins; subdomains C and D are conserved and bind to DNA; the subdomains B and E may confer functional diversity between NAC TFs. If the N-terminus is associated with DNA binding, nuclear localization and the formation of homo- or heterodimers with other NAC proteins (Olsen *et al.,* 2005), the highly divergent C-terminus, which can present a transmembrane motif, has an activation/repression of transcriptional putative regulatory function (Puranik *et al*., 2011).

The investigation on NAC TFs in grapevine, identified by integrated networking analysis based on the global gene expression atlas and a grapevine berry transcriptomic dataset (Palumbo *et al.,* 2014), started from five members of this gene family (D'Incà, 2017). *VviNAC33* and *VviNAC60* have been selected as *switch* genes of the entire plant development; *VviNAC11* and *VviNAC13* have been selected as *switch* genes of berry development. Thanks to the relevance of tomato as model for fruit development and ripening and the availability of collected tomato-ripening mutants such as *NOR*, *VviNAC03* was also considered as it resulted to be one of the two closest tomato *NOR* homologues.

The high number of TFs related to the selected *NACs* supported the existence of a complex regulatory network during plant development. A correlation between *VviNAC03* and *VviNAC11* was found and was noticed that *VviNAC33* was co-expressed with *VviNAC60*. Moreover, several *switch* genes emerged from the analyses, indicating that the *switch* genes could include putative key regulators of the developmental phase transition but also their direct targets. Furthermore, a correlation between *NACs* and secondary metabolic processes was observed.

To study the role of these TFs, transient ectopic expressions of *VviNAC03, VviNAC11, VviNAC13, VviNAC33* and *VviNAC60* in grapevine plants were carried out and microarray global gene analyses of the transcriptomes were performed.

The attention was focused on those genes linked to metabolic pathways with central roles during grapevine maturation. Secondary metabolic processes were up regulated by all the five *VviNACs*; in particular, many genes implicated in phenylpropanoid pathway, anthocyanin and terpenoid accumulation were

identified. Interestingly, *VviNAC60* up regulated *VviNAC03* and *VviNAC03* down regulated *VviNAC60*. Regarding *VviNAC60*, the up regulation of many germin proteins, involved in development, osmotic regulation, photoperiodic oscillation, defense and apoptosis was noticed; concerning *VviNAC33*, the most down regulated genes were related to photosynthesis.
All these findings provided real evidence that *VviNAC03, VviNAC11, VviNAC13, VviNAC33* and *VviNAC60* participate in the regulation of the transcriptome reprogramming during grapevine development.

VviNAC33 and *VviNAC60* were then stably over expressed in grapevine plants showing that *VviNAC33* affected the chlorophyll metabolism in leaves, causing an anticipated leaf chlorosis and senescence, while *VviNAC60* altered the plant growth and caused a higher accumulation in leaves of anthocyanins, secondary metabolites responsible for the red berry coloring; this confirmed the involvement of both VviNAC TFs in the regulation of the organ maturation/senescence associated processes.
To obtain more information about the role of *VviNAC33* and *VviNAC60*, microarray analyses on transgenic stably over expressing leaves were performed.
The number of down regulated genes were higher that the up regulated ones and, moreover, about half of the down regulated genes were shared from both the transcriptomes. Several genes related to the vegetative phase processes, such as photosynthesis, cell cycle and development, were down regulated when plant switched to mature growth, confirming that the transition mainly involved the suppression of vegetative pathways rather than the activation of the maturation ones, as was previously reported (Palumbo *et al.*, 2014).
The results also showed the up regulation of many genes involved in plant development processes associated with ripening and senescence (such as cell wall metabolism, aroma controlling pathways, anthocyanins and stilbenes biosynthesis and ethylene response), suggesting that both *VviNAC33* and *VviNAC60* mainly act as transcriptional activators and confirming, once more, their putative involvement in the regulation of vegetative-to-mature transition in grapevine.

By investigating the result of the microarray analyses performed on both transiently

and stably over expressing plants, the transcription factor activity was present as the most represented functional category. Moreover, a *VviNAC03* up regulation by *VviNAC60* over expression and a *VviNAC60* down regulation by the *VviNAC03* one was seen. Co-expression analyses, which revealed a correlation between *VviNAC03* and *VviNAC11* and between *VviNAC33* and *VviNAC60,* also brought to the hypothesis of a possible regulatory network between VviNACs. Moreover, several *switch* genes emerged in the co-expression and microarray analyses, supporting again the existence of a complex regulatory network during plant development.

To complete the functional analysis of both *VviNAC33* and *VviNAC60* and to obtain more information about their role during ripening, they have been converted into transcriptional repressors fusing their coding sequences with the Ethylene-responsive element-binding factor-Associated amphiphilic Repression motif (EAR motif; Kagale and Rozwadowski, 2011) and stably expressing them in grapevine plants (Bertini, 2018- unpublished). Each *VviNAC-EAR* gene was cloned under the control of its endogenous promoter to inhibit the transcription of putative target genes in the same organs and developmental stage when the endogenous *VviNAC* genes are expressed. Their phenotypic and molecular characterization showed a normal vegetative growth and the down regulation of some genes previously induced in the over expressing plants. These results indicated that the addition of the EAR motif was successful in turning these factors into transcriptional repressor and confirmed some of the preliminary data obtained from the over expressing plants.

Overall, *VviNAC33* and *VviNAC60* seem to be master regulator of the immature-to-mature transition phase. In particular, *VviNAC33* could be considered a negative regulator of photosynthesis, due to the phenotypic effects on stably transformed grapevine leaves, and *VviNAC60* seems to play a role in ripening/senescence process, by considering the developmental growth of stably transformed grapevine plants.

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1.3 THE REGULATION MECHANISMS OF TRANSCRIPTION

Gene expression can be constant or can have a temporal/spatial regulation pattern; in eukaryotic organisms it is multistage, spatially compartmented and controlled by specific factors (Bilas *et al*., 2016). The primary regulation (**Fig. 6**) occurs with the initiation complex assembling on the available DNA strand (euchromatin) and the RNA polymerase II attachment to the initiation site (IS); both stages require the participation of transcription factors (TFs). After elongation and termination, pre-mRNA is formed; the mRNA maturation step comprises splicing (excising of introns and joining of exons) and attachment of 5' cap and 3' poly-A tail to the transcript ends (Bilas *et al*., 2016). Mature mRNAs are then moved to the cytoplasm where translation takes place (Klug and Cummings, 2003; Phillips, 2008). The organization of all eukaryotic genomes is similar and most of the regulatory elements are universal. However, substantial differences occur among elements assigned to tissues/organ specific promoters (Twyman, 2003; Venter and Botha, 2010).

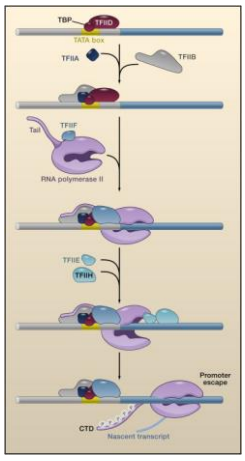

Figure 6: First steps in transcription activation (Levine, 2011).

Generally, the regulators of transcription are classified in terms of their structure as cis regulatory elements (CREs) and trans factors (Bilas *et al*., 2016). CREs are linear nucleotide fragments of non-coding DNA involved in protein-DNA interaction (Venter and Botha 2010). Plant regulatory sequences are located directly in the transcribed DNA strand (promoters, enhancers, silencers) or may be added during post-transcriptional modifications (5′ cap, poly-A tail, signal sequences) (Vaughn *et al*., 2012). Furthermore, specific regulatory proteins, called trans

elements, interact with cis sequences and other proteins to form active complexes (Bilas *et al*., 2016).

CREs directly influence gene regulation; depending on the type, they are present in different copy numbers, as well as variable distances (even 10.000-100.000 bp) and orientation in relation to the gene (Venter and Botha, 2010). CREs are also potentially involved in epigenetic pathways (Lu *et al*., 2018). They are more frequently differentiated based on their activity in each model system and, when they recur in genes ensuring identical functions, are classified as motifs (Bilas *et al*., 2016).

Gene promoters, located upstream of the gene coding sequence, enable the initiation of transcription by the presence of RNA polymerase II binding sites. This enzyme is attached during the sequential binding of specific proteins, called transcription factors, which results in transcription initiation (Russell 1996; Porto *et al*. 2014). The structure of a promoter, containing cis elements to which trans factors can bind to, can be divided into two regions (Klug and Cummings, 2003; Peremarti *et al*., 2010; Porto *et al*., 2014): core promoter and distal region, the last comprising enhancers and silencers. Moreover, the promoters vary in the terms of efficiency, site, and period of action; they may be active throughout all developmental stages in each tissue (constitutive promoters) or in particular tissues or development-stage (tissue-specific promoters). Some promoters may require specific stimuli for activation (inducible promoters) or be operating in the specific developmental stage (Peremarti *et al*., 2010).

The core promoter (**Fig. 7**) may be composed of TATA box, initiator region (Inr), CAAT box and downstream promoter element (DPE); however, not all these regions are always present at each promoter (Juven-Gershon and Kadonaga, 2010; Kadonaga, 2012). The TATA box, located 25–30 bp upstream of the transcription start site (TSS), is usually flanked with GC-rich regions (Lewin, 2001); it is approximately 8 bp in length and composed of AT base pairs with the consensus sequence TATA(A/T)A(A/T) (Twyman, 2003).

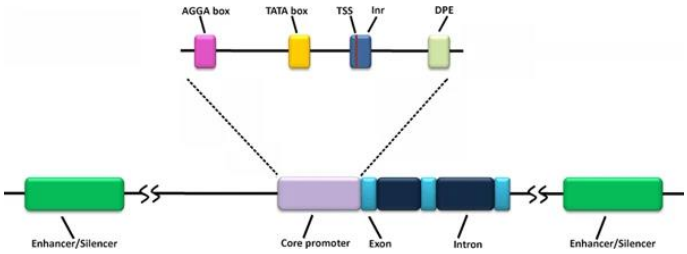

Figure 7: Example of a plant gene organization. Not all the depicted elements are universal; the core promoter may consist of the AGGA box, the TATA box, Inr and DPE. TSS is the transcription start site, Inr is the initiator and DPE is the downstream promoter element. Figure taken from Bilas *et al*., 2016.

The transcription factors and RNA polymerase II bind to the TATA box in a specific order (Bilas *et al*., 2016). The transcription factor TFIID is bound first and forms a complex with TFIIA; TFIIB attaches to this complex via TATA-binding protein (TBP), which is a part of TFIID, and because of direct interaction with the DNA strand. RNA polymerase II is bound in the next step and TFIIF attaches to it. TFIIE and TFIIH join the complex and the full transcription apparatus is formed (**Fig. 8**; Kwak and Lis, 2013).

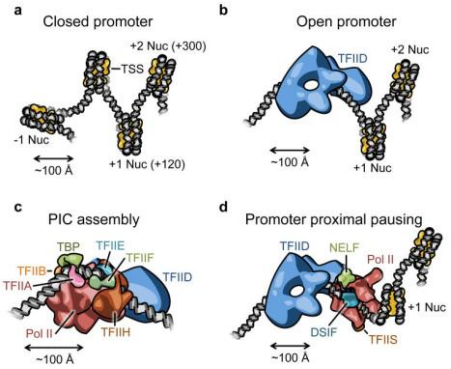

Figure 8: Structure of the promoter and Pol II before and during early elongation (Kwak and Lis, 2013).

A frequently observed conservative promoter element is the CAAT box, located approximately 80 bp upstream of TSS; it is considered as the most important factor as it can act in both directions and is highly susceptible to mutations, significantly influencing the expression efficiency (Bilas *et al*., 2016). In plants, a similar AGGA box has been identified in place of the CAAT box (Roa-Rodríguez, 2003; Porto *et al*., 2014).

In the promoters lacking the TATA box, DPE may be responsible for binding TFIID protein; it is usually located 28–32 bp downstream of adenine A+1 of the Inr (Bilas

et al., 2016). Occasionally TATA, Inr and DPE coexist. Even in the case of a single nucleotide alteration between the DPE and Inr, TFIID cannot bind properly to the promoter and the expression efficiency decreases significantly. In plants, several copies of DPE are present upstream the transcription start site and regulate the response to external stimuli (Kutach and Kadonaga, 2000; Sawant *et al*., 2001).

The 5′ untranslated region sequence (5’ UTR) is a fragment of mRNA transcript located on its 5′ end (Barrett *et al*., 2012). It is encoded in the DNA strand and submits to transcription but not translation; in fact, it provides a connection between protein translational factors, small 40S ribosomal subunit and the transcript (Bock, 2013). The sequence of 5′ UTR comprises the 5′ cap (a single 7-methyloguanosine nucleotide followed by several methylated riboses), the upstream open reading frame (uORF; Hayden and Jorgensen, 2007), the guanine-rich fragment and the internal ribosome entry site (IRES). Some plant UTRs contain a pyrimidine-rich fragment responsible for high transcription levels (Barrett *et al*. 2012). Presence of 5′UTR stabilizes the transcript (**Fig. 9**) during transport from nucleus to cytoplasm where it protects it from endonuclease activity (Bilas *et al*., 2016).

The 3′ UTR sequence is in the transcript between the stop codon and polyadenylation signal; since the 3′ UTR is added after transcription, it is considered an important regulator of translation and act as stabilizers, enhancers and silencers (Bilas *et al*., 2016). The presence of 3′ UTR significantly affect the final gene expression: it influences the transcription termination stage and cooperates with 5′ UTR during loop formation, which facilitates the translation initiation and the translation stabilization through the incorporation of specific factors (Papadakis *et al*., 2004; Vignesh *et al*., 2013). The 3′ UTR also contains sequences complementary to microRNA (miRNA); pairing of complementary sequences between mRNA and miRNA prevents translation, by decreasing the amount of mRNA available in the cytoplasm, and indirectly regulates expression efficiency (Barrett *et al*., 2012).

The poly-A tail is a polynucleotide sequence composed of around 250 adenines. Both poly-A and 3′UTR are added to the transcript during transcription termination and cooperate in stabilization of mRNA structure (**Fig. 10**), affect the transcript

transport and its fate in the cytoplasm (Li *et al*., 2012).

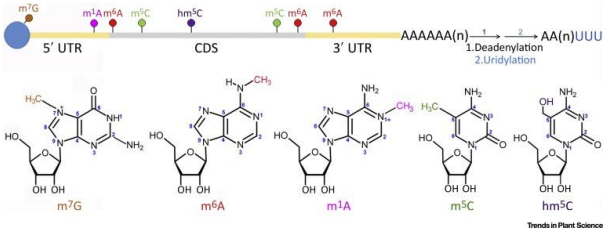

Figure 9: mRNA structure (Shen *et al*., 2019).

Unlike the previously described elements, enhancers and silencers cis elements could be located further from the TSS, could be included in non-coding intron sequences, could be located near their target core promoter or even within the transcribed region of a gene body, and can act under specific environmental conditions or in definite tissues (Peremarti *et al*., 2010; Ong and Corces, 2011; Spitz and Furlong, 2012). Furthermore, enhancers generally do not display universal sequence conservation, making them very challenging to locate (Morton *et al*., 2014; Mejía-Guerra *et al*., 2015). Mostly they are located at considerable distances, upstream or downstream of the promoter sequence, and enhance gene expression through cooperation with specific transcription factors (Mehrotra *et al*., 2011). Transcription efficiency improvement is a result of conformational alteration as chromatin state, including dependence on histone modifications, and DNA looping (Alberts *et al*., 2002). It was recently discovered that many enhancer elements in animal genomes could be identified with relatively high confidence based on a unique combination of flanking histone post translational modifications (PTMs), leading to the annotation of such elements in several animal models and specialized cell types (Heintzman *et al*., 2009; Bonn *et al*., 2012). However, the known correlations between plant CREs and histone PTMs appear to be modest (Zhang *et al*., 2012b; Zhu *et al*., 2015).

A well-known feature of sequence-specific DNA binding proteins is their ability to displace nucleosomes upon DNA binding, with an increase in nuclease accessibility around the binding region (Gross and Garrard, 1988; Henikoff, 2008). The DNase-seq studies in *Arabidopsis* demonstrate that most open chromatin sites (accessible

chromatin regions, ACRs, with low nucleosome occupancy) exist outside of genes, that differences can be identified between tissues and that a large proportion of intergenic ACRs are in fact regulatory (Zhang *et al*., 2012a; Pajoro *et al*., 2014; Zhu *et al*., 2015). More recent studies on species comparisons confirmed that the majority of ACRs exist outside the transcribed regions; they also tended to cluster within several kilobases upstream of the TSS, despite the large differences in intergenic space between genomes (Maher *et al*., 2018). When orthologous genes were compared across species, was found that the number and location of ACRs were highly variable, suggesting that regulatory elements are not statically positioned relative to target genes over evolutionary timescales (Maher *et al*., 2018). However, were found evidence that some sets of gene remain under control of common TFs across species: overall, the suggestion is that the cis regulatory structure of plant genomes is similar, that TF-target gene modules are generally conserved and that the co-regulation of specific gene sets by multiple TFs seems to be frequently maintained across species (Maher *et al*., 2018).
Consequently, CREs are preferentially located inside ACRs (Thurman *et al*., 2012; Lu *et al*., 2018).

Recently published epigenomic datasets revealed that gene distal ACRs are indeed abundant in plant genomes (Lu *et al*., 2017; Bajic *et al*., 2018; Zhu *et al*., 2015; Rodgers-Melnick *et al*., 2016; Sullivan *et al*., 2014; Maher *et al*., 2018; Oka *et al*., 2017; Mei *et al*.,2018). ACRs are highly enriched at transcriptional start and end sites, depleted for cytosine DNA methylation (Haring *et al*., 2010) and exhibit greater GC content compared with nearby intergenic sequences (Wang *et al*., 2012). The proportion of gene distal ACRs scales with genome size and the amount of intergenic space (Mei *et al*., 2018) and the number of ACRs also correlates well with the number of annotated genes within the plant species (Lu *et al.*, 2019).
Although genome size varied greatly among the plant species, the total length of sequence occupied by ACRs is consistent and did not scale linearly with genome size (Lu *et al.*, 2019, **Fig. 10**). Interestingly, an increase in distal ACRs (>2kb from a gene) accompanied by a loss of proximal ACRs (within 2 kb of a gene) is clearly correlated with increasing genome sizes (Lu *et al.*, 2019). In *A. thaliana* (135 Mb genome), approximately 80% of ACRs reside within 2000 bp of genes, whereas in

S. lycopersicum (980 Mb genome), only 50% of ACRs reside within 2000 bp of genes (Maher *et al*., 2018). In *Z. mays* (2.3 Gb genome), a significant proportion of ACRs are found tens to hundreds of kb away from genes (Rodgers-Melnick *et al*., 2016). A major factor that could have contributed to the strong correlation between genome sizes and the distances between ACRs and genes is the differential activities of transposable elements (TEs); although distal ACRs are generally depleted from TEs, species-specific distal ACRs are strongly enriched within TEs (Lu *et al.*, 2019).

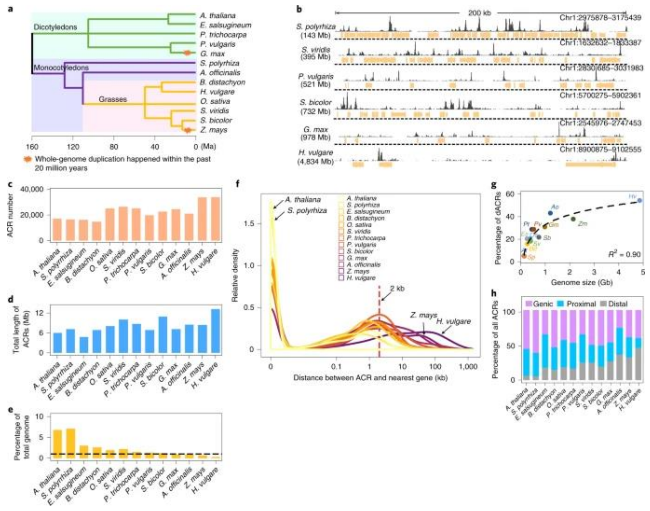

Figure 10: The prevalence of distal accessible regions is a consequence of genome size (Lu *et al*., 2019).

Distal CREs in plant genomes were identified (Weber *et al*., 2016; Marand *et al*., 2017; Dong *et al*., 2017; Li *et al*., 2008; Qiu *et al*., 2016; Zhang *et al*., 2102; Zhang *et al*., 2014; Lu *et al*., 2018) and was seen that sequence variations at several distal CREs cause phenotypic variations that are important for the domestication *(*Salvi *et al., 2007;* Xu *et al., 2017;* Studer *et al., 2011;* Louwers *et al., 2009)* and variation in flowering time *(*Adrian *et al*., 2010; McGarry *et al*., 2008; Yang *et al*., 2005; Liu *et al*., 2014). Although, the mode of action of distal CREs remain unknown.

Plant species have a considerably lower SNP diversity at distal ACRs compared with flanking intergenic regions; moreover, many distal ACRs are in syntenic regions, making it possible to identify orthologous distal ACRs by sequence similarity and/or chromatin accessibility (Lu *et al.*, 2019). In summary, the vast majority of distal ACR sequences are under strong purifying selection between

species, possibly to retain the sequence specificities for TF binding (Lu *et al.*, 2019). Importantly, histone modifications at orthologous distal ACRs are conserved among species. However, changes in gene expression for distal ACRs exhibiting species specificity are observed (Lu *et al.*, 2019). Genic chromatin modifications are similar between plant species. Expressed genes are enriched in H3K4me3, H3K56ac and H2A.Z at the TSS, and enriched in H3K4me1 and H3K36me3 in gene bodies; by contrast, repressed genes are enriched in H3K27me3 and H2A.Z (Lu *et al.*, 2019).

Chromatin can form long-range loops that bring together distal ACRs with cognate genes during both transcriptional activation and repression; strong loops preferentially contained distal ACRs located upstream of and adjacent to their interacting genes, permitting to assign with reasonable confidence the putative distal CREs to target genes (Lu *et al.*, 2019; Ricci *et al*., 2019). Studies suggested that long-range regulatory interactions were predictable based on loop strength, orientation and location relative to target genes (Ricci *et al*., 2019). Moreover, the enhancer activities of distal and proximal ACRs were significantly greater than the activities of control intergenic regions (Ricci *et al*., 2019).

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1.4 THE CISTROME LANDSCAPE

Recent studies have demonstrated that many important aspects of plant development are regulated by heritable changes in gene expression that do not involve changes in DNA sequence. Rather, these regulatory mechanisms involve modifications of chromatin structure, that affect the accessibility of target genes to regulatory factors that can control their expression, and DNA methylation. Indeed, many TFs are active in different tissues and stages of development, and target genes may differ in a tissue-specific manner depending on chromatin accessibility and the availability of cofactors.

The cistrome is the complete set of TFs binding sites (cis elements, CREs) in an organism (O'Malley *et al.*, 2016). Robust methods to construct comprehensive cistrome maps are critical for elucidating complex transcriptional networks (O'Malley *et al.*, 2016). Comprehensive identification of transcription factor binding sites (TFBS) in a genome is essential for the characterization of regulatory elements and TFs functions (Kheradpour and Kellis, 2014; Stamatoyannopoulos *et al.*, 2012). At present, there are two main approaches for CREs research. One approach is the *in vivo* or *in vitro* DNA–protein interactions study; it led to the development of methods for CREs recognition such as DNase I footprinting (Sullivan *et al.*, 2015), electrophoretic mobility shift assays (EMSA, Hellman *et al.*, 2007), chromatin immunoprecipitation sequencing (ChIP-seq, Kidder *et al.*, 2011; Kaufmann *et al.*, 2010), yeast one-hybrid systems (Reece-Hoyes *et al.*, 2012) and DNA affinity purification sequencing (DAP-seq, Bartlett *et al.*, 2017). The second main approach uses in silico algorithms and databases to calculate the probability of predicted regulatory element rightness (Mehrotra *et al.*, 2011), but requires biological confirmation.

The *in vivo* protein-DNA interaction landscape is affected by multiple factors including primary sequence, DNA modifications, and chromatin accessibility, along with stabilizing and destabilizing interactions between proteins associated with the DNA (Lelli *et al.*, 2012; Levo and Segal, 2014).

For this reason, the *in vivo* approach is the most informative considering developmental phase and tissue specificity of plants transcriptomic regulation. Indeed, the ChIP-seq (**Fig. 11**) is a powerful technique used to study interactions

between TFs and DNA *in vivo* (Kaufmann *et al.*, 2010) and, consequently, for TFBS discovery. Unfortunately, genome-wide applications of the ChIP-seq methodology in plants are still relatively scarce. This delay is partly due to technical difficulties in sample preparation related to the properties and complexity of plant tissues. Moreover, ChIP-seq is limited in its throughput by challenging and expensive need to create specific antibodies. As a result, binding site information is available for relatively few TFs and substantial TFBS coverage is only available for humans and several model organisms (O'Malley *et al.*, 2016).

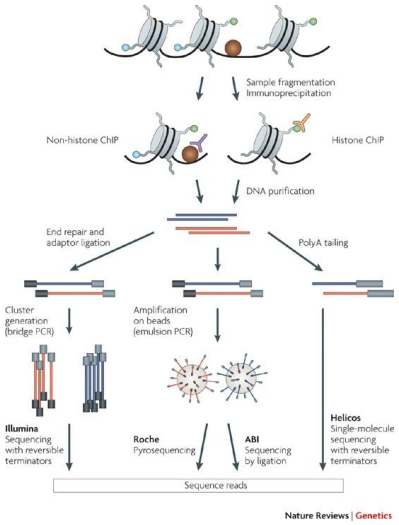

Figure 11: The ChIP-seq assay (Park, 2009).

Methods such as ATAC-seq (Buenrostro *et al.*, 2015a) offer an approach to annotate genome-wide regulatory elements across many organisms and cell types (Buenrostro *et al.*, 2015b; Sullivan *et al.*, 2014; Thurman *et al.*, 2012); however, without comprehensive knowledge of TFs sequence specificity, the targeting TFs of the identified regions cannot be verified.

The *in vitro* mapping of TFBS provides an easier, faster and scalable alternative. In the past the two most used *in vitro* methods were systematic evolution of ligands by exponential enrichment (SELEX, Jolma *et al.*, 2010) and protein binding microarrays (PBM, Berger and Bulyk, 2009). In both methods synthetic DNA oligomers were enriched with an affinity-tagged TF and the preferred binding sequences were used for the identification of binding motifs (Machanick *et al.*, 2011); they permitted to find many TF motifs, but the use of synthetic DNA does

not allow to identify the genomic DNA (gDNA) properties known to impact TF binding *in vivo* (O'Malley *et al.*, 2016).

The gDNA is the native substrate for a TF and therefore ideal for an *in vitro* DNA-TF interaction assay; unlikely synthetic oligomers, gDNA encodes primary sequence and cell-, tissue-, and organism-specific methylation patterns that may impact TFs binding (O'Malley *et al.*, 2016).

Previous TF: DNA binding assays using naked gDNA were effective in identifying motifs and in vivo binding sites (Guertin *et al.*, 2012; Liu *et al.*, 2005; Rajeev *et al.*, 2014), but this approach has not been applied for global TFBS characterization or to investigate the impact of *in vivo* important features for the TFs binding.

Instead, the DAP-seq (**Fig. 12**), a fast and high-throughput assay that couples affinity-purified TFs with next-generation sequencing of a genomic DNA library to discover TFBS, allows to capture the impact of the primary sequence but also of the DNA methylation (a parameter that impact binding *in vivo*) on binding affinities at individual TFBS (Bartlett *et al.*, 2017). The ampDAP-seq, which uses a DNA library in which the DNA modifications are removed by PCR, is a valid method to measure the impact of DNA modifications on TFs binding: a comparison between DAP-seq and ampDAP-seq data could bring to a global understanding of the DNA modifications effects on TFs binding.

Moreover, although the DAP-seq technique lacks tissue-specific chromatin accessibility context, a comparison between its results and the ChIP-seq datasets (Zhang *et al.*, 2008) could be useful to find TFBS and to start understanding how the epigenomic variants influence the regulatory dynamics.

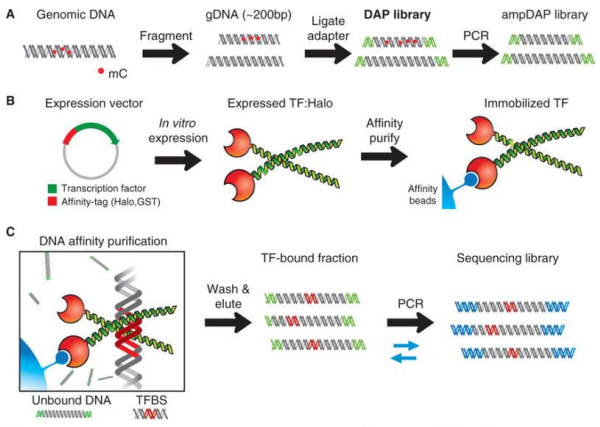

Figure 12: The DAP-seq assay size (O'Malley *et al*., 2016).

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2. *VviNACs* SELECTION AND FUNCTIONAL ANALYSES

2.1 INTRODUCTION

Grapevine is one of the most important fruit crops in the world, reflecting its commercial value in the winemaking, in the vinegar industries and the nutritional benefits of table grapes and raisins.

To provide insight into the transcriptional programs controlling the development of grapevine, at first a global gene expression atlas was generated (Fasoli *et al*., 2012). Afterwards, a set of *switch* genes were identified by genes co-expression networks analyses (Palumbo *et al.*, 2014) generated from the grapevine global gene expression atlas (Fasoli *et al.*, 2012) and from a large berry transcriptomic dataset (Massonnet *et al.*, 2018). These genes could represent key regulators of transcriptome reprogramming during grapevine development as they are up regulated during the transition from vegetative to mature and inversely correlated with many genes suppressed during the mature growth phase. Among them, *VviNAC* genes were identified as a good candidate gene family to study and better understand the regulation of the maturation processes (D'Incà, 2017).

NAC (*NAM, ATAF1,2, CUC2*) are plant specific genes that are often involved in drought and salinity responses. The members of this family are also associated with senescence in different plant organs, including fruits. The *NAC* genes may represent important signalling components in the control of grapevine maturation processes such as late berry developmental and leaves senescence.

In grapevine 74 family members have been identified (Wang *et al.*, 2013). The relationship among them was investigated through a phylogenetic tree that identified 8 separate clades (**Fig. 13**, Wang *et al.*, 2013). All the family members share the highly conserved NAC domain at N-terminus, divided into five subdomains: subdomain A may have an important role in the formation of functional NAC dimeric proteins, subdomains C and D are conserved and bind to DNA, the highly divergent subdomains B and E may confer functional diversity to NAC TFs (**Fig. 13**).

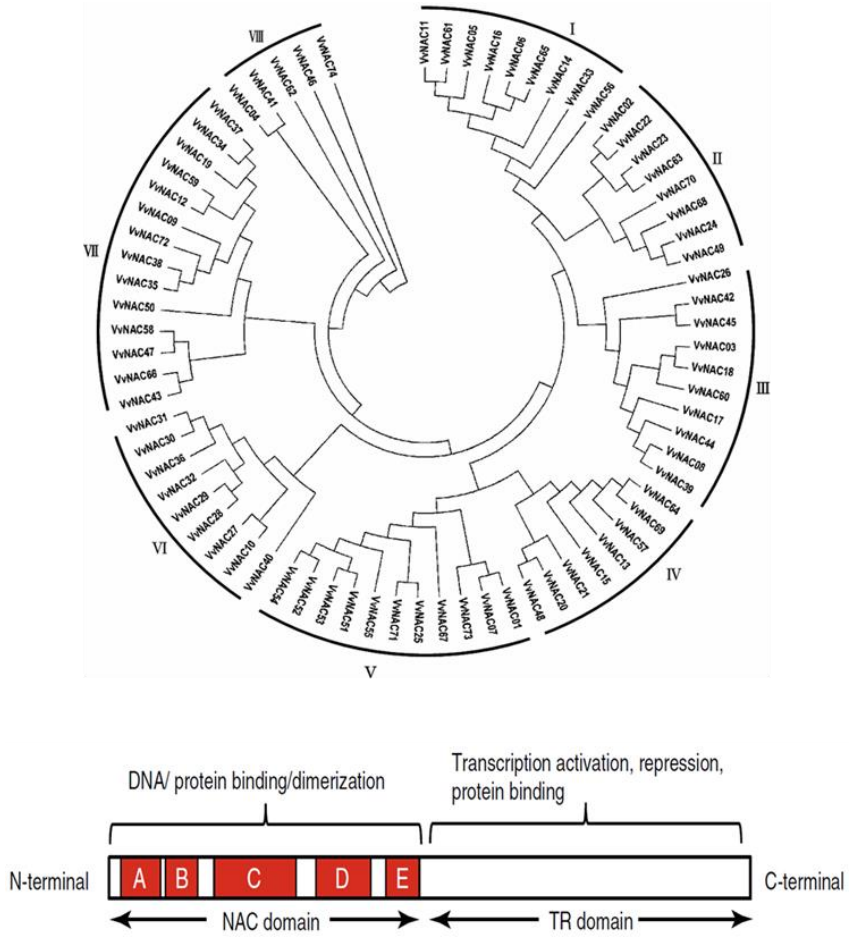

Figure 13: *VviNACs* phylogenetic tree (Wang *et al*., 2013) and *Vvi*NACs domain composition.

At first the attention was focused on *VviNAC33* (VIT_19s0027g00230) and *VviNAC60* (VIT_08s0007g07670), selected as they seem to be master regulators of the organ phase transition in the whole plant (D'Incà, 2017). On the base of their expression profiles, which show a very low expression in the vegetative/green tissues and a significantly high expression in the mature/woody organs (**Fig. 14**), they might represent master regulators for the transition from vegetative-to-mature growth, with particular attention to leaves senescence for *VviNAC33* and to berry ripening for *VviNAC60*.

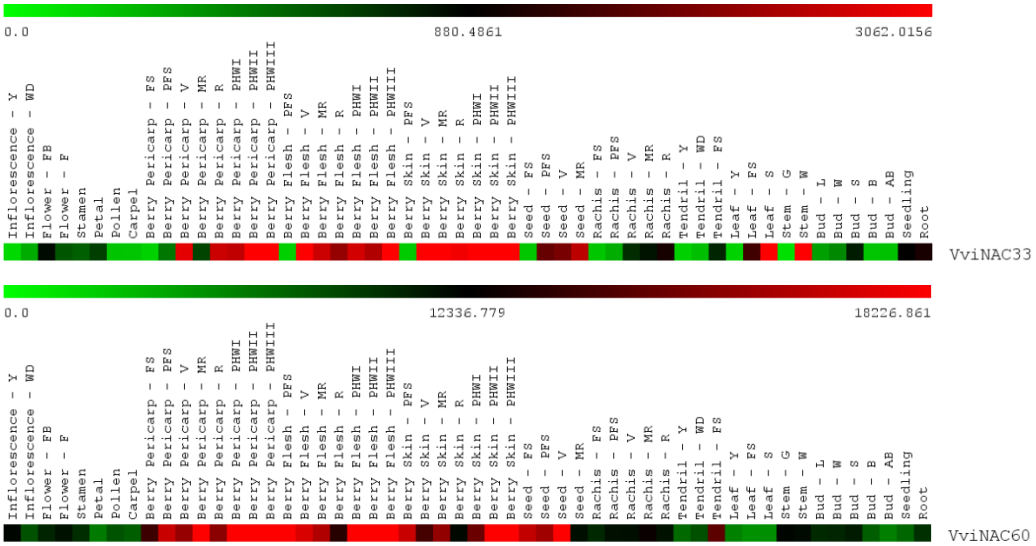

Figure 14: Heatmaps representing the *VviNAC33* and *VviNAC60* expression profiles in all organs and at different developmental stages (data taken from Fasoli *et al.*, 2012).
Description of ATLAS abbreviations (54 developmental stages):
Bud – L = latent bud; – W = winter bud; – S = bud swell; – B = bud burst; – AB = bud after-burst; **Inflorescence** – Y = young inflorescence; – WD = well developed inflorescence; **Flower** – FB = flowering begins; – F = flowering; **Stamen** = pool of stamen from undisclosed flowers; **Pollen** = pollen from disclosed flowers; **Carpel** = pool of carpels from undisclosed flowers; **Petal** = pool of petals from undisclosed flowers; **Tendril** – Y = young tendril; – WD = well developed tendril; – FS = mature tendril; **Leaf** – Y = young leaf; – FS = mature leaf; – S = senescencing leaf; **Berry Pericarp** – FS =fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post- harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III**; Berry Skin** – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post-harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Berry Flesh** – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; – PHWI = post-harvest withering I; –PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Seed** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; **Rachis** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; **Stem** – G = green stem; - W = woody stem; **Root** = in-vitro cultivated roots; **Seedling** = pool of 3 developmental stages.

Moreover, embryogenic calli of *Vitis vinifera* cv Shiraz were transformed with *Agrobacterium tumefaciens* harboring a binary vector in which *VviNAC33* and *VviNAC60* were independently over expressed and transgenic grapevines were successfully generated (D'Incà, 2017). *VviNAC33* over expressing plants show normal growth and development; however, a clear bleaching effect on fully expanded leaves was observed (**Fig. 15**). *VviNAC60* over expressing plants show a slightly reduced growth and earlier stem lignification in comparison to the same-age control plants (**Fig. 15**). These results confirm the hypothesis that *VviNAC33* and *VviNAC60* may have functions in the transition from vegetative to mature development in grapevine since these changes mirror typical behaviors of plants undergoing ripening and/or senescence.

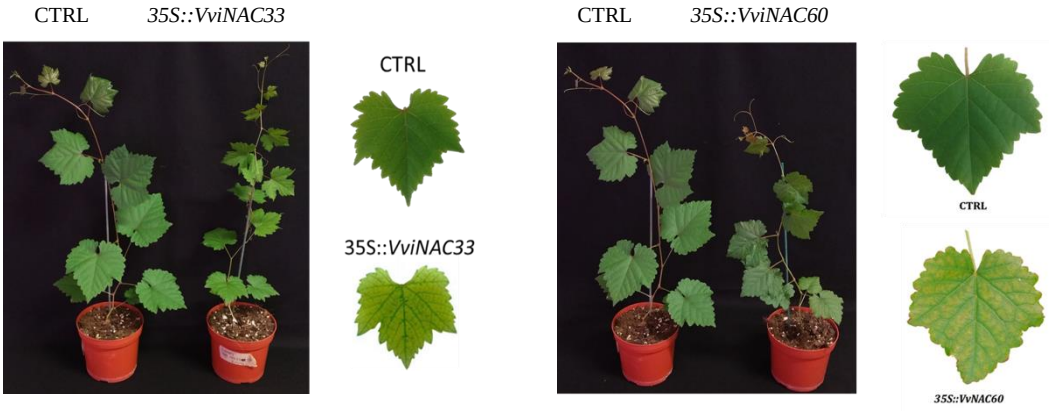

Figure 15: Transgenic plants over expressing *VviNAC33* and *VviNAC60* (D'Incà, 2017).

VviNAC11 (VIT_14s0108g01070) and *VviNAC13* (VIT_02s0012g01040) were also selected as *switch* genes related only to the berry development (D'Incà, 2017); their expression profiles clearly reflect this bioinformatic evidence (**Fig. 16**).

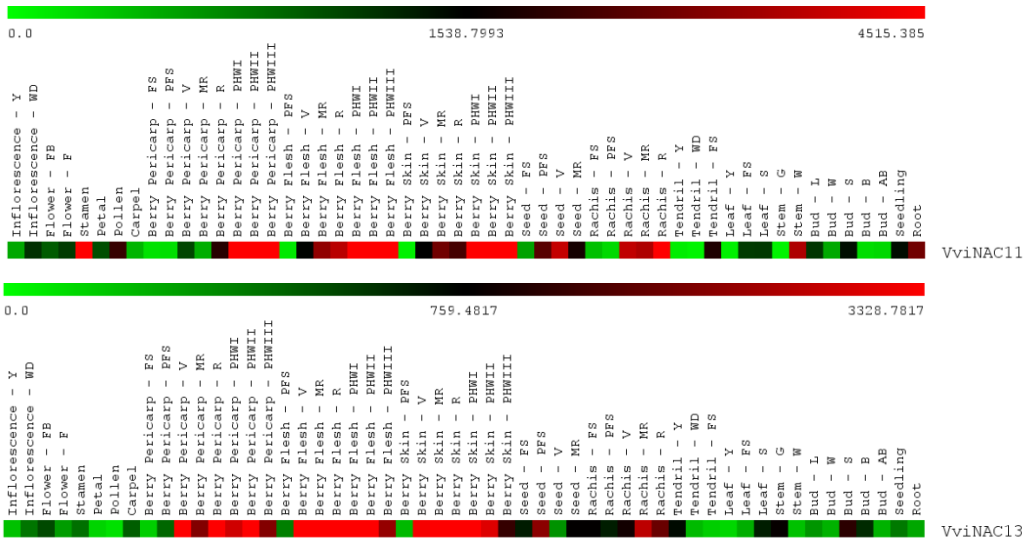

Figure 16: Heatmaps representing the *VviNAC11* and *VviNAC13* expression profiles in all organs and at different developmental stages (data taken from Fasoli *et al.*, 2012).
Description of ATLAS abbreviations (54 developmental stages):
Bud – L = latent bud; – W = winter bud; – S = bud swell; – B = bud burst; – AB = bud after-burst; **Inflorescence** – Y = young inflorescence; – WD = well developed inflorescence; **Flower** – FB = flowering begins; – F = flowering; **Stamen** = pool of stamen from undisclosed flowers; **Pollen** = pollen from disclosed flowers; **Carpel** = pool of carpels from undisclosed flowers; **Petal** = pool of petals from undisclosed flowers; **Tendril** – Y = young tendril; – WD = well developed tendril; – FS = mature tendril; **Leaf** – Y = young leaf; – FS = mature leaf; – S = senescencing leaf; **Berry Pericarp** – FS =fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post- harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III**; Berry Skin** – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post-harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Berry Flesh** – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; – PHWI = post-harvest withering I; –PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Seed** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; **Rachis** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; **Stem** – G = green stem; - W = woody stem; **Root** = in-vitro cultivated roots; **Seedling** = pool of 3 developmental stages.

Since NOR (a NAC-domain transcription factor whose mutation leads to a non-ripening phenotype) has been classified as one of the key regulators of fruit development and ripening in tomato, the two closest grapevine *NOR* orthologues were also selected: *VviNAC03* (VIT_00s0375g00040), already described by D'Incà (2017, **Fig. 17**) and *VviNAC18* (VIT_19s0014g03300).

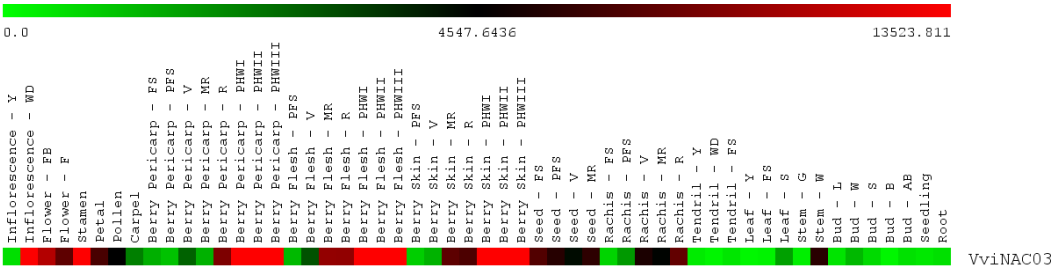

Figure 17: Heatmap representing the *VviNAC03* expression profile in all organs and at different developmental stages (data taken from Fasoli *et al.*, 2012).
Description of ATLAS abbreviations (54 developmental stages):
Bud – L = latent bud; – W = winter bud; – S = bud swell; – B = bud burst; – AB = bud after-burst; **Inflorescence** – Y = young inflorescence; – WD = well developed inflorescence; **Flower** – FB = flowering begins; – F = flowering; **Stamen** = pool of stamen from undisclosed flowers; **Pollen** = pollen from disclosed flowers; **Carpel** = pool of carpels from undisclosed flowers; **Petal** = pool of petals from undisclosed flowers; **Tendril** – Y = young tendril; – WD = well developed tendril; – FS = mature tendril; **Leaf** – Y = young leaf; – FS = mature leaf; – S = senescencing leaf; **Berry Pericarp** – FS =fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post- harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III**; Berry Skin** – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post-harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Berry Flesh** – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; – PHWI = post-harvest withering I; –PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Seed** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; **Rachis** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; **Stem** – G = green stem; - W = woody stem; **Root** = in-vitro cultivated roots; **Seedling** = pool of 3 developmental stages.

As the interest is mainly focused on the berry ripening, to choose other candidates all the expression profiles of the *VviNACs* genes were compared. A hierarchical clustering analysis permitted to find those *VviNACs* with a similar profile to the one of *VviNAC60* (**Fig. 18**). Between the genes of this cluster, *VviNAC03*, *VviNAC11*, *VviNAC13* and *VviNAC18* were also found. Some genes presented interesting expression profiles in all berry tissues (*VviNAC01*, *VviNAC05*, *VviNAC15*, *VviNAC20*, *VviNAC37*, *VviNAC38*, *VviNAC48* and *VviNAC61*), where their expression increase during the ripening. Other genes had a very high expression in seeds at the veraison (*VviNAC08, VviNAC17, VviNAC26* and *VviNAC39)* (**Fig. 18**).

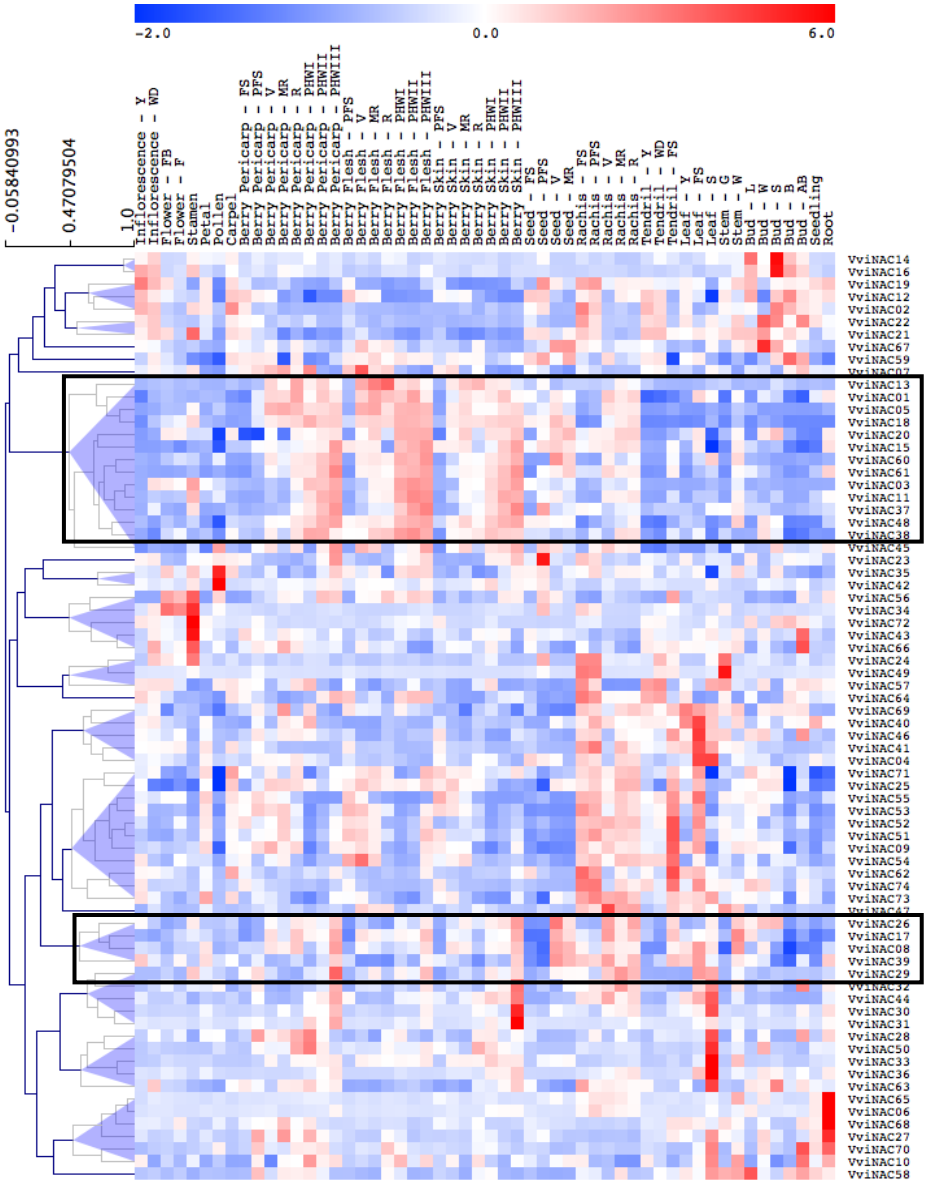

Figure 18: Heatmap representing all the *VviNACs* expression profiles in all organs and at different developmental stages (data taken from Fasoli *et al.*, 2012). The two clusters of selected NACs are highlighted by black rectangles. Description of ATLAS abbreviations (54 developmental stages):
Bud – L = latent bud; – W = winter bud; – S = bud swell; – B = bud burst; – AB = bud after-burst; **Inflorescence** – Y = young inflorescence; – WD = well developed inflorescence; **Flower** – FB = flowering begins; – F = flowering; **Stamen** = pool of stamen from undisclosed flowers; **Pollen** = pollen from disclosed flowers; **Carpel** = pool of carpels from undisclosed flowers; **Petal** = pool of petals from undisclosed flowers; **Tendril** – Y = young tendril; – WD = well developed tendril; – FS = mature tendril; **Leaf** – Y = young leaf; – FS = mature leaf; – S = senescencing leaf; **Berry Pericarp** – FS =fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post- harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III**; Berry Skin** – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post-harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Berry Flesh** – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; – PHWI = post-harvest withering I; –PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Seed** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; **Rachis** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; **Stem** – G = green stem; **-** W = woody stem; **Root** = in-vitro cultivated roots; **Seedling** = pool of 3 developmental stages.

These clustered genes were selected as they could have an important role, even if marginal compared to the one of the *VviNAC60*, in the vegetative-to-mature phase transition. Afterwards, the previously shown phylogenetic analysis (**Fig. 13**, Wang *et al*., 2013) was repeated to identify *VviNACs* clustering in the same *VviNAC60* clade (**Fig. 19**). Between the genes of this cluster, *VviNAC03* and *VviNAC18* were also found. The nearest, some of which were also present in the expression profile cluster, were selected (*VviNAC01*, *VviNAC08*, *VviNAC15*, *VviNAC17*, *VviNAC26*, *VviNAC39*, *VviNAC42*, *VviNAC44* and *VviNAC45)*.

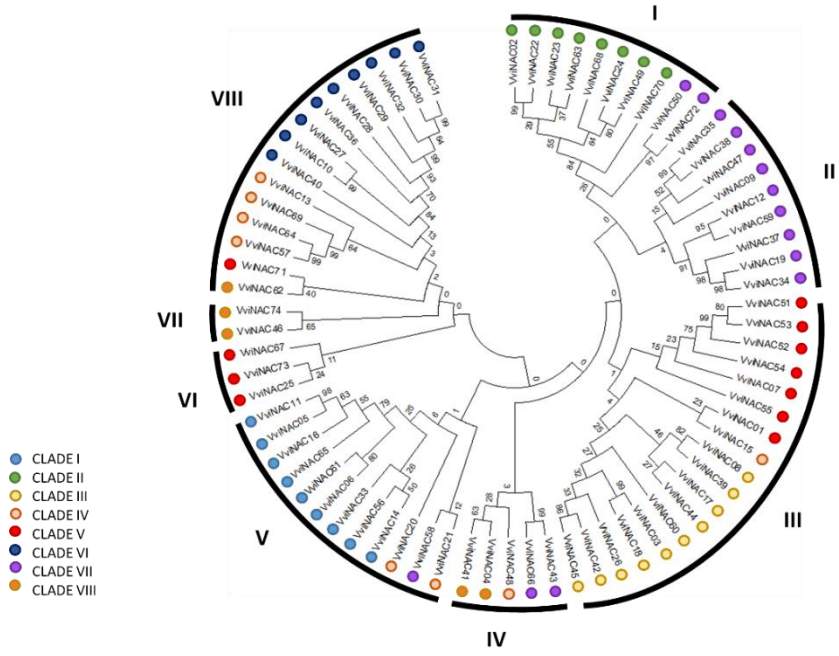

Figure 19: Phylogenetic analysis of the 74 *VviNAC*s genes. The previous clade designation (Wang *et al*., 2013) is shown as a circle of different colours.

Finally, was taken into consideration the new highly detailed transcriptomic map of grape berry development (Fasoli *et al*., 2018), which is focused on transcriptomic rearrangements close to veraison where two rapid and successive transitions at transcriptional level before veraison were found. Positive and negative markers of each transition were identified, and the expression profile of these genes highlighted the transcriptional rearrangements during the two weeks before veraison. The positive markers of the first transition seem to play a major role as triggers being not expressed until two weeks before veraison and then characterized by a dramatic increase in expression. By looking at the first transition markers list, 3 switch genes

were identified: *VviNAC33*, *VvibHLH075* and *VviWRKY19*. Looking at the genes resulted up regulated by the over expression of *VvibHLH075* and *VviWRKY19*, other *VviNACs* genes were found: *VviNAC05*, *VviNAC17*, *VviNAC26* and *VviNAC61* (**Fig. 20**).

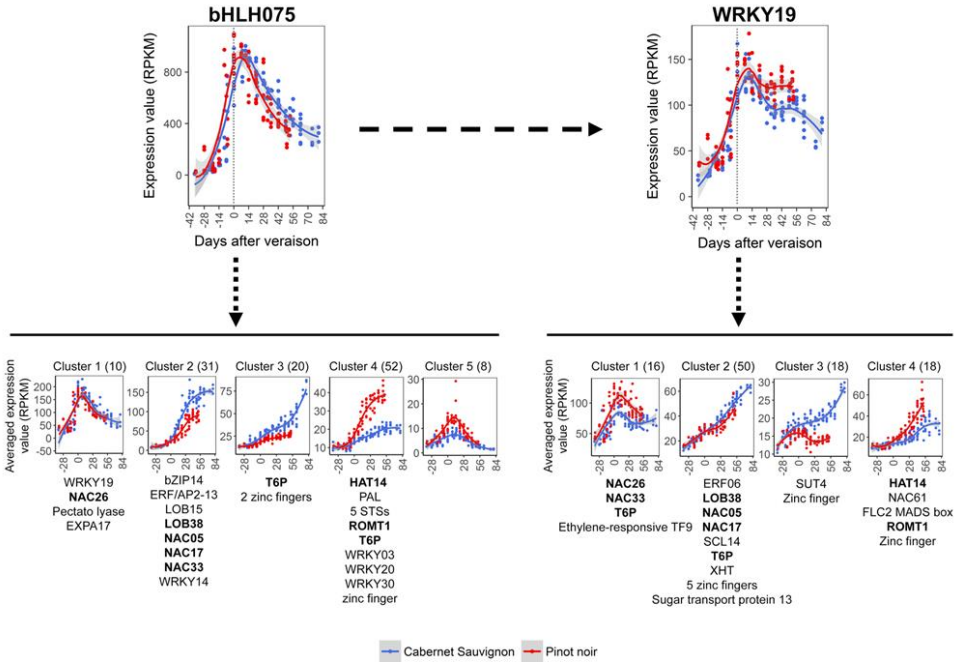

Figure 20: *Vvi*bHLH075 and *Vvi*WRKY19 putative transcriptional activation network (Fasoli *et al*., 2018).

At the end, comparing the set of genes selected by different criteria, a total of 14 *VviNACs* were selected and isolated for functional studies*:* *VviNAC01* (VIT_01s0146g00280), *VviNAC03* (VIT_00s0375g00040), *VviNAC08* (VIT_18s0001g02300), *VviNAC11* (VIT_14s0108g01070), *VviNAC13* (VIT_02s0012g01040), *VviNAC15* (VIT_18s0001g01820), *VviNAC17* (VIT_19s0014g03290), *VviNAC18* (VIT_19s0014g03300), *VviNAC26* (VIT_01s0026g02710), *VviNAC33* (VIT_19s0027g00230), *VviNAC38* (VIT_10s0003g00500), *VviNAC39* (VIT_07s0031g02610), *VviNAC60* (VIT_08s0007g07670) and *VviNAC61* (VIT_08s0007g07640).

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2.2 MATERIAL AND METHODS

Plant material

The ORF of the selected *VviNACs*, except for the already described one (D'Incà, 2017), were isolated from berry pericarp tissue of growth chamber grown *Vitis vinifera* cv Shiraz fruiting cuttings, propagated as described in Mullins *et al*,.1981. This method permits to have plants for sampling during all the year and is useful to avoid environmental influences. Cuttings were rooted in a heated container (26°C at bases of cuttings) in a cold room (4°C). Buds remained dormant. After four weeks, the rooted cuttings were planted in vermiculite-perlite mixture and transferred in a growth chamber (27°C day and 22°C night, 16 hours photoperiod). At bud burst, leaves basal and adjacent to inflorescences were removed as soon as accessible and the shoot tip was excised. These treatments promote inflorescence growth. A lateral shoot was permitted to grow from an axillary bud of one of the defoliated nodes on the main axis. Development of the bunch to maturity is supported by the foliage of the lateral shoot.
For the transient over expression of the *VviNACs* in grapevine, plantlets of *V. vinifera* cv Sultana were *in vitro* micro-propagated and cultivated in HB medium in a growth chamber at 25°C with a 16 h photoperiod.

Co-expression analysis

Using the global gene expression atlas of *V. vinifera* cv Corvina obtained by microarray approach (Fasoli *et al.*, 2012) a co-expression analysis with the Pearson correlation metric was performed using Cor.To (http://www.usadellab.org/cms/index.php?page=corto).

Isolation and Cloning

The total RNA was isolated from 400 mg of ground *Vitis vinifera* cv. Shiraz berry pericarp using Spectrum^{TM} Plant Total RNA kit (Sigma-Aldrich).
RNA samples were quantified with the NanoDrop spectrophotometer (NanoDrop Thermo Scientific Technologies) and 1 µg of RNA was treated with

the TURBO DNA-free™ kit (Ambion). DNase-treated RNA was used for cDNA synthesis using the enzyme Super-Script™ III Reverse Transcriptase (Invitrogen).

The *VviNAC* ORFs were amplified from cDNA using KAPA HiFi DNA polymerase (KAPA Biosystems, Wilmington, MA, USA) and the primers listed in **Table 1**. The PCR products were directionally cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Each ORF of interest was then transferred into the binary over expression vector pK7GW2.0 (Laboratory of Plant Systems Biology, PSB; Ghent University, Belgium) by site specific LR Gateway® recombination.

Gene ID	TF	Direction	Primer	T_a (°C)	Lenght (bp)
VIT_01s0146g00280	VviNAC01	For	5'-CACCATGGAGAAGCTCAATTTTGTGAAG-3'	55	756
		Rev	5'-TTATGGTTTTCTTCTGAAAGGC-3'		
VIT_18s0001g02300	VviNAC08	For	5'-CACCATGACAGCGGAGTTGCA-3'	55	900
		Rev	5'-TCAGAAGGGCTTCTGCA-3'		
VIT_18s0001g01820	VviNAC15	For	5'-CACCATGAAGGTGACAGTGGGT-3'	55	1719
		Rev	5'-TCATGAGGGGAGGCATC-3'		
VIT_19s0014g03290	VviNAC17	For	5'-CACCATGGGTGTACCGGAGAC-3'	55	1002
		Rev	5'-TTACTGCCTATATCCAAATCCAC-3'		
VIT_19s0014g03300	VviNAC18	For	5'-CACCATGGAGAGCACCGATTCATC-3'	55	1062
		Rev	5'-CTAAGCATACCAATTCATGCC-3'		
VIT_01s0026g02710	VviNAC26	For	5'-CACCATGGATGGAAAAGGCAGCT-3'	55	849
		Rev	5'-TCACTGAAATTCATACACTGGG-3'		
VIT_10s0003g00500	VviNAC38	For	5'-CACCATGATGGGGAAGGGTTCC-3'	55	1458
		Rev	5'-TCATGTCAAATAATAGTCACCT-3'		
VIT_07s0031g02610	VviNAC39	For	5'-CACCATGATGAGCGGAGATCAGT-3'	55	885
		Rev	5'-TCAAAATGACTTGTTCGAGA-3'		
VIT_08s0007g07640	VviNAC61	For	5'-CACCATGGAAGAGGCTTCACTTG-3'	55	1212
		Rev	5'-TCAGTAGTCGAGTAAATAATCCA-3'		

Table 1: Primers list for the *VviNACs* isolation from *V. vinifera* cv Shiraz berry pericarp tissue.

Agrobacterium tumefaciens C5851 transformation

A. tumefaciens strain C58C1 was individually transformed by electroporation (Bio-Rad electroporation instrument, 25 µF, 200 Ω and 2.5 kV) with the pK7WG2.0 transformed vectors containing the 35S:*VviNACs* (tests) and the empty pK7WG2.0 (control). The transformed colonies were selected by strain antibiotic resistance (tetracycline) and construct antibiotic resistance (streptomycin and spectinomycin).

VviNACs transient over expression

5-week-old *in vitro* plantlets of *Vitis vinifera* cv Sultana were vacuum infiltrated with each of the transformed bacteria strains (tests and control) at 0.5 OD_{600nm}; 6

plants were immersed in the bacterial suspension and vacuum infiltrated two times for 2 min at 90kPa. Immediately after the agroinfiltration, the plants were put in glass jars with sterile perlite and rinsed with sterile water for the *in vitro* recovery. After 7 day from the infiltration, the apical leaves of the 6 plantlets of each over expression were collected and grounded with liquid nitrogen.

The powders were used for the RNA extraction with the Spectrum^{TM} Plant Total RNA kit (Sigma-Aldrich). The extracted RNAs were quantified with the Nanodrop (Thermo Scientific), checked in their quality with a Bioanalyzer Chip RNA 7500 series II (Agilent) for a RIN value over 8 and used in the qPCR Real-Time control.

VviNAC03, *VviNAC11*, *VviNAC13*, *VviNAC33* and *VviNAC60* transient over expression were already performed by D'Incà (2017).

VviNACs stable over expression

VviNAC33 and *VviNAC60* stable over expression were performed by D'Incà (2017).

qPCR Real-Time analyses

Vitis vinifera cv Sultana plantlets were screened for the confirmation of the *VviNACs* transient over expression by qPCR with the primers reported in **Table 2**.

Gene ID	TF	Direction	Primer	T_a (°C)	Lenght (bp)
VIT_01s0146g00280	VviNAC01	For	5'- TTGCCAGGTGATTTGGAGCA -3'	55	103
		Rev	5'- TCCAGTAACCCGAAACCGTG -3'		
VIT_18s0001g02300	VviNAC08	For	5'- AGCGGAGTTGCAGTTACCTC -3'	55	91
		Rev	5'- TTGCGATGCACATTTACGGC -3'		
VIT_18s0001g01820	VviNAC15	For	5'- AGGCCGTGCTCCAAGTAAAG -3'	55	91
		Rev	5'- CTGCACATTCGGGCATCTCT -3'		
VIT_19s0014g03290	VviNAC17	For	5'- AGAAGTCCAGAGCGGACTCA -3'	57	116
		Rev	5'- CGAACGGGTCGAGTGAGTTA -3'		
VIT_19s0014g03300	VviNAC18	For	5'- TTCCACGACAAAGAGGTTCC -3'	55	120
		Rev	5'- TGGTGCAATGAAGGAGTCTG -3'		
VIT_01s0026g02710	VviNAC26	For	5'- CGCGTGACCGTAAGTATCCC -3'	55	113
		Rev	5'- CCCCACATACTTAGCCCCAC -3'		
VIT_07s0031g02610	VviNAC39	For	5'- CTCTCCAAGGGACCGCAAAT -3'	55	118
		Rev	5'- AATTCCGACCGTCTTGGGTC -3'		
VIT_08s0007g07640	VviNAC61	For	5'- CGACCAGTACCGTAAGAGCC -3'	55	93
		Rev	5'- GCGAGAGGCTGACCATAGAC -3'		

Table 2: Primers used in the qPCR Real-Time analyses for the confirmation of the *VviNACs* over expression.

All the qPCR analyses were performed as described by Zenoni *et al.* (2010) using SYBR green qPCR Master mix (Promega) and a QuantStudio 3 Real-Time PCR System (Thermo Fisher).

The qPCRs were set up and performed according to the instrument and enzyme manufactures instruction with an appropriate T_a for each set of primers.

Each expression value was determined in triplicate and relatively to the housekeeping gene UBIQUITIN (VIT_16s0098g01190), amplified with the following primers:

For 5'-TCTGAGGCTTCGTGGTGGTA-3'

Rev 5'-AGGCGTGCATAACATTTGCG-3'

Amplification efficiency was calculated from raw data using the LingRegPCR software (Ramakers *et al.*, 2003).

The MNE final expression value were calculated according to Simon (2003).

Microarray analyses

Microarray analyses were performed on the transient over expressing plantlets according to the Agilent Microarray-Based Gene Expression Analysis Guide (V 6.5) protocol. The Agilent custom microarray was designed on the 4pack 44K format (Agilent Sure Print HD 4X44K 60-mer G2514F-048771) and was created by using an Agilent's web-based application (https://earray.chem.agilent.com/earray/). 34.651 specific *V. vinifera* 60-mer probes were produced by the software: 29.798 from the *V. vinifera* cv Pinot Noir predicted transcripts V1 version, 4.392 from the new *V. vinifera* cv Pinot Noir loci identified by *V. vinifera* cv Corvina transcriptome reconstruction and analysis and 179 from *V. vinifera* cv Corvina private genes (Venturini *et al.*, 2013). Scanning and Feature Extraction was performed with an Agilent Scanner following the settings and parameters indicated in the instruction manual. For each extraction, the QC report was analyzed to assess the quality of the overall hybridization procedure and a datamatrix was prepared selecting from each single sub-array outcome file the gProcessedSignalvalues, which are the raw fluorescence intensities of each probe. The data were normalized on the 75th percentile and Feature extraction and statistical analysis of the microarray data was conducted as reported in Amato *et al.* (2017). DEGs were identified by TMeV Student's t test ($p<0.05$), assuming equal variance among samples, and selected by fold change |1.5|.

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2.3 RESULTS

Selected *VviNACs* expression profiles

The genes expression patterns of *VviNAC01*, *VviNAC08*, *VviNAC15*, *VviNAC17*, *VviNAC18*, *VviNAC26*, *VviNAC38*, *VviNAC39* and *VviNAC61* were analyzed by consulting the global gene expression atlas (Fasoli *et al.*, 2012). The expression atlas is a whole-genome expression survey of 54 grapevine tissues and organs collected at various developmental stages, obtained by NimbleGen microarray analysis. As described in the **Introduction** part of this chapter, the above mentioned *VviNACs* were selected as they are in some ways related to *VviNAC60*, the hypothetical master regulator of the berry ripening.

For this reason, the expression profiles of the following genes show high similarity with the *VviNAC60* one (**Fig. 21**).

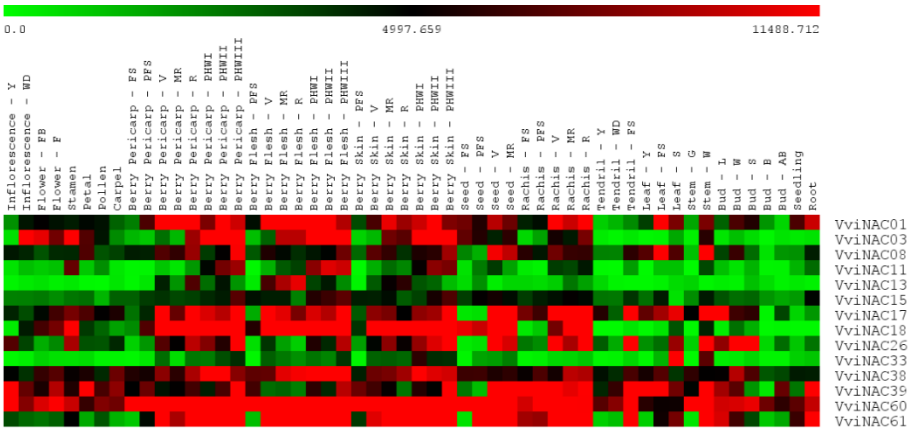

Figure 21: Heatmap representing the selected *VviNACs* expression profiles in all organs and at different developmental stages (data taken from Fasoli *et al.*, 2012).
Description of ATLAS abbreviations (54 developmental stages):
Bud – L = latent bud; – W = winter bud; – S = bud swell; – B = bud burst; – AB = bud after-burst; **Inflorescence** – Y = young inflorescence; – WD = well developed inflorescence; **Flower** – FB = flowering begins; – F = flowering; **Stamen** = pool of stamen from undisclosed flowers; **Pollen** = pollen from disclosed flowers; **Carpel** = pool of carpels from undisclosed flowers; **Petal** = pool of petals from undisclosed flowers; **Tendril** – Y = young tendril; – WD = well developed tendril; – FS = mature tendril; **Leaf** – Y = young leaf; – FS = mature leaf; – S = senescencing leaf; **Berry Pericarp** – FS =fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post- harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III**; Berry Skin** – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post-harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Berry Flesh** – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; – PHWI = post-harvest withering I; –PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Seed** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; **Rachis** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; **Stem** – G = green stem; - W = woody stem; **Root** = in-vitro cultivated roots; **Seedling** = pool of 3 developmental stages.

In details, *VviNAC01* shows an increasing expression during berry developmental phases, in the seeds at veraison, in the rachis from the veraison on, in the mature leaves and in the roots. *VviNAC08* is highly expressed in the berry tissues, in the pericarp and skin from the veraison and in the post-harvesting withering flesh, in the petal, in the seeds from the veraison, in the mature tendril, in the woody stem, in the winter bud and in all the leaf and rachis developmental phases. *VviNAC15* shows an increasing expression during berry development with a peak at post-harvesting withering phase; it is also highly expressed in the seed and rachis all over the growth phases, in the woody stem, in the mature leaf and in the root. *VviNAC17*, has an increased expression in the seed, rachis and berry pericarp and flesh from the veraison on and in the skin at the post-harvesting withering phase III; it is also expressed in the mature tendril, in the woody stem and in the latent bud. *VviNAC18*, is highly expressed in berry tissues after veraison, in the seeds, in the stamen and in the rachis from veraison on ripening. *VviNAC26* shows an increase of expression in the seed, rachis and berry pericarp from veraison on, and in the berry flesh and skin at the post-harvesting withering phases; it also highly expressed in the stamen, in the mature tendril, in the leaves at senescence, in the woody stem and in the bud until burst. *VviNAC38* is expressed in the berry pericarp and skin from the veraison on and in the flesh from the mid-ripening stage on; it also shows high expression in the winter bud and in the mid-ripening stage rachis. *VviNAC39* expression profile shows high expression in the post-harvesting withering phase III of all the berry tissues, in the seed from veraison on, in all the rachis developmental stages, in the mature tendril, in the root, in the young and mature leaves, in the young inflorescences, in the woody stem and in the root. *VviNAC61* increases its expression during the post-harvest withering phases of all the berry tissues but is also highly expressed in the rachis from veraison on, in the mature tendril and in the roots.

Selected *VviNACs* co-expression analyses

To discover genes co-expressed with the selected *VviNACs* and possibly representing their targets or partners, the Atlas transcriptomic dataset was investigated. Thanks to a specific correlation tool named Cor.To, the most

correlated genes with *VviNAC01, VviNAC08, VviNAC15, VviNAC17, VviNAC18, VviNAC26, VviNAC38, VviNAC39* and *VviNAC61* were selected. All the 'no hit' and 'unknown' were eliminated from the list to focus only on the annotated genes with a predicted functional role. At first, a GO enrichment analysis using the on-line tool ShinyGO v0.61 (http://bioinformatics.sdstate.edu/go/) was performed on the co-expressed genes for each selected *VviNACs* (**Appendix A**). Then, for each *VviNAC* member the top-20 positive and negative correlated genes were investigated.

VviNAC01 co-expression analysis presented a lot of genes correlated to different catabolic processes, protein modifications and photosynthesis. Among the highest positively correlated genes, *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR CORRELATED TO APETALA2 4* (VIT_00s0662g00040), *VviNAC05* (VIT_17s0000g06400) and *VviWRKY19* (VIT_07s0005g01710) were identified, while many *CHLOROPLAST PRECURSORS* (VIT_15s0048g00370, VIT_10s0116g00560, VIT_00s0480g00080 and VIT_09s0018g01870) and a *LHCII* (VIT_19s0014g00160) were found among the negatively correlated genes (**Table 3**).

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_15S0046G02300	Beta-cyanoalanine synthase	0.83	VIT_01S0011G03450	Alpha-glucosidase	-0.75
VIT_08S0040G01950	zinc finger (C3HC4-type RING finger)	0.83	VIT_11S0016G05840	protease inhibitor/seed storage/lipid transfer protein (LTP)	-0.74
VIT_00S0404G00040	ARF GTPase activator, ARF-GAP Domain 5	0.81	VIT_17S0000G06880	heparanase protein 2 precursor	-0.73
VIT_00S0662G00040	Ethylene-responsive transcription factor RELATED TO APETALA2 4	0.81	VIT_13S0067G00080	COP1-interacting protein 7	-0.73
VIT_17S0000G06400	NAC domain-containing protein (VvNAC05)	0.79	VIT_02S0025G01040	Receptor protein kinase	-0.73
VIT_14S0060G00410	EDA32 (embryo sac development arrest 32)	0.78	VIT_15S0048G00370	transketolase, chloroplast precursor	-0.73
VIT_01S0127G00590	Protein disulfide isomerase	0.78	VIT_01S0137G00290	oxysterol binding protein	-0.73
VIT_08S0007G08840	Glycosyl transferaseHGA1	0.78	VIT_01S0244G00140	aspartate kinase	-0.73
VIT_10S0003G02450	flavonol synthase	0.77	VIT_18S0001G00470	monocopper oxidase SKS5 (SKU5 Similar 5)	-0.73
VIT_01S0011G05110	major latex protein 22	0.77	VIT_10S0116G00560	polyphenol oxidase II, chloroplast precursor	-0.72
VIT_05S0051G00640	purple acid phosphatase 23- ATPAP23/PAP23	0.77	VIT_00S0480G00080	polyphenol oxidase II, chloroplast precursor	-0.72
VIT_06S0004G07560	ribosomal protein S29 28S	0.77	VIT_01S0011G02030	serine carboxypeptidase S10	-0.72
VIT_12S0028G00930	Glutathione S-transferase (VvGST3)	0.77	VIT_18S0001G05180	beta-D-xylosidase	-0.72
VIT_07S0005G01710	WRKY transcription factor (VvWRKY19)	0.77	VIT_03S0063G02490	glucan endo-1,3-beta-glucosidase 7 precursor	-0.72
VIT_00S0360G00050	Cyclin-related	0.77	VIT_08S0040G00980	tubulin beta-1 chain	-0.72
VIT_02S0025G04560	copper amine oxidase	0.77	VIT_14S0068G00930	chalcone synthase 1	-0.72
VIT_06S0061G00550	xyloglucan endotransglucosylase/hydrolase 32	0.77	VIT_09S0002G02220	Protein kinase CDG1	-0.72
VIT_12S0028G00410	protein phosphatase 2C	0.76	VIT_09S0018G01870	D-3-phosphoglycerate dehydrogenase	-0.71
VIT_02S0025G04880	geraniol 10-hydroxylase	0.76	VIT_19S0014G00160	LHCII type I CAB-1	-0.71
VIT_01S0026G00850	Zinc finger protein 5	0.76	VIT_14S0108G01660	biotin carboxyl carrier protein of acetyl-CoA carboxylase	-0.71

Table 3: *VviNAC01* positive (left) and negative (right) co-expressed top 20 genes.

VviNAC08 co-expressed genes were highly enriched for the cell cycle and cellular organization and regulation processes categories. Among the highest positively correlated genes, *VviNAC17* (VIT_19s0014g03290), two *ERF/AP2* members (VIT_16s0013g01080 and VIT_18s0001g10150) and *VviWRKY16* (VIT_06s0004g07500) were identified, while *CELL ELONGATION PROTEIN/DWARF1* (VIT_01s0010g01200), *TUBULIN BETA-1 CHAIN*

(VIT_08s0040g00980) and *GIBBERELLIN-REGULATED PROTEIN4/GASA4* (VIT_03s0038g00120) resulted among the negatively correlated gene (**Table 4**).

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_19S0014G03290	NAC domain-containing protein (VvNAC17)	0.77	VIT_18S0072G00330	Nucleic acid binding	-0.63
VIT_00S0399G00060	alternative oxidase / immutans protein (IM)	0.75	VIT_05S0077G01060	Aldehyde Dehydrogenase (VvALDH22A1)	-0.62
VIT_11S0016G02580	MAPK activating protein-like	0.74	VIT_06S0004G02480	Cysteine peptidase	-0.61
VIT_16S0013G01080	ERF/AP2 Gene Family (VvERF086)	0.73	VIT_01S0010G01200	cell elongation protein / DWARF1 / DIMINUTO (DIM)	-0.60
VIT_12S0028G01410	heat shock transcription factor A4A	0.73	VIT_14S0108G00380	Protein kinase APK1A	-0.60
VIT_18S0001G12260	Incomplete root hair elongation	0.73	VIT_08S0007G08380	cellulose synthase CESA3	-0.60
VIT_06S0004G06750	armadillo/beta-catenin repeat	0.73	VIT_08S0007G08430	LRR receptor-like kinase 2	-0.60
VIT_09S0002G03610	MYB DIVARICATA	0.71	VIT_10S0003G04560	Auxin-Induced in Root cultures 9 AIR9	-0.60
VIT_06S0004G04950	scarecrow-like transcription factor 14 SCL14	0.71	VIT_08S0007G01760	steroid 5 alpha reductase DET2	-0.59
VIT_13S0019G00830	armadillo repeat-containing protein	0.69	VIT_09S0018G00520	plastocyanin domain-containing protein	-0.59
VIT_02S0025G03520	haloacid dehalogenase hydrolase	0.69	VIT_08S0040G00980	tubulin beta-1 chain	-0.59
VIT_01S0026G00190	armadillo/beta-catenin repeat	0.69	VIT_04S0008G05830	armadillo/beta-catenin repeat	-0.59
VIT_16S0039G01060	CYP89A2	0.69	VIT_07S0005G05510	Kinase-like protein TMKL1	-0.59
VIT_18S0001G10150	ERF/AP2 Gene Family (VvERF006)	0.69	VIT_05S0020G01180	proteasome 26S AAA-ATPase subunit (RPT5a)	-0.59
VIT_18S0001G01830	ATP-NAD kinase	0.68	VIT_09S0002G08960	EREBP-4	-0.58
VIT_18S0122G00320	PLATZ transcription factor	0.68	VIT_00S0218G00090	exostosin family protein	-0.58
VIT_16S0050G00390	4-coumarate-CoA ligase	0.67	VIT_03S0038G00120	gibberellin-regulated protein 4 (GASA4)	-0.58
VIT_14S0060G00430	plant IF (intermediate filament)	0.67	VIT_00S1644G00010	S-locus protein kinase	-0.58
VIT_01S0010G01840	GEM-like protein 5	0.67	VIT_17S0000G02010	atypical receptor kinase MARK	-0.58
VIT_06S0004G07500	WRKY transcription factor (VvWRKY16)	0.67	VIT_03S0088G00380	Tubulin alpha	-0.58

Table 4: *VviNAC08* positive (left) and negative (right) co-expressed top 20 genes.

VviNAC15 genes list showed high enrichment in the regulation of the metabolic processes and the protein localization categories. *VviNAC15* top highly co-expressed genes list presents four interesting elements: *DENN/AEX-3 DOMAIN-CONTAINING PROTEIN* (VIT_11s0016g01240) and *AUTOPHAGY PROTEIN APG6* (VIT_15s0046g00950), as positively correlated, and *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 2* (VIT_18s0001g01250) and *ELIP1-EARLY LIGHT-INDUCIBLE PROTEIN* (VIT_05s0020g04110), as negatively correlated (**Table 5**).

Gene ID	Functional annotation	Pearson
VIT_01S0011G04520	programmed cell death 6-interacting protein	0.89
VIT_16S0039G01480	PHD finger transcription factor	0.87
VIT_11S0016G01240	DENN (AEX-3) domain-containing protein	0.85
VIT_19S0015G00930	hydroxyproline-rich glycoprotein	0.84
VIT_07S0005G02190	Zinc finger (C2H2 type) family	0.84
VIT_15S0046G00950	autophagy protein Apg6	0.84
VIT_05S0102G00160	zinc finger (Ran-binding)	0.83
VIT_17S0000G03430	Adaptor-related protein complex 3, delta 1 sub	0.83
VIT_17S0000G01450	exocyst subunit EXO70 F1	0.83
VIT_05S0077G00650	Poly(A) polymerase	0.83
VIT_01S0011G05350	Cysteine-rich RLK10	0.83
VIT_07S0104G01600	MORC family CW-type zinc finger 4	0.83
VIT_13S0067G01320	Alpha-1,2-mannosyltransferase	0.83
VIT_10S0003G01770	heat shock transcription factor A4A	0.83
VIT_08S0007G03880	Zinc finger (C2H2 type) family	0.83
VIT_00S0231G00050	ATP binding protein	0.82
VIT_06S0004G03180	zinc finger (FYVE type)	0.82
VIT_09S0054G01880	Calreticulin interacted protein	0.82
VIT_05S0077G01350	Mov34 STAM-binding protein	0.82
VIT_06S0009G00250	Large subunit GTPase 1	0.81

Gene ID	Functional annotation	Pearson
VIT_18S0001G06410	Ribosomal protein 60S	-0,679
VIT_14S0068G00230	Spiral 1 like 2	-0,636
VIT_12S0035G01210	cytochrome c	-0,615
VIT_00S1491G00020	lil3 protein	-0,600
VIT_07S0104G01270	kinase interacting family protein	-0,591
VIT_06S0004G05940	Uncoupling protein 1	-0,591
VIT_05S0020G04110	ELIP1 (early light-inducible protein)	-0,582
VIT_15S0046G01130	myb TRIPTYCHON	-0,578
VIT_06S0061G00810	cyclin-dependent kinase regulatory subunit CKS2	-0,574
VIT_02S0087G00810	SWIB complex BAF60b domain-containing protein	-0,568
VIT_18S0001G01250	senescence-inducible chloroplast stay-green protein 2	-0,567
VIT_19S0027G00200	photosystem II 10 kDa polypeptide PSBR	-0,565

Table 5: *VviNAC15* top 20 positive (left) and all the negative (right) co-expressed genes.

VviNAC17 co-expression analysis revealed that the most represented categories were the one regarding the regulation of metabolic and biosynthetic processes.

VviNAC17 co-expressed genes list presented seven interesting elements: *VviNAC08* (VIT_18s0001g02300), *VviNAC26* (VIT_01s0026g02710), two *ERF/AP2* (VIT_10s0003g00140 and VIT_18s0001g10150), *ETHYLENE-RESPONSIVE DEAD BOX RNA HELICASE/RH30* (VIT_17s0000g09120), *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR CORRELATED TO APETALA2 6* (VIT_18s0072g00260) and *VviWRKY16* (VIT_06s0004g07500) as positively correlated (**Table 6**). No interesting genes were found between the negatively correlated.

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_18S0001G02300	NAC domain-containing protein (VvNAC08)	0.77	VIT_05S0077G01060	Aldehyde Dehydrogenase (VvALDH22A1)	-0.64
VIT_01S0026G02710	NAC domain-containing protein (VvNAC26)	0.77	VIT_11S0016G05840	protease inhibitor/seed storage/lipid transfer protein (LTP)	-0.62
VIT_10S0003G00140	ERF/AP2 Gene Family (VvERF064)	0.75	VIT_14S0108G00380	Protein kinase APK1A	-0.61
VIT_17S0000G09120	ethylene-responsive DEAD box RNA helicase (RH30)	0.71	VIT_01S0026G02070	Peptidyl-prolyl cis-trans isomerase cyclophilin-type	-0.61
VIT_16S0050G00390	4-coumarate-CoA ligase	0.71	VIT_15S0048G00370	transketolase, chloroplast precursor	-0.61
VIT_06S0004G05660	glycosyl transferase family 1 protein	0.70	VIT_08S0007G01760	steroid 5 alpha reductase DET2	-0.60
VIT_08S0056G00180	Translation initiation factor IF-2B subunit delta	0.70	VIT_05S0020G01180	proteasome 26S AAA-ATPase subunit (RPT5a)	-0.60
VIT_04S0008G02390	zinc finger (C3HC4-type RING finger)	0.70	VIT_06S0004G04610	fasciclin arabinogalactan-protein (FLA4)	-0.60
VIT_05S0020G01910	1,4-alpha-D-glucan maltohydrolase	0.70	VIT_04S0008G03610	Xylan synthase	-0.60
VIT_00S0587G00030	CBS domain-containing protein	0.70	VIT_17S0000G02010	atypical receptor kinase MARK	-0.60
VIT_18S0001G00180	protein kinase	0.70	VIT_15S0021G00880	ferredoxin-related	-0.60
VIT_01S0146G00410	GEM-like protein 5	0.70	VIT_17S0053G00090	ACCUMULATION AND REPLICATION OF CHLOROPLASTS 6H	-0.59
VIT_11S0016G02580	MAPK activating protein-like	0.70	VIT_12S0028G00310	Protein kinase family	-0.59
VIT_07S0104G00930	gibberellin receptor GID1L2	0.69	VIT_07S0151G00140	NADH dehydrogenase (ubiquinone) Fe-S protein 4	-0.59
VIT_04S0044G00510	GT2-like trihelix DNA-binding protein	0.69	VIT_12S0059G01320	glucan endo-1,3-beta-glucosidase 7 precursor	-0.59
VIT_18S0072G00260	Ethylene-responsive transcription factor RELATED TO APETALA2 6	0.69	VIT_06S0004G08210	Receptor protein kinase	-0.58
VIT_14S0128G00390	Mitochondrial FAD carrier	0.69	VIT_00S0218G00090	exostosin family protein	-0.58
VIT_06S0004G07500	WRKY transcription factor (VvWRKY16)	0.69	VIT_07S0141G00240	NADH dehydrogenase (ubiquinone) flavoprotein 2	-0.58
VIT_13S0156G00170	molybdopterin synthase (CNX2)	0.69	VIT_06S0004G01540	Oxidoreductase	-0.58
VIT_18S0001G10150	ERF/AP2 Gene Family (VvERF006)	0.69	VIT_17S0000G08870	carboxyl-terminal proteinase	-0.58

Table 6: *VviNAC17* positive (left) and negative (right) co-expressed top 20 genes.

The most represented enriched categories for *VviNAC18* were the one correlated to different type of catabolic processes, protein modification and localization and photosynthesis. *VviNAC18* co-expressed genes list presented ten interesting elements: *VviNAC05* (VIT_17s0000g06400), an *ERF/EP2* member (VIT_07s0031g00220) and an *UDP-SUGAR PYROPHOSPHARYLASE* (VIT_18s0001g01640) as positively correlated and *LHCB2.1 PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.1* (VIT_12s0057g00630), *LHCII TYPE I CAB-1* (VIT_19s0014g00160), *CHLOROPHYLL SYNTHETASE* (VIT_08s0105g00590), *LHCA3* and *LHCA4* (VIT_15s0024g00040 and VIT_17s0000g06350), *PHOTOSYSTEM I REACTION CENTER SUBUNIT III PSAF* (VIT_00s0125g00280) and *PHOTOSYSTEM II 22 KDA PROTEIN PSBS* (VIT_18s0001g02740) as negatively correlated (**Table 7**).

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_19S0090G01350	aspartyl protease	0.90	VIT_12S0057G00630	LHCB2.1 (Photosystem II light harvesting complex gene 2.1)	-0.77
VIT_04S0044G01790	Ribosome assembly protein 1	0.88	VIT_18S0001G13570	calcium ion binding protein	-0.77
VIT_19S0015G02880	Glutathione S-transferase 20 GSTU20	0.88	VIT_00S0256G00030	Ankyrin repeat protein	-0.76
VIT_17S0000G05110	CYP78A4	0.88	VIT_19S0014G00160	LHCII type I CAB-1	-0.76
VIT_19S0015G02590	Glutathione S-transferase 25 GSTU25	0.87	VIT_13S0019G02520	subtilisin protease C1	-0.76
VIT_08S0007G00540	haloacid dehalogenase hydrolase	0.87	VIT_12S0142G00420	Copper-transporting ATPase PAA1	-0.76
VIT_11S0016G05700	RabGAP/TBC domain-containing protein	0.87	VIT_18S0041G00950	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase	-0.75
VIT_05S0020G03860	homocysteine S-methyltransferase 3	0.86	VIT_00S0256G00040	Ankyrin repeat protein	-0.75
VIT_08S0040G01950	zinc finger (C3HC4-type RING finger)	0.86	VIT_08S0105G00590	chlorophyll synthetase	-0.75
VIT_17S0000G06400	NAC domain-containing protein (VvNAC05)	0.85	VIT_15S0024G00040	LHCA3 (Photosystem I light harvesting complex gene 3)	-0.75
VIT_01S0011G04370	Phosphatidylserine synthase 2	0.85	VIT_00S0256G00110	Ankyrin repeat protein	-0.75
VIT_07S0031G00220	ERF/AP2 Gene Family (VvAP2-13)	0.85	VIT_00S0411G00020	Ankyrin repeat protein	-0.75
VIT_11S0016G03000	Phosphoinositide 3-kinase regulatory subunit 4	0.85	VIT_15S0046G03670	NADP-dependent malic enzyme	-0.75
VIT_05S0051G00640	purple acid phosphatase 23- ATPAP23/PAP23	0.85	VIT_17S0000G06350	LHCA4 (Photosystem I light harvesting complex gene 4)	-0.75
VIT_18S0001G01640	UDP-sugar pyrophospharylase	0.85	VIT_00S0125G00280	Photosystem I reaction center subunit III (PSAF)	-0.74
VIT_14S0030G01600	DNA-binding storekeeper protein	0.85	VIT_18S0001G02740	photosystem II 22 kDa protein PSBS	-0.74
VIT_08S0040G00770	cysteine protease inhibitor	0.84	VIT_18S0001G00470	monocopper oxidase SKS5 (SKU5 Similar 5)	-0.74
VIT_01S0127G00680	SRO2 (SIMILAR TO RCD ONE 2)	0.84	VIT_02S0012G00570	pseudo-response regulator 2 (APRR2) (TOC2)	-0.74
VIT_02S0025G00030	zinc finger (C3HC4-type RING finger)	0.84	VIT_18S0041G01040	UDP-glycosyltransferase 88A4	-0.74
VIT_04S0023G03120	Histone H3	0.84	VIT_18S0001G09040	LIM domain protein WLIM1	-0.73

Table 7: *VviNAC18* positive (left) and negative (right) co-expressed top 20 genes.

VviNAC26 analysis showed a GO enrichment for the regulation of biosynthetic and metabolic processes categories, but also for photosynthesis. *VviNAC26* co-expressed genes list presented five interesting elements: *VviNAC17* (VIT_19s0014g03290), *ERF/AP2* (VIT_18s0001g10150), *VviMYB15* (VIT_05s0049g01020) and *ETHYLENE-RESPONSIVE DEAD BOX RNA HELICASE RH30* (VIT_17s0000g09120) as positively correlated and *LEAF SENESCENCE PROTEIN* (VIT_14s0083g00400) and *PURINE PERMEASE 1 PUP1* (VIT_18s0001g06950) as negatively correlated (**Table 8**).

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_04S0023G02480	Dehydrin 1b	0.77	VIT_01S0026G02070	Peptidyl-prolyl cis-trans isomerase cyclophilin-type	-0.65
VIT_19S0014G03290	NAC domain-containing protein (VvNAC17)	0.77	VIT_09S0002G01550	lecithine cholesterol acyltransferase	-0.60
VIT_18S0001G10150	ERF/AP2 Gene Family (VvERF006)	0.70	VIT_15S0046G03520	phosphomannomutase	-0.60
VIT_00S0762G00010	S-locus lectin protein kinase family	0.70	VIT_05S0077G01060	Aldehyde Dehydrogenase (VvALDH22A1)	-0.59
VIT_18S0001G11130	calmodulin-binding protein AR781	0.69	VIT_11S0078G00430	L-fucokinase	-0.59
VIT_05S0049G01020	VvMYB15	0.68	VIT_06S0004G05940	Uncoupling protein 1	-0.58
VIT_16S0039G01060	CYP89A2	0.67	VIT_14S0060G00460	Receptor-like protein kinase	-0.58
VIT_05S0051G00140	RRS1 (resistant to ralstonia solanacearum 1)	0.67	VIT_05S0020G02890	GPI-anchored protein-like protein II	-0.57
VIT_00S0347G00060	S-locus lectin protein kinase	0.67	VIT_02S0025G01330	Polygalacturonase GH28	-0.57
VIT_06S0004G05660	glycosyl transferase family 1 protein	0.66	VIT_05S0049G01860	receptor-like protein kinase	-0.57
VIT_18S0001G06950	purine permease 1 (PUP1)	0.66	VIT_11S0037G00930	GDP-L-fucose synthase.	-0.57
VIT_00S0743G00020	S-locus lectin protein kinase	0.66	VIT_06S0080G00250	sialyltransferase	-0.56
VIT_19S0027G01630	disease resistance protein (NBS-LRR class)	0.66	VIT_06S0004G04610	fasciclin arabinogalactan-protein (FLA4)	-0.56
VIT_04S0008G02980	Lupeol synthase	0.66	VIT_03S0038G02520	aquaporin PIP3	-0.56
VIT_04S0023G03630	WD repeat domain 60	0.66	VIT_12S0057G01020	fasciclin-like arabinogalactan protein FLA2	-0.56
VIT_17S0000G09120	ethylene-responsive DEAD box RNA helicase (RH30)	0.66	VIT_14S0030G02180	GDP-mannose 3,5-epimerase 1	-0.55
VIT_05S0051G00230	R protein L6	0.65	VIT_06S0004G01540	Oxidoreductase	-0.55
VIT_08S0007G07730	CYP93A1 2-hydroxyisoflavanone synthase	0.65	VIT_14S0083G00400	Leaf senescence protein	-0.55
VIT_02S0025G03510	bromo-adjacenty (BAH) domain-containing protein	0.65	VIT_19S0090G00020	TOR1 (TORTIFOLIA 1)	-0.55
VIT_02S0025G03290	hydroxyproline-rich glycoprotein	0.64	VIT_09S0002G01710	Zeta-cop, subunit of COP-I complex	-0.54

Table 8: *VviNAC26* positive (left) and negative (right) co-expressed top 20 genes.

VviNAC38 co-expressed genes revealed a GO enrichment for the catabolic processes, protein, macromolecule and organic compounds transport and protein modification categories. Among the positively correlated genes with *VviNAC38* a *ABC TRANSPORTER F MEMBER 6* (VIT_18s0076g00380) was identified, while the *COPPER-TRANSPORTING ATPASE PAA1* (VIT_12s0142g00420) a *BZIP*

TRANSCRIPTION FACTOR (VIT_13s0067g02900), the *LHCB2.1* (VIT_12s0057g00630), the *LHCA4* (VIT_17s0000g06350) and the *PHOTOSYSTEM I REACTION CENTER (PSAF)* resulted negatively correlated with *VviNAC38* (**Table 9**).

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_08S0217G00030	Ran-binding protein 1 RanBP1	0.84	VIT_08S0007G06450	omega-6 fatty acid desaturase, endoplasmic reticulum (FAD2)	-0.70
VIT_02S0025G02760	Ubiquitin thiolesterase 4	0.84	VIT_01S0244G00140	aspartate kinase	-0.67
VIT_13S0064G01090	Zinc finger (CCCH-type) family protein	0.83	VIT_12S0035G00970	evolutionarily conserved C-terminal region 11 ECT11	-0.67
VIT_04S0008G05770	CBL-interacting protein kinase 25 (CIPK25)	0.83	VIT_05S0020G00870	UbiE/COQ5 methyltransferase	-0.66
VIT_16S0098G00890	harpin-induced protein	0.83	VIT_13S0019G05290	acyl-CoA binding	-0.64
VIT_08S0040G00770	cysteine protease inhibitor	0.82	VIT_19S0014G01470	Thioredoxin TTL1 (Tetratricopetide-repeat thioredoxin-like 1)	-0.64
VIT_14S0030G01600	DNA-binding storekeeper protein	0.82	VIT_12S0142G00420	Copper-transporting ATPase PAA1	-0.64
VIT_06S0004G03440	Ulp1 protease	0.82	VIT_18S0001G08250	tubulin alpha-3 chain	-0.64
VIT_18S0076G00380	ABC transporter F member 6	0.82	VIT_03S0038G02790	Ribosomal protein L2, Chloroplast	-0.63
VIT_18S0001G13790	Cytochrome P450, family 83, subfamily B, polypeptide 1	0.82	VIT_04S0008G03610	Xylan synthase	-0.63
VIT_06S0080G00800	Vesicle-associated membrane protein 714	0.81	VIT_13S0067G02900	bZIP transcription factor	-0.63
VIT_11S0016G05600	Zinc finger (ubiquitin-hydrolase) domain-containing	0.81	VIT_04S0044G01670	ankyrin	-0.63
VIT_01S0026G00850	Zinc finger protein 5	0.81	VIT_13S0019G03210	Lectin-like protein	-0.62
VIT_07S0255G00100	Extra-large G-protein (XLG1)	0.81	VIT_12S0057G00630	LHCB2.1 (Photosystem II light harvesting complex gene 2.1)	-0.62
VIT_10S0092G00120	gamma-soluble NSF attachment protein (GSNAP)	0.81	VIT_17S0000G06350	LHCA4 (Photosystem I light harvesting complex gene 4)	-0.62
VIT_14S0068G00840	RBR1 (retinoblastoma-related 1)	0.81	VIT_07S0104G01150	RNA-binding protein	-0.61
VIT_09S0002G02070	Protein kinase PKN/PRK1	0.81	VIT_11S0078G00310	isoamylase-type starch-debranching enzyme 1	-0.61
VIT_01S0011G04370	Phosphatidylserine synthase 2	0.80	VIT_00S0125G00280	Photosystem I reaction center subunit III (PSAF)	-0.61
VIT_14S0083G00740	Amino acid permease	0.80	VIT_06S0004G00040	Pur alpha-1 (purin-rich alpha 1)	-0.61
VIT_12S0035G01090	Regulator of nonsense transcripts 3 UPF3	0.80	VIT_10S0003G02590	SH3 domain-containing protein 3 (SH3P3)	-0.61

Table 9: *VviNAC38* positive (left) and negative (right) co-expressed top 20 genes.

VviNAC39 genes list showed high enrichment in the regulation of the biosynthetic and metabolic processes. *VviNAC39* co-expressed genes list presented eight interesting elements: four *VviWRKYs* (VIT_10s0116g01200, VIT_08s0058g00690, VIT_10s0003g01600 and VIT_06s0004g07500), two *ETHYLENE RESPONSIVE ELEMENTS* (VIT_02s0234g00130 and VIT_18s0072g00260) and *UDP-GLUCOSE: FLAVONOID 7-O-GLUCOSYLTRANSFERASE* (VIT_05s0062g00270) as positively correlated and *AUXIN-INDEPENDENT GROWTH PROMOTER* (VIT_14s0083g00940) as inverse correlated (**Table 10**).

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_10S0116G01200	WRKY transcription factor (VvWRKY29)	0.77	VIT_06S0004G06610	peptidyl-prolyl cis-trans isomerase ROC7 (ROTAMASE CYP 7)	-0.54
VIT_02S0234G00130	Ethylene responsive element binding factor 1	0.74	VIT_08S0007G03490	Mitochondrial Acyl carrier protein 2	-0.54
VIT_19S0015G00130	serine acetyltransferase 3	0.74	VIT_04S0023G00840	Mitochondrial Acyl carrier protein 2	-0.54
VIT_06S0004G04960	scarecrow-like transcription factor 14 SCL14	0.72	VIT_14S0083G00490	Phosphoglycerate mutase	-0.53
VIT_17S0000G03340	Wall-associated kinase 4	0.72	VIT_01S0010G03690	Splicing factor YT521-B	-0.53
VIT_09S0002G03540	MYB DIVARICATA	0.71	VIT_14S0083G00940	auxin-independent growth promoter	-0.53
VIT_00S0370G00010	LRR receptor-like serine/threonine-protein kinase	0.69	VIT_17S0000G08540	lipase GDSL	-0.53
VIT_08S0058G00690	WRKY transcription factor (VvWRKY24)	0.69	VIT_00S0357G00070	beta-hydroxyacyl-ACP dehydratase	-0.52
VIT_06S0004G06710	Cys-3-His zinc finger protein	0.68	VIT_18S0001G06830	Methyltransferase type 11	-0.52
VIT_00S2376G00010	NADH dehydrogenase, putative	0.68	VIT_13S0067G03650	histone H4	-0.51
VIT_09S0002G03610	MYB DIVARICATA	0.67	VIT_07S0151G00140	NADH dehydrogenase (ubiquinone) Fe-S protein 4	-0.51
VIT_10S0003G01600	WRKY transcription factor (VvWRKY30)	0.67	VIT_06S0004G05470	sterol 4-alpha-methyl-oxidase 2 (SMO2)	-0.51
VIT_16S0039G01060	CYP89A2	0.67	VIT_04S0023G00760	biotin carboxyl carrier protein 2 (BCCP2)	-0.50
VIT_10S0003G05450	reticuline oxidase precursor	0.66	VIT_08S0007G03940	histone deacetylase HD2A	-0.50
VIT_05S0062G00270	UDP-glucose:flavonoid 7-O-glucosyltransferase	0.66	VIT_04S0023G02390	Thioredoxin domain-containing protein 9	-0.50
VIT_06S0004G07500	WRKY transcription factor (VvWRKY16)	0.66	VIT_08S0040G00970	plectin (myosin-like)	-0.50
VIT_19S0014G04940	Chitin-inducible gibberellin-responsive protein 1	0.65	VIT_15S0021G00570	acyl carrier protein, mitochondrial	-0.50
VIT_00S0231G00040	RPM1-interacting protein 4	0.65	VIT_17S0000G08840	trans-2-enoyl-CoA reductase	-0.49
VIT_18S0072G00260	Ethylene-responsive transcription factor RELATED TO APETALA2 6	0.65	VIT_00S0357G00020	beta-hydroxyacyl-ACP dehydratase	-0.49
VIT_00S2648G00010	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	0.65	VIT_06S0004G00250	PUMILIO 12 (APUM12)	-0.49

Table 10: *VviNAC39* positive (left) and negative (right) co-expressed top 20 genes.

VviNAC61 co-expressed genes were highly enriched for autophagy, inorganic and organic molecules transport, protein modification and transport, catabolic processes and photosynthesis categories. *VviNAC61* co-expressed genes list presented ten interesting elements: *LATERAL ORGAN BOUNDARIES DOMAIN* 15 (VIT_06s0004g07790) and *MYB DOMAIN PROTEIN 3R2* (VIT_15s0048g02120), as positively correlated, and *LHCB2.1 PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.1* (VIT_12s0057g00630), *LHCA4 PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE 4* (VIT_17s0000g06350), *PHOTOSYSTEM I REACTION CENTER SUBUNIT III PSAF* (VIT_00s0125g00280), *2-CYS PEROXIREDOXIN BAS1, CHLOROPLAST PRECURSOR* (VIT_08s0007g02490), *PECTINACETYLESTERASE* (VIT_05s0020g01110), *FRUCTOSAMINE KINASE* (VIT_15s0048g01810) and *FRUCTOKINASE-2* (VIT_14s0006g01410) and *MAGNESIUM-PROTOPORPHYRIN IX MONOMETHYL ESTER [OXIDATIVE] CYCLASE* (VIT_08s0040g00390), as inverse correlated (**Table 11**).

Gene ID	Functional annotation	Pearson	Gene ID	Functional annotation	Pearson
VIT_06S0004G07790	lateral organ boundaries DOMAIN 15	0.89	VIT_12S0057G00630	LHCB2.1 (Photosystem II light harvesting complex gene 2.1)	-0.73
VIT_14S0108G00890	beta-1,3-galactosyltransferase	0.88	VIT_11S0016G04850	carboxylic ester hydrolase	-0.73
VIT_04S0023G01080	Lys Motif-Type Receptor-Like Kinase LYK4	0.88	VIT_07S0141G00240	NADH dehydrogenase (ubiquinone) flavoprotein 2	-0.72
VIT_14S0128G00180	zinc finger (C3HC4-type RING finger)	0.88	VIT_06S0004G05940	Uncoupling protein 1	-0.72
VIT_12S0028G03580	lectin-receptor like protein kinase 3	0.87	VIT_15S0048G01810	fructosamine kinase	-0.71
VIT_08S0007G05250	cig3	0.87	VIT_01S0244G00140	aspartate kinase	-0.71
VIT_11S0016G03780	CONTINUOUS VASCULAR RING (COV1)	0.86	VIT_17S0000G00290	alpha-mannosidase precursor lysosomal	-0.71
VIT_13S0019G02440	Mitochondrial substrate carrier family protein	0.86	VIT_05S0020G01110	pectinacetylesterase	-0.71
VIT_12S0059G01100	PRLI-interacting factor K	0.86	VIT_17S0000G06350	LHCA4 (Photosystem I light harvesting complex gene 4)	-0.70
VIT_08S0007G01150	Unc51-like kinase	0.86	VIT_14S0108G01420	DEFENSE NO death 1	-0.70
VIT_14S0083G00930	BIM2 (BES1-interacting Myc-like protein 2)	0.85	VIT_14S0006G01410	fructokinase-2	-0.70
VIT_14S0068G01360	GEM-like protein 5	0.85	VIT_08S0040G00390	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase	-0.70
VIT_18S0072G01010	Peptide chain release factor eRF subunit 1	0.85	VIT_18S0001G08250	tubulin alpha-3 chain	-0.70
VIT_14S0006G02240	2-hydroxyacyl-CoA lyase 1	0.84	VIT_12S0035G00910	bile acid sodium symporter	-0.70
VIT_14S0068G02150	kelch repeat-containing F-box family protein	0.84	VIT_11S0016G05840	protease inhibitor/seed storage/lipid transfer protein (LTP)	-0.70
VIT_15S0048G01160	phospholipase D beta 1	0.84	VIT_18S0001G00810	24-methylenesterol C-methyltransferase 2	-0.70
VIT_05S0062G01190	pirin	0.84	VIT_12S0035G00270	ferredoxin, chloroplast (PETF)	-0.70
VIT_01S0127G00680	SRO2 (SIMILAR TO RCD ONE 2)	0.84	VIT_00S0125G00280	Photosystem I reaction center subunit III (PSAF)	-0.70
VIT_11S0118G00360	leucoanthocyanidin dioxygenase	0.84	VIT_08S0007G02490	2-cys peroxiredoxin BAS1, chloroplast precursor	-0.69
VIT_15S0048G02120	myb domain protein 3R2	0.84	VIT_06S0004G05280	tropinone reductase	-0.69

Table 11: *VviNAC61* positive (left) and negative (right) co-expressed top 20 genes.

Selected *VviNACs* isolation

Starting from *V. vinifera* cv Shiraz fruiting cuttings, all the selected *VviNACs* coding sequences were isolated from cDNA obtained from berry pericarp tissue RNA. Afterwards, the isolated sequences were directionally cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) and sequenced.

VviNAC01, VviNAC08, VviNAC15, VviNAC17, VviNAC18, VviNAC26, VviNAC38, VviNAC39 and *VviNAC61* contain a 756, 900, 1719, 1002, 1062, 849, 1458, 885

and 1212 bp open reading frame encoding a protein of 251, 299, 572, 333, 353, 282, 517, 294 and 403 amino acidic residues with a predicted mass of 28.66, 34.09, 63.40, 37.19, 38.92, 32.46, 57.10, 33.47 and 45.44 kDa and a calculated pI = 9.20, 6.66, 4.73, 8.66, 8.55, 7.70, 5.76, 7.06 and 6.52, respectively.

The sequenced genes taken from cv Shiraz were blasted against the cv Pinot Noir reference genome and the analyses revealed that the predicted *VviNACs* gene sequences were practically identical to the one of the reference genome (**Appendix B**). The nucleotide sequences were translated into aminoacids sequences to see if the SNPs found in the different cultivar genes could bring to deleterious mutations. *Vvi*NAC01 reports only one change, from a G (glycine, non-polar) to a D (aspartic acid, negative polar) at the residue 207 (**Fig. 22A**).

*Vvi*NAC15 reports two changes, one from a G (glycine, non-polar) to a D (aspartic acid, negatively polar) at the residue 364 and the other from a I (isoleucine, non-polar) to a M (methionine, non-polar) at the residue 369 (**Fig. 22C**).

*Vvi*NAC38 reports a frameshift nucleotides insertion/duplication of 96 bp that does not affect the protein translation but just adds 32 aminoacids to the final protein (**Fig. 22G**).

*Vvi*NAC39 reports five changes: from a K (lysine, positively polar) to a E (glutamic acid, negatively polar), from a P (proline, non-polar) to a L (leucine, non-polar), from a Q (glutamine, uncharged polar) to a H (histidine, positively polar), from a N (asparagine, uncharged polar) to a D (aspartic acid, negatively polar) and from a V (valine, non-polar) to a A (alanine, non-polar) at the residues 74, 76, 171, 192 and 194 respectively (**Fig. 22H**).

A

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VviNAC01_Shiraz        MEKLNFVKNGVLRLPPGFRFHPTDEELVVQYLKRKAYSCPLPASIIPEVDVCKADPWDLP  60
VviNAC01_Reference     MEKLNFVKNGVLRLPPGFRFHPTDEELVVQYLKRKAYSCPLPASIIPEVDVCKADPWDLP  60
                       ************************************************************

VviNAC01_Shiraz        GDLEQERYFFSTREAKYPNGNRSNRATVSGYWKATGIDKQIVASKGNQVVGMKKTLVFYR  120
VviNAC01_Reference     GDLEQERYFFSTREAKYPNGNRSNRATVSGYWKATGIDKQIVASKGNQVVGMKKTLVFYR  120
                       ************************************************************

VviNAC01_Shiraz        GKPPHGSRTDWIMHEYRLVGAETTPQRKSSTTQSSMAQAENWVLCRIFLKKRGTKNDEEM  180
VviNAC01_Reference     GKPPHGSRTDWIMHEYRLVGAETTPQRKSSTTQSSMAQAENWVLCRIFLKKRGTKNDEEM  180
                       ************************************************************

VviNAC01_Shiraz        MQTNNENRVVQKLRSSRPVFYDFLTRDRDDTNLASSNSSGSSGITEVSNTESEEHEESSS  240
VviNAC01_Reference     MQTNNENRVVQKLRSSRPVFYDFLTRGRDDTNLASSNSSGSSGITEVSNTESEEHEESSS  240
                       *************************.**********************************

VviNAC01_Shiraz        CNSFSPFRRKP        251
VviNAC01_Reference     CNSFSPFRRKP        251
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B

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VviNAC08_Shiraz       MTAELQLPPGFRFHPTDEELVMHYLCRKCASQSIAVPIIAEIDLYKFDPWQLPEMALYGE  60
VviNAC08_Reference    MTAELQLPPGFRFHPTDEELVMHYLCRKCASQSIAVPIIAEIDLYKFDPWQLPEMALYGE  60
                      ************************************************************

VviNAC08_Shiraz       KEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGHPKPVGIKKALVFYAGKAPRGE  120
VviNAC08_Reference    KEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGHPKPVGIKKALVFYAGKAPRGE  120
                      ************************************************************

VviNAC08_Shiraz       KTNWIMHEYRLADVDRSARKKNNSLRLDDWVLCRIYNKKGIVEKQHTAARKSDCSDVEDQ  180
VviNAC08_Reference    KTNWIMHEYRLADVDRSARKKNNSLRLDDWVLCRIYNKKGIVEKQHTAARKSDCSDVEDQ  180
                      ************************************************************

VviNAC08_Shiraz       KPGPLALSRKVGAMPPPPPPSSSTAPTATAALDDLVYFDSSDSVPRLHTDSSCSEHVVSP  240
VviNAC08_Reference    KPGPLALSRKVGAMPPPPPPSSSTAPTATAALDDLVYFDSSDSVPRLHTDSSCSEHVVSP  240
                      ************************************************************

VviNAC08_Shiraz       EFTCEREVQSEPKWKEWENPMDFSYNYMDATVDNAFLSQFPNNQMSPLQDMFMYLQKPF   299
VviNAC08_Reference    EFTCEREVQSEPKWKEWENPMDFSYNYMDATVDNAFLSQFPNNQMSPLQDMFMYLQKPF   299
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C

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VviNAC15_Shiraz       MKVTVGDSSPCFDGDEKSAWPPGFRFHPTDEELVLYYLKKKICRRRLKLDIIAEVDVYKW  60
VviNAC15_Reference    MKVTVGDSSPCFDGDEKSAWPPGFRFHPTDEELVLYYLKKKICRRRLKLDIIAEVDVYKW  60
                      ************************************************************

VviNAC15_Shiraz       DPEDLPGLSKLKTGDRQWFFFSPRDRKYPNGARSNRATRHGYWKATGKDRTISCNIRSVG  120
VviNAC15_Reference    DPEDLPGLSKLKTGDRQWFFFSPRDRKYPNGARSNRATRHGYWKATGKDRTISCNIRSVG  120
                      ************************************************************

VviNAC15_Shiraz       VKKTLVFYKGRAPSKERTDWVMHEYTMDEEELKRCPNVQDYYALYKVFKKSGPGPKNGEQ  180
VviNAC15_Reference    VKKTLVFYKGRAPSKERTDWVMHEYTMDEEELKRCPNVQDYYALYKVFKKSGPGPKNGEQ  180
                      ************************************************************

VviNAC15_Shiraz       YGAPFKEEEWADEDDLDVSNYSVEETPPEQLNGVISVNNSKPNGQDCQADAWDDIWKGLA  240
VviNAC15_Reference    YGAPFKEEEWADEDDLDVSNYSVEETPPEQLNGVISVNNSKPNGQDCQADAWDDIWKGLA  240
                      ************************************************************

VviNAC15_Shiraz       EAPPVVPLRVDDYVNLLAQVIGEEEAQTPLVDSSLNGAFVADPISTVLTPTSQQYAVPEN  300
VviNAC15_Reference    EAPPVVPLRVDDYVNLLAQVIGEEEAQTPLVDSSLNGAFVADPISTVLTPTSQQYAVPEN  300
                      ************************************************************

VviNAC15_Shiraz       VEFTQSASSQLQLHEAPEVTSAPNISEQERGLSEEDFLEMDDLLGPEPIPQNYEKTEENL  360
VviNAC15_Reference    VEFTQSASSQLQLHEAPEVTSAPNISEQERGLSEEDFLEMDDLLGPEPIPQNYEKTEENL  360
                      ************************************************************

VviNAC15_Shiraz       QFEIDGLSMLDLYHDAAMFLRDIGPIDQGTVPHPYLNTIENEMVNQLNYQLQPHSVGADQ  420
VviNAC15_Reference    QFEGDGLSILDLYHDAAMFLRDIGPIDQGTVPHPYLNTIENEMVNQLNYQLQPHSVGADQ  420
                      ***.****:******************************************************

VviNAC15_Shiraz       ISGQLWTLDQSVCTSAESIQGIIGQPTSGVVYASSSTNVPTEGNQNMNGEGGNGAGNRFT  480
VviNAC15_Reference    ISGQLWTLDQSVCTSAESIQGIIGQPTSGVVYASSSTNVPTEGNQNMNGEGGNGAGNRFT  480
                      ************************************************************

VviNAC15_Shiraz       SALWSFVESIPTTPASASENALVNRALVRMSSFSRMRMNALNTNAGNGGAATWKGGINKG  540
VviNAC15_Reference    SALWSFVESIPTTPASASENALVNRALVRMSSFSRMRMNALNTNAGNGGAATWKGGINKG  540
                      ************************************************************

VviNAC15_Shiraz       GFIILSVIGALIAIFWVLMLGPVKMLGRCLPS    572
VviNAC15_Reference    GFIILSVIGALIAIFWVLMLGPVKMLGRCLPS    572
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D

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VviNAC17_Shiraz       MGVPETDPLSQLSLPPGFRFYPTDEELLVQYLCRKVAGQGFSLEIIGEIDLYKFDPWVLP  60
VviNAC17_Reference    MGVPETDPLSQLSLPPGFRFYPTDEELLVQYLCRKVAGQGFSLEIIGEIDLYKFDPWVLP  60
                      ************************************************************

VviNAC17_Shiraz       SKAIFGEKEWYFFSPRDRKYPNGSRPNRVAGSGYWKATGTDKVITTEGRKVGIKKALVFY  120
VviNAC17_Reference    SKAIFGEKEWYFFSPRDRKYPNGSRPNRVAGSGYWKATGTDKVITTEGRKVGIKKALVFY  120
                      ************************************************************

VviNAC17_Shiraz       VGKAPKGTKTNWIMHEYRLLENSRKNGSSKLDDWVLCRIYKKNSNSSKPIAAVLPSKAHS  180
VviNAC17_Reference    VGKAPKGTKTNWIMHEYRLLENSRKNGSSKLDDWVLCRIYKKNSNSSKPIAAVLPSKAHS  180
                      ************************************************************

VviNAC17_Shiraz       NGSSSSSSSHLDDVLESLPEIDDRFFSPNRMNSLRVSQPDEKVNFHNLGSGNFDWATLAG  240
VviNAC17_Reference    NGSSSSSSSHLDDVLESLPEIDDRFFSPNRMNSLRVSQPDEKVNFHNLGSGNFDWATLAG  240
                      ************************************************************

VviNAC17_Shiraz       VSSLQELVSGVQSHAQPPAAVNNSNEMYVPSLPPLIQAEEEVQSGLRTQRVDPVMNQGFF  300
VviNAC17_Reference    VSSLQELVSGVQSHAQPPAAVNNSNEMYVPSLPPLIQAEEEVQSGLRTQRVDPVMNQGFF  300
                      ************************************************************

VviNAC17_Shiraz       PQNSNAFSQSFSNSLDPFGFRYPTQPSGFGYRQ   333
VviNAC17_Reference    PQNSNAFSQSFSNSLDPFGFRYPTQPSGFGYRQ   333
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E

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VviNAC18_Shiraz       MESTDSSSGSPQPQLPPGFRFHPTDEELVVHYLKKKASSAPLPVAIIAEVDLYKFDPWEL  60
VviNAC18_Reference    MESTDSSSGSPQPQLPPGFRFHPTDEELVVHYLKKKASSAPLPVAIIAEVDLYKFDPWEL  60
                      ************************************************************

VviNAC18_Shiraz       PAKASFGEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVLTSGGTQKVGVKKAL  120
VviNAC18_Reference    PAKASFGEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVLTSGGTQKVGVKKAL  120
                      ************************************************************

VviNAC18_Shiraz       VFYGGKPPKGIKTNWIMHEYRLADNKVNTKPPGCDMGNKKNSLRLDDWVLCRIYKKNNTH  180
VviNAC18_Reference    VFYGGKPPKGIKTNWIMHEYRLADNKVNTKPPGCDMGNKKNSLRLDDWVLCRIYKKNNTH  180
                      ************************************************************

VviNAC18_Shiraz       RTLDPDKDDSMDDMLGPVPSSISMGQQSLKLQFPKVPNYSALLENEQSLFEGMINSDGIN  240
VviNAC18_Reference    RTLDPDKDDSMDDMLGPVPTSISMGQQSLKLQFPKVPNYSALLENEQSLFEGMINSDGIN  240
                      ******************:*****************************************

VviNAC18_Shiraz       SSGTISQLACSSSKPDHLSLVAATTSSILPLKRTLPSLYWNDDDTAGPSTTKRFQAENTD  300
VviNAC18_Reference    SSGTISQLACSSSKPDHLSLVAATTSSILPLKRTLPSLYWNDDDTAGPSTTKRFQAENTD  300
                      ************************************************************

VviNAC18_Shiraz       GNIGRTTDGNNSIATLLSQLPQTPSLHQQSMLGSLGEGVFRQPFQLPGMNWYA 353
VviNAC18_Reference    GNIGRTTDGNNSIATLLSQLPQTPSLHQQSMLGSLGEGVFRQPFQLPGMNWYA 353
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F

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VviNAC26_Shiraz       MDGKGSSQLPPGFRFHPTDEELIMYYLKNQATSKPCPVSIIPEVDIYKFEPWELPEKAEF  60
VviNAC26_Reference    MDGKGSSQLPPGFRFHPTDEELIMYYLKNQATSKPCPVSIIPEVDIYKFEPWELPEKAEF  60
                      ************************************************************

VviNAC26_Shiraz       GENEWYFFSPRDRKYPNGARPNRATVSGYWKATGTDKAIYSGAKYVGVKKALVFYKGRPP  120
VviNAC26_Reference    GENEWYFFSPRDRKYPNGARPNRATVSGYWKATGTDKAIYSGAKYVGVKKALVFYKGRPP  120
                      ************************************************************

VviNAC26_Shiraz       KGIKTDWIMHEYRLSDSRPRPKKHNGSMRLDDWVLCRIYKKKHVGRILEEKEENLGPQIP  180
VviNAC26_Reference    KGIKTDWIMHEYRLSDSRPRPKKHNGSMRLDDWVLCRIYKKKHVGRILEEKEENLGPQIP  180
                      ************************************************************

VviNAC26_Shiraz       VTNSDDGGEQHQVKFPRTFSLAHLLDMEYLGPISQLLGDNSYHSAFDFQGTISNIAGTDP  240
VviNAC26_Reference    VTNSDDGGEQHQVKFPRTFSLAHLLDMEYLGPISQLLGDNSYHSAFDFQGTISNIAGTDP  240
                      ************************************************************

VviNAC26_Shiraz       PGVDKFELFQLPCQYNDSTKFQVNQNHIPNQPLFVNPVYEFQ   282
VviNAC26_Reference    PGVDKFELFQLPCQYNDSTKFQVNQNHIPNQPLFVNPVYEFQ   282
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G

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VviNAC38_Shiraz       MMGKGSKSNCKSASHKMFKDKAKNRVDDLQGMFTDLQSARKESRSVDVAVLEEQLHQMLR  60
VviNAC38_Reference    MMGKGSKSNCKSASHKMFKDKAKNRVDDLQGMFTDLQSARKESRSVDVAVLEEQLHQMLR  60
                      ************************************************************

VviNAC38_Shiraz       EWKAELNEPSPASSLQGGSLGSFSSDICRLLQLCEEEDDATSALADGAVPKPEPDAQGHQ  120
VviNAC38_Reference    EWKAELNEPSPASSLQGGSLGSFSSDICRLLQLCEEEDDATSALADGAVPKPEPDAQGHQ  120
                      ************************************************************

VviNAC38_Shiraz       IGASVLFQERYNKGPQEHGFQLVDQCKISPSGAHNMGVHNLEGATQLDYRQFDLQQDFEQ  180
VviNAC38_Reference    IGASVLFQERYNKGPQEHGFQLVDQCKISPSGAHNMGVHNLEGATQLDYRQFDLQQDFEQ  180
                      ************************************************************

VviNAC38_Shiraz       NFFAGYDGTGLCGEDAMPHISSFLPSICLPPSAFLGPKCALWDCPRPAQGMDWCQNYCSS  240
VviNAC38_Reference    NFFAGYDGTGLCGEDAMPHISSFLPSICLPPSAFLGPKCALWDCPRPAQGMDWCQNYCSS  240
                      ************************************************************

VviNAC38_Shiraz       FHATLALSEGPPGMTPVLRPGGIGLKDGLLFAALSAKVQGKDVGIPECEGAATAKSPWNA  300
VviNAC38_Reference    FHATLALSEGPPGMTPVLRPGGIGLKDGLLFAALSAKVQGKDVGIPECEGAATAKSPWNA  300
                      ************************************************************

VviNAC38_Shiraz       PELFDLSVLEGETIREWLFFDKPRRAFESGNRKQRSLPDYSGRGWHESRKQVMNEYGGLK  360
VviNAC38_Reference    PELFDLSVLEGETIREWLFFDKPRRAFESGNRKQRSLPDYSGRGWHESRKQVMNEYGGLK  360
                      ************************************************************

VviNAC38_Shiraz       RSYYMDPQPLNHFEWHLYEYEISKCDACALYRLELKLVDGKKSSKAKATTDSVADLQKQM  420
VviNAC38_Reference    RSYYMDPQPLNHFEWHLYEYEISKCDACALYRLELKLVDGKKSSKAKATTDSV-------  413
                      ************************************************

VviNAC38_Shiraz       GRLTAEFPFTMMGKGSKAKATTDSVADLQKQMGRLTAEFPLDNKRSVKGRTKINMKDGVG  480
VviNAC38_Reference    -------------------------ADLQKQMGRLTAEFPLDNKRSVKGRTKINMKDGVG  448
                                               **********************************

VviNAC38_Shiraz       DVYSTPNRVGPPNQQGDYGVGGPYDYLVENLGDYYLT        517
VviNAC38_Reference    DVYSTPNRVGPPNQQGDYGVGGPYDYLVENLGDYYLT        485
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Figure 22: Alignments of (A) *VviNAC01*, (B) *VviNAC08*, (C) *VviNAC15*, (D) *VviNAC17*, (E) *VviNAC18*, (F) *VviNAC26*, (G) *VviNAC38*, (H) *VviNAC39* and (I) *VviNAC61* aminoacids sequences from Shiraz and Pinot Noir cultivars.

Transient over expression of the selected *VviNACs* in grapevine

To elucidate the functions of *VviNAC01*, *VviNAC08*, *VviNAC15*, *VviNAC17*, *VviNAC18*, *VviNAC26*, *VviNAC38*, *VviNAC39* and *VviNAC61*, a transient over expression of the nine selected *VviNACs* was performed directly in the native species (*V. vinifera* cv. Sultana), avoiding possible problems that may occur with gene expression in heterologous systems. In this way an overview on the putative primary effects of these TFs on grapevine leaf transcriptome was obtain. For each *Vvi*NAC factor, by vacuum *Agrobacterium*-mediated infection six young *in vitro* grown grapevine plantlets were transfected. Other six plantlets were transformed with the same vector containing a non-coding sequence (control). Agroinfiltrated plants were screened for their over expression through qPCR Real Time analyses

and the three plantlets with the highest transcript amount of transgene, in comparison with their respective *VviNAC* expression level in the three control lines, were selected (**Fig.23**).

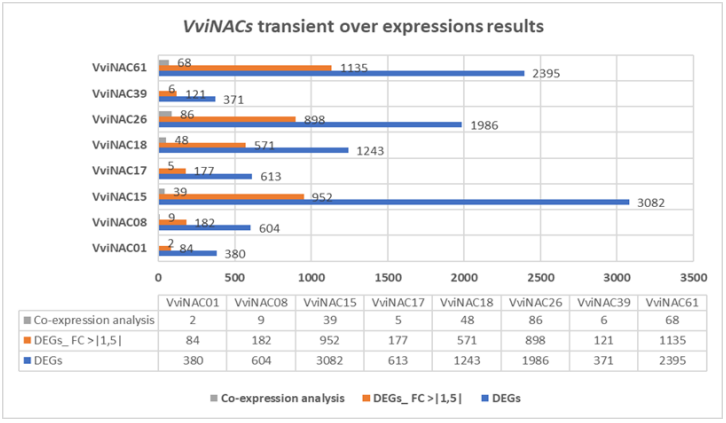
Figure 23: qPCR analysis of *VviNACs* expression level in transiently over expressing leaves and control. Each expression value corresponds to the mean ± standard deviation (SD) of three technical replicates relative to the *VviUBIQUITIN1* (VIT_16s0098g01190) control.

Unfortunately, *VviNAC15* over expression produced only one over expressing plantlet and concerning the *VviNAC38* one no plantlet at all was transfected. The two over expressions will be performed again in the future but, meanwhile, the only *VviNAC15* over expressing plantlet was used for the further analysis. All the *VviNACs* transiently over expressed grapevine plantlets were collected and used for microarray transcriptomic analyses.

Microarray transcriptomic analyses of the selected *VviNACs*

To gain information about the impact of the ectopic expression of the selected *VviNACs* on the leaf transcriptome and to identify the possible targets of these TFs, microarray analyses were performed on the leaf RNA of the selected lines. T-test analyses were carried out with a p correlation value of 0.05 (TMeV 4.3), comparing the over expressing *VviNACs* samples with the control lines. Considering a |fold

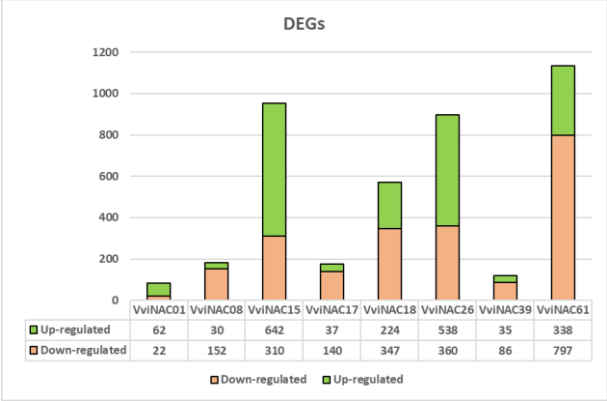
change| > 1.5 and eliminating all the 'no hit' and 'unknow' annotated genes, up and down regulated genes were identified; these filtered DEGs were annotated using V1 version of the 12X draft annotation of the grapevine genome. Comparing the DEGs lists with the co-expressed genes one, only 2, 9, 39, 5, 48 86, 6 and 68 genes were found in common for *VviNAC01*, *VviNAC08*, *VviNAC15*, *VviNAC17*, *VviNAC18*, *VviNAC26*, *VviNAC39* and *VviNAC61*, respectively (**Fig. 24**).



	VviNAC01	VviNAC08	VviNAC15	VviNAC17	VviNAC18	VviNAC26	VviNAC39	VviNAC61
Co-expression analysis	2	9	39	5	48	86	6	68
DEGs_ FC >|1,5|	84	182	952	177	571	898	121	1135
DEGs	380	604	3082	613	1243	1986	371	2395

Figure 24: Summary of the general results of the *VviNACs* transient over expression, integrated with the co-expression datasets.

In detail, in the FC>|1.5| DEGs lists were identified 62, 30, 642, 37, 224, 538, 35 and 338 up regulated genes and 22, 152, 310, 140, 347, 360, 86 and 797 down regulated genes regarding *VviNAC01*, *VviNAC08*, *VviNAC15*, *VviNAC17*, *VviNAC18*, *VviNAC26*, *VviNAC39* and *VviNAC61*, respectively (**Fig. 25**). The long DEGs lists represent a good starting point to investigate the hypothetical *VviNACs* targets and to elucidate the role of the transcription factors in a specific regulatory network.



	VviNAC01	VviNAC08	VviNAC15	VviNAC17	VviNAC18	VviNAC26	VviNAC39	VviNAC61
Up-regulated	62	30	642	37	224	538	35	338
Down-regulated	22	152	310	140	347	360	86	797

Figure 25: Summary of the number of DEGs, divided into up- and down- regulated, of the *VviNACs* transient over expression.

GO enrichment analyses, divided into two gene sub-groups (up and down regulated) were performed on the DEGs obtained by the over expression of each of the eight *VviNACs* (**Appendix C**).

VviNAC01 over expression up regulated genes were mainly enriched in regulation of biosynthetic and metabolic processes categories but were also found genes concerning the aromatic and organic compound biosynthesis and the calcium transport; among the down regulated DEGs, many genes regarding the response regulation to different type of stress and stimuli resulted enriched.

The most relevant information obtained from the *VviNAC01* over expression was related to the up regulated genes (**Table 12**). At the first place *LIPASE CLASS 3* (VIT_10s0003g04210) was found, which is part of the triacylglycerol lipase family involved in lipid degradation, esterification, and transesterification processes in plants (Nurniwalis *et al*., 2015). It is indeed well known that the cuticular wax of the grape berries, which is chemically a mixture of long-chain alcohols, esters of such alcohols and fatty acids, free fatty acids, long-chain aldehydes, and hydrocarbons (Grncarevic *et al*., 1971), is the main barrier to the evaporation of water. Analyses on the grape berry lipid profiles revealed that there is a very high number of double bonds in a fatty acid molecule in skins and juices, which suddenly affect the production of volatile compounds (Arita *et al*., 2017). For example, linoleic acid is the major components in *V. vinifera* berry and C6 and C9 aldehydes, important volatiles responsible for the 'green aroma' in berry and wine, are generated from it by oxidative cleavage (Arita *et al*., 2017).

ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 9 (VIT_12s0028g03270) was also found between the top 20 up regulated genes.

Another important family found between the up regulated genes is the one of *ERF/AP2*, which was represented by 9 different genes (VIT_16s0013g01050, VIT_16s0013g01060, VIT_16s0013g01030, VIT_16s0013g01000, VIT_16s0013g01070, VIT_16s0013g00990, VIT_16s0013g00980, VIT_16s0013g00900 and VIT_16s0013g00950). The *APETALA 2/ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (AP2/ERF)* family is a large group of plant-specific transcription factors involved in plant abiotic stress such as drought, salinity and high temperatures (Mizoi *et al*., 2012). Traditionally, the

expression of ERF genes has been associated with the molecular response to ethylene and therefore have been suggested as being involved in the ripening process in climacteric fruits; grape is a non-climacteric fruit, but ethylene still plays an important role during development and ripening processes such as anthocyanin accumulation and alcohol dehydrogenase expression (Licausi *et al*., 2010). Moreover, 149 *ERF/AP2* genes were identified in the grape genome and characterized by their expression patterns in aerial vegetative, inflorescence and berry skin and flesh at two different ripening stages, *veraison* and full ripening (Licausi *et al*., 2010).

Gene ID	Functional annotation	FC	Gene ID	Functional annotation	FC
VIT_10s0003g04210	Lipase class 3	21.62	VIT_11s0052g01570	MATE efflux family protein	-2.15
VIT_16s0013g01050	ERF/AP2 Gene Family (VvERF107)	10.38	VIT_07s0005g02030	Lipase GDSL	-2.14
VIT_16s0013g01060	ERF/AP2 Gene Family (VvERF108)	10.07	VIT_08s0007g00570	WRKY Transcription Factor (VvWRKY27)	-1.96
VIT_12s0134g00240	Avr9/Cf-9 rapidly elicited protein 20	9.97	VIT_15s0046g01700	Chitinase, class III	-1.86
VIT_06s0080g00170	Aluminium-tolerance ALMT1	8.09	VIT_14s0081g00400	Ankyrin repeat	-1.83
VIT_16s0013g01030	ERF/AP2 Gene Family (VvERF106)	7.68	VIT_13s0156g00450	R protein disease resistance protein	-1.77
VIT_03s0063g00370	Nitrite reductase	7.62	VIT_19s0014g03770	Retrotransposon	-1.75
VIT_16s0013g01000	ERF/AP2 Gene Family (VvERF084)	6.41	VIT_18s0001g15410	Alcohol dehydrogenase 1	-1.73
VIT_16s0013g01070	ERF/AP2 Gene Family (VvERF085)	6.27	VIT_18s0117g00360	R protein L6	-1.65
VIT_16s0013g00990	ERF/AP2 Gene Family (VvERF105)	6.25	VIT_08s0007g00130	Heat shock protein 70	-1.64
VIT_03s0063g01790	Transducin protein	5.81	VIT_10s0092g00380	Multidrug resistance-associated protein 3	-1.63
VIT_16s0013g00980	ERF/AP2 Gene Family (VvERF082)	5.64	VIT_09s0002g06730	Longin	-1.60
VIT_12s0028g03270	Ethylene-responsive transcription factor 9	5.48	VIT_14s0108g01270	Cyclin-dependent kinase CDC2C	-1.58
VIT_16s0013g00900	ERF/AP2 Gene Family (VvERF079)	5.27	VIT_18s0001g10850	Patatin	-1.56
VIT_16s0013g00950	ERF/AP2 Gene Family (VvERF080)	5.00	VIT_18s0001g10910	Patatin	-1.56
VIT_12s0028g03010	Glutaredoxin	4.05	VIT_13s0067g01880	Other LOB domain-containing protein ASL5	-1.56
VIT_09s0002g03220	Acid phosphatase	3.79	VIT_02s0033g00700	Nitrilase	-1.55
VIT_12s0034g00270	R protein L6	3.52	VIT_18s0001g03710	R protein L6	-1.54
VIT_15s0024g00400	R protein MLA10	3.25	VIT_14s0108g00270	Aldose 1-epimerase	-1.53
VIT_10s0116g01390	F-box family protein	3.00	VIT_17s0000g07450	Glycerophosphoryl diester phosphodiesterase	-1.52

Table 12: *VviNAC01* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

VviNAC08 GO enrichment analysis showed phosphorus metabolic processes and transport as significantly enriched gene categories for the up regulated genes and presented a high number of down regulated gene involved in the regulation of the response to salicylic acid, organic compounds and hydrogen peroxide; moreover, a very represented gene category was the cyanide metabolic/catabolic processes one, which is related to the ethylene production. Looking at the up and down regulated genes found in the *VviNAC08* over expressing plants transcriptome, an *ACID PHOSPHATASE* (VIT_09s0018g01800) and *TRANSCRIPTION FACTOR JUMONJI (JMJC) DOMAIN-CONTAINING PROTEIN* (VIT_00s0179g00180) were found in the first places, respectively (**Table 13**).

Acid phosphatases catalyze the hydrolysis of phosphate, an essential but limiting macronutrient important for plant metabolism and development; it is a key structural constituent of important biomolecules such as ATP, NADPH, nucleic

acids, phospholipids, and sugar-phosphates (Tran *et al.*, 2010).

Gibberellins are well known for their growth control function in fruit and seed development and exogenous gibberellic acid (GA) application plays an important role in viticulture. In a recent study was demonstrated that application of GA to grapevine inflorescences at pre-bloom promoted flower opening, and induced fruit coloring and seed abortion; GA treatment brought to the up regulation of genes encoding acid phosphatase (Cheng *et al.*, 2015).

Instead, a subgroup of JmjC domain-containing proteins were identified in *Arabidopsis thaliana* as a novel clock component involved in controlling the circadian period, in particular affecting the flowering phenotypes (Lu *et al.*, 2011).

Gene ID	Functional annotation	FC	Gene ID	Functional annotation	FC
VIT_09s0018g01800	Acid phosphatase	5.34	VIT_00s0179g00180	Transcription factor jumonji (jmjC) domain-containing protein	-7.54
VIT_14s0066g01210	Carbonic anhydrase, chloroplast precursor	2.86	VIT_05s0094g00330	Chitinase, class IV [Vitis vinifera]	-3.61
VIT_14s0066g00850	Nitrate transporter2.5	2.77	VIT_09s0070g00350	R protein disease resistance protein	-3.44
VIT_07s0031g00920	Inositol-3-phosphate synthase	2.33	VIT_04s0008g01860	CYP72A58	-3.43
VIT_11s0016g02350	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE	2.18	VIT_00s0125g00150	SHR5-receptor-like kinase	-3.34
VIT_15s0046g02390	ANTR2 (anion transporter 2)	2.03	VIT_03s0017g01360	UDP-glycosyltransferase 85A8	-3.20
VIT_05s0020g04110	ELIP1 (early light-inducible protein)	2.02	VIT_14s0030g02060	Aldo/keto reductase AKR	-3.19
VIT_17s0000g04030	Endonuclease	1.88	VIT_04s0023g02790	Dehydration-responsive protein	-3.15
VIT_03s0038g04210	Phototropin-2	1.81	VIT_16s0022g02230	Leucine-rich repeat receptor protein kinase EXS	-3.05
VIT_08s0040g03240	Pentatricopeptide (PPR) repeat	1.79	VIT_16s0039g01370	(9,10) (9`,10`) cleavage dioxygenase (CCD4) (VvCCD4c)	-3.00
VIT_16s0050g00990	ferric reduction oxidase 4	1.76	VIT_12s0028g01830	Peroxidase precursor	-2.86
VIT_18s0001g12510	5' nucleotidase	1.74	VIT_14s0030g01990	Perakine reductase aldo/keto reductase	-2.81
VIT_09s0002g02430	ABC Transporter (VvMRP8 - VvABCC8)	1.70	VIT_15s0024g01650	Glutathione S-transferase 8 GSTU8	-2.79
VIT_13s0019g02110	fringe-related protein	1.69	VIT_14s0081g00350	Ankyrin repeat	-2.78
VIT_07s0005g02470	Cellulose synthase CESA3	1.66	VIT_10s0003g04250	Glutathione S-transferase GSTU22	-2.76
VIT_01s0026g01710	Zinc knuckle	1.66	VIT_19s0015g02470	Zinc finger (C3HC4-type ring finger)	-2.74
VIT_02s0012g01240	PHD finger transcription factor	1.62	VIT_11s0118g00040	Receptor Like Protein 9	-2.73
VIT_07s0031g02160	Protein phosphatase 2C DBP	1.61	VIT_08s0040g02600	F-box protein	-2.63
VIT_17s0000g06610	Zinc finger (C3HC4-type ring finger)	1.61	VIT_02s0025g04890	CYP76B1	-2.63

Table 13: *VviNAC08* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

VviNAC15 over expression up regulated genes were mainly enriched in metabolic, catabolic processes and transport categories; among the down regulated were found may genes related to the response to hormones and to protein catabolic processes.

In the up regulated genes list of the *VviNAC15* over expression transcriptome, at the first place the *SEVEN IN ABSENTIA SINA5* (VIT_19s0014g00880) was found (**Table 14**). A recent study on a parthenocarpic somatic variant of a seeded cultivar found that a polymorphism on the *SEVEN-IN-ABSENTIA SINA5* (VIT_02s0025g01660) gene was the one potentially responsible for its parthenocarpic phenotype (Rojo *et al.*, 2016).

Many up regulated *GERMININ-LIKE PROTEIN 3* genes (VIT_14s0128g00540, VIT_14s0128g00660, VIT_14s0128g00610 and VIT_14s0128g00630) were also found. Germin and germin-like proteins (GLPs) constitute a ubiquitous family of plant proteins. They were first discovered in wheat seeds as specific markers of

germination but *GLPs* genes are expressed in all types of organs including leaves, cotyledons, stems, roots, embryos, flowers and seeds, and are involved in the developmental processes (Lu *et al*., 2010). Moreover, recent studies revealed a close connection between *GLPs* genes and disease resistance phenotypes and the mechanism by which GLPs influence plant defense is likely related to ROS (reactive oxygen species) production (Li *et al*., 2016).

As for *VviNAC08* over expression, a *TRANSCRIPTION FACTOR JUMONJI (JMJC) DOMAIN-CONTAINING PROTEIN* (VIT_00s0179g00180) was found down regulated also in *VviNAC15* DEGs list.

Fruit ripening involves the regulation of numerous biochemical pathways associated with pigmentation, cell wall metabolism, and the production of aromatic and nutritionally important compounds but, additionally, ripening is also characterized by a major increase in susceptibility to necrotrophic pathogens (Wang *et al*., 2017).

Knowing that the regulatory networks that contribute to fruit ripening are almost always associated with disease resistance, finding *R protein MLA10* (VIT_09s0002g08020) gene as the most down regulated was not strange. Indeed, MLA10 activity in disease resistance and cell death signalling was already demonstrated in barley and *Nicotiana benthamiana* (Bai *et al.*, 2012).

Gene ID	Functional annotation	FC	Gene ID	Functional annotation	FC
VIT_19s0014g00880	Seven in absentia SINA5	122.25	VIT_09s0002g08020	R protein MLA10	-10.40
VIT_03s0088g00560	Citrate synthase	25.81	VIT_13s0047g00230	Pectinesterase family	-9.33
VIT_14s0128g00570	Germin	24.41	VIT_10s0092g00240	Alpha-glucosidase 1 (AGLU1)	-8.73
VIT_08s0007g05100	Glycosyl hydrolase family 31 protein	19.95	VIT_19s0014g02900	Ring finger protein 185	-7.38
VIT_14s0128g00540	Germin-like protein 3 [Vitis vinifera]	18.98	VIT_04s0069g00510	WD-40 repeat family protein / beige-related	-6.31
VIT_03s0063g01290	Gibberellin 20 oxidase 2	16.92	VIT_02s0025g02610	basic helix-loop-helix (bHLH) family	-5.81
VIT_14s0128g00680	Germin	16.50	VIT_16s0022g00860	Invertase/pectin methylesterase inhibitor	-5.41
VIT_13s0064g00750	Polygalacturonase GH28	16.08	VIT_09s0070g00350	R protein disease resistance protein	-5.23
VIT_11s0016g04160	Sulfate transporter 3.5	15.55	VIT_12s0035g00610	CYP82M1v3	-4.33
VIT_03s0063g01330	Nodulin 1A, senescence-associated	15.14	VIT_19s0085g00010	Auxin-regulated protein	-4.19
VIT_03s0063g01280	Gibberellin 20 oxidase 2	14.73	VIT_14s0081g00370	Ankyrin repeat	-4.15
VIT_14s0128g00600	Germin-like protein 3 [Vitis vinifera]	12.98	VIT_08s0007g07140	Esterase/lipase/thioesterase family protein	-4.10
VIT_07s0005g05770	Tetratricopeptide repeat domain male sterility MS5	12.82	VIT_19s0014g01360	Curculin (mannose-binding) lectin	-4.02
VIT_14s0128g00660	Germin-like protein 3 [Vitis vinifera]	12.44	VIT_19s0014g00440	PMR5 (powdery mildew resistant 5)	-3.66
VIT_14s0128g00610	Germin-like protein 3 [Vitis vinifera]	11.99	VIT_11s0052g01510	MATE efflux family protein	-3.61
VIT_14s0128g00630	Germin-like protein 3 [Vitis vinifera]	11.80	VIT_00s0179g00180	Transcription factor jumonji (jmjC) domain-containing protein	-3.55
VIT_05s0020g03860	Homocysteine S-methyltransferase 3	10.99	VIT_11s0052g01560	MATE efflux family protein	-3.51
VIT_14s0066g01290	Exonuclease	10.76	VIT_11s0052g01540	MATE efflux family protein	-3.50
VIT_11s0052g01650	Pathogenesis-related protein 1 precursor (PRP 1)	10.60	VIT_10s0116g00560	Polyphenol oxidase II, chloroplast precursor	-3.49
VIT_09s0002g05280	2-oxoglutarate-dependent dioxygenase	10.38	VIT_06s0004g00070	Expansin (VvEXPA5)	-3.42

Table 14: *VviNAC15* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

VviNAC17 over expression brought to a high number of up regulated genes related to the regulation of biosynthetic and primary metabolic processes and to the transmembrane transport, while the down regulated clustered into only two categories: generation of precursor metabolites and energy and oxidation-reduction

processes. *VviNAC17* over expression led to an up regulation of many *VviWRKY* genes (VIT_12s0028g01700, VIT_12s0055g00340 and VIT_12s0028g01690), which are involved in the regulation of various physiological processes, such as development and senescence, and in plant response to many biotic and abiotic stresses (Wang et al., 2014); moreover, they resulted important in regulating vacuolar transport and flavonoid biosynthesis (Amato *et al*., 2017) (**Table 15**).
Among the *VviNAC17* over expression down regulated genes was found again an *ACID PHOSPHATASE* (VIT_09s0018g01800) but, at the first place, a *STEROID 5ALPHA-REDUCTASE* (VIT_19s0014g00080) was present. *STEROID 5ALPHA-REDUCTASE* inhibition has been already studied in relation to the increase of condensed tannins (Liu *et al*., 2008). Tannins are a type of bitter and astringent polyphenols that occur abundantly in nature; as natural products in plants, they act as a barrier against insects, pathogens and animals because of their ability to react with proteins and their antioxidant properties. In grapes, condensed tannins are mainly found in the stem, skin, flesh and seeds of the berries (Watrelot *et al*., 2020).

ID	Functional annotation	FC	ID	Functional annotation	FC
VIT_14s0108g00630	Amino acid permease	2.84	VIT_19s0014g00080	Steroid 5alpha-reductase	-3.89
VIT_14s0108g00690	Amino acid permease	2.76	VIT_05s0020g04110	ELIP1 (early light-inducible protein)	-3.28
VIT_18s0001g11630	Allene oxide synthase (jasmonates from fatty acids)	2.63	VIT_09s0018g01800	Acid phosphatase	-3.06
VIT_05s0049g00100	DNA-binding protein	2.24	VIT_04s0008g02670	Cryptochrome DASH	-2.90
VIT_16s0098g00900	Pseudo-response regulator 5 (APRR5)	2.19	VIT_12s0035g02150	ferric reduction oxidase 7 FRO7	-2.50
VIT_12s0028g01700	WRKY DNA-binding protein 65	2.14	VIT_18s0041g01150	Lectin protein kinase	-2.48
VIT_15s0048g01540	CYP76B1	2.12	VIT_12s0059g02280	Spg1p (septum-promoting GTPase)	-2.46
VIT_16s0022g00860	Invertase/pectin methylesterase inhibitor	2.03	VIT_16s0022g00470	Peroxisomal biogenesis factor 11 (PEX11)	-2.36
VIT_12s0055g00340	WRKY Transcription Factor (VvWRKY39)	1.87	VIT_13s0156g00090	Protease inhibitor/seed storage/lipid transfer protein (LTP)	-2.35
VIT_19s0014g01120	Curculin (mannose-binding) lectin	1.82	VIT_19s0015g01720	fructose-bisphosphate aldolase, cytoplasmic isozyme 1	-2.30
VIT_01s0011g04790	1-phosphatidylinositol-3-phosphate 5-kinase	1.82	VIT_08s0007g03240	Carbonic anhydrase precursor	-2.28
VIT_00s0400g00020	HcrVf1 protein	1.81	VIT_04s0044g01530	UDP-glucuronic acid:anthocyanin glucuronosyltransferase	-2.28
VIT_12s0028g01690	WRKY DNA-binding protein 65	1.80	VIT_06s0004g06170	Thylakoid soluble phosphoprotein	-2.26
VIT_05s0062g01120	PIN1	1.80	VIT_00s0291g00060	Inorganic phosphate transporter 2-1, chloroplast precursor	-2.24
VIT_13s0064g01510	Auxin-independent growth promoter	1.79	VIT_00s0475g00020	Zinc finger (C3HC4-type ring finger)	-2.22
VIT_08s0058g00700	F-box protein (FBL3)	1.77	VIT_07s0151g00860	Hydrolase, alpha/beta fold	-2.16
VIT_09s0002g02700	basic helix-loop-helix (bHLH) family	1.76	VIT_10s0003g03310	EMB2756 (embryo defective 2756)	-2.16
VIT_13s0067g03260	Pheophorbidase	1.76	VIT_06s0061g00080	Saccharopine dehydrogenase	-2.16
VIT_00s0179g00170	Indeterminate(ID)-domain 12	1.73	VIT_11s0016g03830	Protein kinase	-2.15
VIT_10s0003g00130	ERF Domain protein 12	1.71	VIT_10s0116g00600	RIC10 (ROP-Interactive crib motif-containing)	-2.15

Table 15: *VviNAC17* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

The genes related to the phosphate-containing compounds metabolic processes and activity are highly represented in the *VviNAC18* up regulated genes sub dataset. Many genes were also related to the defense response, an important aspect know to be up regulated during the ripening process. As for *VviNAC08*, a very represented gene category was the cyanide metabolic/catabolic processes one. In the *VviNAC18* DEGs list, another ethylene-related gene, *ERF/AP2 GENE FAMILY VVERF022*, *DEHYDRATION RESPONSIVE ELEMENT-BINDING TRANSCRIPTION*

FACTOR VVDREB23 (VIT_16s0100g00380), was found as the most up regulated (**Table 16**).

Among the down regulated genes, the *STEROID 5ALPHA-REDUCTASE* (VIT_19s0014g00080) and the *ACID PHOSPHATASE* (VIT_09s0018g01800) were found again, but the most down regulated one was *MINI ZINC FINGER 2 MIF2* (VIT_08s0056g01130). A recent study has demonstrated that *Arabidopsis thaliana MINI ZINC FINGER2 (AtMIF2)* and its homolog in tomato, *INHIBITOR OF MERISTEM ACTIVITY (SlIMA)*, participate in the floral meristem termination process; the number of carpels arising from the floral meristem, and consequently the number of fruit locules, is determined during floral meristem termination (Bollier *et al*., 2018).

ID	Functional annotation	FC	ID	Functional annotation	FC
VIT_16s0100g00380	ERF/AP2 Gene Family (VvERF022)	18.02	VIT_08s0056g01130	Mini zinc finger 2 MIF2	-5.65
VIT_12s0028g03260	Rooty/superroot1	8.48	VIT_08s0007g05130	UDP-glucoronosyl/UDP-glucosyl transferase	-5.24
VIT_05s0094g00360	Chitinase class IV	5.85	VIT_19s0014g00080	Steroid 5alpha-reductase	-4.88
VIT_15s0048g00510	Pectinesterase family	5.44	VIT_09s0018g01800	Acid phosphatase	-4.51
VIT_16s0100g01070	Stilbene synthase (VvSTS35)	4.45	VIT_01s0026g00570	Bet v I allergen	-4.42
VIT_07s0104g00850	Pto kinase interactor	4.41	VIT_05s0077g01670	s3_Pathogenesis protein 10 [Vitis vinifera]	-4.17
VIT_05s0077g01600	s8_Pathogenesis protein 10 [Vitis vinifera]	4.20	VIT_13s0156g00090	Protease inhibitor/seed storage/lipid transfer protein (LTP)	-4.11
VIT_13s0019g01380	Integral membrane protein	4.16	VIT_09s0002g06750	ERF/AP2 Gene Family (VvERF042)	-4.08
VIT_04s0044g00270	Monooxygenase (MO3)	4.12	VIT_19s0015g00510	Pectate lyase 2	-3.79
VIT_09s0002g05410	ABC Transporter (VvPDR11 - VvABCG41)	4.04	VIT_05s0020g04110	ELIP1 (early light-inducible protein)	-3.38
VIT_16s0100g01200	Stilbene synthase (VvSTS48)	3.90	VIT_11s0052g01620	Pathogenesis-related protein 1 precursor (PRP 1)	-3.30
VIT_04s0008g00110	TIFY gene family (VvJAZ3)	3.77	VIT_03s0063g01880	Acyl-CoA synthetase long-chain member 2	-3.28
VIT_02s0025g04420	MATE efflux family protein	3.73	VIT_00s0230g00080	Copper-binding family protein	-3.20
VIT_19s0014g04650	Avr9/Cf-9 rapidly elicited protein 20	3.70	VIT_09s0002g03140	Lipase GDSL	-3.17
VIT_01s0026g02390	Glutathione S-transferase 10 GSTU10	3.69	VIT_03s0038g03770	Auxin-induced SAUR	-3.12
VIT_10s0003g03790	Jasmonate ZIM domain-containing protein (VvJAZ5)	3.69	VIT_04s0069g00750	AHK5 (Cytokinin independent 2)	-3.08
VIT_16s0100g00910	Stilbene synthase (VvSTS21)	3.62	VIT_17s0000g08680	IMP dehydrogenase/GMP reductase	-3.07
VIT_01s0026g02400	Glutathione S-transferase 10 GSTU10	3.57	VIT_17s0000g10060	Lipase GDSL	-3.05
VIT_18s0041g02010	12-oxophytodienoate reductase 1	3.44	VIT_16s0050g00100	Myosin-related	-2.99
VIT_01s0026g02370	Glutathione S-transferase 10 GSTU10	3.40	VIT_15s0045g00040	Copper-binding family protein	-2.95

Table 16: *VviNAC18* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

VviNAC26 over expression up regulated genes were mainly enriched in response to different stimuli, transmembrane transport and biosynthetic processes categories; among the down regulated dataset were found the lipid catabolism category and, again, many genes related to cyanide metabolic/catabolic processes. Between the up regulated genes related to the *VviNAC26* over expression, some already described genes were present: *SEVEN IN ABSENTIA SINA5* (VIT_19s0014g00880) and *ACID PHOSPHATASE* (VIT_09s0018g01800) (**Table 17**).

Moreover, *CITRATE SYNTHASE* (VIT_03s0088g00560) was found; it plays an important role in the Krebs cycle, in β-oxidation of fatty acids and in photo-respiratory glycolate pathways, can affect fruit ripening (Liu *et al*., 2013).

Among the down regulated *ALPHA-GLUCOSIDASE 1 AGLU1* (VIT_10s0092g00240) and, again, a *R PROTEIN MLA10* (VIT_09s0002g08020) were found. *ALPHA-GLUCOSIDASE 1 AGLU1* occur widely in plants but their function is unknown.

Gene ID	Functional annotation	FC	Gene ID	Functional annotation	FC
VIT_19s0014g00880	Seven in absentia SINA5	88.46	VIT_10s0092g00240	Alpha-glucosidase 1 (AGLU1)	-13.49
VIT_09s0018g01800	Acid phosphatase	46.57	VIT_13s0047g00230	Pectinesterase family	-8.10
VIT_03s0088g00560	Citrate synthase	20.48	VIT_09s0002g08020	R protein MLA10	-8.10
VIT_11s0052g01650	Pathogenesis-related protein 1 precursor (PRP 1)	19.08	VIT_16s0022g00860	Invertase/pectin methylesterase inhibitor	-7.32
VIT_03s0063g02680	Radialis-like protein 5	10.16	VIT_19s0014g02900	Ring finger protein 185	-7.03
VIT_04s0023g00610	Protein kinase CRK1 CRK1 protein(Cdc2-related kinase 1)	6.58	VIT_00s0179g00180	Transcription factor jumonji (jmjC) domain-containing protein	-6.02
VIT_03s0063g00370	Nitrite reductase	6.15	VIT_16s0039g01370	(9,10) (9',10') cleavage dioxygenase (CCD4) (VvCCD4c)	-5.38
VIT_01s0127g00400	Polygalacturonase GH28	5.93	VIT_02s0025g04890	CYP76B1	-5.19
VIT_19s0090g00080	Ubiquitin-conjugating enzyme E2 variant	5.86	VIT_00s0371g00100	Mannitol dehydrogenase	-4.78
VIT_19s0090g00120	Ubiquitin-conjugating enzyme E2 variant	5.80	VIT_06s0061g00120	Beta-1,3-glucanase [Vitis riparia]	-4.49
VIT_12s0035g01050	Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	5.60	VIT_09s0070g00350	R protein disease resistance protein	-4.34
VIT_12s0035g02150	ferric reduction oxidase 7 FRO7	5.56	VIT_14s0081g00400	Ankyrin repeat	-4.25
VIT_03s0063g02620	Myb RAD (Transcription factor RAD)	5.50	VIT_03s0038g00360	Isochorismatase hydrolase	-4.20
VIT_11s0016g02350	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE	5.48	VIT_11s0052g01560	MATE efflux family protein	-4.07
VIT_17s0000g03890	Peptidyl-prolyl isomerase C	5.33	VIT_19s0014g00250	Bile acid:sodium symporter	-3.96
VIT_00s0572g00020	Linalool synthase (VvTPS62)	5.08	VIT_11s0052g01510	MATE efflux family protein	-3.93
VIT_11s0016g02860	Nudix hydrolase 8	4.75	VIT_02s0025g02610	basic helix-loop-helix (bHLH) family	-3.85
VIT_19s0090g01350	Aspartyl protease	4.56	VIT_08s0007g08910	Cis-zeatin O-beta-D-glucosyltransferase	-3.83
VIT_14s0030g00420	Inosine-uridine preferring nucleoside hydrolase family protein	4.50	VIT_10s0042g00480	ABC transporter g family pleiotropic drug resistance 12 PDR12	-3.79
VIT_09s0002g06970	Palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase	4.49	VIT_13s0067g02130	Dehydration-induced protein (ERD15)	-3.61

Table 17: *VviNAC26* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

The genes related to the regulation of transcription and pathogenesis were highly represented in the *VviNAC39* up regulated genes sub dataset. Many genes were also related to the regulation of biosynthetic and metabolic and to the light response. Concerning the down regulated genes, the most represented categories were the response to different type of stress. *VviNAC39* over expression brought to the up regulation of two *SERINE CARBOXYPEPTIDASE II* (VIT_08s0040g01130 and VIT_08s0040g01040) (**Table 18**). Although the biological role remains to be determined, some studies suggest that they are likely to function in a broad range of biochemical pathways including those involved in secondary metabolite biosynthesis (Fraser *et al*., 2005).

Also, four pathogenesis related genes were up-regulated: three *PORE-FORMING TOXIN-LIKE PROTEIN HFR-2s* (VIT_07s0005g06090, VIT_07s0005g06000 and VIT_07s0005g06060) and a *DISEASE RESISTANCE PROTEIN NBS-LRR CLASS* (VIT_14s0030g00520). Moreover, a *PECTATE LYASE 2* (VIT_19s0015g00510), related to fruit softening (Zenoni *et al*., 2016) and a *MINI ZINC FINGER 2 MIF2* (VIT_08s0056g01130) were found down regulated. Interestingly a *VviNAC* and a *VviMYB* family member, *VviNAC74* (VIT_06s0080g00780) and *MYB RAD* (VIT_03s0063g02620), resulted down regulated too.

Gene ID	Functional annotation	FC
VIT_08s0040g01130	Serine carboxypeptidase II	5.93
VIT_07s0005g06090	Pore-forming toxin-like protein Hfr-2	5.34
VIT_07s0005g06000	Pore-forming toxin-like protein Hfr-2	4.96
VIT_08s0040g01040	Serine carboxypeptidase II	4.74
VIT_07s0005g06060	Pore-forming toxin-like protein Hfr-2	3.62
VIT_06s0009g03670	F-box family protein	3.12
VIT_09s0002g05590	ABC Transporter (VvPDR16 - VvABCG46)	2.97
VIT_14s0030g00520	Disease resistance protein (NBS-LRR class)	2.41
VIT_10s0071g00430	GRAM domain-containing protein / ABA-responsive	2.30
VIT_09s0054g00530	Zinc finger (B-box type)	2.24
VIT_13s0106g00610	Tetracycline transporter	2.19
VIT_01s0010g02730	Chaperone BCS1 mitochondrial	2.11
VIT_10s0071g00390	GRAM domain-containing protein / ABA-responsive	2.02
VIT_01s0026g02620	Expansin (VvEXPA1)	2.02
VIT_16s0022g00510	Heat shock 22 kDa protein	2.00
VIT_04s0008g01110	Heat shock transcription factor A6B	1.99
VIT_10s0071g00500	GRAM domain-containing protein / ABA-responsive	1.89
VIT_09s0018g00620	Co-chaperone-curved DNA binding protein A	1.87
VIT_16s0050g00020	Cullin-4	1.83
VIT_00s0179g00150	Heat shock transcription factor A6B	1.83

Gene ID	Functional annotation	FC
VIT_19s0015g00510	Pectate lyase 2	-7.23
VIT_08s0056g01130	Mini zinc finger 2 MIF2	-6.24
VIT_01s0026g00490	Nodulin	-4.29
VIT_16s0100g00380	ERF/AP2 Gene Family (VvERF022)	-3.80
VIT_13s0139g00460	Annexin ANN4	-3.49
VIT_00s0131g00180	Annexin ANN4	-3.48
VIT_07s0151g00250	Chlorophyllase	-3.43
VIT_13s0139g00420	Annexin ANN4	-3.27
VIT_09s0002g03520	Arabidopsis histidine phosphotransfer AHP4	-3.25
VIT_07s0031g02270	Tonoplast monosaccharide transporter2	-3.02
VIT_08s0007g02450	Aspartic Protease (VvAP22)	-3.00
VIT_03s0038g01830	Proline-rich protein 4	-2.92
VIT_06s0080g00780	NAC domain-containing protein (VvNAC74)	-2.78
VIT_13s0019g02160	Laccase	-2.74
VIT_11s0052g01620	Pathogenesis-related protein 1 precursor (PRP 1)	-2.69
VIT_08s0007g01680	Ceramidase	-2.69
VIT_14s0030g02090	Calcium-transporting ATPase 12 ACA12	-2.68
VIT_03s0063g02620	Myb RAD (Transcription factor RAD)	-2.63
VIT_13s0019g02760	Heat shock protein 17.6 kDa class I	-2.53
VIT_07s0005g01860	Patatin	-2.38

Table 18: *VviNAC39* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

VviNAC61 over expression brought to a high number of up regulated genes related to chitin, amino sugars, lignin, aromatic compounds and cyanide (ethylene related) metabolic processes, while the down regulated mainly clustered into photosynthesis and response to hormones categories.

The findings concerning the *VviNAC61* over expression were perfectly in line with what has already been reported by Zenoni *et al*. (2016) concerning the withering processes, where this *Vvi*NAC TF (together with *VviNAC60*) seems to play a major role (**Table 19**).

Among the up-regulated DEGs list were found an ethylene-related gene *ERF/AP2 GENE FAMILY VVERF072* (VIT_15s0021g01630), many *LACCASE* genes (VIT_18s0117g00590, VIT_18s0075g00780, VIT_18s0117g00550, VIT_18s0001g00680 and VIT_18s0117g00480), the pathogenesis related gene *R PROTEIN MLA10* (VIT_09s0002g08020), and the *UDP-GLUCORONOSYL AND UDP-GLUCOSYL TRANSFERASE* (VIT_06s0004g07250), which is s essential and one of the last steps in anthocyanin pigment biosynthesis.

Among the down regulated, two genes related to the photosynthetic process were found, *CHLOROPHYLLASE* (VIT_07s0151g00250) and *CHLOROPHYLLASE 1* (VIT_07s0151g00190); two *MYB* genes, *MYB RAD* (VIT_03s0063g02620) and *MYB FAMILY* (VIT_04s0023g01910); a *PECTATE LYASE 2* (VIT_19s0015g00510) was also found.

Gene ID	Functional annotation	FC	Gene ID	Functional annotation	FC
VIT_05s0020g05020	Inhibitor of trypsin and hageman factor (CMTI-V)	62.11	VIT_07s0151g00250	Chlorophyllase	-7.89
VIT_18s0117g00590	Laccase	55.76	VIT_01s0026g00490	Nodulin	-7.46
VIT_18s0075g00780	Laccase	51.12	VIT_03s0063g02620	Myb RAD (Transcription factor RAD)	-7.40
VIT_18s0117g00550	Laccase	51.09	VIT_05s0062g01160	Pectinesterase family	-6.02
VIT_10s0003g04910	Sinapyl alcohol dehydrogenase	43.85	VIT_16s0022g02090	Embryo-specific 3	-5.73
VIT_18s0001g00680	Laccase	32.56	VIT_03s0091g00880	Endoxylanase	-5.57
VIT_05s0020g05040	Proteinase inhibitor 1 PPI3B2	29.05	VIT_19s0015g00510	Pectate lyase 2	-5.34
VIT_14s0060g02170	GLUTATHIONE S-TRANSFERASE TAU 8	22.81	VIT_02s0025g00250	SP1L5 (SPIRAL1-like5)	-5.27
VIT_07s0129g00800	CYP81E1 Isoflavone 2'-hydroxylase	21.24	VIT_09s0002g06750	ERF/AP2 Gene Family (VvERF042)	-4.98
VIT_09s0002g08020	R protein MLA10	16.52	VIT_14s0030g02090	Calcium-transporting ATPase 12 ACA12	-4.92
VIT_06s0004g07250	UDP-glucoronosyl and UDP-glucosyl transferase	15.57	VIT_07s0151g00190	Chlorophyllase 1	-4.76
VIT_08s0007g06160	Basic Leucine Zipper Transcription Factor (VvbZIP28)	14.78	VIT_09s0002g03520	Arabidopsis histidine phosphotransfer AHP4	-4.48
VIT_15s0021g01630	ERF/AP2 Gene Family (VvERF072)	13.79	VIT_11s0016g02860	Nudix hydrolase 8	-4.47
VIT_13s0019g02180	Tropinone reductase	12.89	VIT_05s0020g00950	Aspartic Protease (VvAP10)	-4.45
VIT_00s0301g00150	Late embryogenesis abundant protein	11.80	VIT_08s0056g01130	Mini zinc finger 2 MIF2	-4.40
VIT_18s0117g00480	Laccase	11.31	VIT_14s0128g00210	Xyloglucanase inhibitor	-4.40
VIT_05s0094g00360	Chitinase class IV	10.86	VIT_04s0023g01910	Myb family	-4.29
VIT_08s0040g01130	Serine carboxypeptidase II	9.38	VIT_04s0023g03040	Lactoylglutathione lyase	-4.28
VIT_00s0301g00120	Late embryogenesis abundant protein	8.76	VIT_11s0103g00050	High-affinity K+ transporter 1 (HKT1)	-4.26
VIT_01s0010g03040	Calcium-binding protein CML	7.97	VIT_08s0007g02450	Aspartic Protease (VvAP22)	-4.17

Table 19: *VviNAC61* (A) top 20 up-regulated and (B) top 20 down-regulated DEGs.

Then, the attention was focused on the DEGs lists to see if any *VviNACs* would be present as up or down regulated genes under a specific *VviNAC* TF over expression. Interestingly, it was found that the *VviNAC15* and *VviNAC18* over expressions seem to have a role in the up regulation of other *VviNACs*, while the over expression of *VviNAC26*, *VviNAC39* and *VviNAC61* seem to bring to a down regulation of the same family TFs (**Table 20**). For *VviNAC01*, *VviNAC08* and *VviNAC17* no interesting *VviNAC* regulation was found.

VviNAC15 over expression seems to up regulate *VviNAC03* (VIT_00s0375g00040), *VviNAC13* (VIT_02s0012g01040), *VviNAC17* (VIT_19s0014g03290), *VviNAC56* (VIT_07s0005g03610) and *VviNAC61* (VIT_08s0007g07640, this one aslo present a positive Pearson correlation from the co-expression analysis).

VviNAC18 over expression seems to up regulate *VviNAC46* (VIT_18s0041g00700), which results down regulated by the over expression of *VviNAC39* and *VviNAC61*.

VviNAC39 over expression seems to down regulate *VviNAC64* (VIT_04s0079g00280) and *VviNAC74* (VIT_06s0080g00780), but the *VviNAC74* down regulation does not match with the positive Pearson correlation found in the coexpression analysis.

VviNAC61 over expression seems to down regulate *VviNAC19* (VIT_11s0016g02880).

Moreover, an important evidence is that *VviNAC26* over expression seems to up regulate *VviNAC33* (VIT_19s0027g00230).

Looking at the D'Incà (2017) microarray results, was found that *VviNAC13* over

expression seems to up regulate *VviNAC53* (VIT_15s0048g02300) and the *VviNAC33* one seems to up regulate *VviNAC11* (VIT_14s0108g01070); whereas, the over expression of *VviNAC60* seems to down regulate *VviNAC65* (VIT_04s0008g02710).

No *VviNACs* regulation matches were found for *VviNAC03* and *VviNAC11*.

Over expressed *VviNAC*	Regulated *VviNAC*			
	Gene ID	Functional annotation	FC	Pearson
VviNAC01				
VviNAC03				
VviNAC08				
VviNAC11				
VviNAC13	VIT_15s0048g02300	NAC domain-containing protein (VvNAC53)	1,50	
VviNAC15	VIT_00s0375g00040	NAC domain-containing protein (VvNAC03)	1,82	
	VIT_02s0012g01040	NAC domain-containing protein (VvNAC13)	2,77	
	VIT_19s0014g03290	NAC domain-containing protein (VvNAC17)	1,51	
	VIT_07s0005g03610	NAC domain-containing protein (VvNAC56)	4,05	
	VIT_08s0007g07640	NAC domain-containing protein (VvNAC61)	1,56	0,747
VviNAC17				
VviNAC18	VIT_18s0041g00700	NAC domain-containing protein (VvNAC46)	2,09	
VviNAC26	VIT_19s0027g00230	NAC domain-containing protein (VvNAC33)	-2,25	
VviNAC33	VIT_14s0108g01070	NAC domain-containing protein (VvNAC11)	2,65	
VviNAC39	VIT_18s0041g00700	NAC domain-containing protein (VvNAC46)	-1,52	
	VIT_04s0079g00280	NAC domain-containing protein (VvNAC64)	-1,81	
	VIT_06s0080g00780	NAC domain-containing protein (VvNAC74)	-2,78	0,400
VviNAC60	VIT_04s0008g02710	NAC domain-containing protein (VvNAC65)	-2,00	
VviNAC61	VIT_11s0016g02880	NAC domain-containing protein (VvNAC19)	-1,52	
	VIT_18s0041g00700	NAC domain-containing protein (VvNAC46)	-1,60	

Table 20: *VviNACs* found in the over expression analyses. The *VviNAC03*, *VviNAC11*, *VviNAC13*, *VviNAC33* and *VviNAC60* data were taken from D'Incà (2017).

Taking together all these results showed that each *VviNAC* seems to have a different role in the ripening processes that needs to be better investigated to be fully understood.

The transcriptome analyses were just a first step to elucidate the specific role of all the *Vvi*NAC family members but will be useful, integrated with other methods, to select some hypothetical candidates as direct targets of the studied TFs.

Moreover, the final evidence suggested how important could the interaction between *Vvi*NACs TF be in the complicated grapevine maturation regulatory network.

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3. THE GRAPEVINE CISTROME DISCOVERING

3.1 INTRODUCTION

The DNA of a eukaryotic cell must be compacted to fit into the nucleus. Cells can pack their genetic information still maintain it accessible to regulatory proteins that can activate or repress specific genes, repair damage, mediate recombination and replicate the DNA during the cell cycle.

The term chromatin is used to describe fold DNA. The basic unit of chromatin is the nucleosome, which contains approximately 146 bp of DNA wrapped almost twice around a core histone octamer, composed of two molecules each of the histones H2A, H2B, H3 and H4, organized into an H3–H4 tetramer and two H2A–H2B dimers (Luger *et al*., 1997). Each nucleosome has a diameter of around 10 nm, although the least compacted chromatin appears in the electron microscope as a 30 nm diameter fiber, which appears to correspond to euchromatin, the component of a eukaryotic genome that is actively transcribed (Goodrich and Tweedie, 2002).

Instead, heterochromatin is typically transcriptionally silent and is characterized by higher order packaging of the nucleosomes; it can be reversibly relaxed into euchromatin, to allow activation of gene expression, or may be permanently inactive, as found in gene-poor regions of the genome (peri-centromeric regions).

The regulation of chromatin structure has a key role in the epigenetic control of gene expression. The core histones can be modified by acetylation, methylation, phosphorylation, ubiquitination or ADP-ribosylation; actively transcribed genes are predominantly associated with highly acetylated histones whereas inactive genes are often characterized by the presence of hypoacetylated histones in the nucleosomes that are associated with them (Fischle et al., 2003; Jenuwein and Allis, 2001; Strahl and Allis, 2000).

In addition, the DNA itself can be modified, most commonly by cytosine methylation, which often characterizes inactive genes (Martienssen and Colot, 2001; Ng and Adrian, 1999). Furthermore, RNA has also been found associated with the heterochromatin structure (Maison et al., 2002).

Gene expression is controlled by not only DNA sequence but also by the interaction of DNA-binding proteins with higher order chromatin structure; indeed, transcription factors play an important part in the initiation of gene expression by

interacting with gene promoters.

ChIP-seq represents a powerful way to study TF-DNA interactions *in vivo*; this methodology has produced numerous applications in studying the composition and dynamics of chromatin landscapes and TF binding, as well as assessing the interplay between different factors in gene regulation under different conditions and developmental phases. ChIP-seq approach can be used to study DNA methylation, chromatin structure/histone modifications, and the cooperative binding of TFs.
The first ChIP protocol was developed by Gilmour and Lis (1984) to monitor RNA polymerase II/ DNA association in *Escherichia coli* and *Drosophila*.
Genome-wide applications of the ChIP-seq methodology in the plant field are still relatively scarce. This delay is partly due to technical difficulties in ChIP sample preparation related to the complexity of plant tissues for the presence of rigid cell walls, high levels of cellulose and lignin and large vacuoles. The establishment of an efficient ChIP-seq protocols for plant systems is hard and many optimizations are required if different types of tissues are sampled or for ChIP performed in different plant species.

A general ChIP assay (**Fig. 26**) consists in the TF-DNA crosslinking by formaldehyde vacuum infiltration of the fresh living tissue, the isolation of chromatin from a nuclear extract, the fragmentation of chromatin (average fragment size varying from 200 to 1000 bp), the precipitation of any insoluble material (pre-clearing step), the immunoprecipitation of the TF-DNA complexes with a specific antibody, the reverse crosslinking for the recovery of DNA and the identification of associated DNA sequences through the sequencing, which provides a powerful method to map protein–DNA interactions genome-wide.
Sequencing libraries are prepared from the immunoprecipitated (IP) DNA as well as from the INPUT (not immunoprecipitated sheared chromatin, control). Sequenced data of the IP and INPUT are then analyzed and compared using bioinformatics.

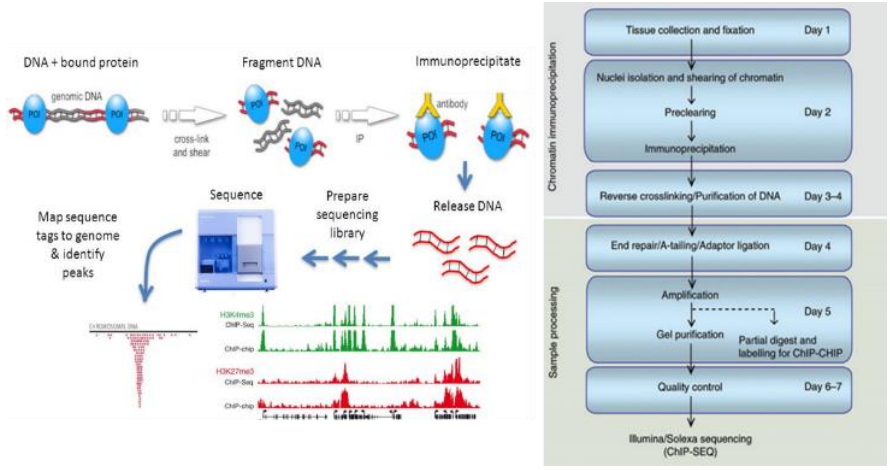

Figure 26: ChIP-seq workflow (Kaufmann *et al.*, 2010).

As *Vitis vinifera* is one of the most important fruit crops in the world, playing a central role in the economy of many developed countries, the regulation of berry ripening has been studied intensely to identify the physiological, biochemical and molecular features that influence fruit quality. Transcriptomic data obtained during berry development and integrated network analyses allowed the identification of several members of the *VviNAC* gene family, functionally involved in a large variety of plant growth and development related programs, as candidate genes for the regulation of the onset of berry ripening. Between these genes, *VviNAC60* was identified as *switch* gene that might represent a master regulator for the transition from vegetative-to-mature growth, since its expression is low in vegetative/green tissues but significantly higher in mature/woody organs and shows a high negatively correlation with genes that are down regulated during ripening.
With the aim to characterize the function of VviNAC60, we undertook a ChIP-seq approach on *Vitis vinifera* cv Shiraz plants to identify putative targets of this transcription factor, making a first step in the disclosure of its regulation mechanism. The analysis was performed on berries before veraison, characterized by a very low expression of *VviNAC60*, and berries after veraison, characterized by a high expression of the NAC candidate; given the absence of *VviNAC60* expression in young leaves, these were used as negative control.

The characterization of a specific TF binding profile in the reference genome is essential to identify its regulatory mechanisms of action, especially considering that

the coordination between different TFs via local and/or proximal binding sites influences gene expression. ChIP-seq is the leading method in the *in vivo* determination of TFBS. However, the method is limited in its throughput by the need to create specific antibodies, which can be technically challenging and expensive. Moreover, characterizing TFBS for all TFs within a specific family is crucial to expand the knowledges of complex gene expression networks but is quite difficult and expensive through the ChIP-seq technique.

To obtain cistrome maps of the selected *Vvi*NAC TFs, the DAP-seq (**Fig. 27**) was performed as it is a fast, high-throughput, inexpensive, and more easily scaled *in vitro* method than ChIP-seq. The binding site data generated by DAP-seq shares similarities to the ChIP-seq one; indeed, DAP-seq data are analysed by standard peak-calling and motif-characterization software, typically allowing uniquely mapped reads to be used in subsequent analyses, effectively masking repeat regions that may give false peaks (Bartlett *et al.*, 2017).

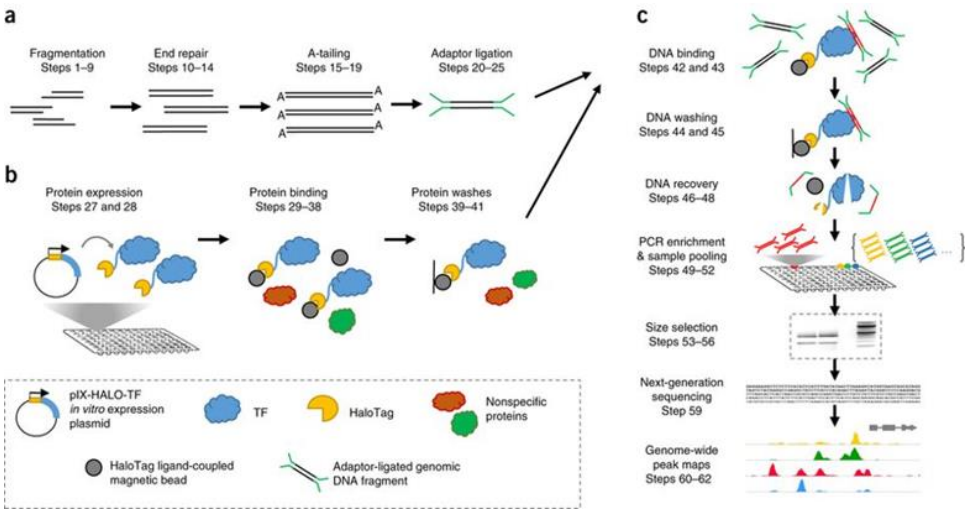

Figure 27: DAP-Seq workflow. (Bartlett *et al*., 2017). (a) An adaptor-ligated DNA library is prepared by shearing native genomic DNA into ~200-bp fragments and ligating Illumina-based sequencing adaptors to the repaired ends. (b) Transcription factor (TF) ORF clones fused to the Halo affinity tag are expressed in vitro and bound to ligand-coupled beads, whereas nonspecific proteins are washed away. (c) Halo Tag-TF fusion proteins are incubated with an adaptor-ligated genomic DNA library, and unbound DNA fragments are washed away. (d) Samples are heated to release TF-bound DNA, and the recovered DNA is PCR-amplified to attach indexed sequencing primers. (e) Indexed DNA samples are subsequently combined and size-selected to remove residual adaptor dimers. (f) Purified DNA libraries are sequenced using next-generation sequencing, and the resulting genome-wide binding events are analysed.

DAP-seq DNA libraries were constructed using gDNA from young leaves of *Vitis vinifera* cv Shiraz, preserving the cytosine methylations that are known to impact

TFs binding. The resulting gDNA libraries were incubated with single HALO-tagged *in vitro* expressed *Vvi*NAC TFs, and TF-DNA complexes were purified using magnetic separation of the affinity tag. The bound gDNA was eluted and sequenced. Sequence reads were mapped to the *Vitis vinifera* cv Pinot Noir reference genome, identifying genome-wide binding locations for each *Vvi*NAC TF. For the expression of the affinity-tagged TFs, individual TF ORFs were transferred into the Gateway-compatible pIX-HALO expression vector, which contains an N-terminal HALO affinity tag sequence. The pIX-HALO-TF constructs were then expressed using rabbit reticulocyte based *in vitro* transcription/translation coupled. The use of a negative control sample (pIX-HALO-GST vector) as background substantially reduced false peak signals. A good *in vitro* expression of the tagged protein is crucial for a successful experiment; for this reason, a Western blot analysis can be performed to verify the expression.

Although DAP-seq retains many of the tissue/cell line-specific secondary modifications and features present in gDNA, the effects of chromatin accessibility and histone modifications are not reflected in DAP-seq datasets. For this reason, a comparison between DAP-seq and ChIP-seq data could be useful to fully uncover the possible CREs of a specific TF, defining its role and mechanism of action.

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3.2 MATERIAL AND METHODS

Plant material

The isolated promoting sequences used for the Dual Luciferase Reporter Assay, the ChIP-seq and the DAP-seq data were obtained by chamber-grown *Vitis vinifera* cv. Shiraz fruiting cuttings, propagated as described in Mullins *et al*,.1981.

Nicotiana benthamiana, plants were grown from seeds in a greenhouse with temperature between 21 °C and 30°C, relative humidity of approximately 32-50% and a 15h/9h light/dark cycle.

Anti-*Vvi*NAC60 polyclonal antibody

The anti-VviNAC60 polyclonal antibodies design has been performed thanks to an initial sequence alignment of the *VviNAC60* against all the other *VviNAC* genes to find the most specific sub-sequences of our gene of interest.

Then, a preliminary structure prediction of the VviNAC60 TF, to evaluate if the selected peptides would have been potentially exposed and could be able to interact with the antibody, has been created thanks to the Robetta server (https://robetta.bakerlab.org/); it uses a fully automated structure prediction procedure that produces a model for an entire protein sequence in the presence or absence of sequence homology to protein(s) of known structure. The protein was visualized by UCSF Chimera (https://www.cgl.ucsf.edu/chimera/).

The anti-VviNAC60 polyclonal antibodies was finally produced by Biotem Company (https://www.biotem-antibody.com/) and was used for the immunoprecipitation step in the ChIP-Seq protocol.

ChIP-seq protocol

1) Tissue collection and fixation

- Collect the plant material (leaves/ berries) and keep it on ice.
- Wash 2 times for 1 minute with 40 ml sterile MilliQ in a 50 ml falcon tube.
- Sliced the tissue (berries must be without seeds) and put it on ice in a 50 ml falcon tube.

  LEAVES: 1g

  BERRIES: 3g
- Immediately add to the 50 ml falcon tube 36 ml of MC Buffer and 1 ml of 37% formaldehyde (C_f = 1% formaldehyde).

NB Better prepare the MC Buffer the evening before the fixation (under laminar flow hood) and keep it at 4°C.

MC Buffer		
C_i	V_i	C_f
Na_3PO_4 1M pH7	10 ml	Na_3PO_4 10mM pH7
NaCl 5M	10 ml	NaCl 50mM
Sucrose 2M	50 ml	Sucrose 0,1M
H_2O	930 ml	H_2O
	1L	

NB Na_3PO_4 1M pH7 is prepared adding to the Na_2HPO_4 1M solution the NaH_2PO_4 1M solution until a pH of 7 is reached (57,7 ml Na_2HPO_4 1M + 42,3 ml NaH_2PO_4 1M).

- Vacuum infiltrate keeping the sample on ice and submerged in the buffer (use a tinfoil ball).

  LEAVES: 15 minutes+ 15 minutes (with a pause in the middle to mix the sample)

  BERRIES: 4 x 10 minutes (with a pause in the middle to mix the sample)
- Add 3,7 ml of Glycine 1.25M and mix.
- Vacuum infiltrate for 5 minutes keeping the sample on ice and submerged in the buffer (use a tinfoil ball).
- Wash 3 times for 1 minute with 30 ml MC Buffer.
- Wash 1 time for 1 minute with 40 ml sterile MilliQ.

- Dry the plant tissue on tissue paper.
- Grind the sample in a mortar with liquid nitrogen.
- Make a pull and put the sample in a 50 ml falcon tube.
- Store at -80°C.

2) Nuclei isolation and chromatin shearing

- Do not let the sample defrost and add 25 ml of cold M1 Buffer to a 50 ml falcon tube containing 1 g of leaves/berries powder. Mix by inverting the tube until the sample is totally defrost. Keep it on ice for 10 minutes.

NB Better prepare the M1 Buffer the evening before (under laminar flow hood) and keep it at 4°C. Add the β-ME e the protease inhibitors just before use.

M1 Buffer		
C_i	V_i	C_f
Na_3PO_4 1M pH7	250µl	Na_3PO_4 10mM pH7
NaCl 5M	500µl	NaCl 0,1M
2-methyl 2,4 pentanediol	3,2 ml	
β-ME	17,5µl	
Protease inhibitor	1/2 tablet	
	25 ml	(to volume with sterile MilliQ)

- Use a 55µm sterile filter (the one that is used in the lab for protoplasts) and a sterile Petri dish to collect the nuclei and discard the tissue residues.
- Collect the filtrate in a new 50 ml falcon tube and keep it on ice.
- Centrifuge at 1.000 g for 20 minutes at 4°C.
- Wash the pellet with 5 ml of cold M2 Buffer.

NB Better prepare the M2 Buffer the evening before (under laminar flow hood) and keep it at 4°C. Add the β-ME e the protease inhibitors just before use.

M2 Buffer		
C_i	V_i	C_f
Na_3PO_4 1M pH7	250µl	Na_3PO_4 10mM pH7
NaCl 5M	500µl	NaCl 0,1M
2-methyl 2,4 pentanediol	3,2 ml	
β-ME	17,5µl	
Protease inhibitor	1/2 tablet	
$MgCl_2$ $6H_20$ 1M	250µl	$MgCl_2$ $6H_20$ 10mM
Triton X-100 20%	625µl	Triton X-100 0,5%
	25 ml	(to volume with sterile MilliQ)

- Centrifuge at 1.000 g for 10 minutes at 4°C.
- Discard the supernatant and repeat the wash with the cold M2 Buffer 4 more times.
- Discard the supernatant and wash the pellet with 5 ml of cold M3 Buffer.

NB Better prepare the M3 Buffer the evening before (under laminar flow hood) and keep it at 4°C. Add the β-ME e the protease inhibitors just before use.

M3 Buffer		
C_i	V_i	C_f
Na_3PO_4 1M pH7	250µl	Na_3PO_4 10mM pH7
NaCl 5M	500µl	NaCl 0,1M
β-ME	17,5µl	
Protease inhibitor	1/2 tablet	
	25 ml	(to volume with sterile MilliQ)

- Centrifuge at 1.000 g for 10 minutes at 4°C.
- Discard the supernatant and resuspend the pellet in 1 ml of cold Sonic Buffer.

NB Prepare, filter and aliquote the Sonic Buffer in 2 ml eppy tubes and store at -20°C. Keep it on ice before use.

Sonic Buffer		
C_i	V_i	C_f
Na_3PO_4 1M pH7	500µl	Na_3PO_4 10mM pH7
NaCl 5M	1ml	NaCl 0,1M
Sarkosyl 5%	250mg	
EDTA 0,5M pH8	1ml	EDTA 10mM pH8
Protease inhibitor	1/2 tablet	
	50 ml	(to volume with sterile MilliQ)

- Transfer the solution in a 2 ml eppy tube and keep it on ice.
- Aliquote 130 µl of the solution in Covaris microTUBEs and sonicate for the maximum time (20 minutes) as describe below.

NB Check the sonication on an agarose gel. The DNA smear should be concentrated between 200 and 1000 bp.

Appendix A: AFA Focused Ultrasonicator Operating Conditions

Low Tissue Mass Chromatin Shearing Protocol						
Instrument	**M220**	**ME220**	**S220**	**E220 Evolution**	**E220**	**LE220**
Target Size (bp)	200-700	200-700	200-700	200-700	200-700	200-700
PIP	75	75	105	105	105	300
Duty Factor (%)	5	5	2	2	2	15
CPB	200	1000	200	200	200	200
Treatment Time (minutes)	2-20	2-20	2-12	2-12	2-12	2-12
Setpoint Temperature (C) [1]	7	9	6	6	6	6
Min/Max Temperature (C)	4/10	6/12	3/9	3/9	3/9	3/9
Degassing Mode	NA	NA	Continuous	Continuous	Continuous	Continuous
Max Cell Number (Million)	3M	3M	3M	3M	3M	3M
AFA Intensifier Required [2]	NA	NA	Integrated	Yes	Yes	NA
Water Level (run) [3]	Full	9	12	6	6	6
Sample Volume (µl) [4]	130	130	130	130	130	130

Important Notes

1. If using the S220, E220 Evolution, E220, or LE220, set the temperature on the external chiller 3C below the setpoint temperature for the run. The min/max is set in SonoLab
2. If intensifier is required, please ensure PN 500141 is used
3. Water level should always be 1mm below the neck of the microTUBE–130 cap 4. Always fill the microTUBE-130 with 130 µl of sample

- Transfer the sonicated solution in a new 2 ml eppy tube.
- Centrifuge at top speed for 20 minutes at 4°C.
- Transfer the supernatant in a new 2 ml eppy tube measuring the correct volume.
- Add an equal volume of cold IP Buffer.

NB Prepare, filter and aliquote the IP Buffer in 2 ml eppy tubes and store at -20°C. Keep it on ice before use.

IP Buffer		
C_i	V_i	C_f
HEPES 0,5M pH7.5	5ml	HEPES 50mM pH7.5
NaCl 5M	1,5ml	NaCl 150mM
$MgCl_2$ $6H_20$ 1M	250µl	$MgCl_2$ $6H_20$ 5mM
$ZnSO_4$ 100mM	5µl	$ZnSO_4$ 10µM
Triton X-100 20%	2,5ml	Triton X-100 1%
SDS 10%	250µl	SDS 0,05%
	50 ml	(to volume with sterile MilliQ)

- Put aside in a 2 ml eppy tube 120 µl of sheared chromatin as control (“input DNA”) and store at -20°C.
- Aliquote 800µl of the remaining sheared chromatin in 1,5 ml low adhesion eppy tube.

3) Chromatin immunoprecipitation

- Prepare the Protein-A Agarose Beads (Protein-A agarose beads (Santa Cruz, cat. no. sc-2001) in a 1,5 ml low-adhesion eppy tube.

  NB 160µl beads per sample are needed, plus 100µl of extra beads in total.
  Es. 3 samples = 160µl * 3 → 480µl → +100µl → 580µl beads in total

- Wash the beads with 1ml IP Buffer (independently from the volume of the beads) inverting the eppy tube several times.
- Centrifuge at 3.800 g for 3 minutes at 4°C.
- Discard 1ml of supernatant.
- Repeat the wash of the beads with the IP Buffer.
- Add 80µl of Protein-A Agarose Beads to the sample using a sterile cut tip.

  NB This step is important to remove all the DNA linked to protein containing an IgG before adding the antibody (that is produced in rabbit and contains an IgG) and to avoid false positive. Use a 1,5 ml eppy tube to better visualize the beads pellet.

- Incubate on a rotating wheel for 2h at 4°C.
- Centrifuge at 3.800 g for 5 minutes at 4°C.
- Carefully transfer the supernatant in a new 1,5 ml low adhesion eppy tube (avoid all the beads).
- Add 3µg of antibody to the sample (anti-*Vv*NAC60: 1,4mg/ml→ 2,2µl).
- Incubate on a rotating wheel for 2h at room temperature.
- Add 80µl of Protein-A Agarose Beads to the sample using a sterile cut tip.
- Incubate on a rotating wheel for 2h at 4°C.
- Centrifuge at 3.800 g for 5 minutes at 4°C.
- Discard the supernatant without touching the beads.
- Wash the beads 3 times with 1ml IP Buffer leaving the eppy tube in rotation at room temperature for 8 minutes; centrifuge every time at 3.800 g for 3 minutes at room temperature and discard 1 ml of supernatant.

- Add 100 µl of cold Elution Buffer to the beads.

NB Prepare, filter and aliquote the Elution Buffer in 2 ml eppy tubes and store at -20°C. Keep it on ice before use.

Elution Buffer (pH 2.8 with HCl)		
C_i	V_i	C_f
Glycine 1,25M	4ml	Glycine 0,1M
NaCl 5M	5ml	NaCl 0,5M
Tween 20	25µl	
	50 ml	(to volume with sterile MilliQ)

- Incubate with vigorous shaking at 37°C for 1 minute.
- Centrifuge at maximum speed for 1 minute at room temperature.
- Transfer the supernatant in a new 1,5 ml eppy tube and add 50µl of Tris-HCl 1M pH9.
- Add 100µl of cold Elution Buffer to the beads.
- Incubate with vigorous shaking at 37°C for 1 minute.
- Centrifuge at maximum speed for 1 minute at room temperature.
- Transfer the supernatant in the 1,5 ml eppy tube and add 50µl of Tris-HCl 1M pH9.
- Add 100µl of cold Elution Buffer to the beads.
- Incubate with vigorous shaking at 37°C for 4 minutes.
- Centrifuge at maximum speed for 1 minute at room temperature.
- Transfer the supernatant in the 1,5 ml eppy tube and add 50µl of Tris-HCl 1M pH9.

NB The final volume in the 1,5 ml eppy tube should be of 450µl.

- Centrifuge at maximum speed for 2 minutes at room temperature.
- Transfer the supernatant in a new 2 ml eppy tube.
- Recover the 120 µl of "input DNA" and add 330 µl of TE Buffer to them.

TE Buffer		
C_i	V_i	C_f
EDTA 0,5M pH8	100µl	EDTA 1mM pH8
Tris-HCl 1M pH8	500µl	Tris-HCl 10mM pH8
	50 ml	(to volume with sterile MilliQ)

- Add 11,25 µl of Proteinase K (C_i = 20mg/ml → C_f =0,5mg/ml) to the sample and to the "input DNA".
- Incubate at 37°C o/n.
- Add a second aliquote of Proteinase K (11,25µl).
- Incubate at 65°C for 6 h.
- Add 2.5 volumes of 100% EtOH, 1/10 volume of 3M NaAc pH 5.4 and 1 µl of Glycogen.
- Let the DNA precipitate at -20°C o/n.
- Centrifuge at maximum speed for 30 minutes at 4°C.
- Resuspend the pellet with 100 µl of sterile MilliQ.
- Purify the DNA using the Qiagen PCR Purification Kit and elute in 30 µl of EB in a new 1,5 ml low adhesion eppy tube.

4) Libraries preparation

The ChIP-seq libraries were prepared using the KAPA Hyper Prep Kit (Roche), starting from 500pg of IP and from 2,5ng of INPUT, using for the purification clean-up the AMPure XP beads (Beckman Coulter) and amplifying the IP and the INPUT libraries for 13 and 10 cycles, respectively.

Finally, the libraries were quantified through qPCR Real Time analysis.

pIX-HALO cloning

The previously isolated *VviNACs* ORF and the one isolated by D'Incà (2017), *VviNAC03*, *VviNAC11*, *VviNAC13*, *VviNAC33* and *VviNAC60*, were transferred from the pENTR/D-TOPO to the Gateway-compatible pIX-HALO destination vector (Bartlett *et al.*, 2017) following the site specific LR recombination manual of the Gateway^R Technology system. A pIX-HALO vector containing the GST sequence, instead of the TF CDS, was used as negative control.

Genomic DNA extraction (from Thomas *et al.*, 1993)

1. Put 1 g of grinded tissue in a 50 ml falcon tube.
2. Add 12,5 ml of CNB buffer (with β-ME added) and vortex.

CNB Crude Nuclei Buffer pH7.6		
C_i	C_f	Volumes (V_f = 100ml)
5 M NaCl	0.25 M	5 ml
1 M Tris-HCl pH 7.6	0.2 M	20 ml
PVP 40.000	2,5%	2,5 g
0.5 M Na_2EDTA pH 8	0.05 M	10 ml
β-ME	0.1 %	100 µl
H_20		to a final volume of 100 ml
β-ME must be added just before use		
Store at 4°C		

3. Centrifuge at 4.000 rpm for 10 minutes at 4°C and discard the supernatant.
4. Add 5 ml of EtOH/Sark and resuspend the pellet by vortexing.

DNA Extraction Buffer EtOH/Sark		
C_i	C_f	Volumes (V_f = 100ml)
5 M NaCl	0.5 M	10 ml
1 M Tris-HCl pH 8	0.2 M	20 ml
PVP 40.000	2,5%	2,5 g
0.5 M Na_2EDTA pH 8	0.05 M	10 ml
N-Lauroyl-Sarcosine	3%	3 g
EtOH	20%	20 ml
β-ME	1%	1 ml
H_20		to a final volume of 100 ml
β-ME and EtOH must be added just before use		
Store at 4°C		

5. Put the falcon tube at 55°C for 30 minutes with frequent shaking.
6. Add 5 ml of chloroform and vortex.
7. Transfer the solution to a 15 ml falcon tube to better visualize the phase separation.
8. Centrifuge at 5.000 rpm for 15 minutes at 20°C.
9. Add 2,5 ml of isopropanol into a new 50 ml falcon tube.

10. Transfer the upper phase of the 15 ml falcon tube into the 50 ml falcon tube with isopropanol.
11. Mix very well by inverting the 50 ml falcon tube until a white precipitate is seen.
12. Leave at room temperature for 15 minutes.
13. Centrifuge at 5.000 rpm for 15 minutes at 20°C and discard the supernatant.
14. Put the 50 ml falcon tube upside down on some paper layers to better discard the supernatant drops.
15. Wash the pellet with 5 ml of 70% EtOH.
16. Centrifuge at 5.000 rpm for 10 minutes at 20°C and discard the supernatant.
17. Put the 50 ml falcon tube upside down on some paper layers to better discard the supernatant drops.
18. Let the pellet air dry.
19. Resuspend the pellet with 600 µl of TE buffer added with RNase (3µl of 20mg/ml stock).
20. Place the 50 ml falcon tube at 4°C O/N.
21. Transfer the gDNA solution in a 1,5 ml eppy tube.
22. Add 300 µl of 7.5 M NH_4OAc.
23. Centrifuge at 13.000 rpm for 10 minutes at room temperature.
24. Transfer the supernatant to a new 1,5 ml eppy tube.
25. Add 500 µl of isopropanol.
26. Mix by inverting until a precipitate is seen.
27. Centrifuge at 13.000 rpm for 10 minutes at room temperature and discard the supernatant.
28. Wash the pellet with 1 ml of 70% EtOH.
29. Centrifuge at 13.000 rpm for 10 minutes at room temperature and discard the supernatant.
30. Let the pellet air dry.
31. Resuspend the pellet with 300 µl of TE buffer.
32. Leave the eppy tube at 4°C O/N for a better resuspension.

DAP-seq protocol

- **gDNA library preparation (from Galli *et al.*, 2018)**

1) Fragmentation:

a. Measure gDNA concentration using Invitrogen Qubit (Quant-iT™ Assays with Qubit™ dsDNA BR Assay Kit, 2-1000ng, using only thin-wall clear 0,5ml PCR tubes).

b. Set up Covaris S2: chill and degas the water (~1h).

c. Dilute 7,5µg of gDNA with EB (Elution Buffer, Qiagen) in a finale volume of 130µL and load it into a snap-cap Covaris tube (Covaris cat. 520045, microTUBEs AFA Fiber Pre-Slit Snap-Cap 6X16mm).

d. Run 200bp fragment program.

200bp Covaris settings:
Mode: Frequency Sweeping
Duty Cycle: 10%
Intensity: 5
Cycles/Burst: 200
Time: 60sec
Number of cycles: 3
Water level: 12 (between 10-15 on the Covaris)
T: <10°C (better around 4°C)

e. Transfer 125µl of sheared sample to a 1,5ml low- bind eppy tube.

f. AMPure XP Beads clean (1X→ i.e.: 125µL DNA + 125µL beads).

i. Add beads and vortex 10 seconds; quick spin. Incubate at RT for 10 minutes.

ii. Place on magnetic rack (Invitrogen, Dynal, Beads separation) for 5min.

iii. Remove most of supernatant with pipet.

iv. Immediately add 500µl 80% EtOH (freshly made), remove and rotate tube 180 degrees on the magnet for 6 times to wash beads. Wait 1 minute.

v. Pipet off EtOH and repeat wash as in iv.

vi. Pipet off all remaining EtOH.

vii. Dry tubes with caps open at 37°C on the magnet until cracks form in beads (~10 minutes).

viii. Resuspend beads in 35µl EB and mix well by pipetting. Incubate at RT for 10 minutes.

ix. Place on magnet for 3 minutes and then aspirate supernatant into new tube being careful not to transfer beads.

g. Check concentration on Qubit to ensure good yield before continuing (optional).

2) End Repair:

a. 50µL reaction (End-It kit; Lucigen cat. ER0720 or ER81050):

DNA sample	34µL
10x Buffer	5µL
dNTP Mix	5µL
ATP	5µL
End-it Enzyme	1µL

b. Incubate at RT for 45min.
c. Qiaquick PCR purify (QIAquick PCR Purification Kit; Cat No./ID: 28104).

3) A-tail:

a. 50µL reaction:

DNA sample	32µL
NEBuffer2	5µL
1mM dATP	10µL
Klenow (3'-5'exo-; NEB)	3µL

-Klenow (3'-5' exo-) M0212L 5,000U/ml NEB
-dATP Solution N0440S 100mM NEB

b. Incubate at 37°C for 30min.
c. Qiaquick PCR purify and elute off column in 32µl EB.

4) Adapter Ligation:

a. 50µL reaction:

DNA sample	30µL
10x ligase Buffer	5µL
30µM Y-Adapter	10µL
T4 DNA Ligase (NEB)	5µL

-T4 DNA Ligase M0202L 400,000U/ml NEB
-Y-Adapter:

5'-/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'
5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'

b. Incubate at 16°C for overnight.

c. Heat inactivate at 70°C for 10min.

d. Bead clean using commercial AMPure XP beads as described above (1X→ i.e.: 50µL DNA + 50µL beads).

e. Elute in 32µl EB.

f. Check concentration with Qubit (Quant-iT™ Assays with Qubit™ dsDNA BR Assay Kit using only thin-wall clear 0,5ml PCR tubes).

g. Store at -20°C.

- **TF in vitro expression (from Bartlett et al., 2017)**

5) In vitro protein expression

a) Use 1µg of pIX-HALO-ORF plasmid in a 50µl TNT Coupled Reticulocyte Lysate System reaction *(Promega, Catalog number L4601)*.

TNT® Coupled Reticulocyte Lysate Systems	**(X1)** µl
TNT Rabbit Reticulocyte Lysate	25
TNT Reaction Buffer	2
Minus Met 1mM	1
Minus Leu 1mM	1
RNasin Ribonuclease Inhibitor	1
TNT RNA Polymerase (SP6)	1
pIX-HALO-TF + EB (1ug)	10
H_20	9
	50

RNasin Ribonuclease Inhibitor *(Promega, Catalog number N251B)* is not in the kit and must be ordered separately.

b) Aliquot 40µl of master mix to each PCR.

c) Add 10µl of EB diluted pIX-HALO-TF and pipet slowly up and down to mix.

d) Quick spin.

e) Incubate at 30°C for 2 hours and then let the samples stay at RT.

- **DAP-seq (from Galli et al., 2018)**

6) DAP

a) Wash 10µl of Magne Halo-Tag beads (*Promega, Catalog number G7282*) per DAP sample in 1ml PBSNP wash buffer (1xPBS +0.005%NP40; add 7.5µl of 20% NP40 to 30ml 1xPBS).
b) Flick tube to mix.
c) Place on magnet (*Invitrogen Dynal Beads Separation*) and remove supernatant. Repeat three times.
d) Resuspend beads in volume that allows each sample to contain 45µl of beads.
e) Save 5µl of each TNT Coupled Reticulocyte Lysate System reaction to check the correct TFs expression on a Western blot. Store at -20°C.
f) Add 45µl of washed Magne Halo-Tag beads to each of the remaining 45µl of TNT Coupled Reticulocyte Lysate System reactions.
g) Rotate at RT for 1 hour.
h) Place tube on a PCR tubes magnet.
i) Remove supernatant and wash with 90µl PBSNP wash buffer flicking gently the tube to mix. Let the beads settle. Put on the magnet. Repeat for 4 times.
j) Final resuspend in 40µl of PBST.
k) Dilute 500ng of adapter ligated gDNA library in EB to a final volume of 40µl.
l) Add the 40µl of EB diluted gDNA library to the 40µl of PBSNP resuspended beads (total final volume of 80µl sample).
m) Rotate at RT for 1 hour.
n) Place PCR tubes on magnet, remove supernatant.
o) Wash beads with 90µl of PBST as above for 6 times.
p) Resuspend beads in 25µl EB, transfer to new PCR tubes.
q) Heat tubes at 98°C in PCR machine for 10 min to release DNA and denature protein.

r) Place on ice 5 min.

s) Place on magnet, transfer supernatant to new PCR tubes.

The DAP step was performed using 500 ng of libraries for VviNAC01, VviNAC03, VviNAC11, VviNAC13, VviNAC15, VviNAC18, VviNAC38, VviNAC39 and VviNAC61, whereas for VviNAC08, VviNAC17, VviNAC26, VviNAC33 and VviNAC60 1000 ng were used.

7) PCR Enrichment

a) 50µL reaction (*Phusion High-Fidelity DNA Polymerase, NEB, M0530S or M0530L*).

Enrichment PCR	(X1)
DAP sample	25,0
5x HF Buffer	10,0
10mM dNTPs	2,5
Primer i5* (25µM)	1,0
Primer i7* (25µM)	1,0
Phusion polymerase	1,0
H_2O	9,5
	50,0

95°C X 2 min
98°C X 30 sec
98°C X 15 sec
60°C X 30 sec
72°C X 1 min
X20
72°C X 10 min
4°C X ∞

b) Transfer the PCR reactions in 1,5ml eppy tubes.

c) Perform 1x AMPure beads clean-up (i.e. 50µl of beads + 50µl PCR reaction).

d) Elute in 22µl EB.

e) Measure the final library concentration using Qubit dsDNA HS kit (*Invitrogen, Catalog number Q32854*).

f) Check library quality using bioanalyzer or KAPA Library Quantification Kit (Illumina platforms) to confirm absence of adapter dimers prior to sequencing.

g) Store at -20°C.

Sequencing

The DAP-seq and ChIP-seq datasets were obtained through a NextSeq500 sequencing system which gave a total of 60 (ChIP-seq) and 3,5 (DAP-seq) million 75 bp single reads for each sample; VviNAC08, VviNAC17 and VviNAC26 DAP-seq datasets were obtained from a 150 bp paired-end sequencing.

Read mapping, filtering, peak calling and motif analysis

Fastq files were trimmed using trimmomatic (Bolger *et al.*, 2014) with the following parameters: ILLUMINACLIP = TruSeq3–, SE = 2:30:10. LEADING = 3, TRAILING = 3, SLIDINGWINDOW = 4:20, MINLEN = 50. Trimmed reads were mapped to the grape reference genome version 12X.2 (nuclear chromosomes only) at URGI (https://urgi.versailles.inra.fr/Species/Vitis/Annotations) using bowtie2 v2.3.4.3 (Langmead and Salzberg, 2012). Reads mapping to multiple locations were filtered, removing all reads with the XS:i field present in the BAM file. Uniquely mapped reads were used for all subsequent steps. Peaks were called using GEM v3.4 (Guo *et al.*, 2012) applying the following parameters: --d Read_Distribution_default.txt --k_min 6 --k_max 20 –outNP; for the DAP-seq samples, a GST-HALO negative control was used for background subtraction. To reduce the number of false positives, in the DAP-seq datasets all peaks with a sample/control ratio < 5 fold were removed. The remaining peaks were associated with nearest genes using the ChipSeeker R package (Yu *et al.*, 2015). For gene annotation, we used the V1 on the 12X.0 assembly transposed to the 12X.2 assembly. The gff3 files were downloaded from URGI. For visualization in the Integrative Genome Browser (IGV), bam files were converted to bigwig files using deepTools v3.4.3, bamCoverage with a 10-bp bin size, and FPKM normalization. Motifs were detected and assigned using RSAT Plants NGS ChIP-Seq peak-motifs analysis and RSAT Plants Motif Discovery dyad-analysis (http://rsat.eead.csic.es/plants/index.php). The analyses were performed in collaboration with Prof. Nicola Vitulo (Università degli Studi di Verona).

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3.3 RESULTS

The selected *VviNACs* sequences were transferred into the pIX-Halo vector and *in vitro* translated; afterwards, they were used in a DAP-seq assay to study the specific cistrome for each of them.

Considering the enormous datasets derived from the DAP-seq assays, the decision was to keep analyzing only the FC>5 (compared to GST-Halo expressed protein, the negative control) filtered peaks obtained from each VviNAC, which correspond to more than the 90% average of the starting dataset.

Unexpectedly, most of the binding sites were not found in the 'standard' studied promoting regions; indeed, many features were found in the 'distal intergenic' and in the 'intron' regions. Considering the recent discoveries concerning the mechanisms of transcription regulation, it is extremely risky not to give relevance to all these genic regions as a lot of information could be obtained from newly studied long-distance features; despite this consideration, these type of regulation mechanisms are still unknown or not well defined and it will be difficult to presume a hypothetical regulatory network based on them.

For this reason, the attention for further considerations about the VviNACs targets was focused only on the 'promoter' (up to 2Kb from the transcription start site) and '5'UTR' genic regions. These 'standard' and well-known transcription promoting regions are the most intuitive and obligatory starting point in the difficult but fascinating world of the fine VviNACs regulatory network.

Between the target of each TF, a lot of *switch* genes (Palumbo *et al*., 2014) and transition markers (Fasoli *et al*., 2018) were found, indicating that the selected VviNACs represent the regulators of many maturation-related genes.

Interestingly but also in line with the well know cooperative family role of the VviNAC TFs, many genes were found to be targets of more than one of the selected VviNACs.

All the filtered DAP-seq datasets were also crossed with the transient and stable over expression results to highlight some particularly interesting genes.

The results clearly show how the over expression could reveal the general and final principal effects of a TF on the plant biological processes, without necessarily

highlighting the effective direct targets of the TF transcriptional regulation, which are correctly recognized and reported in the DAP-seq assay.

VviNAC01

The DAP-seq assay reported 20730 binding sites (peaks), which were reduced to 20153 after the FC>5 filtering; even considering the very stringent filter used, the loss was very low, only the 3% (**Fig. 28**). The distribution of peaks revealed that 24% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 1% were in 5′ untranslated regions (UTRs), 12% were located in exons, 18% were located within introns, 3% were located in 3′ UTRs, 7% were located in the 3 kb downstream region, and 32% were intergenic.

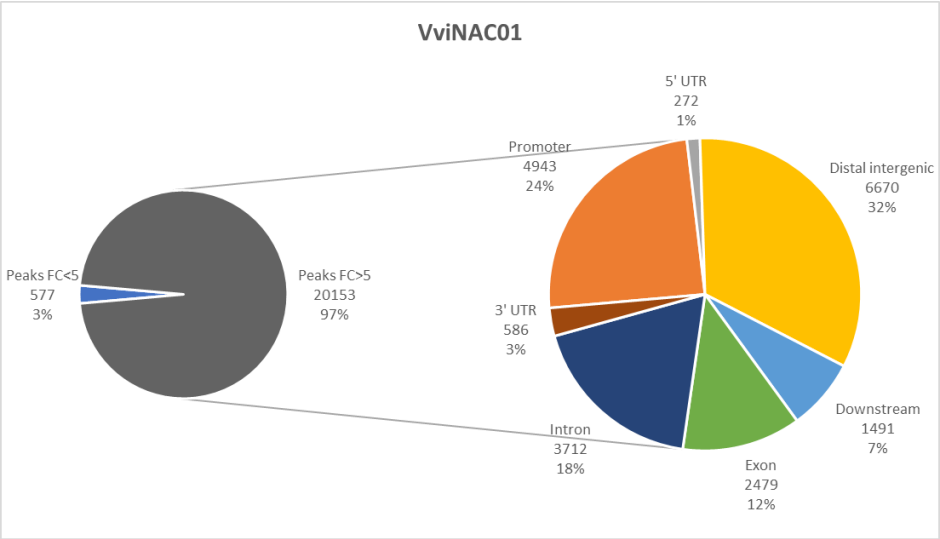

Figure 28: Double pie chart of the VviNAC01 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Three major binding motifs (TT[G/A][C/A][G/T]TG[T/A]) were identified with strong significance (**Fig. 29**) and the phylogenetic footprints correlate with ANAC092, considered the key positive regulator of leaf senescence, involved in a delicately balanced feed-forward loop that promotes ethylene-mediated chlorophyll degradation (Kim *et al.*, 2009).

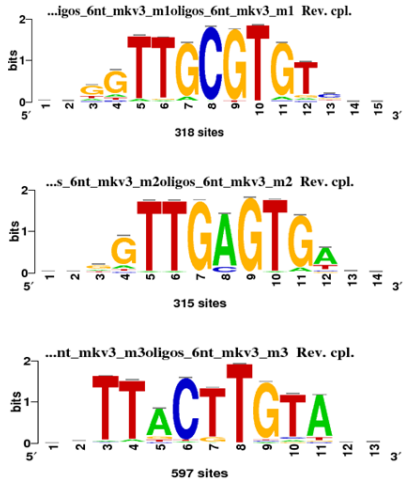

Figure 29: RSAT Plants NGS ChIP-Seq peak motifs analysis on the *Vvi*NAC01 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 25% of the final analyzed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 5215 sites; this percentage, at first considered low compared to the number of sites, was perfectly in line with other DAP-seq published papers.

After the elimination of all the 'no hit' and 'unknown' elements, 4215 binding sites were obtained for a total of 3545 unique genes regulated by VviNAC01.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to general categories such as biological regulation, in particular to the regulation of different biological and metabolic processes and transcription (**Fig. 30**).

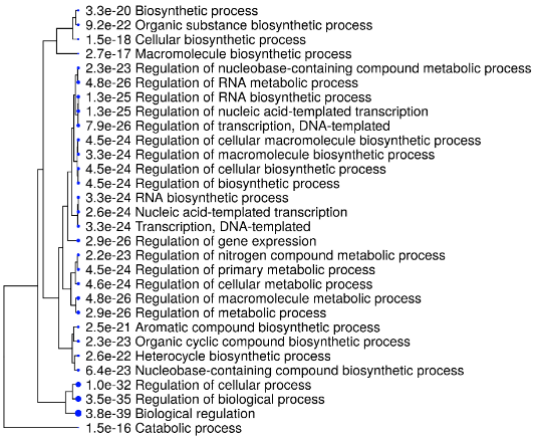

Figure 30: GO enrichment analysis of the VviNAC01 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 21**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
114,67	Promoter (1-2kb)	VIT_16s0098g01150	-1172	**Auxin-responsive SAUR29 ***
83,07	Promoter (1-2kb)	VIT_07s0191g00050	-1919	Peroxidase 17 *
78,94	Promoter (<=1kb)	VIT_07s0255g00110	-145	WD40
77,17	Promoter (<=1kb)	VIT_01s0026g01030	-974	Zinc finger (C3HC4-type ring finger)
76,64	Promoter (1-2kb)	VIT_13s0067g00600	-1194	Arginine-tRNA-protein transferase 1
73,98	Promoter (<=1kb)	VIT_17s0000g06970	-424	Diacylglycerol kinase 1
69,67	Promoter (<=1kb)	VIT_18s0001g14950	-543	EMB2170 (embryo defective 2170)
68,94	Promoter (<=1kb)	VIT_04s0044g00710	-159	UTP--glucose-1-phosphate uridylyltransferase *
67,78	Promoter (<=1kb)	VIT_06s0061g01190	-132	SWI/SNF matrix-associated regulator of chromatin sbfamily A mber 3 2
65,87	Promoter (1-2kb)	VIT_08s0056g01480	-1033	Cation exchanger, CAX7
65,13	Promoter (1-2kb)	VIT_12s0059g00870	-1271	MAPKKK15
64,48	Promoter (1-2kb)	VIT_18s0001g09420	-1850	Progesterone 5-beta-reductase
62,39	Promoter (<=1kb)	VIT_18s0001g08300	0	Tubulin alpha-6 chain
62,17	Promoter (<=1kb)	VIT_10s0003g00140	-733	ERF/AP2 Gene Family (VvERF064)
61,59	Promoter (<=1kb)	VIT_19s0177g00070	-505	Polyubiquitin (UBQ14)
60,66	Promoter (1-2kb)	VIT_18s0001g14980	-1611	3-methyl-2-oxobutanoate dehydrogenase
60,66	Promoter (1-2kb)	VIT_11s0118g00220	-1417	Chloride channel protein CLC
60,1	Promoter (<=1kb)	VIT_06s0004g04180	-452	Zinc finger (C2H2 type) protein (ZAT11) *
59,8	Promoter (<=1kb)	VIT_17s0000g06410	-525	MYB transcription factor MIXTA-like 2
59,52	Promoter (<=1kb)	VIT_06s0080g00970	-157	NAC domain-containing protein (VvNAC67)

Table 21: Top 20 (q-value based) represented direct target genes of VviNAC01. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

The most sequenced represented gene was an auxin-related gene, the *AUXIN-RESPONSIVE SAUR29* (VIT_16s0098g01150). Moreover, in agreement with what found in **Chapter 2**, the ethylene-related gene *ERF/AP2 GENE FAMILY VvERF064* (VIT_10s0003g00140) was found. Two transcription factors, *MYB TRANSCRIPTION FACTOR MIXTA-LIKE 2* (VIT_17s0000g06410) and *VviNAC67* (VIT_06s0080g00970), were also present in the list.

Afterwards, the list of the VviNAC01 targets was used to find all the possible *VviNACs* targets of the TF. 23 *VviNACs* target genes were found (**Table 22**), some of which harboring multiple VviNAC01 binding sites, *VviNAC29* (VIT_19s0027g00880), *VviNAC34* (VIT_12s0028g03050) and *VviNAC37* (VIT_10s0003g00350), for a total of 28 *Vvi*NAC01-*VviNACs* defined interactions. Intriguingly, between them *VviNAC01* itself was found, indicating an autoregulation of the TF. Moreover, *VviNAC08*, *VviNAC17*, *VviNAC39* and *VviNAC61* were also found.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
59,52	Promoter (<=1kb)	VIT_06s0080g00970	-157	NAC domain-containing protein (VvNAC67)
27,26	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
25,66	Promoter (<=1kb)	VIT_11s0016g02880	0	NAC domain-containing protein (VvNAC19)
23,31	Promoter (1-2kb)	VIT_13s0019g05240	-1179	NAC domain-containing protein (VvNAC20)
21,71	Promoter (<=1kb)	VIT_12s0028g03050	-353	NAC domain-containing protein (VvNAC34)
16,86	Promoter (1-2kb)	VIT_07s0031g02610	-1560	NAC domain-containing protein (VvNAC39)
15,38	Promoter (<=1kb)	VIT_10s0003g00350	0	NAC domain-containing protein (VvNAC37)
15,31	Promoter (<=1kb)	VIT_18s0001g10250	-5	NAC domain containing protein 19
14,8	Promoter (1-2kb)	VIT_14s0068g01490	-1124	NAC domain-containing protein (VvNAC73)
13,63	Promoter (<=1kb)	VIT_10s0003g00350	-905	NAC domain-containing protein (VvNAC37)
12,84	Promoter (<=1kb)	VIT_17s0000g06400	0	NAC domain-containing protein (VvNAC05)
9,34	Promoter (<=1kb)	VIT_12s0028g03050	-229	NAC domain-containing protein (VvNAC34)
8,79	Promoter (<=1kb)	VIT_01s0146g00280	-6	NAC domain-containing protein (VvNAC01)
8,12	Promoter (<=1kb)	VIT_18s0089g01120	-84	NAC domain-containing protein (VvNAC70)
7,79	Promoter (<=1kb)	VIT_13s0019g05230	-5	NAC domain-containing protein (VvNAC21)
7,34	Promoter (<=1kb)	VIT_08s0007g07660	0	NAC domain-containing protein (VvNAC60bis)
7	Promoter (<=1kb)	VIT_07s0005g03610	-885	NAC domain-containing protein (VvNAC56)
5,22	Promoter (1-2kb)	VIT_04s0044g01500	-1416	NAC domain-containing protein (VvNAC47)
3,69	Promoter (1-2kb)	VIT_04s0079g00280	-1293	NAC domain-containing protein (VvNAC64)
3,21	Promoter (<=1kb)	VIT_19s0014g03290	-502	NAC domain-containing protein (VvNAC17)
2,97	Promoter (1-2kb)	VIT_18s0072g01060	-1329	NAC domain-containing protein (VvNAC63)
2,63	Promoter (<=1kb)	VIT_10s0003g00350	-272	NAC domain-containing protein (VvNAC37)
2,59	Promoter (1-2kb)	VIT_02s0025g03020	-1632	NAC domain-containing protein (VvNAC25)
2,4	Promoter (<=1kb)	VIT_18s0001g02300	-327	NAC domain-containing protein (VvNAC08)
2,4	Promoter (<=1kb)	VIT_08s0007g02940	-106	NAC domain-containing protein (VvNAC62)
2,25	Promoter (<=1kb)	VIT_15s0048g02300	-246	NAC domain-containing protein (VvNAC53)
2,2	Promoter (<=1kb)	VIT_19s0027g00880	-819	NAC domain-containing protein (VvNAC29)
2,02	Promoter (<=1kb)	VIT_19s0027g00880	0	NAC domain-containing protein (VvNAC29)

Table 22: VviNAC01 DAP-seq *VviNACs* targets genes.

Then, the previously reported *VviNAC01* transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 20 correlated binding sites (15 unique genes), all of them resulted up regulated (**Table 23**); none of them were present between the top 20 reported genes.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE FC
45,37	Promoter (<=1kb)	VIT_03s0038g02680	-40	Zeta-carotene desaturase	1,88
27,53	Promoter (<=1kb)	VIT_12s0028g03270	-102	Ethylene-responsive transcription factor 9	5,48
15,32	Promoter (1-2kb)	VIT_18s0001g05250	-1274	Dehydration Responsive Element-Binding Transcription Factor (VvDREB27)	1,80
10,77	Promoter (1-2kb)	VIT_08s0058g00790	-1700	Secoisolariciresinol dehydrogenase	1,58
9,05	Promoter (<=1kb)	VIT_01s0026g00550	-883	Nodulin MtN21 family	2,07
7,79	Promoter (1-2kb)	VIT_13s0019g02280	-1890	Regulator of chromosome condensation (RCC1)	1,87
7,3	Promoter (<=1kb)	VIT_17s0000g04030	-74	Endonuclease	2,10
7,26	Promoter (<=1kb)	VIT_00s0620g00010	-685	Triacylglycerol lipase	2,82
6,5	Promoter (1-2kb)	VIT_13s0019g02280	-1444	Regulator of chromosome condensation (RCC1)	1,87
6,25	Promoter (<=1kb)	VIT_04s0023g00580	-172	Auxin-responsive SAUR32	2,19
5,52	Promoter (<=1kb)	VIT_03s0063g01790	0	Transducin protein	5,81
5,52	Promoter (<=1kb)	VIT_01s0026g01460	-238	Thioredoxin H-type 2 (Trx-H-2)	1,56
5,28	Promoter (<=1kb)	VIT_08s0058g00790	-911	Secoisolariciresinol dehydrogenase	1,58
4,03	Promoter (<=1kb)	VIT_01s0026g01460	-507	Thioredoxin H-type 2 (Trx-H-2)	1,56
3,76	Promoter (1-2kb)	VIT_08s0007g03530	-1095	Zf A20 and AN1 domain-containing stress-associated protein 4	2,00
3,67	Promoter (<=1kb)	VIT_18s0001g00560	-183	Alpha-amylase / 1,4-alpha-D-glucan glucanohydrolase	1,53
2,48	Promoter (1-2kb)	VIT_03s0063g01790	-1685	Transducin protein	5,81
2,25	Promoter (1-2kb)	VIT_11s0052g01210	-1084	Xyloglucan endotransglycosylase 6	1,84
2,2	Promoter (<=1kb)	VIT_17s0000g01010	-119	Lysophospholipase homolog	1,72
2,19	Promoter (<=1kb)	VIT_00s0620g00010	-145	Triacylglycerol lipase	2,82

Table 23: VviNAC01 DAP-seq targets genes which have a match in the *VviNAC01* transient over expression dataset.

At the first place *ZETA-CAROTENE DESATURASE* (VIT_03s0038g02680), which catalyzes the conversion of zeta-carotene to lycopene, was found and resulted up regulated by the *VviNAC01* transient over expression. An *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 9* (VIT_12s0028g03270), with a very high fold change of up-regulation, was also found in the list, reflecting the already reported *VviNAC01* role described in **Chapter 2**.

Moreover, with the highest fold change value, two binding sites on the *TRANSDUCIN PROTEIN* (VIT_03s0063g01790) were determined. Transducin proteins mediate diverse protein-protein interactions; they serve as multi-interacting platforms in cellular networks for the assembly of protein complexes or mediators of transient interplay among other proteins (Gachomo *et al.*, 2014).Transducin repeated domains have been reported to play central roles in biological processes such as cell division, apoptosis, light signalling and vision, flowering, floral development, meristem organization, protein trafficking, chromatin modification and transcriptional mechanism (Gachomo *et al.*, 2014).

VviNAC03

The DAP-seq assay reported 3461 binding sites (peaks), which were reduced to 3276 after the FC>5 filtering; even considering the very stringent filter used, the loss was very low, only the 5% (**Fig. 31**). The distribution of peaks revealed that 30% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 2% were in 5′ untranslated regions (UTRs), 20% were located in exons, 9% were located within introns, 3% were located in 3′ UTRs, 7% were located in the 3 kb downstream region, and 24% were intergenic.

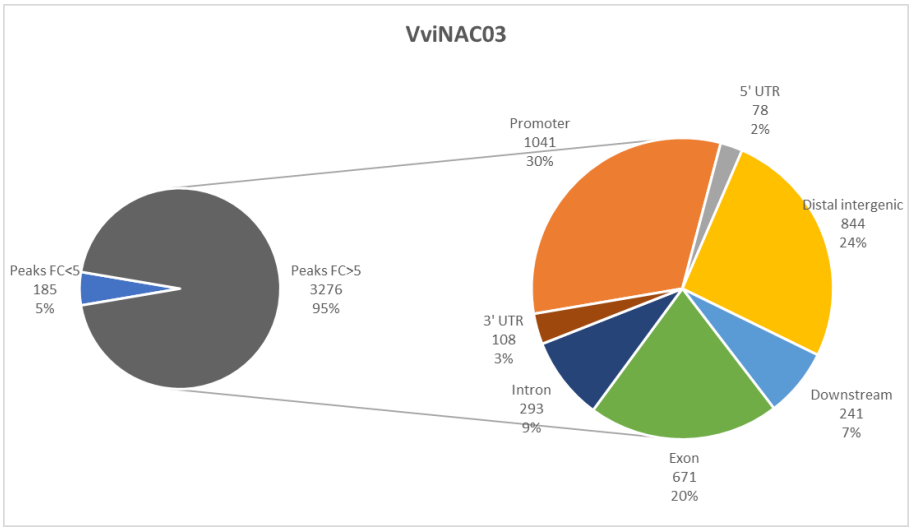

Figure 31: Double pie chart of the VviNAC03 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Three major binding motifs (GT[T/G][G/A]CGT[G/A][T/A]C) were identified with strong significance (**Fig. 32**) and the phylogenetic footprints correlate with ANAC047, which is involved in embryo development ending in seed dormancy, positive regulation of ethylene biosynthetic process and regulation of transcription (https://www.arabidopsis.org/servlets/TairObject?type=locus&name=At3g04070). Moreover, a recent work reported that ANAC047 plays a decisive role in regulating root waterlogging-induced leaf movement by directly or indirectly stimulating localized cell expansion at the abaxial petiole side through ethylene formation (Rauf *et al.*, 2013).

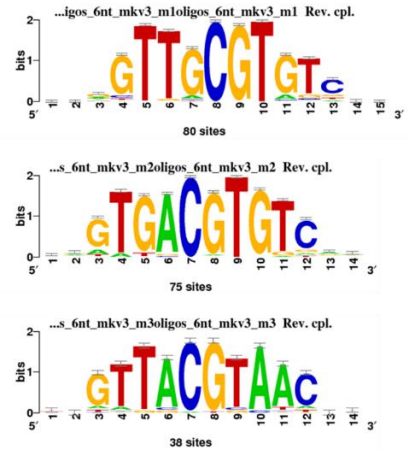

Figure 32: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC03 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 32% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 1119 sites; this percentage was even higher than the one found in other DAP-seq published papers.

After the elimination of all the 'no hit' and 'unknown' elements, 909 binding sites were obtained for a total of 870 unique genes regulated by VviNAC03.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to the general categories of regulation of cellular and biological processes, but also genes important for the cellular differentiation, the homeostatic processes, localization, transport and the response to stimulus (**Fig. 33**).

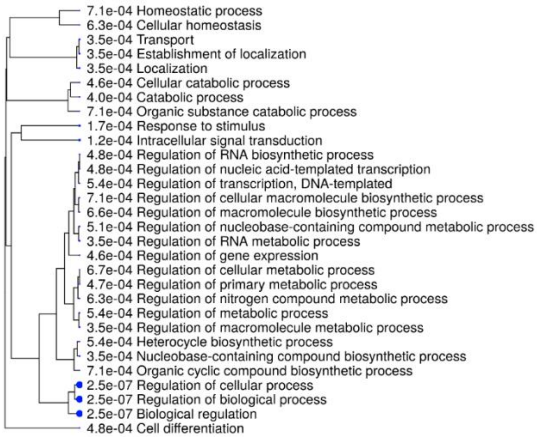

Figure 33: GO enrichment analysis of the VviNAC03 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 24**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
22,62	Promoter (<=1kb)	VIT_15s0048g01740	-376	Growth-regulating factor 9
22,42	Promoter (1-2kb)	VIT_18s0001g14980	-1600	3-methyl-2-oxobutanoate dehydrogenase
20,39	Promoter (1-2kb)	VIT_05s0062g01120	-1261	PIN1
18,55	Promoter (1-2kb)	VIT_18s0001g09420	-1844	Progesterone 5-beta-reductase
18,16	5' UTR	VIT_17s0000g05610	502	Isopiperitenol dehydrogenase
18,04	Promoter (<=1kb)	VIT_05s0020g02310	0	Pyruvate,orthophosphate dikinase
16,94	Promoter (<=1kb)	VIT_17s0000g06410	-525	MYB transcription factor MIXTA-like 2
16,01	Promoter (1-2kb)	VIT_02s0025g04130	-1354	Rhomboid ATRBL2
16	Promoter (<=1kb)	VIT_05s0020g05060	-953	Cellulose synthase CSLG2
15,23	Promoter (<=1kb)	VIT_13s0019g00550	-32	Cofilin
14,08	Promoter (<=1kb)	VIT_07s0255g00110	-148	WD40
14,07	Promoter (<=1kb)	VIT_15s0048g02260	-118	Calcium-binding EF hand
14,01	Promoter (<=1kb)	VIT_01s0011g04760	-74	myb domain protein 4 (VvMybC2-L1)
13,31	Promoter (<=1kb)	VIT_10s0003g04650	-226	Nuclear transcription factor Y subunit A-7
12,99	Promoter (<=1kb)	VIT_16s0098g01830	0	Transcription factor jumonji (jmjC) domain-containing protein
12,16	Promoter (<=1kb)	VIT_17s0000g06970	-421	Diacylglycerol kinase 1
12,15	Promoter (<=1kb)	VIT_16s0050g00120	-973	B57
11,93	Promoter (<=1kb)	VIT_18s0001g14950	-544	EMB2170 (embryo defective 2170)
11,66	Promoter (<=1kb)	VIT_08s0058g00450	-775	Substrate carrier, Mitochondrial
11,51	Promoter (<=1kb)	VIT_05s0020g02160	0	Squamosa promoter-binding protein (VvSBP6)

Table 24: Top 20 (q-value based) represented direct target genes of VviNAC03.

The most sequenced represented gene was the *GROWTH-REGULATING FACTOR 9* (VIT_15s0048g01740), which is demonstrated to exerts a negative effect on leaf growth controlling the final leaf dimensions by restricting cell number in the leaf primordium, while the size of the leaf cells remains unaltered (Omidbakhshfard et al., 2018). Moreover, two *MYB* genes were found, *MYB TRANSCRIPTION FACTOR MIXTA-LIKE 2* (VIT_17s0000g06410) and *MYB DOMAIN PROTEIN 4 VvMybC2-L1* (VIT_01s0011g04760), together with other two transcription factors, *NUCEAR TRANSCRIPTION FACTOR Y SUBUNIT A-7* (VIT_10s0003g04650) and *jmjC* (VIT_16s0098g01830).

Another interesting gene was the *PIN1* (VIT_05s0062g01120), an auxin efflux facilitator which controls auxin transport and signalling, regulating plant growth and development (Kong *et al*., 2019).

Afterwards, the list of the VviNAC03 targets was used to find all the possible *VviNACs* targets of the TF. 2 *VviNACs* target genes were found (**Table 25**), *VviNAC05* (VIT_17s0000g06400) and *VviNAC73* (VIT_14s0068g01490).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
6,66	Promoter (1-2kb)	VIT_17s0000g06400	-1859	NAC domain-containing protein (VvNAC05)
2,82	Promoter (1-2kb)	VIT_14s0068g01490	-1111	NAC domain-containing protein (VvNAC73)

Table 25: VviNAC03 DAP-seq *VviNACs* targets genes.

Then, the *VviNAC03* transient over expression results (D'Incà, 2017) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 7 correlated binding sites (7 unique genes), 3 up regulated and 4 down regulated (**Table 26**); none of them were present between the top 20 reported genes.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
11,09	Promoter (1-2kb)	VIT_13s0019g02080	-1041	DNA-binding protein	-1,59
6,11	Promoter (<=1kb)	VIT_05s0062g00710	-879	UDP-glucose:flavonoid 7-O-glucosyltransferase	4,67
4,26	Promoter (<=1kb)	VIT_16s0022g01210	-797	myb domain protein 85	-3,24
3,71	Promoter (1-2kb)	VIT_16s0098g01350	-1315	Oligopeptide transporter 6	-1,75
3,7	Promoter (<=1kb)	VIT_13s0019g04060	-261	MLO1	1,82
3,18	Promoter (1-2kb)	VIT_00s0125g00180	-1318	Zinc finger (C3HC4-type ring finger)	-2,07
2,9	Promoter (1-2kb)	VIT_14s0006g02530	-1122	Non-specific lipid-transfer protein 2 (LTP 2)	2,54

Table 26: VviNAC03 DAP-seq targets genes which have a match in the *VviNAC03* transient over expression dataset.

With a high positive fold change and at second place in the list (q-value based), the *UDP-GLUCOSE:FLAVONOID 7-O-GLUCOSYLTRANSFERASE* (VIT_05s0062g00710) was found; glycosylation of flavonoids, which have roles in UV protection, pathogen defense and coloration, is usually mediated by the glycosyltransferases, which use UDP-sugars as the glycosyl donor (Kim *et al*., 2006). Also, a *MYB* gene was found down regulated, *MYB DOMAIN PROTEIN 85* (VIT_16s0022g01210), and *MLO1* (VIT_13s0019g04060), another plant immunity related gene (Acevedo-Garcia *et al*., 2014), resulted up regulated.

VviNAC08

The DAP-seq assay reported 150 binding sites (peaks), which were reduced to 116 after the FC>5 filtering; the loss was a little bit higher than the standard of the other assays, about the 23% (**Fig. 34**). The distribution of peaks revealed that 7% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 2% were in 5′ untranslated regions (UTRs), 5% were in exons, 4% were located within introns, no gene was located in 3′ UTRs, 1% were located in the 3 kb downstream region, and 58% were intergenic.

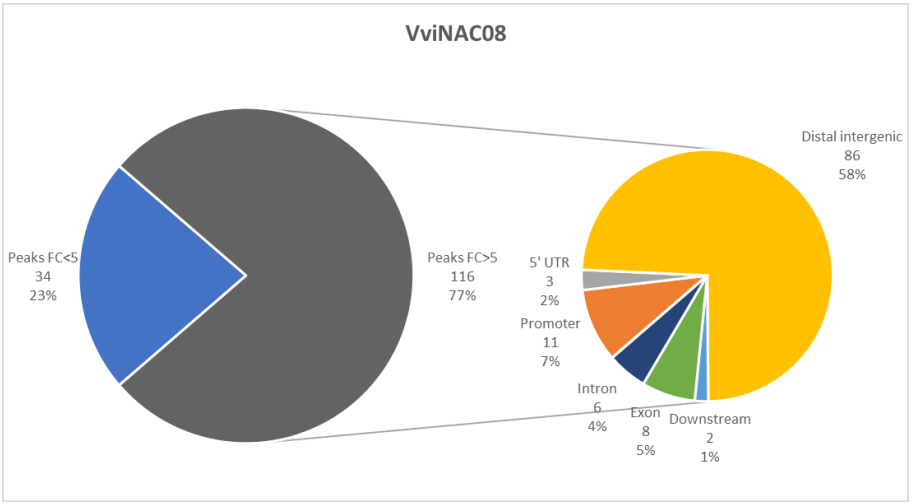

Figure 34: Double pie chart of the VviNAC08 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Due to the low number of sequenced reads, only one binding motif (CGGTACTC) was identified and not with a strong significance (**Fig. 35**); moreover, no phylogenetic footprint was found.

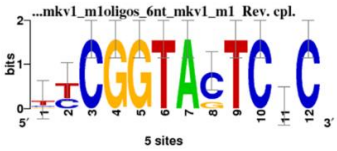

Figure 35: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC08 DAP-seq results. Top-ranking binding motif identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

Focusing the attention only on the 9% of the final analyzed binding sites, which

belongs to the ‘promoter’ and ‘5’UTR’ genic regions and presents a total of 14 sites, would not have been possible to perform further analyses. Indeed, this percentage is very low considering the what was normally found in the other DAP-seq assays. However, the DAP-seq was performed two times on this specific TF, and nothing changed; this means that the low number of binding sites reported is not due to a technical issue but could be related to a VviNAC08- specific folding difficulty during the *in vitro* translation or to the biological role of this TF. Trying to better investigate the cistrome landscape of VviNAC08, the GO enrichment analysis was performed on the whole FC>5 DAP-seq dataset (**Fig. 36**). The most represented categories were the one of regulation of biosynthetic processes; the aromatic compound biosynthetic processes, protein catabolic processes and ubiquitination, and electron transport chain categories were also found. Moreover, eliminating all the ‘no hit’ and ‘unknown’ elements, only 8 binding sites were obtained for a total of 8 unique genes regulated by VviNAC08 (**Table 27**). No matches were found between the DAP-seq and the transient over expression results and no *VviNAC* gene was found to be regulated by this TF. However, in the small final targets list an interesting gene was found: the *UDP-GLUCURONOSYL/UDP-GLUCOSYLTRANSFERASE* (VIT_06s0004g07280). Glycosyltransferases catalyze the transfer of a sugar portion from an activated sugar donor to acceptor molecules and this sugar conjugation results in increased stability and water solubility (Bonisch *et al*., 2014). In grapevine, several classes of phenylpropanoids, including flavonols, anthocyanidins, flavanones, flavones, isoflavones, a stilbene, simple phenols, and monoterpenols, were among the substrates glucosylated, indicating that glycosylation in grape has a big impact on the berries composition, which results in the wine flavor (Bonisch *et al*., 2014).

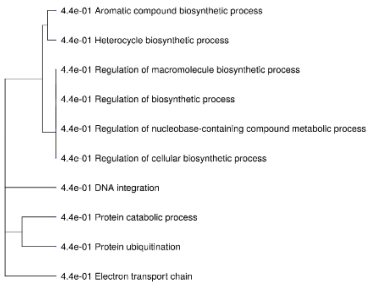

Figure 36: GO enrichment analysis of all the VviNAC08 targets. The analysis was performed on ShinyGO.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
662	Promoter (1-2kb)	VIT_18s0001g14980	-1668	3-methyl-2-oxobutanoate dehydrogenase
571	Promoter (<=1kb)	VIT_06s0004g07280	-103	UDP-glucuronosyl/UDP-glucosyltransferase
344	5' UTR	VIT_09s0054g00520	196	Membrane protein ycf1 (RF1)
238	Promoter (1-2kb)	VIT_09s0002g00280	-1021	Ubiquitin-conjugating enzyme E2 J2
237	Promoter (<=1kb)	VIT_00s2634g00010	-833	Receptor kinase RK20-1
221	5' UTR	VIT_13s0064g01680	112	RNA polymerase beta''
216	Promoter (<=1kb)	VIT_17s0000g09270	-803	MATE efflux family protein
214	Promoter (1-2kb)	VIT_03s0063g01470	-1137	Steroid nuclear receptor, ligand-binding

Table 27: Direct target genes of VviNAC08.

VviNAC11

The DAP-seq assay reported 2678 binding sites (peaks), which were reduced to 2459 after the FC>5 filtering; even considering the very stringent filter used, the loss was low, only the 8 (**Fig. 37**). The distribution of peaks revealed that 28% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 2% were in 5′ untranslated regions (UTRs), 12% were located in exons, 13% were located within introns, 3% were located in 3′ UTRs, 7% were located in the 3 kb downstream region, and 27% were intergenic.

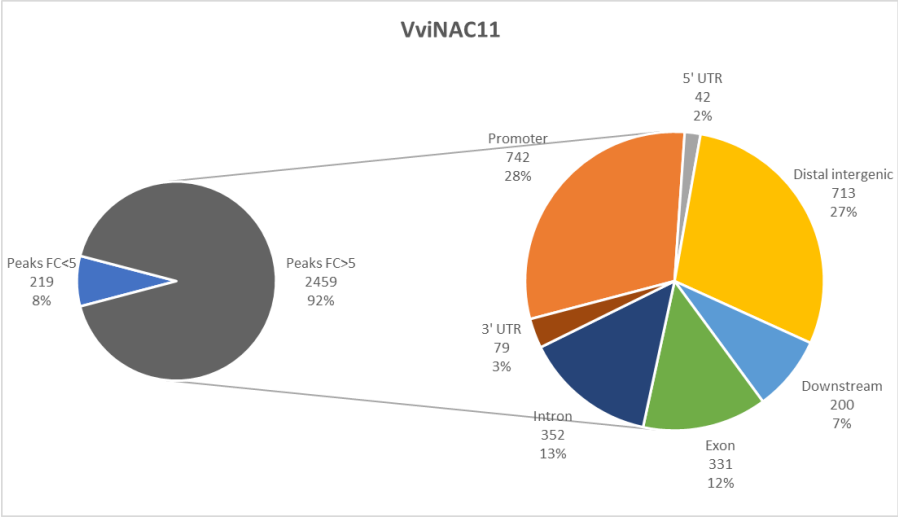

Figure 37: Double pie chart of the VviNAC11 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Two major binding motifs (GGTTGCGTGT and ACACGCAACCA) were identified with strong significance (**Fig. 38**) and the phylogenetic footprints correlate with ANAC092 and ANCA046, respectively. As said before, ANAC092

is considered the key positive regulator of leaf senescence (Kim *et al*., 2009); ANAC046 too is a positive regulator of chlorophyll degradation and senescence in *Arabidopsis* leaves (Oda-Yamamizo *et al*., 2016).

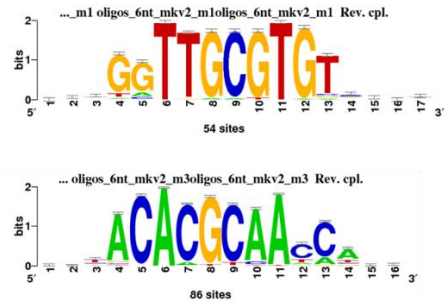

Figure 38: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC11 DAP-seq results. Two top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 30% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 784 sites. This percentage was perfectly in line with other DAP-seq published papers, even higher.

After the elimination of all the 'no hit' and 'unknown' elements, 640 binding sites were obtained for a total of 610 unique genes regulated by VviNAC11.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to the general categories of biological regulation and regulation of metabolic and biosynthetic processes; moreover, developmental and homeostatic processes genes were also highly represented (**Fig. 39**).

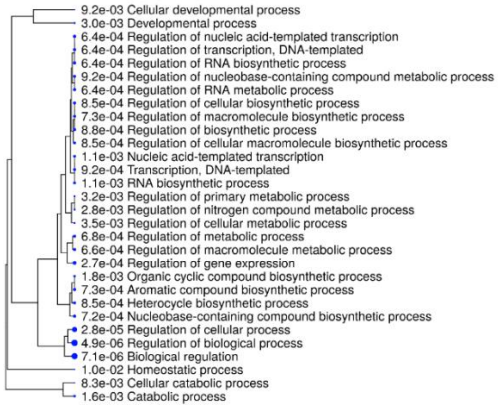

Figure 39: GO enrichment analysis of the VviNAC11 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 28**).

At the first place (q-value based) the *ARGININE_TRNA-PROTEIN TRANSFERASE 1 ATE1* (VIT_13s0067g00600) was found. It is involved in the post-translational conjugation of arginine to the N-terminal aspartate or glutamate of a protein. This arginylation is required for degradation of the protein via the ubiquitin pathway. Moreover, was also seen that the delayed-senescence phenotypes are associated with undetectable levels of *AtATE1* transcripts (Yoshida *et al*., 2002).

The *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 1* (VIT_02s0025g04660) is involved in chlorophyll and photosystem degradation.

VviWRKY52 (VIT_17s0000g01280) is associated with the transcriptional regulation of three genes putatively involved in the jasmonic acid signalling pathway and in the reduced susceptibility to downy mildew infection (Vannozzi *et al*., 2018); moreover, it is a red berry *switch* gene (Massonnet *et al*., 2017).

UDP-GLUCOSE:ISOFLAVONE 7-O-GLUCOSYLYTRANSFERASE (VIT_16s0050g01670), as said before, has a role in the coloration (Kim *et al*., 2006).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
28,52	Promoter (1-2kb)	VIT_13s0067g00600	-1194	Arginine-tRNA-protein transferase 1
26,82	Promoter (1-2kb)	VIT_16s0098g01150	-1172	**Auxin-responsive SAUR29 ***
26,73	Promoter (1-2kb)	VIT_17s0000g09210	-1926	No pollen (Osnop)
26,73	Promoter (<=1kb)	VIT_02s0025g04660	-164	Senescence-inducible chloroplast stay-green protein 1
26,21	Promoter (1-2kb)	VIT_18s0001g14980	-1615	3-methyl-2-oxobutanoate dehydrogenase
22,74	Promoter (<=1kb)	VIT_03s0038g00750	0	Ubiquitin fusion degradation protein UFD1
22,7	Promoter (<=1kb)	VIT_06s0061g01190	-122	SWI/SNF matrix-associated regulator of chromatin sbfamily A mber 3 2
21,81	Promoter (<=1kb)	VIT_13s0019g02180	0	Tropinone reductase
21,6	Promoter (<=1kb)	VIT_04s0044g00710	-159	UTP--glucose-1-phosphate uridylyltransferase *
21,31	Promoter (<=1kb)	VIT_05s0020g02310	-134	Pyruvate,orthophosphate dikinase
20,15	Promoter (<=1kb)	VIT_17s0000g01280	-76	**WRKY Transcription Factor (VvWRKY52)**
18,9	Promoter (<=1kb)	VIT_11s0052g00180	-746	Las1
17,03	Promoter (<=1kb)	VIT_12s0057g01420	-9	Exostosin
17,03	Promoter (<=1kb)	VIT_05s0020g02160	0	Squamosa promoter-binding protein (VvSBP6)
16,43	Promoter (1-2kb)	VIT_11s0118g00220	-1415	Chloride channel protein CLC
16,42	Promoter (<=1kb)	VIT_00s0207g00200	0	Integral membrane protein, putative
15,9	Promoter (1-2kb)	VIT_08s0058g00960	-1899	basic helix-loop-helix (bHLH) family
15,46	Promoter (1-2kb)	VIT_16s0050g01670	-1436	UDP-glucose:isoflavone 7-O-glucosyltransferase
14,56	Promoter (<=1kb)	VIT_18s0001g11990	-88	Methyltransferase-like protein 2, meth_7
13,71	Promoter (1-2kb)	VIT_09s0002g02490	-1105	Dof zinc finger protein 1

Table 28: Top 20 (q-value based) represented direct target genes of VviNAC11. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

Afterwards, the list of the VviNAC11 targets was used to find all the possible *VviNACs* targets of the TF. 6 *VviNACs* target genes were found (**Table 29**): *VviNAC19* (VIT_11s0016g02880), *VviNAC34* (VIT_12s0028g03050), *VviNAC37* (VIT_10s0003g00350), *VviNAC45* (VIT_08s0040g02110), *VviNAC47* (VIT_04s0044g01500) and, interestingly, *VviNAC61* (VIT_08s0007g07640).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
4,13	Promoter (<=1kb)	VIT_11s0016g02880	0	NAC domain-containing protein (VvNAC19)
3,26	Promoter (<=1kb)	VIT_12s0028g03050	-353	NAC domain-containing protein (VvNAC34)
3,25	Promoter (1-2kb)	VIT_04s0044g01500	-1416	NAC domain-containing protein (VvNAC47)
2,75	Promoter (<=1kb)	VIT_10s0003g00350	0	NAC domain-containing protein (VvNAC37)
2,55	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
2,05	Promoter (1-2kb)	VIT_08s0040g02110	-1157	NAC domain-containing protein (VvNAC45)

Table 29: VviNAC11 DAP-seq *VviNACs* targets genes.

Then, the previously reported *VviNAC11* transient over expression (D'Incà, 2017) results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 5 correlated binding sites (5 unique genes), all of them resulted up regulated with exception for *GROWTH-REGULATING FACTOR RELATED* (VIT_08s0007g06690) which was down regulated (**Table 30**); none of them were present between the top 20 reported genes.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
3,65	Promoter (<=1kb)	VIT_17s0000g03740	-443	Constans-like 16	1,86
3,42	Promoter (<=1kb)	VIT_07s0031g02780	-315	Cyclase/dehydrase	1,58
2,59	Promoter (<=1kb)	VIT_08s0007g06690	-975	Growth-regulating factor related	-1,65
2,54	Promoter (<=1kb)	VIT_05s0020g04040	-565	Chlorophyllase (CLH2)	2,14
2,22	Promoter (<=1kb)	VIT_06s0004g04000	-140	Beta-ketoacyl-CoA synthase	1,64

Table 30: VviNAC11 DAP-seq targets genes which have a match in the *VviNAC11* transient over expression dataset.

Concerning the up regulated genes, very informative features were found.

CONSTANS-LIKE 16 (VIT_17s0000g03740), which was already reported to enhance chlorophyll accumulation in petals and leaves (Ohmiya *et al*., 2019) was found at the first place (q-value based) of the list.

CHLOROPHYLLASE CLH2 (VIT_05s0020g04040), which catalyzes the hydrolysis of chlorophyll to chlorophyllide and phytol and possesses a predicted signal-peptide for chloroplast localization, was for a very long time considered as the first enzyme of chlorophylls breakdown during leaf senescence and fruit ripening (Schenk et al., 2007). However, a very recent study demonstrated that CLH2 is not involved in chlorophylls breakdown during senescence in *Arabidopsis* (Hu *et al*., 2020).

BETA-KETOACYL-COA SYNTHASE (VIT_06s0004g04000) represents the key enzyme in very-long-chain fatty acids biosynthesis (Wang *et al*., 2017). Knowing that cuticular waxes are complex mixtures of very-long-chain fatty acids and their derivatives, forming a natural barrier on aerial surfaces of terrestrial plants against biotic and abiotic stresses, this gene could be an interesting element in the berry

maturation mechanisms.

The only down regulated gene found, the *GROWTH-REGULATING FACTOR RELATED GRF* (VIT_08s0007g06690), is a plant-specific transcription factor, conserved in all land plants, which has growth-promoting activities in lateral organs; indeed, loss-of-function mutations of *GRFs* resulted in small narrow leaves and petals (Lee *et al.*, 2018).

VviNAC13

The DAP-seq assay reported 1670 binding sites (peaks), which were reduced to 1619 after the FC>5 filtering; even considering the very stringent filter used, the loss was very low, only the 3% (**Fig. 40**). The distribution of peaks revealed that 20% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 2% were in 5′ untranslated regions (UTRs), 11% were located in exons, 23% were located within introns, 2% were located in 3′ UTRs, 7% were located in the 3 kb downstream region, and 32% were intergenic.

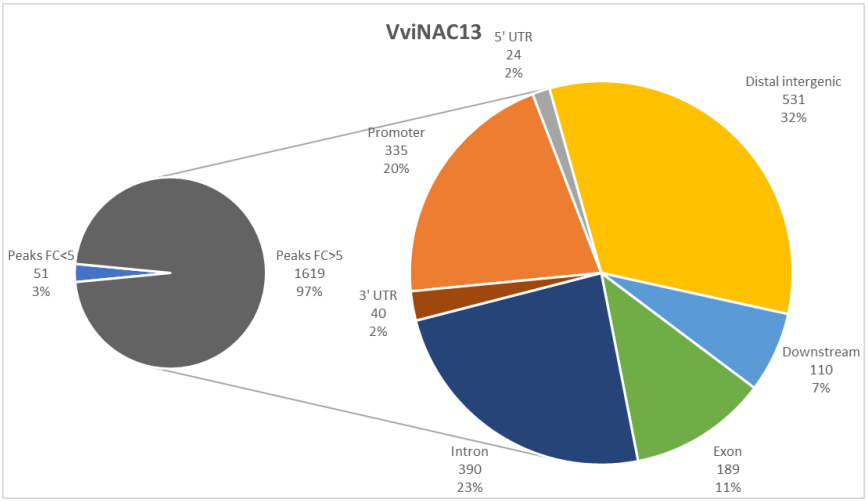

Figure 40: Double pie chart of the VviNAC13 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Three major binding motifs (TT[A/G/T]CTT[G/C]) were identified with strong significance (**Fig. 41**) and the phylogenetic footprints correlate with ANAC055, which may function as transcription activator to regulate jasmonic acid-induced

expression of defense genes (Bu *et al*., 2008).

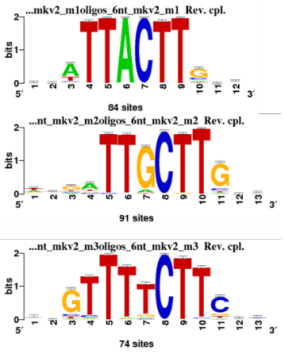

Figure 41: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC13 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 22% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 359 sites. This percentage is a little bit lower compared to the number of sites normally found in other DAP-seq published papers. After the elimination of all the 'no hit' and 'unknown' elements, 283 binding sites were obtained for a total of 275 unique genes regulated by VviNAC13. A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to regulation of biological and metabolic processes and to catabolic processes; moreover, the response to stimulus and to chitin, the lignin metabolic processes, the phenylpropanoid metabolic processes and the defense response to Gram negative bacterium categories were present (**Fig. 42**).

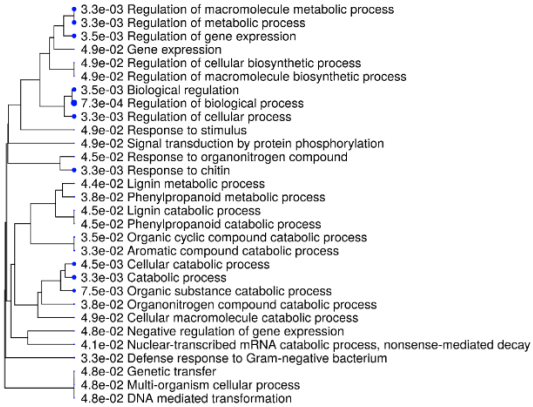

Figure 42: GO enrichment analysis of the VviNAC13 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 31**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
35,28	Promoter (<=1kb)	VIT_12s0057g01420	0	Exostosin
31,33	Promoter (1-2kb)	VIT_13s0067g00600	-1191	Arginine-tRNA-protein transferase 1
27,99	Promoter (<=1kb)	VIT_18s0001g08290	-172	Poly(ADP-ribose) polymerase *
25,06	Promoter (1-2kb)	VIT_01s0011g01300	-1061	Polygalacturonase QRT3
21,24	Promoter (1-2kb)	VIT_14s0066g00620	-1362	Chitinase, class III
21,23	Promoter (<=1kb)	VIT_07s0005g00310	-149	Zinc finger (CCCH type) helicase family protein
21,21	Promoter (<=1kb)	VIT_06s0061g01210	-619	Lysine histidine transporter 1
18,54	Promoter (1-2kb)	VIT_08s0007g04510	-1907	RPG related protein 1 RR1
18,19	Promoter (1-2kb)	VIT_09s0002g02490	-1114	Dof zinc finger protein 1
16,89	Promoter (<=1kb)	VIT_17s0000g06970	-438	Diacylglycerol kinase 1
16,8	Promoter (<=1kb)	VIT_06s0061g01190	-132	SWI/SNF matrix-associated regulator of chromatin sbfamily A mber 3 2
14,32	Promoter (1-2kb)	VIT_18s0001g14980	-1605	3-methyl-2-oxobutanoate dehydrogenase
13,97	Promoter (<=1kb)	VIT_08s0058g01360	-96	Vacuolar sorting receptor 1
13,82	Promoter (<=1kb)	VIT_01s0011g05210	-965	Phosphoglucomutase
13,78	Promoter (<=1kb)	VIT_18s0122g00500	0	Basic Leucine Zipper Transcription Factor (VvbZIP41)
13,55	Promoter (1-2kb)	VIT_13s0019g05240	-1179	NAC domain-containing protein (VvNAC20)
13,52	Promoter (<=1kb)	VIT_12s0142g00130	-530	Mitogen-activated Protein Kinase (VvMPK8)
12,95	Promoter (<=1kb)	VIT_14s0068g01710	0	Metal-nicotianamine transporter YSL6
12,2	Promoter (<=1kb)	VIT_18s0001g09230	-761	Salt tolerance zinc finger
11,6	Promoter (1-2kb)	VIT_06s0009g02500	-1409	Plastocyanin domain-containing protein

Table 31: Top 20 (q-value based) represented direct target genes of VviNAC13. The asterisk (*) refers to the transition markers.

The most sequenced represented gene was the *EXOSTOSIN* (VIT_12s0057g01420), which is an animal glycosyltransferase that catalyze the synthesis a glycosaminoglycan with many roles in cell differentiation and development; this also suggests that the plant cell wall, as the animal extracellular matrix, is synthesized by evolutionarily related enzymes even though the structures of the polysaccharides are different from each other (Madson *et al*., 2003).

ARGININE-TRNA-PROTEIN TRANSFERASE 1 (VIT_13s0067g00600), described before as senescence phenotypes associated (Yoshida *et al*., 2002), was again found.

Chitinases constitute a group of defense molecules for which a direct activity against pathogens has been demonstrated. In the DAP-seq list a *CHITINASE, CLASS III* (VIT_14s0066g00620) was found; class III chitinases possess a similar domain to the prokaryotic chitinases one and display lysozyme activity (Robert *et al*., 2002).

A *BASIC LEUCINE ZIPPER TRANSCRIPTION FACTOR VvbZIP41* (VIT_18s0122g00500) was found. bZIP transcription factors are candidates for the regulation of ABA-mediated fruit ripening. Indeed, in a recent study was seen that the bZIP *VvABF2* was ubiquitously expressed in different grape organs and its transcript accumulated just before the onset of grape berry ripening, when ABA concentrations increase (Nicolas *et al*., 2014).

Moreover, was very interesting to see that in the top 20 list *VviNAC20* (VIT_13s0019g05240) was present.

Afterwards, the list of the VviNAC13 targets was used to find all the possible *VviNACs* targets of the TF.
6 *VviNACs* target genes were found (**Table 32**): *VviNAC19* (VIT_11s0016g02880), *VviNAC20* (VIT_13s0019g05240) and *VviNAC21* (VIT_13s0019g05230), VviNAC34 (VIT_12s0028g03050) and VviNAC37 (VIT_10s0003g00350). *VviNAC37* harbors multiple *VviNAC13* binding sites.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
13,55	Promoter (1-2kb)	VIT_13s0019g05240	-1179	NAC domain-containing protein (VvNAC20)
9,64	Promoter (<=1kb)	VIT_10s0003g00350	0	NAC domain-containing protein (VvNAC37)
9,17	Promoter (<=1kb)	VIT_13s0019g05230	-3	NAC domain-containing protein (VvNAC21)
8,71	Promoter (<=1kb)	VIT_11s0016g02880	0	NAC domain-containing protein (VvNAC19)
6,76	Promoter (<=1kb)	VIT_12s0028g03050	-200	NAC domain-containing protein (VvNAC34)
2,3	Promoter (<=1kb)	VIT_10s0003g00350	-926	NAC domain-containing protein (VvNAC37)

Table 32: VviNAC13 DAP-seq *VviNACs* targets genes.

Then, the D'Incà (2017) reported *VviNAC13* transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 4 correlated binding sites (4 unique genes, **Table 33**); none of them were present between the top 20 reported genes.
The two up regulated genes were *ZINC METALLOPROTEASE PITRILYSIN SUB A* (VIT_03s0063g00680) and *FERREDOXIN* (VIT_04s0008g04240), whereas the down regulated one were *HEAT SHOCK PROTEIN 70* (VIT_13s0019g01430) and *RETROTRANSPOSON PROTEIN, UNCLASSIFIED* (VIT_18s0075g00520).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
3,2	Promoter (<=1kb)	VIT_13s0019g01430	-178	Heat shock protein 70	-1,62
2,63	Promoter (<=1kb)	VIT_03s0063g00680	0	Zinc metalloprotease pitrilysin sub A	1,54
2,43	Promoter (<=1kb)	VIT_04s0008g04240	-935	ferredoxin	1,95
2,32	Promoter (<=1kb)	VIT_18s0075g00520	0	Retrotransposon protein, Unclassified	-2,21

Table 33: VviNAC13 DAP-seq targets genes which have a match in the *VviNAC13* transient over expression dataset.

VviNAC15

The DAP-seq assay reported 3461 binding sites (peaks), which were reduced to 3321 after the FC>5 filtering; even considering the very stringent filter used, the loss was very low, only the 4% (**Fig. 43**). The distribution of peaks revealed that 20% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 2% were in 5′ untranslated regions (UTRs), 17% were located in exons, 20% were located within introns, 2% were located in 3′ UTRs, 6% were located in the 3 kb downstream region, and 29% were intergenic.

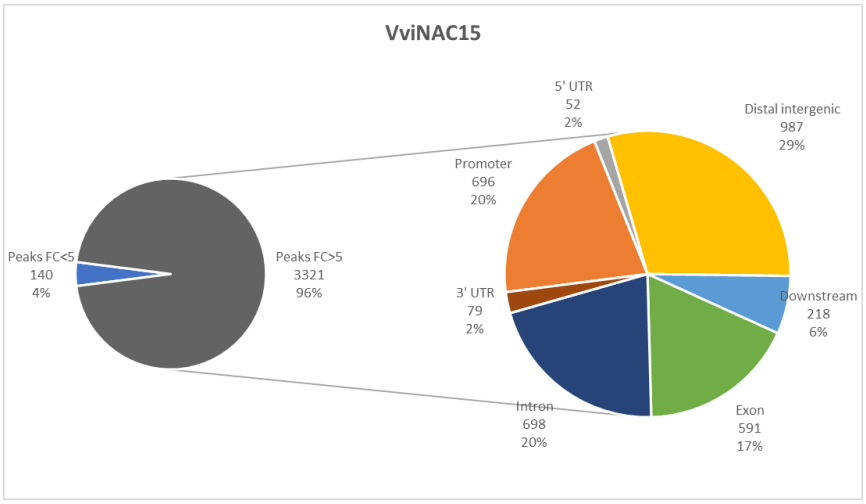

Figure 43: Double pie chart of the VviNAC15 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Three major binding motifs (TT[A/T]CTTG) were identified with strong significance (**Fig. 44**) and the phylogenetic footprints correlate with ANAC076, which is also known as VND2 and belongs to a group of NAC domain transcription factors that function as master regulators of xylem vessel element differentiation in cotyledons (Tan *et al.*, 2018).

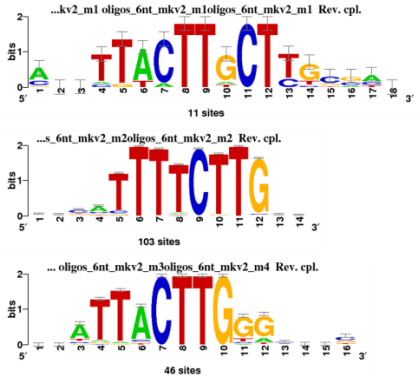

Figure 44: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC15 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 22% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 748 sites. This percentage is a little bit lower compared to the number of sites normally found in other DAP-seq published papers.

After the elimination of all the 'no hit' and 'unknown' elements, 590 binding sites were obtained for a total of 573 unique genes regulated by VviNAC15.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to regulation of gene expression, metabolic (also aromatic compound) and cellular processes (**Fig. 45**).

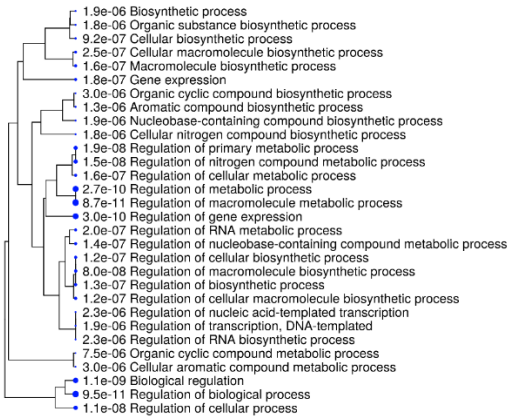

Figure 45: GO enrichment analysis of the VviNAC15 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 34**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
78,08	Promoter (1-2kb)	VIT_18s0001g14980	-1618	3-methyl-2-oxobutanoate dehydrogenase
46,74	Promoter (<=1kb)	VIT_06s0004g04180	-455	Zinc finger (C2H2 type) protein (ZAT11)
37,53	Promoter (<=1kb)	VIT_17s0000g01280	-68	**WRKY Transcription Factor (VvWRKY52)**
37,15	Promoter (<=1kb)	VIT_12s0035g00650	-293	Zinc Finger Homeodomain Transcription Factor (VvZHD5)
36,55	Promoter (1-2kb)	VIT_17s0000g09890	-1345	Tetratricopeptide repeat (TPR)-containing
31,77	Promoter (<=1kb)	VIT_12s0028g02510	-2	DNA-binding bromodomain-containing protein
31,76	Promoter (<=1kb)	VIT_13s0019g00840	0	UDP-glucuronate decarboxylase.
31,03	Promoter (<=1kb)	VIT_16s0050g00700	-198	Zinc finger (C2H2 type) family
29,21	Promoter (1-2kb)	VIT_06s0004g07030	-1246	MRH1 (morphogenesis of root hair 1)
26	Promoter (1-2kb)	VIT_06s0004g08190	-1315	ERF/AP2 Gene Family (VvERF055)
25,45	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
24,42	Promoter (<=1kb)	VIT_18s0001g14960	-324	Pentatricopeptide (PPR) repeat-containing
24,22	Promoter (1-2kb)	VIT_10s0003g00390	-1237	Glutaredoxin
19,83	Promoter (<=1kb)	VIT_09s0070g00210	0	Metal transporter Nramp1
19,83	Promoter (<=1kb)	VIT_03s0038g00750	0	Ubiquitin fusion degradation protein UFD1
19,66	Promoter (<=1kb)	VIT_04s0008g07010	0	Dynamin-like protein 2b
19,05	Promoter (<=1kb)	VIT_10s0042g01180	-30	ARGONAUTE 2 (AGO2)
18,79	Promoter (1-2kb)	VIT_11s0016g00350	-1111	Sec34/COG3
18,23	Promoter (<=1kb)	VIT_17s0000g01920	-595	Nuclear transcription factor, X-box binding 1
17,94	Promoter (1-2kb)	VIT_13s0067g01740	-1194	Receptor protein kinase

Table 34: Top 20 (q-value based) represented direct target genes of VviNAC15. In bold are reported the *switch* genes.

An ethylene-related gene, *ERF/AP2 GENE FAMILY VvERF055* (VIT_06s0004g08190), was found. Moreover, many transcription factors were present in the list as two *ZINC FINGER* (VIT_06s0004g04180, VIT_12s0035g00650 and VIT_16s0050g00700) and a *NUCLEAR TRANSCRIPTION FACTOR, X-BOX BINDING 1* (VIT_17s0000g01920); however, the main interest is upon *VviWRKY52* (VIT_17s0000g01280), an already mentioned red berry *switch* (Massonnet *et al*., 2017), known to be involved in the jasmonic acid signalling pathway (Vannozzi *et al*., 2018), and one of the selected *VviNAC* family member, *VviNAC61* (VIT_08s0007g07640).

Afterwards, the list of the VviNAC15 targets was used to find all the possible *VviNACs* targets of the TF. 12 *VviNACs* target genes were found (**Table 35**) and, interestingly, two of the selected *VviNACs* were in this group, *VviNAC39 (VIT_07s0031g02610)* and, as said before, *VviNAC61 (VIT_08s0007g07640)*.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
25,45	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
17,55	Promoter (<=1kb)	VIT_12s0028g03050	-203	NAC domain-containing protein (VvNAC34)
14,99	Promoter (1-2kb)	VIT_13s0019g05240	-1176	NAC domain-containing protein (VvNAC20)
12,25	Promoter (<=1kb)	VIT_11s0016g02880	0	NAC domain-containing protein (VvNAC19)
7,52	Promoter (<=1kb)	VIT_10s0003g00350	-3	NAC domain-containing protein (VvNAC37)
5,53	Promoter (<=1kb)	VIT_15s0048g02300	-177	NAC domain-containing protein (VvNAC53)
5,02	Promoter (<=1kb)	VIT_15s0048g02320	-280	NAC domain-containing protein (VvNAC51)
4,63	Promoter (1-2kb)	VIT_07s0031g02610	-1604	NAC domain-containing protein (VvNAC39)
3,37	Promoter (<=1kb)	VIT_16s0098g00760	0	NAC domain-containing protein (VvNAC71)
3,08	Promoter (<=1kb)	VIT_15s0048g02290	-308	NAC domain-containing protein (VvNAC54)
2,31	Promoter (<=1kb)	VIT_18s0001g10250	-14	NAC domain containing protein 19
2,1	Promoter (<=1kb)	VIT_06s0080g00970	-191	NAC domain-containing protein (VvNAC67)

Table 35: VviNAC15 DAP-seq *VviNACs* targets genes.

Then, the already reported *VviNAC15* transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 27 correlated binding sites (27 unique genes, **Table 36**).

3 of these genes, all up regulated, were also present between the top 20 reported genes: *VviWRKY52* (VIT_17s0000g01280), *VviNAC61* (VIT_08s0007g07640) and *METAL TRANSPORTER NRAMP1* (VIT_09s0070g00210).

For the last one, unfortunately, not much information is present in literature; one single study has correlated its role in plants growth under low manganese conditions (Cailliatte et al., 2010).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
37,53	Promoter (<=1kb)	VIT_17s0000g01280	-68	**WRKY Transcription Factor (VvWRKY52)**	1,94
25,45	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)	1,56
19,83	Promoter (<=1kb)	VIT_09s0070g00210	0	Metal transporter Nramp1	2,26
16,4	Promoter (<=1kb)	VIT_17s0000g07670	-240	Betaine-aldehyde dehydrogenase	-1,59
14,15	Promoter (<=1kb)	VIT_07s0104g00150	0	Outer envelope membrane protein	-1,64
10,37	Promoter (<=1kb)	VIT_13s0019g05200	-667	MATE efflux family protein	2,36
9,5	Promoter (<=1kb)	VIT_05s0020g04130	-270	Perakine reductase aldo/keto reductase *	2,06
8,79	Promoter (1-2kb)	VIT_04s0023g00700	-1553	Serine carboxypeptidase S10	2,10
8,24	Promoter (<=1kb)	VIT_16s0098g00890	-737	Harpin-induced protein	1,55
7,24	Promoter (<=1kb)	VIT_13s0019g02180	0	Tropinone reductase	3,24
5,74	Promoter (<=1kb)	VIT_07s0005g01760	-183	Glycerol-3-phosphate acyltransferase 3 (AtGPAT3)	-1,95
5,49	Promoter (1-2kb)	VIT_01s0026g01050	-1888	Myb family *	1,91
5,29	Promoter (<=1kb)	VIT_12s0059g02510	-96	**Zinc finger (B-box type)**	2,29
5,17	Promoter (<=1kb)	VIT_14s0219g00120	-502	PQ-loop repeat / transmembrane	1,79
5,16	Promoter (<=1kb)	VIT_13s0019g02160	-275	Laccase	-1,72
5,07	Promoter (<=1kb)	VIT_06s0004g05710	0	Glutathione S-transferase GSTU7	-1,74
4,02	Promoter (<=1kb)	VIT_11s0016g02800	-138	Myo-inositol oxygenase	1,83
3,92	Promoter (<=1kb)	VIT_13s0019g01430	-181	Heat shock protein 70	1,66
3,92	Promoter (1-2kb)	VIT_13s0019g01940	-1220	Laccase	-2,13
3,11	Promoter (<=1kb)	VIT_07s0005g03470	-84	Structural maintenance of chromosomes (SMC1)	1,65
2,85	Promoter (<=1kb)	VIT_18s0001g11740	-299	Ring zinc finger ariadne protein ARI2	-1,76
2,37	Promoter (1-2kb)	VIT_10s0003g03490	-1640	GA 2-oxidase	5,13
2,3	Promoter (1-2kb)	VIT_15s0046g00740	-1068	Tetratricopeptide repeat (TPR)-containing	1,81
2,27	Promoter (1-2kb)	VIT_08s0007g02860	-1373	RNA-binding protein	1,54
2,17	Promoter (<=1kb)	VIT_18s0157g00020	0	GIGANTEA protein	1,65
2,1	Promoter (1-2kb)	VIT_01s0011g06650	-1820	Beta-Ig-H3/fasciclin	2,42
2,1	Promoter (<=1kb)	VIT_07s0197g00080	-598	SNF1 protein kinase 2-3 AKIP OST1	-2,14

Table 36: VviNAC15 DAP-seq targets genes which have a match in the VviNAC15 transient over expression dataset. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

Moreover, in the list an up regulated *MYB* gene, *MYB FAMILY* (VIT_01s0026g01050) was present and two down regulated *LACCASE* (VIT_13s0019g02160 and VIT_13s0019g01940), which have a role in the flavonoid biosynthesis in grapes (Braidot *et al.*, 2008).

VviNAC17

The DAP-seq assay reported 8599 binding sites (peaks), which were reduced to 8307 after the FC>5 filtering; even considering the very stringent filter used, the loss was very low, only the 3% (**Fig. 46**). The distribution of peaks revealed that

29% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 2% were in 5′ untranslated regions (UTRs), 14% were located in exons, 12% were located within introns, 3% were located in 3′ UTRs, 8% were located in the 3 kb downstream region, and 29% were intergenic.

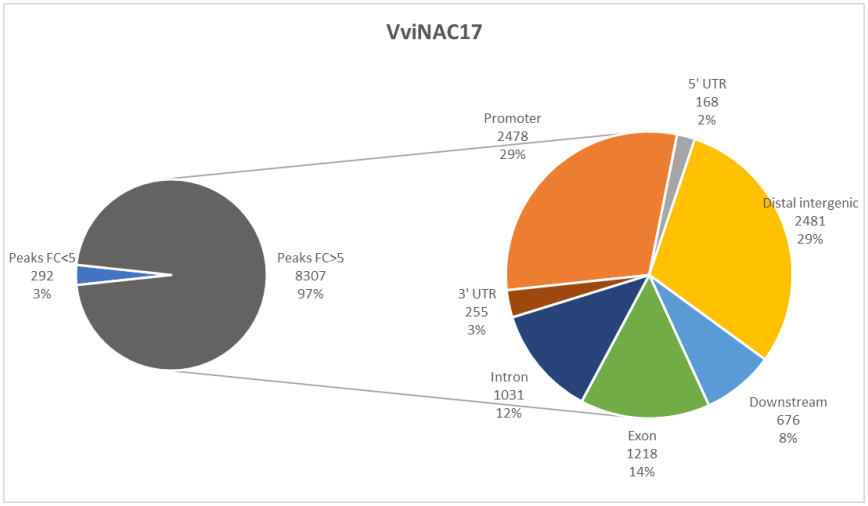

Figure 46: Double pie chart of the VviNAC17 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Three major binding motifs ([T/A][T/G][A/C]CGT[G/A]) were identified with strong significance (**Fig. 47**) and the phylogenetic footprints correlate with ANAC055, which may function as transcription activator to regulate jasmonic acid-induced expression of defense genes (Bu *et al*., 2008).

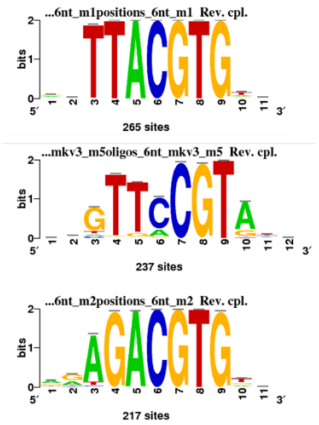

Figure 47: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC17 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 31% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 2646 sites. This was a very high percentage, compared to the number of sites normally found in other DAP-seq published papers.

After the elimination of all the 'no hit' and 'unknown' elements, 2149 binding sites were obtained for a total of 1913 unique genes regulated by VviNAC17.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to regulation of biological and metabolic processes, aromatic compound biosynthesis, response to stimuli and transmembrane transport (**Fig. 48**).

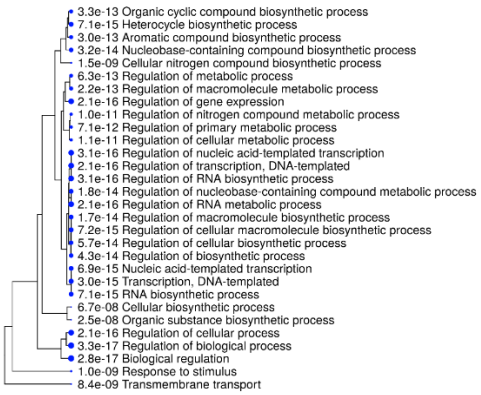

Figure 48: GO enrichment analysis of the VviNAC17 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 37**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
14868	Promoter (<=1kb)	VIT_15s0048g01740	-347	Growth-regulating factor 9
10149	Promoter (<=1kb)	VIT_19s0177g00070	-529	Polyubiquitin (UBQ14)
7108	Promoter (1-2kb)	VIT_18s0041g02010	-1916	12-oxophytodienoate reductase 1
5621	Promoter (<=1kb)	VIT_05s0020g02310	0	Pyruvate,orthophosphate dikinase
5442	Promoter (<=1kb)	VIT_17s0000g06970	-412	Diacylglycerol kinase 1
5223	Promoter (1-2kb)	VIT_18s0001g14980	-1611	3-methyl-2-oxobutanoate dehydrogenase
5028	Promoter (<=1kb)	VIT_11s0016g03000	-20	Phosphoinositide 3-kinase regulatory subunit 4
4861	5' UTR	VIT_03s0038g04390	24	**Dehydrin 1**
4601	Promoter (<=1kb)	VIT_19s0015g02950	0	Secologanin synthase CYP72A1
4515	5' UTR	VIT_17s0000g05610	473	Isopiperitenol dehydrogenase
4402	Promoter (<=1kb)	VIT_17s0000g01280	-77	**WRKY Transcription Factor (VvWRKY52)**
4384	Promoter (<=1kb)	VIT_05s0062g00710	-892	UDP-glucose:flavonoid 7-O-glucosyltransferase
4304	Promoter (<=1kb)	VIT_05s0077g00220	0	Expansin A4
4256	Promoter (<=1kb)	VIT_07s0031g00100	0	flavonol synthase
4202	Promoter (<=1kb)	VIT_00s0207g00200	0	Integral membrane protein, putative
4158	Promoter (<=1kb)	VIT_01s0011g04760	-66	myb domain protein 4 (VvMybC2-L1)
4122	5' UTR	VIT_09s0002g04290	40	Hydroxyphenylpyruvate reductase (HPPR)
4064	Promoter (<=1kb)	VIT_07s0255g00110	-146	WD40
3742	Promoter (<=1kb)	VIT_14s0006g02620	-273	Cgi67 serine protease *
3648	Promoter (<=1kb)	VIT_07s0005g01240	-594	Triacylglycerol lipase

Table 37: Top 20 (q-value based) represented direct target genes of VviNAC17. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

As the most represented gene the *GROWTH-REGULATING FACTOR 9* (VIT_15s0048g01740) was found; it controls the final leaf dimensions by restricting cell number in the leaf primordium (Omidbakhshfard et al., 2018).
In the list two red berry *switch*es were found (Massonnet *et al*., 2017), the *DEHYDRIN 1* (VIT_03s0038g04390), which is related to the acquisition of desiccation tolerance in plants and is generally accumulated during the late stages of seed development (Liu et al., 2017) and the many times presented *VviWRKY52* (VIT_17s0000g01280).
Moreover, a MYB gene, *MYB DOMAIN PROTEIN 4 VvMybC2-L1* (VIT_01s0011g04760) and *UDP-GLUCOSE:FLAVONOID 7-O-GLUCOSYLTRANSFERASE* (VIT_05s0062g00710), which have roles in UV protection, pathogen defense and coloration (Kim *et al*., 2006), were also important features.

Afterwards, the list of the VviNAC17 targets was used to find all the possible *VviNACs* targets of the TF.
10 *VviNACs* target genes were found (**Table 38**). Interestingly, between them also *VviNAC08* (VIT_18s0001g02300) and *VviNAC61* (VIT_08s0007g07640) were found.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
3045	Promoter (1-2kb)	VIT_14s0068g01490	-1125	NAC domain-containing protein (VvNAC73)
1869	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
853	Promoter (1-2kb)	VIT_17s0000g06400	-1911	NAC domain-containing protein (VvNAC05)
663	Promoter (<=1kb)	VIT_06s0080g00970	-155	NAC domain-containing protein (VvNAC67)
652	Promoter (1-2kb)	VIT_13s0019g05240	-1180	NAC domain-containing protein (VvNAC20)
423	Promoter (1-2kb)	VIT_04s0044g01500	-1417	NAC domain-containing protein (VvNAC47)
253	Promoter (<=1kb)	VIT_12s0055g00510	-271	NAC domain-containing protein (VvNAC58)
208	Promoter (<=1kb)	VIT_15s0048g02300	-245	NAC domain-containing protein (VvNAC53)
204	Promoter (<=1kb)	VIT_18s0001g10250	-81	NAC domain containing protein 19
204	Promoter (<=1kb)	VIT_18s0001g02300	-29	NAC domain-containing protein (VvNAC08)

Table 38: VviNAC17 DAP-seq *VviNACs* targets genes.

Afterwards, the already reported *VviNAC17* transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 19 correlated binding sites (17 unique genes, **Table 39**).
PIN1 (VIT_05s0062g01120), present with two VviNAC17 binding sites on its promoting region, is related to the auxin transport and signalling (Kong *et al*., 2019).

Also, a *VviWRKY* gene was present in the list, *VviWRKY45* (VIT_14s0108g01280); unfortunately, no information is available concerning this TF. However, *WRKY* gene family is well known to be involved in the regulation of development and senescence (Wang et al., 2014).

Moreover, the presence of *ALLENE OXIDE SYNTHASE - JASMONATES FROM FATTY ACIDS* (VIT_18s0001g11630), which also resulted up regulated, confirm the correlation with ANAC055, found with the motif analysis.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
2027	Promoter (1-2kb)	VIT_13s0019g02080	-1368	DNA-binding protein	1,52
1775	Promoter (1-2kb)	VIT_13s0019g02080	-1042	DNA-binding protein	1,52
1732	Promoter (<=1kb)	VIT_16s0100g00010	-878	Carboxyesterase 20 CXE20	1,68
1473	Promoter (<=1kb)	VIT_01s0011g06280	-356	Oxidoreductase	1,50
1398	Promoter (1-2kb)	VIT_05s0062g01120	-1260	PIN1	1,80
983	Promoter (<=1kb)	VIT_09s0002g06970	-148	**Palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, chloroplast**	-1,94
839	Promoter (<=1kb)	VIT_05s0020g01670	-430	GTP1/OBG	-1,53
599	Promoter (<=1kb)	VIT_18s0001g11630	-304	Allene oxide synthase (jasmonates from fatty acids)	2,63
424	5' UTR	VIT_08s0007g04550	119	NCS1 nucleoside transporter family protein	-1,77
357	Promoter (<=1kb)	VIT_10s0116g01730	-138	Soluble starch synthase 3, chloroplast precursor	-1,68
331	5' UTR	VIT_08s0007g00920	12	Tropinone reductase	-2,00
276	Promoter (<=1kb)	VIT_05s0062g01120	-708	PIN1	1,80
253	Promoter (1-2kb)	VIT_14s0108g01280	-1509	WRKY DNA-binding protein 4 (WRKY-18), WRKY Transcription Factor (VvWRKY45) *	1,51
253	5' UTR	VIT_04s0008g07310	14	AarF domain-containing kinase ABC1	-1,64
244	Promoter (<=1kb)	VIT_16s0050g02520	0	RNA polymerase sigma subunit SigE (sigE)	-1,65
242	Promoter (<=1kb)	VIT_09s0018g01800	-55	Acid phosphatase	-3,06
241	Promoter (1-2kb)	VIT_11s0016g02350	-1639	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE	-1,75
204	Promoter (<=1kb)	VIT_14s0066g00160	-655	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE *	-1,90
203	Promoter (<=1kb)	VIT_07s0129g01000	-616	F-box family protein	1,66

Table 39: VviNAC17 DAP-seq targets genes which have a match in the *VviNAC17* transient over expression dataset. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

VviNAC18

The DAP-seq assay reported 1409 binding sites (peaks), which were reduced to 1233 after the FC>5 filtering; this time the loss was higher, about the 12% (**Fig. 49**). The distribution of peaks revealed that 29% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 3% were in 5′ untranslated regions (UTRs), 15% were located in exons, 8% were located within introns, 3% were located in 3′ UTRs, 6% were located in the 3 kb downstream region, and 24% were intergenic.

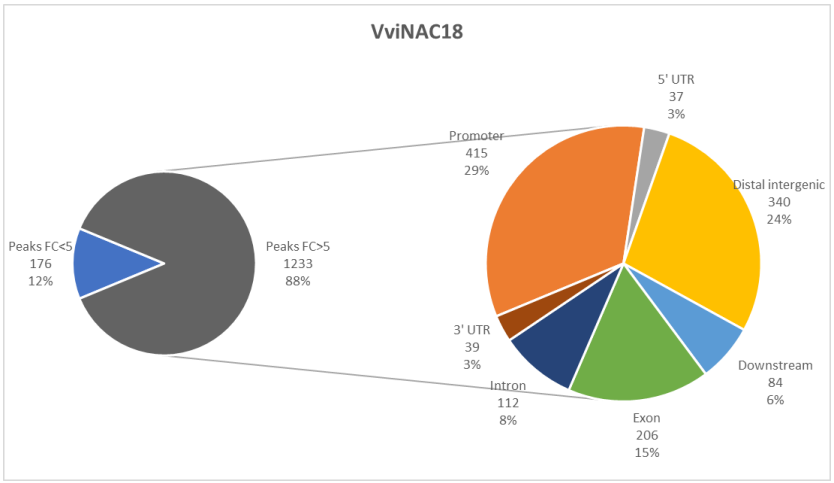

Figure 49: Double pie chart of the VviNAC18 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Three major binding motifs (GT[T/G][G/A/C]CGTG) were identified with strong significance (**Fig. 50**) and the phylogenetic footprints correlate with ANAC058 (no information available).

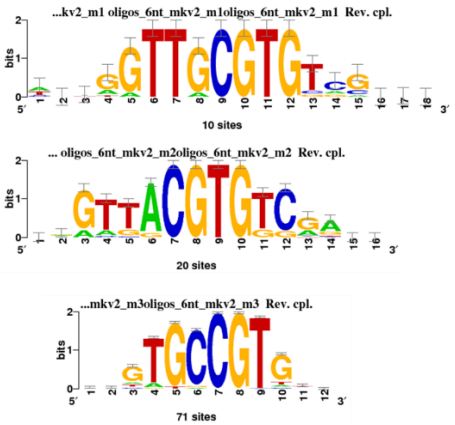

Figure 50: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC18 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 32% of the final analyzed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 452 sites; the percentage of sites belonging to these regulatory regions is, in this case, very high.

After the elimination of all the 'no hit' and 'unknown' elements, 359 binding sites were obtained for a total of 351 unique genes regulated by VviNAC18.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to regulation of cellular and biological processes, transport, localization, vesicle docking and cold acclimatation; moreover, many categories of genes concerning to different types of responses were found (**Fig. 51**).

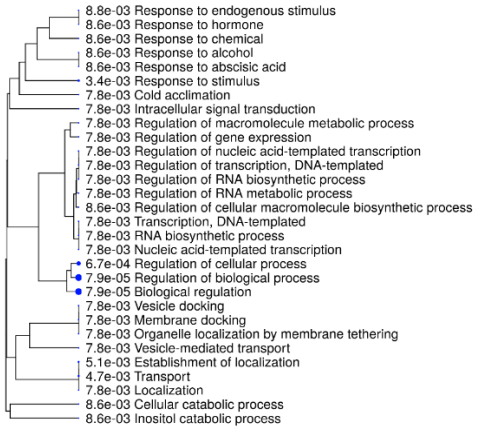

Figure 51: GO enrichment analysis of the VviNAC18 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 40**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
13,92	Promoter (<=1kb)	VIT_16s0050g00120	-973	B57
13,7	5' UTR	VIT_17s0000g05610	502	Isopiperitenol dehydrogenase
13,18	Promoter (<=1kb)	VIT_15s0048g01740	-376	Growth-regulating factor 9
12,95	Promoter (<=1kb)	VIT_19s0015g00930	-208	Hydroxyproline-rich glycoprotein
10,62	5' UTR	VIT_06s0004g01140	764	**GRIK1 (geminivirus rep interacting kinase 1)**
9,89	Promoter (1-2kb)	VIT_17s0000g08950	-1069	D111/G-patch
9,55	Promoter (<=1kb)	VIT_05s0020g02160	0	Squamosa promoter-binding protein (VvSBP6)
9,15	5' UTR	VIT_10s0003g01300	128	Transcription factor
7,91	Promoter (1-2kb)	VIT_05s0062g01120	-1261	PIN1
7,6	Promoter (1-2kb)	VIT_00s0179g00140	-1186	Protein phosphatase 2C / PP2C
7,04	Promoter (<=1kb)	VIT_10s0003g04650	-226	Nuclear transcription factor Y subunit A-7
6,87	Promoter (<=1kb)	VIT_18s0001g00360	-370	Dehydrin (VvDHN2)
6,61	Promoter (<=1kb)	VIT_17s0000g06410	-525	MYB transcription factor MIXTA-like 2
6,37	Promoter (<=1kb)	VIT_18s0001g08300	0	Tubulin alpha-6 chain
6,36	Promoter (1-2kb)	VIT_16s0098g01150	-1172	**Auxin-responsive SAUR29 ***
6,32	Promoter (1-2kb)	VIT_18s0001g09420	-1844	Progesterone 5-beta-reductase
6,26	Promoter (<=1kb)	VIT_06s0004g03870	0	CCT motif constans-like
6,17	Promoter (<=1kb)	VIT_05s0020g05060	-953	Cellulose synthase CSLG2
6,17	Promoter (<=1kb)	VIT_00s0181g00230	-75	LSD ONE like 2
6,15	Promoter (<=1kb)	VIT_13s0019g00550	-32	Cofilin

Table 40: Top 20 (q-value based) represented direct target genes of VviNAC18. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

GRIK1 (VIT_06s0004g01140), a white berry *switch*, is a kinase that can activate SnRK1, which plays a central role in coordinating energy balance and nutrient metabolism in plants (Shen et al., 2009).

PIN1 (VIT_05s0062g01120), *GROWTH-REGULATING FACTOR 9* (VIT_15s0048g01740) and *MYB TRANSCRIPTION FACTOR MIXTA-LIKE 2* (VIT_17s0000g06410) were also found again between the targets even of this TF.

Only one *VviNAC* target genes was found concerning the VviNAC18-*VviNACs* defined interactions: *VviNAC05* (VIT_17s0000g06400).

Then, the previously reported *VviNAC18* transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 9 correlated binding sites (9 unique genes; **Table 41**); none of them were present between the top 20 reported genes.

Between the five up regulated genes, the already described *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 1 SGR1* (VIT_02s0025g04660) returned. Interestingly and in line with literature, the *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 2 SGR2* (VIT_18s0001g01210) was also found and, on the opposite of its companion gene SGR1 (VIT_02s0025g04660), it resulted down regulated. *SGRs are* nuclear gene encoding a chloroplast protein and are senescence-associated genes; they are constitutively expressed at low levels and low amounts of protein are always detected during leaf development (Park *et al*., 2007). SGR1 interacts with chlorophyll catabolic enzymes and light-harvesting complex II at the thylakoid membrane and acts as a key regulator of leaf yellowing (Sakuraba et al., 2014).

Under senescence-inducing conditions, *SGR2* expression is highly up regulated, similarly to *SGR1* expression; SGR1 and SGR2 formed homo- or heterodimers, strongly suggesting a role for SGR2 in negatively regulating chlorophylls degradation by possibly interfering with the proposed chlorophyll catabolic enzymes recruiting function of SGR1 (Sakuraba et al., 2014).

Moreover, *STEROID SULFOTRANSFERASE* (VIT_13s0084g00240), a white berry *switch* and a negative second transition marker, was also found down regulated.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
3,74	Promoter (<=1kb)	VIT_12s0142g00610	-8	Citrate synthase, glyoxysomal precursor	1,55
3,29	Promoter (<=1kb)	VIT_02s0025g04660	-196	Senescence-inducible chloroplast stay-green protein 1	2,58
2,95	Promoter (<=1kb)	VIT_11s0016g03180	0	ABI1 (ABA insensitive 1)	-1,51
2,5	Promoter (1-2kb)	VIT_13s0067g00260	-1302	Nematode-resistance protein	2,41
2,47	Promoter (<=1kb)	VIT_05s0077g00850	-84	Ubiquitin-conjugating enzyme E2 O	1,73
2,45	Promoter (<=1kb)	VIT_18s0001g01210	-44	Senescence-inducible chloroplast stay-green protein 2	-2,20
2,44	Promoter (1-2kb)	VIT_17s0000g08080	-1008	Armadillo/beta-catenin repeat protein / U-box domain-containing protein	2,31
2,31	Promoter (<=1kb)	VIT_16s0098g01790	-477	Calmodulin-binding region IQD21	-1,53
2,01	Promoter (<=1kb)	VIT_13s0084g00240	-8	**Steroid sulfotransferase ***	-1,51

Table 41: VviNAC18 DAP-seq targets genes which have a match in the *VviNAC18* transient over expression dataset. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

VviNAC26

The DAP-seq assay reported 12245 binding sites (peaks), which were reduced to 11795 after the FC>5 filtering; even considering the very stringent filter used, the loss was very low, only the 4% (**Fig. 52**). The distribution of peaks revealed that 30% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 2% were in 5′ untranslated regions (UTRs), 15% were located in exons, 11% were located within introns, 3% were located in 3′ UTRs, 8% were located in the 3 kb downstream region, and 27% were intergenic.

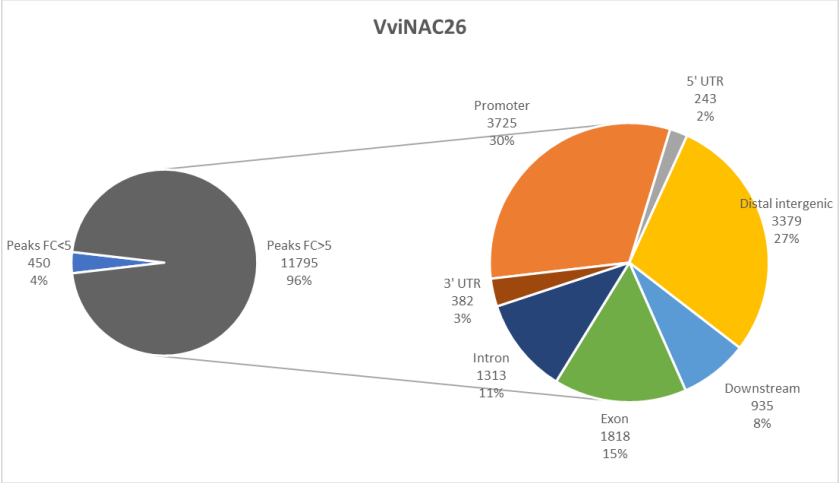

Figure 52: Double pie chart of the VviNAC26 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Two major binding motifs ([G/T]ACGT[G/A]) were identified with strong significance (**Fig. 53**) and the phylogenetic footprints correlate with ANAC055, which may function as transcription activator to regulate jasmonic acid-induced expression of defense genes (Bu *et al*., 2008).

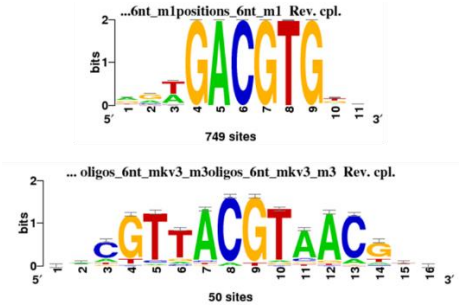

Figure 53: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC26 DAP-seq results. Two top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 32% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 3968 sites. The percentage of sites belonging to these regulatory regions is, in this case, very high.

After the elimination of all the 'no hit' and 'unknown' elements, 3236 binding sites were obtained for a total of 2765 unique genes regulated by VviNAC26.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to regulation of biological processes category; many regulations of metabolic processes genes, also concerning the aromatic compounds, were present (**Fig. 54**).

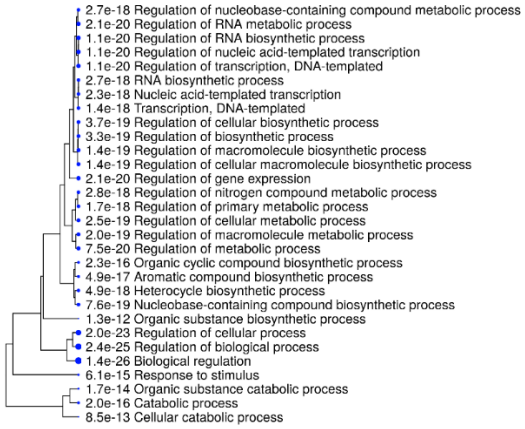

Figure 54: GO enrichment analysis of the VviNAC26 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 42**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
18661	Promoter (<=1kb)	VIT_15s0048g01740	-377	Growth-regulating factor 9
18556	Promoter (1-2kb)	VIT_18s0001g14980	-1611	3-methyl-2-oxobutanoate dehydrogenase
16898	Promoter (<=1kb)	VIT_19s0177g00070	-496	Polyubiquitin (UBQ14)
16516	Promoter (<=1kb)	VIT_07s0255g00110	-145	WD40
14631	Promoter (<=1kb)	VIT_05s0020g02310	0	Pyruvate,orthophosphate dikinase
14485	Promoter (1-2kb)	VIT_18s0001g09420	-1843	Progesterone 5-beta-reductase
13904	Promoter (<=1kb)	VIT_01s0011g04760	-73	myb domain protein 4 (VvMybC2-L1)
13529	Promoter (1-2kb)	VIT_13s0019g02080	-1042	DNA-binding protein
12778	Promoter (<=1kb)	VIT_17s0000g01280	-65	**WRKY Transcription Factor (VvWRKY52)**
12245	Promoter (1-2kb)	VIT_05s0062g01120	-1260	PIN1
11953	Promoter (<=1kb)	VIT_12s0142g00610	0	Citrate synthase, glyoxysomal precursor
11061	5' UTR	VIT_03s0038g04390	24	**Dehydrin 1**
10904	Promoter (<=1kb)	VIT_05s0062g00710	-875	UDP-glucose:flavonoid 7-O-glucosyltransferase
10775	Promoter (<=1kb)	VIT_16s0039g02750	-834	NADH glutamate dehydrogenase
10659	Promoter (1-2kb)	VIT_18s0041g02010	-1897	12-oxophytodienoate reductase 1
10566	Promoter (<=1kb)	VIT_15s0048g02260	-126	Calcium-binding EF hand
10384	Promoter (<=1kb)	VIT_18s0001g08300	0	Tubulin alpha-6 chain
10134	Promoter (1-2kb)	VIT_13s0067g00570	-1194	En/Spm transposon protein
10071	Promoter (<=1kb)	VIT_14s0006g02620	-273	Cgi67 serine protease *
9248	5' UTR	VIT_17s0000g05610	464	Isopiperitenol dehydrogenase

Table 42: Top 20 (q-value based) represented direct target genes of VviNAC26. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

The most sequenced represented gene was the already mentioned *GROWTH-REGULATING FACTOR 9* (VIT_15s0048g01740).

Other genes continually return in the targets lists and were present also in the VviNAC26 dataset: *MYB DOMAIN PROTEIN 4 VvMYBC2-L1* (VIT_01s0011g04760), *PIN1* (VIT_05s0062g01120) and *UDP-GLUCOSE:FLAVONOID 7-O-GLUCOSYLTRANSFERASE* (VIT_05s0062g00710).

Moreover, two red berry *switch*es were present in this small list; *VviWRKY52* (VIT_17s0000g01280) returned also in this *Vvi*NAC TF targets, together with the *DEHYDRIN 1* (VIT_03s0038g04390).

Afterwards, the list of the VviNAC26 targets was used to find all the possible *VviNACs* targets of the TF. 13 *VviNACs* target genes were found (**Table 43**), three of which harboring multiple Vvi*NAC26* binding sites: *VviNAC05* (VIT_17s0000g06400), *VviNAC40* (VIT_12s0035g02020) and *VviNAC61* (VIT_08s0007g07640), for a total of 17 *Vvi*NAC26-*VviNACs* defined gene interactions. Moreover, *VviNAC08*, one of the studied TFs, was also found.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
4704	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
1976	Promoter (1-2kb)	VIT_17s0000g06400	-1867	NAC domain-containing protein (VvNAC05)
1855	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
1812	Promoter (1-2kb)	VIT_07s0031g02610	-1550	NAC domain-containing protein (VvNAC39)
1353	Promoter (1-2kb)	VIT_12s0035g02020	-1029	NAC domain-containing protein (VvNAC40)
1295	Promoter (<=1kb)	VIT_17s0000g06400	0	NAC domain-containing protein (VvNAC05)
1113	Promoter (<=1kb)	VIT_04s0008g06550	0	NAC domain-containing protein (VvNAC66)
729	Promoter (<=1kb)	VIT_06s0080g00970	-179	NAC domain-containing protein (VvNAC67)
636	Promoter (<=1kb)	VIT_18s0001g02300	-341	NAC domain-containing protein (VvNAC08)
471	Promoter (<=1kb)	VIT_12s0055g00510	-303	NAC domain-containing protein (VvNAC58)
404	Promoter (1-2kb)	VIT_12s0035g02020	-1485	NAC domain-containing protein (VvNAC40)
352	Promoter (<=1kb)	VIT_18s0001g02300	-29	NAC domain-containing protein (VvNAC08)
348	Promoter (<=1kb)	VIT_12s0028g03050	-390	NAC domain-containing protein (VvNAC34)
306	Promoter (<=1kb)	VIT_18s0001g10250	0	NAC domain containing protein 19
279	Promoter (1-2kb)	VIT_14s0068g01490	-1139	NAC domain-containing protein (VvNAC73)
4	Promoter (1-2kb)	VIT_04s0023g03110	-1736	NAC domain-containing protein (VvNAC23)
2	Promoter (1-2kb)	VIT_06s0004g02350	-1933	NAC domain-containing protein 25

Table 43: VviNAC26 DAP-seq *VviNACs* targets genes.

Then, the previously reported *VviNAC26* transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 155 correlated binding sites (132 unique genes), 82 of them up regulated (**Table 44**) and 50 down regulated (**Table 45**); one of them was also present between the top 20 reported genes, *DNA-BINDING PROTEIN* (VIT_13s0019g02080).

Many *switch* genes and transition markers were found between the up and down regulated genes; moreover, other maturation related genes were present.

For example, between the up regulated, the *SUCROSE-PHOSPHATE SYNTHASE* (VIT_05s0029g01140) and a *FRUCTOSE-1,6-BISPHOSPHATASE, CYTOSOLIC* (VIT_18s0072g00770) are related to the sucrose biosynthetic pathways and are important in the leaves and berries maturation processes (Wu *et al*., 2011 and Daie, 1993). Even more interesting is the presence in the list of *VvMYBA6* (VIT_14s0006g01290), which has a role in the control of anthocyanin synthesis (Matus *et al*., 2017), and a *bHLH* gene (VIT_11s0052g00100), which are important TFs mainly implicated in the jasmonic acid signalling and in the response to stresses (Heim *et al*., 2003, Goossnes *et al*., 2017 and Sun *et al*., 2018).

Looking at the down regulated genes, a NAC domain containing protein (VIT_18s0001g10250) and other two *bHLH* (VIT_07s0031g00450 and VIT_02s0025g02610) are also direct target of VviNAC26.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
5982	Promoter (<=1kb)	VIT_16s0098g01790	-478	Calmodulin-binding region IQD21	1,74
4646	Promoter (<=1kb)	VIT_00s0207g00200	0	Integral membrane protein, putative	1,69
4247	Promoter (<=1kb)	VIT_01s0026g01030	-962	Zinc finger (C3HC4-type ring finger)	1,76
3167	Promoter (<=1kb)	VIT_17s0000g09540	0	CYP71A26	3,17
2248	Promoter (<=1kb)	VIT_19s0090g01350	-201	**Aspartyl protease**	4,56
2058	Promoter (1-2kb)	VIT_01s0011g05600	-1997	Receptor-like protein kinase	2,77
1871	Promoter (1-2kb)	VIT_07s0005g00210	-1400	Thiamin pyrophosphokinase	1,61
1785	Promoter (1-2kb)	VIT_14s0066g01240	-1951	L-aspartate oxidase	1,78
1743	Promoter (1-2kb)	VIT_01s0011g04630	-1409	Ribose 5-phosphate isomerase	2,23
1723	Promoter (<=1kb)	VIT_19s0090g01350	0	**Aspartyl protease**	4,56
1691	Promoter (<=1kb)	VIT_02s0012g00530	-20	Ribose-phosphate pyrophosphokinase 1	1,96
1664	Promoter (<=1kb)	VIT_13s0019g02200	-523	Protein phosphatase 2CA AHG3 PP2CA (VvPP2C-3)	2,17
1637	Promoter (<=1kb)	VIT_00s0227g00140	0	MLO1	2,57
1379	5' UTR	VIT_08s0007g04550	122	NCS1 nucleoside transporter family protein	2,57
1362	Promoter (<=1kb)	VIT_19s0090g00120	-198	Ubiquitin-conjugating enzyme E2 variant	5,80
1267	Promoter (<=1kb)	VIT_11s0016g03180	-7	ABI1 (ABA insensitive 1)	3,37
1254	Promoter (<=1kb)	VIT_12s0028g03010	-56	Glutaredoxin	1,62
1227	Promoter (<=1kb)	VIT_00s0216g00060	0	Nuclear transport factor 2 (NTF2)	2,16
1213	Promoter (<=1kb)	VIT_08s0007g08290	-664	GTP-binding protein TypA/BipA	2,32
1104	Promoter (<=1kb)	VIT_05s0029g01140	-26	Sucrose-phosphate synthase	4,37
1094	Promoter (1-2kb)	VIT_15s0046g01440	-1776	Basic Leucine Zipper Transcription Factor (VvbZIP40)	2,00
1042	Promoter (<=1kb)	VIT_05s0077g02150	0	Chalcone reductase *	2,93
1042	Promoter (1-2kb)	VIT_05s0020g01150	-1504	Cation/hydrogen exchanger (CHX19)	2,69
1022	Promoter (<=1kb)	VIT_17s0000g09570	-321	CYP71A26	3,05
1012	Promoter (<=1kb)	VIT_17s0000g06610	0	Zinc finger (C3HC4-type ring finger)	3,40
995	Promoter (<=1kb)	VIT_05s0077g00280	-252	Beta-amylase	2,25
914	Promoter (1-2kb)	VIT_01s0011g04630	-1788	Ribose 5-phosphate isomerase	2,23
884	Promoter (<=1kb)	VIT_13s0084g00310	-301	ACI112	1,57
834	Promoter (<=1kb)	VIT_02s0012g01240	-233	PHD finger transcription factor	2,21
781	Promoter (<=1kb)	VIT_16s0050g00990	0	ferric reduction oxidase 4	2,91
767	Promoter (<=1kb)	VIT_07s0031g01530	0	Stress enhanced protein 2 (SEP2)	2,22
738	5' UTR	VIT_08s0007g04980	22	Phosphoadenosine phosphosulfate (PAPS) reductase	1,56
729	Promoter (1-2kb)	VIT_01s0026g00300	-1441	Zinc finger (C3HC4-type ring finger)	1,88
626	Promoter (<=1kb)	VIT_14s0066g01200	-25	Protein kinase beta-1 subunit 5'-AMP-activated	1,58
625	Promoter (1-2kb)	VIT_04s0008g06050	-1396	Phytosulfokine receptor precursor	1,69
612	Promoter (<=1kb)	VIT_07s0005g00180	-407	TOC159 (translocon outer membrane complex 159)	1,68
578	5' UTR	VIT_02s0012g01240	255	PHD finger transcription factor	2,21
578	Promoter (<=1kb)	VIT_12s0034g02390	0	T-complex protein 11	1,60
576	Promoter (<=1kb)	VIT_01s0137g00560	0	**CYP71B34**	1,79
573	Promoter (<=1kb)	VIT_03s0132g00150	-135	GTP cyclohydrolase I	1,89
573	Promoter (<=1kb)	VIT_05s0020g04210	-663	Sulfate adenylyltransferase 3	1,73
554	Promoter (<=1kb)	VIT_11s0016g03180	-379	ABI1 (ABA insensitive 1)	3,37
523	Promoter (<=1kb)	VIT_05s0020g03970	-697	Sulfate transporter 3.1 (AST12) (AtST1)	3,83
471	Promoter (<=1kb)	VIT_07s0005g03680	-56	CCR4-NOT complex subunit CAF16	2,43
471	Promoter (<=1kb)	VIT_01s0127g00220	-7	PMR5 (powdery mildew resistant 5)	1,97
471	Promoter (<=1kb)	VIT_19s0090g01440	-53	Fimbrin 2	1,94
471	Promoter (<=1kb)	VIT_00s0477g00030	-371	MATE efflux family protein ripening responsive	1,78
458	5' UTR	VIT_16s0050g00390	260	**4-coumarate-CoA ligase ***	2,40
448	Promoter (<=1kb)	VIT_08s0007g08290	0	GTP-binding protein TypA/BipA	2,32
448	Promoter (<=1kb)	VIT_05s0077g00280	-16	Beta-amylase	2,25
426	Promoter (1-2kb)	VIT_01s0011g00250	-1298	6-phosphogluconolactonase	1,93
426	Promoter (<=1kb)	VIT_17s0000g01680	0	Adenylate kinase family	1,83
424	Promoter (<=1kb)	VIT_09s0002g06480	-668	CYP81B2v1	4,05
424	Promoter (1-2kb)	VIT_06s0004g02140	-1083	MATE efflux family protein	2,94
421	Promoter (<=1kb)	VIT_09s0002g06970	-148	**Palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, chloroplast ***	4,49
421	Promoter (<=1kb)	VIT_13s0019g02200	-839	Protein phosphatase 2CA AHG3 PP2CA (VvPP2C-3)	2,17
395	Promoter (<=1kb)	VIT_02s0012g00530	-269	Ribose-phosphate pyrophosphokinase 1	1,96
394	Promoter (<=1kb)	VIT_18s0072g00770	-580	fructose-1,6-bisphosphatase, cytosolic	2,45
389	Promoter (<=1kb)	VIT_08s0056g01230	-683	Cycling DOF factor 2	2,07
382	Promoter (1-2kb)	VIT_06s0004g00260	-1318	Shoot1 protein	2,66
376	Promoter (<=1kb)	VIT_18s0089g01410	-295	Purine permease 4 PUP4	2,44
352	Promoter (<=1kb)	VIT_03s0063g00370	-682	Nitrite reductase	6,15
344	Promoter (1-2kb)	VIT_19s0015g00130	-1259	Serine acetyltransferase 3	2,63
344	Promoter (<=1kb)	VIT_00s0216g00060	-197	Nuclear transport factor 2 (NTF2)	2,16
344	Promoter (<=1kb)	VIT_08s0040g01470	-39	Cis-zeatin O-beta-D-glucosyltransferase	1,89
322	Promoter (1-2kb)	VIT_13s0084g00310	-1095	ACI112	1,57
321	Promoter (<=1kb)	VIT_13s0019g02200	-156	Protein phosphatase 2CA AHG3 PP2CA (VvPP2C-3)	2,17
319	Promoter (<=1kb)	VIT_06s0004g06540	-7	Undecaprenyl pyrophosphate synthetase	2,33
306	Promoter (<=1kb)	VIT_05s0077g00810	-622	Sodium-inducible calcium-binding protein (ACP1)	2,34
295	Promoter (<=1kb)	VIT_11s0052g01480	-347	Salt-inducible protein kinase	1,89
295	Promoter (<=1kb)	VIT_14s0030g02230	0	Unfertilized embryo sac 12 UNE12	1,59
283	Promoter (<=1kb)	VIT_14s0066g01240	-286	L-aspartate oxidase	1,78
278	Promoter (<=1kb)	VIT_05s0020g04430	0	MPBQ/MSBQ methyltransferase 1	1,65
269	Promoter (<=1kb)	VIT_00s0187g00380	-998	Phosphatidate cytidylyltransferase	1,53
247	Promoter (<=1kb)	VIT_19s0015g00130	-952	Serine acetyltransferase 3	2,63
247	Promoter (1-2kb)	VIT_11s0037g01210	-1702	Eceriferum 1 (CER1 protein) Sterol desaturase	2,52
247	Promoter (<=1kb)	VIT_00s0207g00010	0	Anthranilate N-benzoyltransferase protein 1	2,19
247	Promoter (<=1kb)	VIT_14s0030g01340	-777	Beta-ketoacyl-CoA synthase	1,79
239	Promoter (<=1kb)	VIT_16s0050g00140	-847	Heavy-metal-associated domain-containing protein	2,83
239	Promoter (1-2kb)	VIT_11s0016g05740	-1675	Calmodulin-5 (CAM5)	1,61
239	Promoter (<=1kb)	VIT_08s0032g00790	-763	ABC Transporter (VvWBC18 - VvABCG18)	1,58
239	Promoter (<=1kb)	VIT_08s0007g04980	0	Phosphoadenosine phosphosulfate (PAPS) reductase	1,56
239	Promoter (1-2kb)	VIT_01s0011g04830	-1352	Extensin	1,51
218	Promoter (<=1kb)	VIT_11s0052g00100	0	basic helix-loop-helix (bHLH) family	3,03
201	Promoter (<=1kb)	VIT_14s0006g01290	-12	myb domain protein 113 (VvMYBA6)	3,56
201	Promoter (1-2kb)	VIT_00s0218g00100	-1901	Metal transporter Nramp6	3,37
201	Promoter (1-2kb)	VIT_08s0007g04890	-1790	ACT domain-containing protein	2,32
201	Promoter (<=1kb)	VIT_18s0001g14310	-403	flavanone-3-hydroxylase 2 (F3H2) [Vitis vinifera]	1,66
198	Promoter (<=1kb)	VIT_13s0019g02200	-379	Protein phosphatase 2CA AHG3 PP2CA (VvPP2C-3)	2,17
121	Promoter (<=1kb)	VIT_18s0001g01030	-547	Protein disulfide isomerase *	2,05
82	Promoter (1-2kb)	VIT_00s0304g00080	-1774	Trehalose-6-phosphate phosphatase	2,07
71	5' UTR	VIT_10s0523g00030	23	Photoregulatory zinc-finger protein COP1	1,72
63	Promoter (<=1kb)	VIT_02s0012g01240	-295	PHD finger transcription factor	2,21
57	Promoter (<=1kb)	VIT_11s0016g04580	-528	ABC Transporter (VvPDR2 - VvABCG32)	1,88
43	Promoter (<=1kb)	VIT_14s0060g00770	-704	Glycosyl transferase family 8 protein	3,67
43	Promoter (1-2kb)	VIT_06s0009g02640	-1772	Hydroxymethylglutaryl-CoA lyase	2,05
42	Promoter (1-2kb)	VIT_11s0016g02350	-1642	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE	5,48
36	Promoter (<=1kb)	VIT_17s0000g09350	-394	Dehydration-induced 19	1,85
3	Promoter (1-2kb)	VIT_11s0016g02350	-1545	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE	5,48
3	Promoter (<=1kb)	VIT_12s0028g03350	-777	Squamosa promoter-binding protein (VvSBP11) *	2,16

Table 44: VviNAC26 DAP-seq up regulated targets genes. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
13529	Promoter (1-2kb)	VIT_13s0019g02080	-1042	DNA-binding protein	-1,62
7261	Promoter (<=1kb)	VIT_06s0004g07930	0	Nucleotidyltransferase family	-1,65
2952	Promoter (<=1kb)	VIT_07s0031g00450	-796	Basic helix-loop-helix protein SPATULA	-2,18
2164	Promoter (<=1kb)	VIT_06s0004g03870	0	CCT motif constans-like	-1,53
1751	Promoter (<=1kb)	VIT_12s0057g00910	-242	Sterile apetala	-1,69
1581	5' UTR	VIT_03s0017g00090	1287	Serine/threonine-protein kinase bri1	-2,13
1296	Promoter (<=1kb)	VIT_05s0020g04850	0	H1flk	-1,78
1287	Promoter (<=1kb)	VIT_15s0048g01000	-690	2'-hydroxy isoflavone/dihydroflavonol reductase	-1,68
1147	Promoter (1-2kb)	VIT_13s0019g02080	-1368	DNA-binding protein	-1,62
1131	Promoter (1-2kb)	VIT_16s0022g02230	-1541	Leucine-rich repeat receptor protein kinase EXS	-2,88
961	Promoter (<=1kb)	VIT_19s0015g00710	-374	Cellulose synthase CSLE1	-2,95
947	Promoter (<=1kb)	VIT_04s0008g00610	-508	Zinc finger (CCCH-type) family protein	-2,05
941	Promoter (1-2kb)	VIT_06s0004g05710	-1438	Glutathione S-transferase GSTU7	-2,43
941	Promoter (<=1kb)	VIT_17s0000g00240	-649	Mei2 AML1	-1,88
888	Promoter (1-2kb)	VIT_17s0000g07520	-1068	Ribosomal-protein S6 kinase p70	-1,58
875	Promoter (<=1kb)	VIT_04s0023g02970	0	UPF0183 protein	-1,59
834	Promoter (1-2kb)	VIT_07s0141g00360	-1803	Lipase class 3	-1,87
657	Promoter (<=1kb)	VIT_12s0034g02220	-839	RKF3 (receptor-like kinase IN in flowers 3)	-1,91
653	Promoter (<=1kb)	VIT_02s0012g00410	0	Naringenin,2-oxoglutarate 3-dioxygenase	-1,58
629	Promoter (1-2kb)	VIT_02s0025g02610	-1990	basic helix-loop-helix (bHLH) family	-3,85
603	Promoter (<=1kb)	VIT_12s0059g01450	-307	Zinc finger CCCH domain-containing protein ZFN-like 4	-1,52
549	Promoter (1-2kb)	VIT_11s0118g00480	-1843	Leucine-rich repeat	-1,52
548	Promoter (1-2kb)	VIT_07s0031g01270	-1220	Ring-H2 finger A2A	-1,73
523	Promoter (<=1kb)	VIT_01s0011g05660	-831	Zinc finger (Ran-binding) family	-1,50
513	Promoter (1-2kb)	VIT_15s0048g01530	-1401	Geraniol 10-hydroxylase	-1,71
505	Promoter (<=1kb)	VIT_06s0004g06290	-62	Binding	-2,52
497	Promoter (<=1kb)	VIT_18s0001g06950	-367	Purine permease 1 (PUP1)	-1,74
497	Promoter (<=1kb)	VIT_04s0044g01620	0	RGLG1 (ring Domain LIGASE1)	-1,57
472	Promoter (<=1kb)	VIT_06s0004g06290	-307	Binding	-2,52
448	Promoter (1-2kb)	VIT_02s0025g04890	-1766	CYP76B1	-5,19
445	Promoter (<=1kb)	VIT_03s0038g00360	-72	Isochorismatase hydrolase	-4,20
403	Promoter (1-2kb)	VIT_19s0014g02900	-1756	Ring finger protein 185	-7,03
403	Promoter (<=1kb)	VIT_18s0001g10910	-423	Patatin	-1,84
366	5' UTR	VIT_07s0104g00830	36	Sugar transporter ERD6-like 7 *	-2,29
344	Promoter (<=1kb)	VIT_19s0014g02900	-243	Ring finger protein 185	-7,03
344	Promoter (1-2kb)	VIT_17s0000g08010	-1616	Trehalose 6-phosphate synthase	-1,73
343	Promoter (1-2kb)	VIT_07s0104g00830	-1410	Sugar transporter ERD6-like 7 *	-2,29
329	Promoter (<=1kb)	VIT_05s0020g02710	-457	Slingshot	-1,80
321	Promoter (<=1kb)	VIT_13s0064g01370	-369	Polygalacturonase inhibiting protein 1 PGIP1	-2,89
306	Promoter (<=1kb)	VIT_18s0001g10250	0	NAC domain containing protein 19	-1,52
295	Promoter (<=1kb)	VIT_06s0004g06290	-598	Binding	-2,52
266	Promoter (1-2kb)	VIT_07s0129g00490	-1291	Hydrogenobyrinic acid a,c-diamide synthase	-1,70
247	Promoter (<=1kb)	VIT_18s0122g00620	-383	Cinnamoyl-CoA reductase *	-2,55
247	Promoter (<=1kb)	VIT_15s0045g00370	-575	Phosphatidylglycerol specific phospholipase C	-2,53
223	Promoter (<=1kb)	VIT_03s0063g01780	-315	Extra-large G-protein (XLG1)	-3,39
223	Promoter (<=1kb)	VIT_03s0017g02110	-125	Anthocyanidin 3-O-glucosyltransferase	-2,79
223	Promoter (<=1kb)	VIT_01s0127g00910	-109	AERO1 (arabidopsis endoplasmic reticulum oxidoreductins 1)	-2,43
223	Promoter (1-2kb)	VIT_04s0008g03660	-1566	Embryonic flower 1	-1,61
219	Promoter (1-2kb)	VIT_13s0067g03820	-1605	chalcone isomerase 1 [Vitis vinifera] (VvCHI1) *	-1,78
201	Promoter (<=1kb)	VIT_05s0094g00290	-797	Chitinase, class IV [Vitis vinifera]	-2,31
158	Promoter (<=1kb)	VIT_06s0004g06930	-171	Zinc finger (C3HC4-type ring finger)	-2,13
43	Promoter (<=1kb)	VIT_09s0054g00860	-228	Lipase GDSL	-1,78
34	Promoter (1-2kb)	VIT_18s0001g04500	-1775	Enhanced EM level EEL (VvABF-2), Basic Leucine Zipper Transcription Factor (VvbZIP43)	-2,00
4	Promoter (<=1kb)	VIT_07s0031g02250	-911	Zinc finger (C3HC4-type ring finger)	-1,86

Table 45: VviNAC26 DAP-seq down regulated targets genes. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

VviNAC33 (New Phytologist, Volume: 231, Issue: 2, Pages: 726-746, First published: 10 February 2021, DOI: (10.1111/nph.17263))

VviNAC33 promotes organ de-greening and represses vegetative growth during the vegetative-to-mature phase transition in grapevine

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Summary
• Plants undergo several developmental transitions during their life cycle. In grapevine, a perennial woody fruit crop, the transition from vegetative/green to mature/woody growth involves transcriptomic reprogramming orchestrated by a small group of genes encoding regulators, but the underlying molecular mechanisms are not fully understood.
• We investigated the function of the transcriptional regulator *VviNAC33* by generating and characterizing transgenic overexpressing grapevine lines and a chimeric repressor, and by exploring its putative targets through DAP-seq approach combined with transcriptomic data.
• We demonstrated that VviNAC33 induces leaf de-greening, inhibits organ growth and directly activates the expression of STAY-GREEN PROTEIN 1 (SGR1), involved in chlorophyll and photosystem degradation, and AUTOPHAGY 8f (ATG8f) involved in the maturation of autophagosomes. Furthermore, we show that VviNAC33 directly inhibits AUXIN EFFLUX FACILITATOR PIN1 and RopGEF1 and ATP SYNTHASE GAMMA CHAIN 1T (ATPC1) involved in photosystem II integrity and activity.
• Our results show that VviNAC33 plays a major role in terminating photosynthetic activity and organ growth as part of a regulatory network governing the vegetative-to-mature phase transition.

Introduction
Plants develop through a succession of distinct growth phases and the correct timing of the transitions is important for plant fitness and agronomic performance (Demura and Ye, 2010). In seed plants, shoots pass through a vegetative growth phase, further divided into juvenile and adult stages, characterized by an increase in photosynthetic capacity and organ size. A reproductive phase follows, during which the vegetative shoot apical meristem assumes an inflorescence meristem identity (Huijser and Schmid, 2011). An essential transition to survive adverse environmental conditions is the shift from an active to a dormant state involving the cessation of growth, the repression of auxin responses, cell wall thickening, and adaptations to survive cold temperatures. For example, seed dormancy prevents germination out of season whereas bud dormancy and shoot lignification in perennial woody plants allows overwinter survival (Schrader *et al*., 2004).
In fleshy fruit species, the transition from unripe fruit (that must be protected against herbivory) to ripe fruit (that must appeal to the same herbivores to facilitate seed dispersal) is another dramatic shift in survival strategy. Fruit ripening involves a tightly coordinated sequence of chemical and physiological alterations (Giovannoni *et al*., 2017).
Senescence is the last part of the plant developmental program before cell death (Woo *et al*., 2013). The transition to senescence involves the ordered disassembly of molecules and cellular components that accumulated during vegetative growth (Lim *et al*., 2007). The earliest and most significant change in cell structure is the disassembly of the chloroplast, an organelle that contains up to 70% of the total leaf protein content. Like fruit ripening, leaf senescence is a complex genetic and epigenetic program that integrates endogenous developmental signals and environmental cues.
In recent years it has become evident that the genetic networks underlying these phase transitions share certain common factors. For example, the juvenile-to-adult and vegetative-to-reproductive phase transitions involve two evolutionarily conserved microRNAs (miR156 and miR172) and their targets (Huijser and Schmid, 2011; Ma *et al*., 2020). Recently, certain NAM/ATAF/CUC (NAC) transcription factors were found to be key regulators of both fruit ripening and senescence, such as tomato NOR, a well-characterized master regulator of the onset of fruit ripening and a positive regulator of leaf senescence (Ma *et al*., 2019).
NAC proteins form a large plant-specific family of transcription factors (TFs) that control the

expression of target genes by forming heterodimers with other NAC proteins as well as other TF families (Kim *et al*., 2016). The analysis of NAC genes in different crops has shed light on their role in integrating developmental age and environmental signals during plant development (Liang *et al*., 2014; Podzimska-Sroka *et al*., 2015). For example, genetic studies have identified ANAC092/ORESARA1 (ORE1), ANAC029/Arabidopsis NAC-LIKE Activated by AP3/PI (AtNAP), ANAC059/ORESARA1 SISTER1 (ORS1), and ANAC016 as positive regulators of leaf senescence (Kim *et al*., 2009; Guo *et al*., 2006; Balazadeha *et al*., 2011; Sakuraba *et al*., 2015), whereas ANAC042/JUNGBRUNNEN1 (JUB1) and ANAC083/VND-INTERACTING2 (VNI2) are negative regulators (Wu *et al*., 2012; Yang *et al*., 2011). ANAC092/ORE1 is considered the key positive regulator of leaf senescence, involved in a delicately balanced feed-forward loop that promotes ethylene-mediated chlorophyll degradation.
Grapevine (Vitis vinifera L.) is an important perennial fruit crop and a useful model of developmental phase transition in woody species, featuring all the major developmental and physiological processes and benefiting from a well characterized fruit development process. We previously revealed major transcriptomic reprogramming in all the organs and tissues during the shift from vegetative/green to mature/woody development (Fasoli *et al*., 2012). Co-expression network analysis uncovered a small group of genes that potentially act as switches for this phase transition (Palumbo *et al*., 2014; Massonnet *et al*., 2017), including the NAC family member *VviNAC33* whose sudden expression was among the earliest molecular signals of the onset of berry ripening (Fasoli *et al*., 2018). Preliminary results indicate that VviNAC33 is controlled by two transcription factors, VvibHLH075 and VviWRKY19, previously identified as potential regulators of berry phase transition (Fasoli *et al*., 2018).
Here we characterize the activity of VviNAC33 by characterizing transient and stable overexpression lines and the creation of a chimeric repressor in planta. We also used the DAP-seq coupled with transcriptomic analysis to identify putative direct targets of VviNAC33, revealing that it acts as an activator and repressor of transcription. We propose that VviNAC33 functions in a regulative network controlling the de-greening and the suppression of organ growth during the transition from the vegetative to the mature phase of development.

Material and Methods

Plant material
N. benthamiana plants, *V. vinifera* cv. Sultana plantlets, cv. Syrah embryogenic calli for the *VviNAC33* genetic transformation, cv. Garganega embryogenic calli for the *VviNAC33-EAR* genetic transformation, *VviNAC33* and *VviNAC33-EAR* transgenic plants were grown as described in Amato *et al*. (2019). For the DAP-seq analysis, cv. Syrah fruiting cuttings were propagated as described in Mullins *et al*. (1981).

Protoplast isolation
Protoplasts were prepared from 0.5 g of leaf tissue as described by Zhao *et al*. (2016). For VviNAC33 subcellular localization, protoplasts were isolated from a pool of four leaves each from three cv. Corvina plants. For chloroplast distribution analysis, protoplasts were isolated from a pool created by four fully expanded leaves each taken from three plants of every transgenic overexpressing line plus a control.

Bioinformatics
NAC protein sequences from different species were aligned using MUSCLE with default settings. The unrooted phylogenetic tree was constructed in MEGA v7.0 (http://www.megasoftware.net/; Kumar *et al*., 2016) using the neighbour-joining method based on the following parameters: pairwise deletion and bootstrap analysis with 1000 replicates. Conserved motifs were identified using the MEME suite (http://memesuite.org/tools/meme) (Bailey *et al*., 2009) with standard parameters, maximum 20 motifs and an optimum motif width of 4–60 amino acids. Transcriptomic data for gene expression analysis were retrieved from the Corvina atlas and two berry-specific expression maps (Fasoli *et al*., 2012; Massonnet *et al*., 2017; Fasoli *et al*., 2018). Co-expression analysis based on Pearson's correlation coefficient was carried out using CorTo software and *VviNAC33* (VIT_19s0027g00230) as the bait in the Corvina atlas. ShinyGO v0.61 was used for enrichment analysis (Ge *et al*., 2020).

Isolation and cloning

The *VviNAC33* coding sequence (with 3′ UTR) and *VviNAC33-EAR* were amplified from cv. Corvina mid-ripening and ripening berry skin using KAPA HiFi DNA polymerase (KAPA Biosystems, Wilmington, MA, USA) and the primers used listed in **Table S1**. The PCR products were directionally cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). For agroinfiltration in N. benthamiana and the transient expression, the *VviNAC33* sequence was transferred into the binary overexpression vector pK7GW2.0 (Laboratory of Plant Systems Biology, Ghent University, Belgium) by site-specific LR recombination. For stable overexpression, the sequence was transferred to the modified binary vector pK7GW2.0 containing an eGFP expression cassette driven by the Arabidopsis ubiquitin 10 promoter. The *VviNAC33-EAR* chimeric repressor was transferred into the modified pK7WG2 vector, harbouring the 1320-bp endogenous *VviNAC33* promoter (isolated from cv. Corvina genomic DNA) instead of the CaMV 35S promoter. For subcellular localization, the GFP sequence was fused to the C-terminus of *VviNAC33*.

Subcellular localization

Protoplasts were transfected with 50 µg of the pK7FWG2:*VviNAC33* vector or the control vector (pK7FWG2:eGFP) as described by Amato *et al*. (2019).

DAP-seq

Genomic DNA was extracted from 1 g of ground young *V. vinifera* cv. Syrah leaves as described by Thomas *et al*. (1993) and the Illumina libraries were prepared as described in Galli *et al*. (2018). The *VviNAC33* sequence was transferred from the pENTR/D-TOPO to the Gateway-compatible destination vector pIX-HALO (Bartlett *et al*., 2017). The HALO-VviNAC33 and GST-HALO (used as negative control) fusion proteins were in vitro translated using the TNTR SP6 coupled reticulocyte lysate system (Promega). The DAP-seq was performed as described in Galli *et al*. (2018) and a total of 3.5 million of 75 bp single reads were obtained for each sample.

Read mapping, filtering, peak calling and motif analysis

Fastq files were trimmed using trimmomatic (Bolger *et al*., 2014) with the following parameters: ILLUMINACLIP = TruSeq3–, SE = 2:30:10. LEADING = 3, TRAILING = 3, SLIDINGWINDOW = 4:20, MINLEN = 50. Trimmed reads were mapped to the grape reference genome version 12X.2 (nuclear chromosomes only) at URGI (https://urgi.versailles.inra.fr/Species/Vitis/Annotations) using bowtie2 v2.3.4.3 (Langmead and Salzberg, 2012). Reads mapping to multiple locations were filtered, removing all reads with the XS:i field present in the BAM file. Uniquely mapped reads were used for all subsequent steps. Peaks were called using GEM v3.4 (Guo *et al*., 2012) and the GST-HALO negative control sample for background subtraction, applying the following parameters: --d Read_Distribution_default.txt --k_min 6 --k_max 20 --outNP. To reduce the number of false positives, we removed all peaks with a sample/control ratio < 5-fold. The remaining peaks were associated with nearest genes using the ChipSeeker R package (Yu *et al*., 2015). For gene annotation, we used the V1 on the 12X.0 assembly transposed to the 12X.2 assembly. The gff3 files were downloaded from URGI. For visualization in the Integrative Genome Browser (IGV), bam files were converted to bigwig files using deepTools v3.4.3, bamCoverage with a 10-bp bin size, and FPKM normalization. Motifs were detected and assigned using RSAT Plants NGS ChIP-Seq peak-motifs analysis (http://rsat.eead.csic.es/plants/peak-motifs_form.cgi) and RSAT Plants Motif Discovery dyad-analysis (http://rsat.eead.csic.es/plants/dyad-analysis_form.cgi).

Transient expression

For *N. benthamiana*, pK7WG2.0 vectors containing 35S:*VviNAC33* or a non-coding sequence (negative control) were transferred to *A. tumefaciens* strain C58C1 by electroporation (Hellens *et al*., 2000) and three fully expanded leaves each from four plants were syringe infiltrated for both vectors. Phenotypic analysis was carried out 3 days after agroinfiltration. The same vectors were used for transient expression in cv Sultana. 5-week-old *in vitro* plantlets (eight plants for *VviNAC33* overexpression and seven for the control) were vacuum infiltrated as previously described (Amato *et al*., 2017). Molecular analyses were carried out on leaf samples collected 7 days after the agroinfiltration.

Transgenic plants
The pK7WG2.0 vectors containing 35S:*VviNAC33* or *VviNAC33-EAR* or the eGFP sequence (negative control) were transferred to *A. tumefaciens* strain EHA105 by electroporation. The genetic transformations were performed as described in Amato *et al.* (2019).

Leaf area measurement
Leaf area was estimated as proposed by Smith and Kliewer (1984) by measuring maximum length and width of three laminae of mature leaves (at the 6th/7th node from apex) each from four plants per transgenic line plus control. Mean ± Standard Deviation (SD) of 12 biological replicates was calculated.

Pigment analysis and photosynthetic parameters
For *N. Benthamiana*, four biological replicates each consisting of a pool of infiltrated leaves from each plant were analyzed. For OXNAC33 and EARNAC33 transgenic plants, four biological replicates each consisting of a pool of three young leaves for each OX1,2,3 lines plus control and four biological replicates each consisting of a pool of three fully expanded leaves for each EAR1, 2, 3 lines plus control were analyzed.Pigments were measured according to Cazzaniga *et al*. (2020). Photosynthetic parameters, and OJIP curves were measured using a DUAL-PAM-100 fluorimeter (Heinz–Walz, Effeltrich, Germany) as previously described (Baker, 2008). Proton motive force was measured by electrochromic shift (ECS) using a MultispeQ V2.0 (PhotosynQ, East Lansing, MI, USA) as previously described (Kuhlgert *et al*., 2016). For each analysis mean ± SD of four biological replicates was calculated.

Thylakoid preparation and immunoblotting
For OX2 line plus control four separated pools of leaves, chosen with the same criteria used for pigment analysis, were analyzed. Thylakoid membranes were isolated and fractionated as previously described (Pinnola *et al*., 2015). Proteins were separated by SDS-PAGE in a Tris-Tricine buffer system (Schägger and von Jagow, 1987). Immnunotitration was performed as described in (Cazzaniga *et al*., 2020) with antibodies (Agrisera Vännäs, Sweden) against the proteins CP47 (AS11 1787), PSAA (AS06 172) LHCII (AS01 004) and AtpC (AS08312). For each analysis mean ± SD of four biological replicates was calculated.

Chloroplast distribution
Protoplast images were captured using a Leica DM2500 microscope equipped with a DFC7000T camera. The distribution of chloroplasts was analysed with ImageJ software on 70 images for each of the three transgenic lines and the control.

Plant NAA treatment
In vitro cv. Syrah stems of OX2 line and control, consisting of two apical leaves, one fully expanded and the other newly developing, were transferred to MS medium without or with two different 1-Naphthaleneacetic acid (NAA) concentrations (5 and 20 mg/L). For each treatment, four stems of OX2 line and four stems of control were maintained in a growth chamber at 25°C with a 16 h photoperiod. Phenotype was observed 14 days after hormone treatment.

Real time qPCR
For transient expression RNA was extracted from a pool of two apical well-expanded leaves from each plant. For OXNAC33 and EARNAC33 transgenic plants, RNA was extracted from leaves collected and pooled following the same criteria used for pigment analysis. Total RNA was isolated from 50-100 mg of ground material using the Spectrum Plant Total RNA kit. Gene expression analysis by qPCR was performed as previously described (Zenoni *et al*., 2011) using the primer sequences listed in **Table S1**. Each value corresponds to the mean ± SD of three technical replicates relative to the VviUBIQUITIN1 (VIT_16s0098g01190) control.

Transcriptomic analysis
The microarray analysis was performed with the RNA isolated for qPCR. For transient expression, the four most highly overexpressing plants (#3, #5, #6 and #7) were selected and used as biological replicates, while for OXNAC33 transgenic lines, the OX1, 2 and 3 lines were used as biological replicates. The cDNA synthesis, labelling, hybridization and washing reactions were performed

according to the Agilent Microarray-Based Gene Expression Analysis Guide (V 6.5). Each sample was hybridized to an Agilent custom microarray four-pack 44K format (Agilent Sure Print HD 4X44K 60-mer; G2514F-048771; Dal Santo *et al*., 2016) and scanned using an Agilent Scanner (Agilent Technologies; G2565CA). Feature extraction and statistical analysis of the microarray data was conducted as reported in Amato *et al*., 2017. DEGs were identified by Student's t test ($\alpha = 0.05$), assuming equal variance among samples, and selected by fold change |1.5|).

Dual luciferase assay

The VviNAC33 (1320 bp), SGR1 (1558 bp), ATG8f (1616 bp), RopGEF1 (1604 bp), PIN1 (1637 bp) and ATPC1 (1573 bp) promoter regions were amplified by PCR from Corvina genomic DNA using KAPA HiFi DNA polymerase. The cloning steps and Dual Luciferase Reporter Assay were carried out as described by Amato *et al*. (2019), using as effector vectors 35S:*VviNAC33* with 35S:*VviWRKY19* (VIT_07s0005g01710) and 35S:*VviBHLH75* (VIT_17s0000g00430), both previously reported by Fasoli *et al*. (2018). Mean ± SD of four biological replicates was calculated.

Data availability

Microarray data for the transient expression experiments are available at GEO under the series entry GSE155037 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155037). Microarray data for the transgenic plants are available at GEO under the series entry GSE156105 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE156105). DAP-seq data are available at GEO under the series entry GSE155445 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155445).

Results

VviNAC33 is upregulated during the vegetative-to-mature transition of several organs and is strongly expressed in leaves undergoing senescence

The analysis of previously released transcriptomic databases (Fasoli *et al*., 2012) revealed that *VviNAC33* is expressed at high levels during senescence in leaves and in woody stems, but only weakly expressed in the young leaf, green stem, flower organs, pollen, bud and tendrils. *VviNAC33* expression is also induced before veraison in developing seeds and at veraison in berries (**Fig. 1a**), and a similar sharp increase was observed during berry softening in different grapevine genotypes and during the 10 days before veraison (**Figs S1a,b**; Massonnet *et al*., 2017; Fasoli *et al*., 2018). Moreover, the *VviNAC33* expression profile during berry development belonged to a stage-specific cluster (Dal Santo *et al*., 2018), thus unaffected by environmental conditions or G×E interactions (**Figs S1c,d**).

Co-expression analysis using a grapevine expression atlas (Fasoli *et al*., 2012) revealed 92 genes positively correlated to *VviNAC33*, given a Spearman coefficient > 0.7 (**Dataset S1**). The top-30 strongly co-expressed genes with putative functional annotations formed two clusters based on their expression profiles during organ development. The first was characterized by a sharp increase in expression during berry and seed development, similar to that observed during leaf senescence (**Fig. 1b**; upper cluster). This large cluster included another NAC member, *VviNAC60*, and genes involved in stress responses and cellular processes, such as *AUTOPHAGY 8f* (*ATG8f*). The second was characterized by a stronger induction of expression during senescence (**Fig. 1b**; lower cluster). This smaller cluster included the α6 tubulin chain gene, the *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 1* (*SGR1*), and genes encoding the S-locus lectin protein kinase and the UDP-glycosyltransferase 85A8.

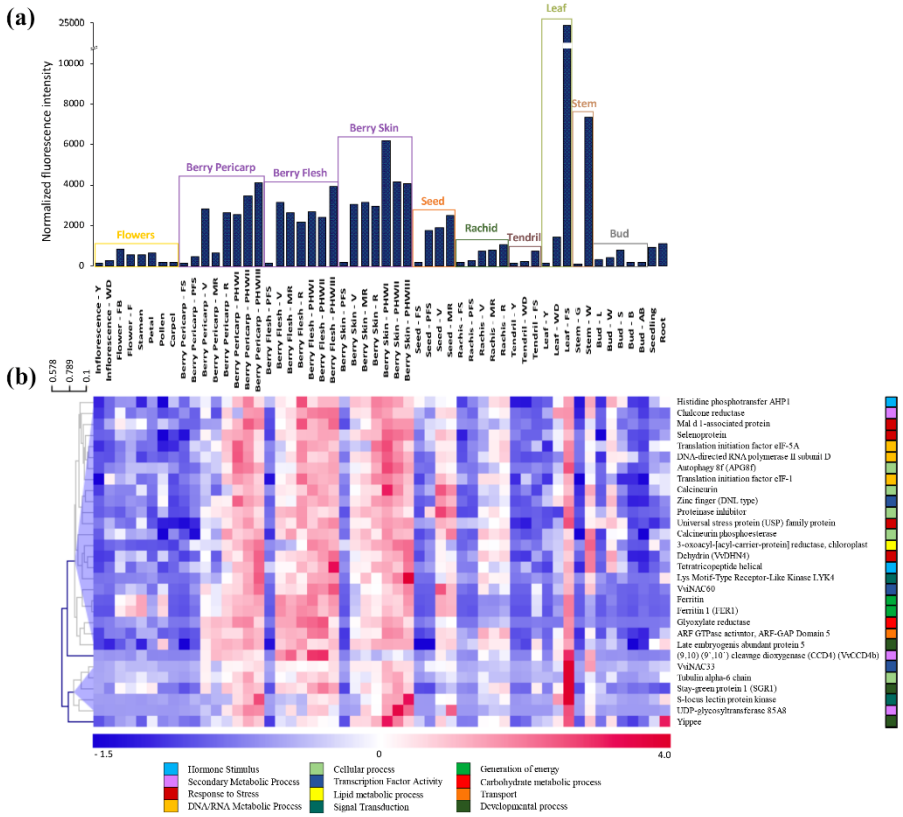


Fig. 1 VviNAC33 expression analysis in grapevine organs. (a) VviNAC33 expression profile in Vitis vinifera cv. Corvina in 54 grape organs during development (Fasoli et al., 2012). (b) Heat map for the set of 30 genes closely co-expressed with VviNAC33. This core set was defined using VvNAC33 as bait in the Corvina atlas (Dataset S1). The heat map shows the expression profiles of the 30 genes during grapevine organs development. Clusters were generated by hierarchical clustering in TMeV software, considering the expression value of each gene in different organs. Abbreviations: FS, fruitset; PFS, post-fruitset; V, veraison; MR, mid-ripening; R, ripening; Bud-L, latent bud; Bud-W, winter bud; Bud-S, bud swell; Bud-B, bud burst; Bud-AB, bud after burst; Inflorescence-Y, young; Inflorescence-WD, well-developed; Flower-FB, flowering begins; Flower-F, flowering; Tendril-Y, young; Tendril-WD, well-developed; Tendril-FS, mature; Leaf-Y, young; Leaf-FS, mature; Leaf-S, senescing leaf; Stem-G, green; Stem-W, woody.

VviNAC33 belongs to the same clade as ANAC092/ORE1, a key activator of leaf senescence

The full-length coding region of *VviNAC33* was amplified from Corvina berry cDNA. The 888-bp open reading frame encodes a protein of 296 amino acids with a predicted mass of 33.38 kDa and a calculated pI of 6.33 (**Fig. S2**). A phylogenetic tree was constructed for *VviNAC33* and the other 73 known grapevine *NAC* genes (Wang *et al*., 2013) whose sequences were downloaded from the new VCost.v3 genome annotation (Canaguier *et al*., 2017) (**Table S2**). The phylogenetic tree revealed some differences with the previous phylogenetic analysis (Wang *et al*., 2013), both in terms of clade designation and gene distribution among clades. *VviNAC33* belongs to clade V and is closely related to *VviNAC56* and *VviNAC14* (**Fig. S3**). However, the three *NAC* genes showed different expression profiles, with *VviNAC33* abundantly expressed in leaves undergoing senescence, *VviNAC56* strongly expressed in stamens, and *VviNAC14* expressed in buds (**Fig. S4**).

In order to explore the role of *VviNAC33*, a phylogenetic tree was constructed with VviNAC33 and 39 additional NAC proteins that have been functionally characterized in different plant species (**Fig. 2a**). The closest matches to VviNAC33 were ONAC011 in rice, a positive regulator of heading and senescence (El Mannai *et al*., 2017), and the Arabidopsis protein CUP SHAPED COTYLEDON 3 (CUC3), involved in shoot meristem formation (Vroemen *et al*., 2003). The Arabidopsis CUC1 and CUC2 and the Petunia hybrida protein NAM, all involved in initiating shoot apical meristem and establishing organ boundaries (Souer *et al*., 1996; Raman *et al*., 2008), also belong to the same clade of VviNAC33, along with the proteins ANAC092/ORE1, a key positive regulator of leaf senescence in Arabidopsis (Kim *et al*., 2016; Xi *et al*., 2019), and SlORE1S02 in tomato (Lira *et al*., 2017). Interestingly, several members of the VviNAC33 cluster are targets of *miR164* (Fig. 2; Sun *et al*., 2012), supporting a similar transcriptional regulation of these NACs.

The predicted sequence of the VviNAC33 protein (**Fig. S5**) contains seven motifs, including two conserved C-terminal motifs shared only with proteins of the VviNAC33 clade (**Fig. 2b**), and a monopartite N-terminal nuclear localization signal (PKGKKTD) (**Fig. S2**).

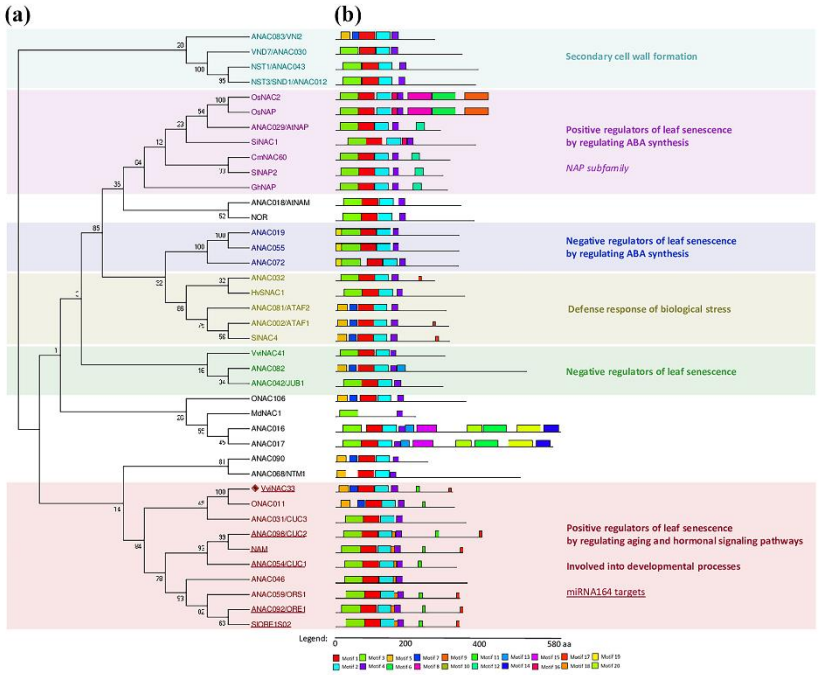


Fig. 2 Phylogenetic relationships and motif compositions of VviNAC33 and 39 additional NAC protein sequences. (a) Phylogenetic tree (neighbour-joining method) of VviNAC33 and 36 additional NAC protein sequences with functional annotations from various species prepared in MEGA v7 (Kumar et al., 2016). The numbers next to the nodes are bootstrap values from 1000 replicates. VviNAC33 is labelled with a red rhombus. The following GenBank accession numbers were used: Arabidopsis thaliana ATAF1/ANAC002 (AT1G01720), ATAF2 (AT5G08790), ANAC016 (AT1G34180), ANAC017 (AT1G34190), ANAC019 (AT1G52890), ANAC032 (AT1G77450), ANAC046 (AT3G04060), ANAC055 (AT3G15500), ANAC072 (AT4G27410), ANAC082 (AT5G09330), ANAC090 (AT5G22380), AtNAM (AT1G52880), AtNAP/ANAC029 (AT1G69490), CUC1 (AT3G15170), CUC2 (AT5G53950), CUC3 (AT1G76420), JUB1/ANAC042 (AT2G43000), ORE1/ANAC092 (AT5G39610), ORS1/ANAC059 (AT3G29035), NTM1 (AT4G01540), NST1/ANAC043 (AT2G46770); NST3/SND1/ANAC012 (AT1G32770); VND7/ANAC030 (AT1G71930); VNI2/ANAC083 (AT5G13180); Cucumis melo CmNAC60 (XM_008448163); Hordeum vulgare HvSNAC1 (AEG21060.1); Gossypium hirsutum GhNAP (ALG62640.1); Oryza sativa ONAC011 (Os06g0675600), ONAC106 (Os08g0433500), OsNAC2 (Os03g0327800), OsNAP (Os03g0327800); Malus domestica MdNAC1 (MF401514.1); Petunia hybrida NAM (CAA63101); Setaria italica SiNAC1 (XP_004967928.1); Solanum lycopersicum NOR (Solyc10g006880), SlNAC4 (Solyc11g017470), SlNAP2 (Solyc04g005610.2.1), SlORE1S02 (Solyc02g088180).(b) Schematic representation of motifs in the same set of NAC proteins identified by MEME analysis. Each color represents a specific motif. See also sequence LOGO in Figure S5.

VviNAC33 localizes in the nucleus and binds to a conserved NAC motif

The subcellular localization of VviNAC33 was assessed through protoplasts transformation with the construct 35S:VviNAC33-GFP or the control vector 35S:GFP. In protoplasts transformed with the control vector, GFP fluorescence was detected in both the nucleus and cytosol, but in protoplasts transformed with 35S:VviNAC33-GFP the GFP fluorescence was concentrated in the nuclei and co-localized with the nuclear stain Hoechst 33342 (**Fig. 3a**).

Next, we carried out DNA affinity purification sequencing (DAP-seq) assays to identify the putative direct targets of VviNAC33. We initially observed 4427 enriched peaks (**Dataset S2**), which was reduced to 3910 by removing those with a sample/control ratio < 5 (**Dataset S3**). The distribution of peaks revealed that 25% were located within promoter regions (up to 2 kb upstream from a transcription start site), 2% were located in 5′ untranslated regions (UTRs), 14% were located in exons, 17% were located within introns, 3% were located in 3′ UTRs, 7% were located in the 3-kb downstream region, and 32% were intergenic (**Fig. 3b**), leading to 3457 candidate target genes.

We identified three major binding motifs (GTTG[C/A][T/G]TGT) with strong significance (**Fig. 3c**) and phylogenetic footprints correlating with ANAC092/ORE1 (**Fig. S6a**). Distribution analysis revealed that 1024 peaks (27% of the total) were in the upstream region (promoter or 5′ UTR) of 971 genes, highlighting potential VviNAC33 direct targets. We focused our analysis on this specific set and found three slightly different top-ranking binding motifs, but the correlation with ANAC092/ORE1 was maintained (**Fig. S6b**). We found three top VviNAC33 dimeric binding motifs, confirming that the well-known characteristic of NAC TFs to bind DNA as stable homodimers or heterodimers is preserved in grapevine (**Fig. S6b**). GO enrichment analysis of the 971 putative direct target genes revealed a significant overrepresentation (FDR < 0.01) of genes in categories including ‘Regulation of biosynthetic process’, ‘Regulation of transcription’, ‘Regulation of gene expression’, ‘Auxin-activated signaling pathway’ and ‘Cellular response to auxin stimulus’ (**Fig. 3d**).

By ranking the putative targets by p value, we found that the top position was occupied by the

photosystem II gene *PsbA*, followed by the regulator of chlorophyll degradation *SGR1* (**Dataset S3**). Interestingly, the 971 putative VviNAC33 targets included 14 switch genes from the 113 identified in the atlas dataset (Fasoli *et al*., 2012), 17 of the 225 berry-specific switch genes reported by Massonnet *et al*. (2017) and several markers of the transcriptional transition characterizing the onset of berry ripening (**Dataset S3**).

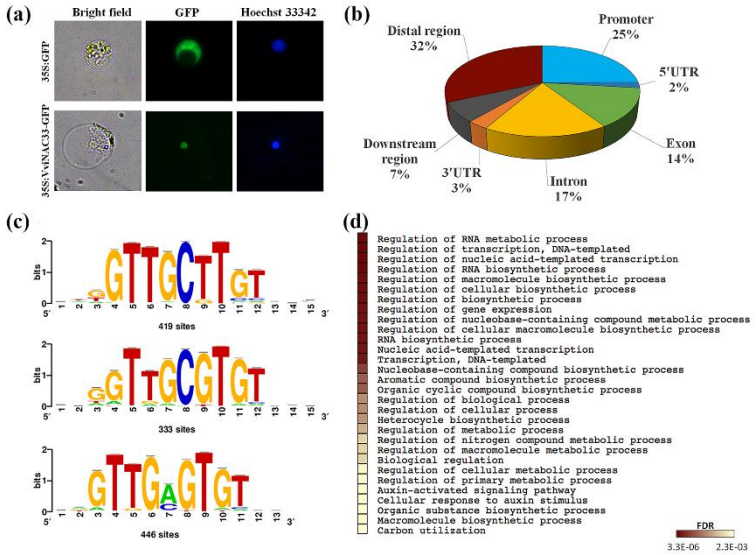
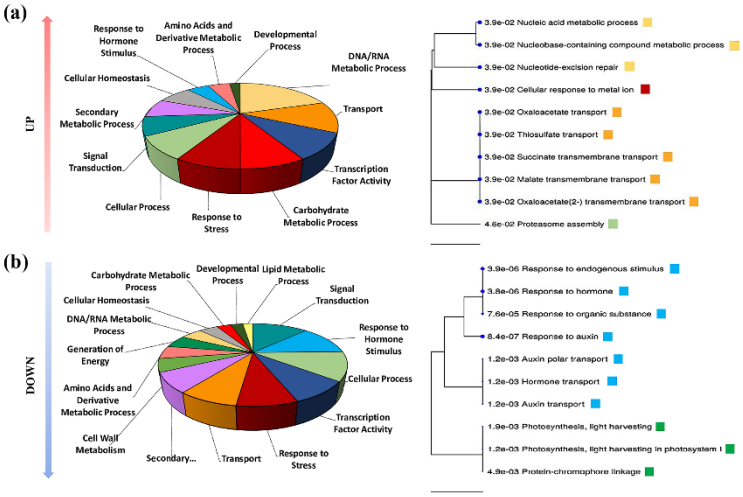


Fig. 3 VviNAC33 subcellular localization and DAP-seq results. (a) Subcellular localization of VviNAC33. Grapevine protoplasts isolated from Corvina leaves were transformed with the 35S:GFP and 35S:VviNAC33-GFP constructs. (b) Distribution of VviNAC33 binding sites. (c) VviNAC33 binding to three core motifs identified by RSAT Plants. (d) GO enrichment analysis on 971 putative VviNAC33 targets. Hierarchical clustering tree summarizes correlations among the 20 most significant pathways (p-value cut-off, FDR ≤ 0.01). Enrichment analysis was based on hypergeometric distribution followed by FDR correction. Pathways with many shared genes are clustered together. Bigger dots indicate more significant p-values.

Transient overexpression of VviNAC33 identifies positively and negatively regulated targets

To investigate the ability of VviNAC33 to activate or inhibit gene expression, we infiltrated grapevine plantlets (cv. Sultana) with *Agrobacterium tumefaciens* (Amato *et al*., 2017) carrying the 35S:*VviNAC33* overexpression construct. The infiltrated plants did not show any overt phenotype, so we used qRT-PCR to quantify transgene expression and selected four lines strongly overexpressing *VviNAC33* for transcriptomic analysis (**Fig. S7**). We identified 1027 differentially expressed genes (DEGs) based on a t-test with a significance threshold of $p < 0.05$ (**Dataset S4**), 379 of which satisfied the fold-change criterion ($|FC| > 1.5$). This comprised 122 upregulated and 257 downregulated genes. GO distribution and enrichment analysis showed that the upregulated genes mainly represented the functional categories ‘DNA/RNA metabolic process’, ‘Transport’ and ‘Cellular response to ions’ (**Fig. 4a**), whereas the downregulated genes mainly represented the functional categories ‘Response to hormone stimulus’, ‘Auxin transport and signaling’ and ‘Photosynthesis’ (**Fig. 4b**). Interestingly, some of the most strongly upregulated DEGs were co-expressed with *VviNAC33*, including *SGR1*, *IAGLU* (indole-3-acetate β-glucosyltransferase), *VviCCD4b*, encoding a (9,10) (9′,10′) cleavage dioxygenase, and the α6 tubulin chain gene (**Dataset S4**).

By aligning the 1027 DEGs with the 971 putative VviNAC33 targets determined by DAP-seq analysis, we identified 40 genes common to both lists, 24 of which were upregulated and 16 downregulated, suggesting VviNAC33 acts directly as both an activator and repressor (**Fig. 4c**). *SGR1* was the most strongly upregulated direct target candidate with the highest DAP-seq signal, with *IAGLU* and the α6 tubulin chain gene also ranking highly. Downregulated putative targets included several genes encoding signaling components such as *RopGEF1*, an activator of plant Rho GTPases (ROPs) involved in the polar distribution of auxin influx carrier AUX1 and the accumulation of PIN efflux carriers (Liu *et al*., 2017).



Gene ID	Functional annotation	Gene Ontology	FC Signal Value
VIT_02s0025g04660	Stay-green protein 1 (SGR1)	Developmental Process/Chlorophyll catabolism	
VIT_06s0004g07230	Indole-3-acetate beta-glucosyltransferase	Response to Hormone Stimulus/Auxin inactivation by conjugation	
VIT_18s0001g08300	Tubulin alpha-6 chain	Cellular Process/Microtubule organization and biogenesis	
VIT_06s0004g04650	Metallothionein	Response to Stress/Heavy metal ion homeostasis	
VIT_05s0020g01830	UPF0497 family	Unknown	
VIT_06s0061g01210	Lysine histidine transporter 1	Transport/Amino Acid/Auxin Permease	
VIT_19s0177g00070	Polyubiquitin (UBQ14)	Cellular Homeostasis/Ubiquitin-mediated proteolysis	
VIT_04s0008g02750	Basic Leucine Zipper Transcription Factor (VvbZIP09)	Transcription Factor Activity	
VIT_08s0032g01080	Ubiquitin carboxyl-terminal hydrolase 8	Cellular Process/Proteolysis	
VIT_13s0019g02080	DNA-binding protein	DNA/RNA Metabolic Process	
VIT_01s0010g03780	Histidine kinase (AHK3)	Response to Hormone Stimulus/Cytokinin-mediated Signaling pathway	
VIT_13s0019g05360	KH domain-containing protein NOVA	DNA/RNA Metabolic Process	
VIT_17s0000g01950	Dihydropyrimidine dehydrogenase	Cellular Amino Acids and Derivative Metabolic Process	
VIT_16s0039g02170	D-xylose-proton symporter-like 2	Transport/Sugar Porter	
VIT_19s0015g00750	Sucrase	Carbohydrate Metabolic Process/Sucrose catabolism	
VIT_02s0025g01420	Preprotein translocase YidC subunit	Cellular Homeostasis/Protein export	
VIT_01s0011g01570	Coatomer subunit delta	Transport	
VIT_14s0060g00110	Glutamyl-tRNA synthetase, cytoplasmic	Generation of Energy/Tetrapyrrole metabolism	
VIT_07s0031g00330	MAP3K protein kinase	Signal Transduction	
VIT_06s0004g04390	UDP-glucuronic acid decarboxylase 1	Carbohydrate Metabolic Process/Starch and sucrose metabolism	
VIT_14s0066g01410	Peptidylprolyl isomerase	Cellular Homeostasis/Protein folding.	
VIT_06s0004g06240	Zinc finger (C2H2 type) family	Transcription Factor Activity	
VIT_04s0044g00720	S-receptor kinase	Signal Transduction	
VIT_18s0001g13870	Calmodulin binding protein	Signal Transduction/Calcium sensors and Signaling	
VIT_09s0002g03450	Taurine dioxygenase	Cellular Amino Acids and Derivative Metabolic Process	
VIT_03s0038g03620	Rho guanyl-nucleotide exchange factor ROPGEF1	Signal Transduction/Auxin distribution	
VIT_10s0003g00330	HSL1 (HASSA-like 1)	Signal Transduction/Development	
VIT_06s0004g02640	Receptor protein kinase PERK1	Signal Transduction	
VIT_18s0041g00010	Wall-associated receptor kinase 2	Signal Transduction	
VIT_18s0001g01630	Calcium-binding protein CML	Signal Transduction/Stress response	

Fig. 4 Transcriptomic analysis of grapevine leaves in transient expression experiments. (a) GO distribution (left) and GO enrichment (right) analysis of the 122 upregulated genes in leaves transiently overexpressing VviNAC33 (threshold |FC| > 1.5.) (b) GO distribution (left) and GO enrichment (right) analysis of 257 downregulated genes in in leaves transiently overexpressing VviNAC33 (|FC| > 1.5). The hierarchical clustering trees summarize correlations among the 10 most significant pathways (FDR ≤ 0.05). Pathways with many shared genes are clustered together. Bigger dots indicate more significant p-values. (c) Common genes in the list of DEGs identified by transient expression and the list of putative VviNAC33 targets based on DAP-seq analysis. Only the 33 functionally annotated genes are reported.

The stable overexpression of VviNAC33 induces leaf de-greening by modulating photosynthetic complexes and leaf expansion

The preliminary functional analysis of *VviNAC33* in *Nicotiana benthamiana* leaves by transient expression revealed the acceleration of leaf de-greening compared to vector controls, confirmed by a significant reduction in chlorophyll amount, chlorophyll/carotenoid ratio and maximum quantum efficiency (Fv/Fm) of PSII (**Figs S8a-d**).

We therefore generated transgenic grapevine plants expressing *VviNAC33* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (OXNAC33 lines). We recovered 40 PCR-positive OXNAC33 plantlets and 10 vector controls (data not shown). Ten of the OXNAC33 transgenic lines expressed *VviNAC33* at significantly higher levels than the control lines and we observed a positive correlation between phenotypic alterations and level of transgene expression (**Figs S9a,b**). Three independent lines with the highest *VviNAC33* expression (#4, #5 and #8) were selected for further analysis and renamed OX1, OX2, OX3 for simplicity. Southern blot analysis revealed that all three lines as well as the vector control contained a single-copy transgene (**Fig. S9c**).

The growth rate, height and general habitus of the OXNAC33 transgenic lines were similar to the controls (**Fig. 5a**). However, as described above for *N. benthamiana*, mature leaves exhibited a clearly visible de-greening effect leading to a green/pale green variegated phenotype that became more apparent with age. Pigment analysis revealed a sharp reduction (~40%) in the levels of chlorophyll and carotenoids (**Fig. 5b**). The leaf area of the transgenic plants was ~35% lower than the controls (**Fig. S9d**). We isolated protoplasts from transgenic and control leaves and found a significant (~20%) reduction in the cell area occupied by chloroplasts in the transgenic lines, which only partially explained the loss of pigments (**Fig. 5c**). We therefore measured the chlorophyll a/b and chlorophyll/carotenoid ratios, both of which were lower in the OXNAC33 transgenic lines.

More detailed analysis of the carotenoid content revealed that the lower chlorophyll/carotenoid ratio in the transgenic plants was almost entirely due to the higher levels of lutein. The analysis of the fluorescence yield in dark-adapted leaves revealed a significant decrease in Fv/Fm in the three transgenic lines. Whereas Fm (normalized to the chlorophyll content) remained stable, F0 was significantly higher in the transgenic leaves (**Figs 5d-g**).

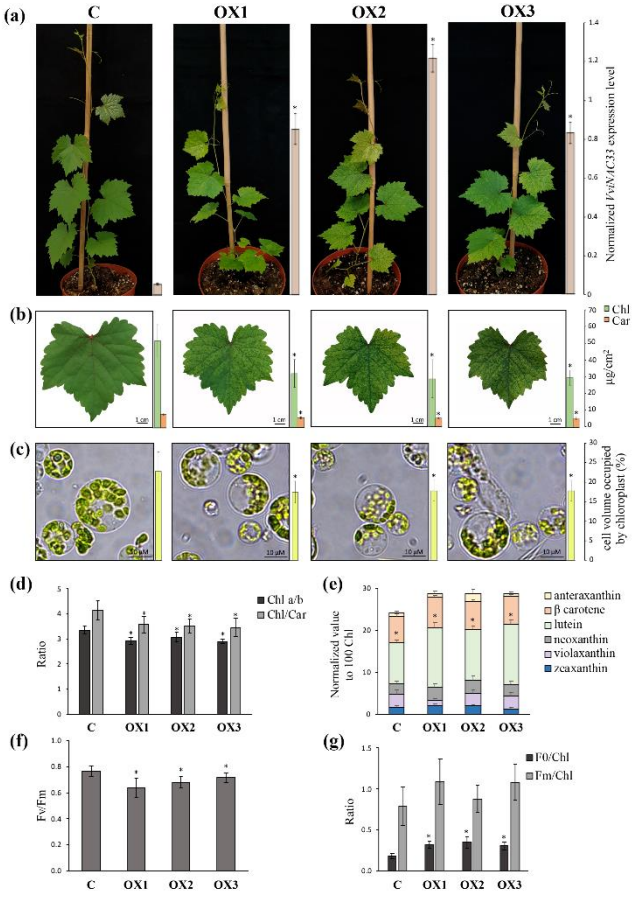


Fig. 5 Phenotypic changes in transgenic grapevine plants overexpressing VviNAC33. (a) Whole-plant phenotype caused by the stable expression of VviNAC33 in selected OXNAC33 lines compared to vector controls. The expression levels detected by qPCR are indicated by the bars next to the pictures. Each value corresponds to the mean ± standard deviation (SD) of three technical replicates relative to the VviUBIQUITIN1 (VIT_16s0098g01190) control. (b) Fully expanded leaves showing the phenotype caused by the stable overexpression of VviNAC33 in the three OXNAC33 lines compared to the vector control. Total chlorophyll (chl) and carotenoid (car) levels are indicated by the bars next to the pictures. (c) Distribution of chloroplasts in protoplasts isolated from the three OXNAC33 lines compared to the vector control. The proportion of cell volume occupied by chloroplasts, as determined by light microscopy, is indicated by the bars next to the pictures. (d) Chlorophyll a and b (Chl a/b) and chlorophyll carotenoid (Chl/Car) ratio. (e) Carotenoid content, with individual carotenoid values were normalized to 100 chlorophyll equivalents. (f) F0 and Fm normalized to Chl. (g) PSII maximum quantum efficiency (Fv/Fm). All pigment and photosynthetic performance data are expressed as mean ± SD (n = 4). Asterisks (*) indicate significant differences (t-test; $p < 0.05$) in the OXNAC33 lines compared to the vector control.

We selected line OX2, which expressed *VviNAC33* at the highest level, for the analysis of photosynthetic parameters in more detail at different light intensities. The PSII operating efficiency (ΦPSII), electron transport rate (ETR) and electrochemical proton gradient (determined by ECS) were significantly lower in OX2 compared to the control at all light intensities, whereas the 1-qL parameter representing the redox state of the plastoquinone pool was higher in OX2, suggesting the plastoquinone pool was more reduced by electrons (**Figs 6a-d**). Non-photochemical quenching (NPQ) was enhanced in the OX2 leaves at higher light intensities (**Fig. S10a**). Finally, dark-adapted leaves were exposed to saturating actinic light and the OJIP curve was registered in order to monitor the polyphasic rise from F0 to Fm (Stirbet *et al.*, 2018). The steeper O–J part of the curve (**Fig. S10b**) indicates lower electron-trapping efficiency in the OX2 leaf and thus an increase in the dissipation of absorbed light energy by fluorescence (Stirbet and Govindjee, 2011).

We then measured the relative amounts of photosynthetic components by immunoblotting on isolated thylakoid membranes. We found that CP47 (PSII core), PSAA (PSI core) and ATPC1 were significantly depleted in OX2 leaves compared to controls whereas LHCII was present at similar levels. We also fractionated the native chlorophyll binding complexes on a sucrose gradient following thylakoid solubilization. The analysis of free pigments, monomeric LHCB, trimeric

LHCII, PSII core and PSI-LHCI confirmed a relative increase in the content of antenna subunits, particularly LHCII, and a relative decrease in the PSII and PSI factions in OX2 compared to the control (**Figs 6e-g**).

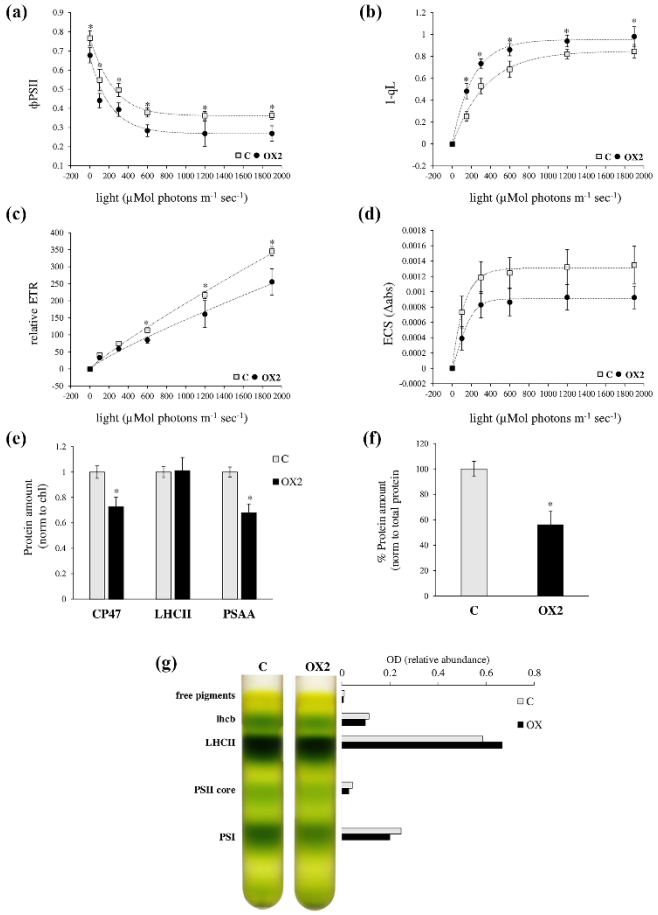


Fig. 6 Photosynthetic parameters and organization of thylakoid membranes. (a) Dependence of PSII operating efficiency (ΦPSII), (b) 1-qL (estimates the fraction of PSII centers with reduced QA), (c) electron transport rate (ETR) and (d) total proton motive force (ECS) on actinic light intensity for the OX2 line compared to the vector control. (e) Immunotitration of thylakoid proteins using specific antibodies against PSAA, LHCII and PSAA corrected for chlorophyll content, and (f) ATPC1 corrected for total protein content. (g) Sucrose density gradient fractionation. The composition of the green bands is shown on the left, and the relative abundance of the band (normalized to total green content on the gradient) is shown on the right. All data are expressed as mean ± standard deviation (SD, n = 4). Asterisks (*) indicate significant differences (t-test; $p < 0.05$) between the OX2 line and vector control.

Transcriptomic analysis of transgenic leaves reveals additional putative direct targets of VviNAC33

We analyzed leaves of OXNAC33 transgenic lines and controls for genes that are differentially expressed before the phenotype appears. In total, we identified 2650 DEGs (t-test, $p < 0.05$), 617 of which were upregulated and 1388 downregulated with a $|FC| > 1.5$ (**Dataset S5**). GO enrichment analysis showed that 'Glutamate signaling pathway', 'Transport' and 'Carbohydrate metabolic process' were the most significant overrepresented functional categories for the upregulated genes whereas 'Response to auxin', 'Developmental growth', 'Cell morphogenesis' and 'Signaling pathway' were the most significant overrepresented functional categories for the downregulated genes (**Fig. 7a**), in agreement with our transient expression experiments (**Figs 4a,b**).
We identified 127 common DEGs among the 2650 identified in transgenic plants and the 1027 identified by transient expression (**Fig. 7b**, **Table S3**, **Fig. 7c**).
By combining the DEGs identified in the transgenic plants and transient expression experiments with the DAP-seq data, we resolved to a list of 139 genes that are most likely to be direct targets of VviNAC33 (**Fig. 7b**, **Table S4**). All 139 genes were screened against the three leaf development stages included in the transcriptomic atlas, revealing four major expression trends: upregulation at senescence, downregulation at senescence, peak of expression in the mature leaf, and trough of expression in the mature leaf. There was consistency between the behaviour of these genes in the expression atlas and transgenic plants, with those induced by VviNAC33 tending to be upregulated at senescence or in the mature leaf and those suppressed by VviNAC33 tending to be downregulated

at senescence or in the mature leaf (**Fig. 7d**; **Table S4**). Interestingly, most of the DEGs identified by transient expression corresponded to those modulated at senescence, indicating that transient expression highlights the impact of VviNAC33 at its highest expression level. Genes strongly expressed at senescence included *SGR1*, *IAGLU* and the α6 tubulin chain gene (**Table S4**).

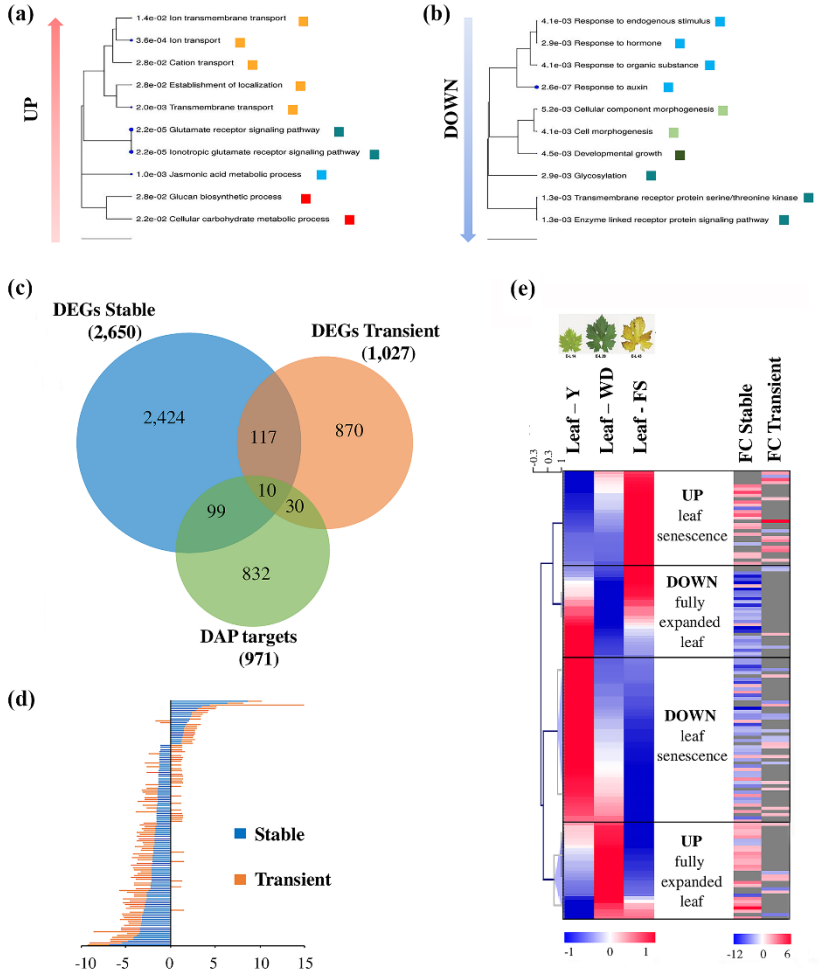


Fig. 7 Transcriptomic analysis of transgenic grapevine plants overexpressing VviNAC33. (a) GO enrichment analysis of the upregulated (left) and downregulated (right) genes in transgenic leaves with |FC| > 1.5. (b) Venn diagram showing genes common to the lists of differentially expressed genes (DEGs) in the transgenic plants and transient expression experiments, and to the targets identified by DAP-seq analysis. (c) Histogram showing the fold changes (FC) in expression of the genes classed as DEGs by both transient expression experiments and transgenic plants. (d) Heat map of the 139 putative direct targets of VviNAC33 in three leaf developmental stages (Fasoli et al., 2012). Clusters were generated by hierarchical clustering in TMeV, considering the expression value of each gene in the different stages (left) and the FC of the 139 putative targets. Boxes indicated four expression trends: the upregulated and downregulated genes at senescence and in the fully expanded leaves.

Putative target genes upregulated by stable VviNAC33 overexpression included several involved in nutrient transport, metabolism and recycling, such as genes encoding a substrate carrier, D xylose proton symporter-like 2, sucrase, and ATG8f (**Table S4**). On the other hand, putative targets downregulated by stable *VviNAC33* overexpression included several genes involved in auxin signaling, such as *RopGEF1* and *PIN1*, as well as *ATPC1*, encoding the ATP synthase γ chain 1t involved in photosystem II integrity and activity.

In addition to putative direct targets, in OXNAC33 transgenic lines we found several downregulated genes involved in the synthesis of chlorophyll and carotenoids, in the spatial regulation of chloroplast division and in auxin signaling (**Dataset S5**).

Considering several clues about the possible role of VviNAC33 in controlling auxin signaling and metabolism emerged from DAP-seq and transcriptomic analyses (**Table S5**), we tested if exogenous auxin treatment was able to rescue the de-greening phenotype in stable VviNAC33 overexpressing plants. We treated *in vitro* shoots of the OX2 and control line with two NAA concentrations (**Fig. S11**). After two weeks, we found that both NAA concentrations were able to restore the green phenotype in newly formed leaves, that appeared similar to the controls, while the fully expanded leaves maintained the de-greening effect (**Fig. S11**).

A VviNAC33-EAR chimeric repressor boosts the chlorophyll content and growth of transgenic grapevine leaves

To create a dominant suppressor which overcomes the activity of endogenous VviNAC33, we fused the C-terminal domain of VviNAC33 to the plant-specific EAR repression domain (Ohta *et al*., 2001; Hiratsu *et al*., 2002) and placed the chimeric repressor under the control of the endogenous *VviNAC33* promoter. The chimeric repressor construct was used to stably transform embryogenic grapevine callus and produce transgenic vines. We recovered four PCR-positive plantlets containing *VviNAC33-EAR* and two containing the vector control (data not shown). Transgene expression was confirmed by qRT-PCR in fully expanded leaves, when the expression of endogenous *VviNAC33* begins to increase. Three independent EARNAC33 lines (#1, #2 and #4) were selected based on their strong transgene expression and were renamed EAR1, EAR2, EAR3 for simplicity (**Figs S12a,b**).
The three EARNAC33 lines showed normal vegetative growth, but the fully expanded leaves were significantly larger than controls (**Figs 8a,b**, **S12c**). Pigment content analysis revealed a significant increase in the chlorophyll and carotenoid content of the transgenic lines (**Fig. 8b**). However, the chlorophyll a/b and chlorophyll/carotenoid ratios, Fv/Fm, F0/chlorophyll and Fm/chlorophyll were similar in transgenic plants and vector controls (**Figs S13a-c**), possibly reflecting the partial but not complete dominance of the chimeric repressor.
The chimeric repressor system was also used to evaluate the expression of six putative VviNAC33 direct target genes by qPCR analysis, comparing transgenic lines OX2 and EAR2 and their controls. We assessed the upregulated candidate genes *SGR1*, the sucrase gene, and *ATG8f* and the downregulated candidate genes *PIN1*, *RopGEF1* and *ATPC1*. We found that *SGR1*, *ATG8f* and the sucrase gene were strongly upregulated by VviNAC33 but inhibited by VviNAC33-EAR, conversely, *PIN1*, *RopGEF1* and *ATPC1* were strongly downregulated by VviNAC33 but not inhibited by VviNAC33-EAR (**Fig. 8c**). This indicates that the addition of the EAR motif could have impaired the VviNAC33 repressor activity possibly by destabilizing putative interactions with binding partners involved in repression or by inhibiting the expression of a repressor that is itself transcriptionally activated by VviNAC33.

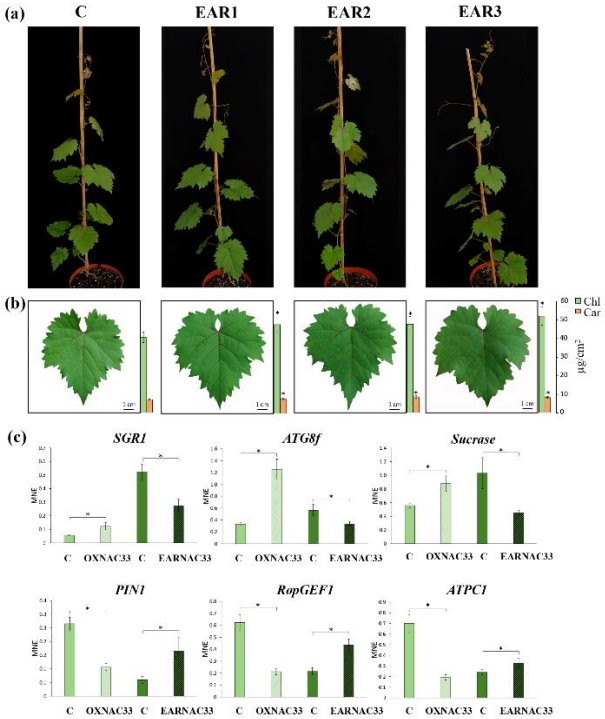


Fig. 8 Phenotypic changes in transgenic grapevine plants expressing the repressor NAC33- EAR and the expression level of VviNAC33 targets. (a) Whole-plant phenotype caused by the stable expression of VviNAC33-EAR in the three EARNAC33 lines compared to the vector control. (b) Fully expanded leaf phenotype caused by the stable expression of VviNAC33-EAR in the three EARNAC33 lines compared to the vector control. Total chlorophyll (Chl) and carotenoid (Car) levels are indicated by the bars next to the pictures. Data are mean ± standard deviations (SD, n = 4). Asterisks (*) indicate significant differences (t-test; $p < 0.05$) between the EARNAC33 lines and vector control. (c) Expression levels of SGR1, ATG8f, sucrase, PIN1, RopGEF1 and ATPC1 in the leaves of transgenic plants overexpressing VviNAC33 (OXNAC33) or expressing VviNAC33-EAR (EARNAC33) determined by qPCR. Each value corresponds to the mean ± SD of three technical replicates relative to the VviUBIQUITIN1 (VIT_16s0098g01190) control. Asterisks (*) indicate significant differences (t-test ; $p < 0.05$) between the OXNAC33 or EARNAC33 lines and the vector control. MNE, mean expression level.

We then tested the ability of VviNAC33 to directly induce or repress the expression of the putative target genes validated by qPCR by a dual-luciferase assay in *N. benthamiana* leaves. The upstream regions of these genes containing a NAC33 DAP-seq peaks (**Fig. 9a**) were cloned upstream of a 35S promoter-Luciferase cassette. Sequence analysis of these putative promoter regions showed several putative VviNAC33 binding sites, including a conserved motif near the summit of each DAP-seq peak (**Fig. S14**). The dual-luciferase assay showed that VviNAC33 significantly activates the *SGR1* and *ATG8f* promoters and represses the *PIN1*, *RopGEF1* and *ATPC1* promoters (**Fig. 9b**).
Finally, we investigate the ability of VvibHLH75 and VviWRKY19, two TFs previously suggested to regulate *VviNAC33* in grape berry (Fasoli *et al*., 2018), to activate the VviNAC33 expression. The analysis showed that *VviNAC33* is positively regulated by both VvibHLH75 and VviWRKY19 (**Fig. S15**).

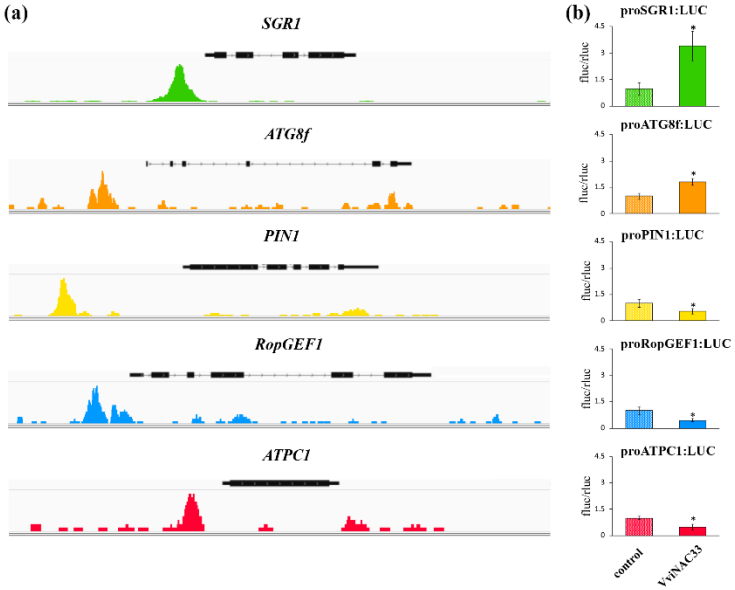


Fig. 9 VviNAC33 binding sites and regulation of SGR1, ATG8f, PIN1, RopGEF1 and ATPC1. (a) IGV images of the VviNAC33 DAP-seq reads mapping to the promoters of the grapevine SGR1, ATG8f, PIN1, RopGEF1 and ATPC1 genes and (b) promoter activation tested by dual-luciferase reporter assays in infiltrated Nicotiana benthamiana leaves. The activity of these promoters was tested in the presence and absence of the 35S:VviNAC33 effector vector. LUC values are reported relative to the REN value and normalized against the control (empty effector vector). LUC values represent the mean of four biological replicates ± standard deviation (SD). Asterisks (*) indicate significant differences in promoter activation compared with the control (t-test; $p < 0.01$).

Discussion

In this work we characterized the function of VviNAC33 in regulating vegetative/green to mature/woody growth shift (Fasoli *et al*., 2012; Palumbo *et al*., 2014). By transgenic approaches we found that VviNAC33 induces organ de-greening and suppresses vegetative growth. DAP-seq and transcriptomic analyses evidenced that 139 genes, bound by VviNAC33 in the upstream region, were differentially expressed in plants stably or transiently expressing VviNAC33, thus likely representing direct targets of regulation.
SGR1 was one of the putative direct targets of VviNAC33 and was strongly co-expressed with *VviNAC33* throughout grapevine organ development. We demonstrated that VviNAC33 directly activates *SGR1* expression, as previously shown for the Arabidopsis close homolog ANAC092/ORE1 (Qiu *et al*., 2015). SGR1 removes magnesium from chlorophyll a as the first step to initiate its degradation in multiple species (Park *et al*., 2007; Sato *et al*., 2007; Hortensteiner, 2009; Zhou *et al*., 2011). The phenotypes of OXNAC33 and EARNAC33 transgenic leaves showed that VviNAC33 induces leaf de-greening by reducing the total chlorophyll content. There was also a significant depletion of the PSI and PSII core relative to antenna subunits in OXNAC33 leaves. Given that chlorophyll b is bound only by antenna proteins whereas chlorophyll a is bound both by core and antenna subunits, this result agrees with the lower chlorophyll a/b ratio in the OXNAC33 lines. In Arabidopsis, ectopic expression of *SGR1* in fully greened leaves reduces the abundance of chlorophyll-binding proteins in PSI/II and LCH, indicating that SGR1 directly attacks the pigment–protein complexes and the chlorophyll-depleted apoproteins may then be immediately degraded in

the thylakoid membranes (Shimoda *et al*. 2016). The loss of pigment-binding proteins in our OXNAC33 leaves supports this hypothesis.

We found that leaf de-greening in our transgenic plants was, at least in part, associated with a smaller area occupied by chloroplasts. A similar phenotype was observed in Arabidopsis *glk1 glk2* double mutants, which featured pale green leaves with mesophyll cells containing small chloroplasts with sparse thylakoid membranes that failed to form grana (Waters *et al*., 2009). GOLDEN2-LIKE1 (GLK1) and GLK2 activate genes responsible for key steps in chlorophyll biosynthesis, contributing to photosystem assembly and chloroplast development and maintenance (Waters *et al*., 2009).

The gene encoding the PSII central core reaction protein D1 (*PsbA*) showed the highest p value in our DAP-seq data, and additional genes in this dataset included LOW PSII accumulation 1 (*LPA1*) and photosystem one 3 (*APO3*), which are required for efficient PSII and PSI assembly (**Dataset S3**). Although the control of these putative targets by VviNAC33 requires confirmation, our results strongly indicate that VviNAC33 plays a key role in the assembly and stability of the photosynthetic apparatus. In this context, we also found that VviNAC33 negatively regulates the expression of *ATPC1*, which encodes the ATP synthase γ chain 1t involved in the production of ATP from ADP in the presence of a proton gradient across the membrane. Interestingly, the inactivation of this gene in Arabidopsis abolishes photophosphorylation and alters PSII activity by increasing NPQ and F0 while reducing Fv/Fm (Dal Bosco *et al*., 2003). Our data clearly demonstrated that VviNAC33 directly activates *SGR1* expression and suppresses *ATPC1*, which combined with the identification of *PsbA* and other PSII component genes among putative VviNAC33 targets strongly indicates that VviNAC33 is directly involved in shutting down photosynthesis in greened organs that enter the vegetative-to-mature phase transition.

Chlorophyll degradation is an integral aspect of senescence occurring during the final phase of development (Lim *et al*., 2007). Senescence involves a massive cellular proteolysis carried out by organelles known as autophagosomes, which form under the control of ATG proteins to break down damaged and superfluous proteins (Zhai *et al*., 2016; Fu *et al*., 2020). A role of VviNAC33 in the control of autophagy is suggested by the fact that one of the direct targets of VviNAC33 is *ATG8f*, and by the upregulation of the *ATG8d* and *ATG18d* genes (Mizushima and Komatsu, 2011; Su *et al*., 2020) in OXNAC33 leaves. Autophagy includes steps of ubiquitination (Mizushima *et al*., 1998; Ichimura *et al*., 2000) and delivery of autophagosomes to the vacuole, via the microtubule cytoskeleton. Interestingly, the polyubiquitin gene *UBQ14* and the α6-tubulin chain, both strongly induced during senescence, were identified among the VviNAC33 targets (**Table S4**). Our data suggest that VviNAC33 directly orchestrates the elimination of chloroplasts by controlling the expression of genes involved in autophagy and photosynthetic activity.

The leaves of the OXNAC33 plants were significantly smaller than controls whereas those of the EARNAC33 plants were significantly larger, suggesting that VviNAC33 controls organ growth. In Arabidopsis plants overexpressing *ANAC092/ORE1*, the length of the root meristem differed from control plants and the primary roots were shorter, reflecting a slower rate of meristematic cell division based on the suppression of *PIN*, *YUCCA2*, and *ARF* (Xi *et al*., 2019). Although we did not determine the cause of the OXNAC33 and EARNAC33 leaf phenotypes, we hypothesize that VviNAC33 and ANAC092/ORE1 play similar roles in the regulation of organ growth. Indeed, several auxin-related genes were found among the 139 direct target candidates that were downregulated (**Table S4**). Furthermore, we demonstrated that VviNAC33 directly binds to the auxin influx carrier *PIN1* promoter, inhibiting its expression. Two other PIN genes were downregulated in OXNAC33 leaves, and *PIN8* has a VviNAC33-binding site within an exon. We also found that the expression of *ARF3*, *ARF4*, *ARF6* and *ARF8* was modulated by VviNAC33 in the OXNAC33 lines, although we did not find VviNAC33 sites in their promoters. These data provide strong evidence that VviNAC33 is a negative regulator of the auxin pathway, thus suppressing cell division during leaf development.

We also demonstrated that *RopGEF1* is directly suppressed by VviNAC33. RopGEF1 maintains polar auxin transport by regulating the properties of auxin influx and efflux carriers such as AUX1 and PIN proteins, thereby influencing auxin-dependent growth and development (Swarup and Péret, 2012; Liu *et al*., 2017). Interestingly, *AUX1* was downregulated in leaves overexpressing VviNAC33, supporting the role of VviNAC33 as a negative regulator of auxin transport and distribution by inhibiting *PIN1* and *RopGEF1*.

The *CRY1* gene encoding cryptocrome 1, a blue light photoreceptor, was also one of the putative VviNAC33 direct targets and was upregulated by the overexpression of VviNAC33. *CRY1* negatively regulates the phytochrome-interacting transcription factors PIF4 and PIF5, which in turn

activate PIN genes (Ma *et al*., 2016; Pedmale *et al*., 2016; Boccaccini *et al*., 2020).
The ability of exogenous auxin treatment to rescue the de-greening phenotype of VviNAC33 overexpressing shoots, further supports that the senescence-like phenotype of transgenic leaves could be due to an impairment of auxin signaling triggered by VviNAC33. Senescence is controlled by multiple hormones; ethylene, jasmonic acid (JA), salicylic acid, abscisic acid and brassinosteroids act as inducers, while cytokinins, gibberellic acid and auxin, as inhibitors (Gan and Amasino, 1997). Given this complexity, the involvement of other hormones besides auxin in the leaf de-greening phenotype and a role of VviNAC33 in their control, could not be ruled out.
We provided several lines of evidence showing that VviNAC33 is a key regulator of the shift toward organ senescence acting through molecular mechanisms similar to those previously described for the Arabidopsis protein ANAC092/ORE1 (Kim *et al*., 2006; Balazadeh *et al*., 2010). Both activate genes such as *SGR1* that trigger chlorophyll degradation (Qiu *et al*., 2015) while repressing the expression of auxin transporter *PIN* genes to control growth (Xi *et al*., 2019). The *VviNAC33* and *ANAC092/ORE1* genes are also regulated in a similar manner, both being repressed by *miRNA164* (Kim *et al*., 2009; Sun *et al*., 2012). Interestingly, *miR164* is expressed strongly in young grapevine leaves but only weakly in old leaves and fruits, the inverse of *VviNAC33* (Belli Kullan *et al*., 2015).
There is strong evidence that some tomato NAC TFs involved in the control of leaf senescence are also involved in fruit ripening. SlORE1S02, SlORE1S03, and SlORE1S06 induce leaf senescence and source–sink sugar partitioning, affecting the final sugar level (Lira *et al*., 2017). Recently, NOR, a NAC TF that regulates fruit ripening in tomato, was shown to induce leaf senescence (Ma *et al*., 2019). While we could not determine the effects of VviNAC33 or VviNAC33-EAR in transgenic berries because neither the transgenic plants nor vector controls flower under our growing conditions, a role for VviNAC33 at the onset of berry ripening is indicated by increased expression level over a few days before veraison.
Consistently, several genes previously identified as markers of the onset of berry ripening together with VviNAC33 (Fasoli *et al*., 2018) were highlighted in this work among the DAP-seq hits as potential direct targets of VviNAC33. In addition, we confirmed that the two berry ripening markers VvibHLH75 and VviWRKY19 are able to directly activate the expression of VviNAC33.
Our data defined VviNAC33 as a central hub in a regulatory network that controls de-greening and growth of grapevine organs during the vegetative to mature phase transition (**Fig. 10**). It represents a key step towards a comprehensive understanding of the intricate molecular circuits that combine developmental and environmental cues in grapevine.

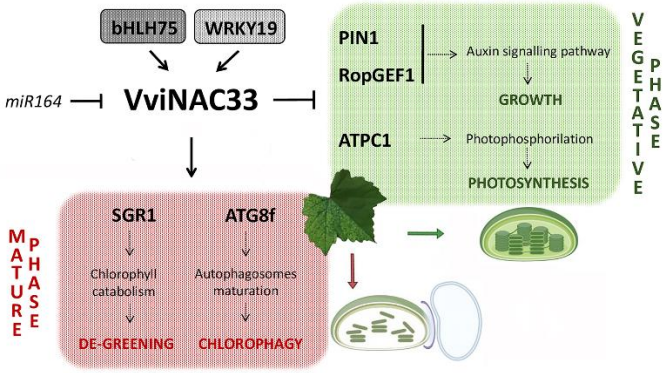


Fig. 10 Proposed regulatory network model of the VviNAC33 mechanisms of action. Induced and repressed VviNAC33 targets identified in this work and the biological processes they are involved in, are indicated together with the transcription factors controlling VviNAC33 expression. The negative regulation of VviNAC33 by miR164 was demonstrated by Sun et al. (2012). The figure also shows the chloroplast images corresponding to vegetative and mature phase (lower right corner). The chloroplast images were created with BioRender.com.

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Author Contributions
E.D., S.C., and C.F. performed the research. E.D., S.C, and S.Z. analysed the data. M.G., A.G., and N.V. contributed new analytic and computational tools. S.Z., G.B.T., and M.P. designed the research. S.Z., E.D., and G.B.T. wrote the paper.

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VviNAC38

The DAP-seq assay reported only 68 binding sites (peaks), which remain the same after the FC>5 filtering (**Fig. 55**). The distribution of peaks revealed that 56% were located within the promoter regions (up to 2 kb upstream from a transcription start site), no genes were in 5′ untranslated regions (UTRs), 10% were located in exons, 7% were located within introns, 3% were located in 3′ UTRs, 6% were located in the 3 kb downstream region, and 18% were intergenic.

Even though the number of regulatory sites was very low, it was curious but significant that more than the half of these peaks belonged to the promoter regions; from this observation many questions concerning the success or failure of the experiment came out. Considering that also the transient over expression of the gene did not work and will be repeated, probably the DAP-seq is going to be redo on this specific TF to validate (or not) this first result.

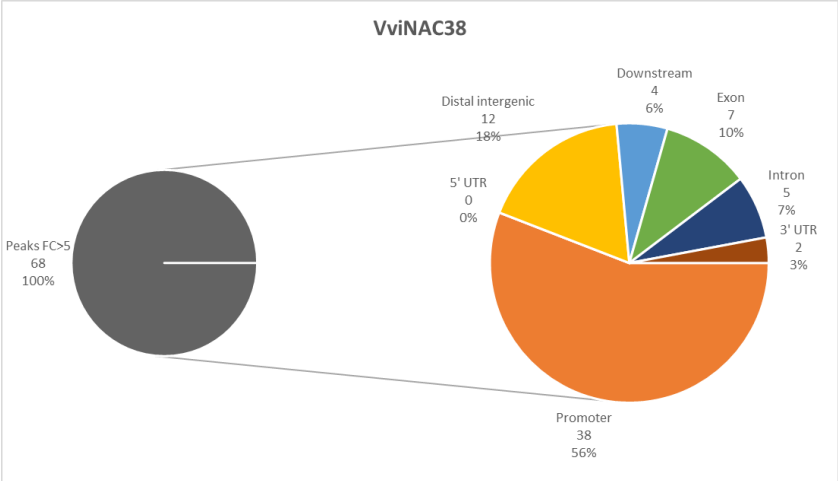


Figure 55: Double pie chart of the VviNAC38 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Three top binding motifs (GCGTG[G/C]A) were identified but not with a very high significance (**Fig. 56**); the phylogenetic footprints correlate with ANAC046, a positive regulator of chlorophyll degradation and senescence in *Arabidopsis* leaves (Oda-Yamamizo *et al*., 2016).

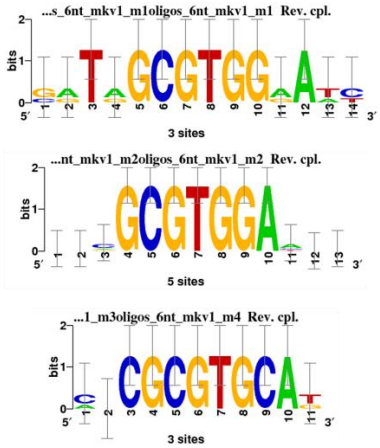


Figure 56: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC38 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was not focused only on the 56% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions and were 38 sites in total, but all the DAP-seq resulting dataset was used to obtain a not limited view of the few regulated genes.

However, the 'no hit' and 'unknown' elements were eliminated, and 55 binding sites were obtained for a total of 48 unique genes regulated by VviNAC38.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to protein modification and cellular response to stress and stimulus (**Fig. 57**).

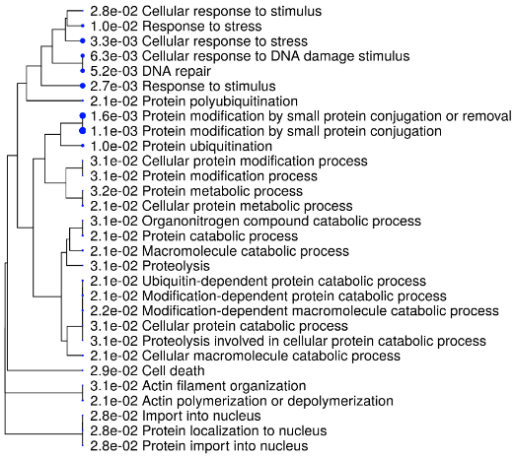


Figure 57: GO enrichment analysis of the VviNAC38 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the remaining binding sites, many transition markers were present (**Table 46**) but no *VviNACs* target.

Unfortunately, as said before, no transient over expression results (FC>|1.5|) were available to be crossed with the DAP-seq promoter and 5'UTR sub-dataset. The cv Sultana transient over expression needs to be repeated to be able to obtain at least three over expressing plantlets and complete the study concerning these *VviNAC* family members.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
137,37	Promoter (1-2kb)	VIT_08s0040g03360	-1144	Cofilin
127,97	Intron (mRNA24461/VIT_08s0040g02470, intron 1 of 8)	VIT_08s0040g02470	247	Inorganic pyrophosphatase
96,43	Promoter (<=1kb)	VIT_04s0044g00710	-165	UTP--glucose-1-phosphate uridylyltransferase *
96,26	Promoter (<=1kb)	VIT_06s0004g02750	-879	Auxin response factor 16
95,45	Exon (mRNA8524/VIT_03s0038g00790, exon 1 of 3)	VIT_03s0038g00790	42	V-type H+-transporting ATPase 16kDa proteolipid subunit
71,2	Promoter (<=1kb)	VIT_07s0031g00160	-132	Ubiquitin-conjugating enzyme E2 I *
67,26	Distal Intergenic	VIT_11s0016g02790	-3336	Zinc finger (CCCH-type) family protein *
61,32	Distal Intergenic	VIT_13s0064g01510	-3624	Auxin-independent growth promoter
56,51	Promoter (<=1kb)	VIT_02s0087g00790	-33	F-box domain containing protein *
54,59	Exon (mRNA6780/VIT_06s0004g03710, exon 1 of 2)	VIT_06s0004g03710	472	PRLI-interacting factor G
48,74	Promoter (<=1kb)	VIT_07s0130g00190	-742	Suppressor of gene silencing 3 (SGS3)
25,17	Promoter (<=1kb)	VIT_04s0008g00490	0	Profilin 5 (PRO5) (PRF3)
24,88	Promoter (<=1kb)	VIT_07s0130g00190	-631	Suppressor of gene silencing 3 (SGS3)
21,37	Downstream (<1kb)	VIT_14s0060g00200	2117	Pentatricopeptide repeat-containing protein
19,64	Promoter (<=1kb)	VIT_11s0016g04780	0	Ras GTP-binding protein (RAN2)
18,93	Promoter (<=1kb)	VIT_04s0008g05020	0	Ras GTP-binding protein (RAN3)
17,37	Promoter (<=1kb)	VIT_03s0038g00750	0	Ubiquitin fusion degradation protein UFD1
14,64	Intron (mRNA3084/VIT_18s0001g08890, intron 1 of 3)	VIT_18s0001g08890	87	Haemolysin-III related
13,8	Promoter (<=1kb)	VIT_02s0012g00400	-55	1-aminocyclopropane-1-carboxylate oxidase
11,14	Promoter (<=1kb)	VIT_04s0008g04210	0	GLB3 (2-on-2 hemoglobin like gene 3)
10,6	Promoter (1-2kb)	VIT_09s0002g02490	-1135	Dof zinc finger protein 1
10,41	Promoter (<=1kb)	VIT_14s0108g00140	-330	Ubiquitin-conjugating enzyme E2 A
10,03	Promoter (<=1kb)	VIT_13s0019g01980	0	Aspartic Protease (VvAP32)
8,79	3' UTR	VIT_02s0025g04170	12575	Heat shock transcription factor B2B
8,71	Exon (mRNA26154/VIT_08s0040g02950, exon 1 of 1)	VIT_08s0040g02950	316	Zinc finger (C3HC4-type ring finger)
7,91	Promoter (<=1kb)	VIT_19s0090g00420	-93	Translation initiation factor eIF-1
7,65	Promoter (1-2kb)	VIT_05s0029g00070	-1797	Calcium-binding protein CML
7,41	Intron (mRNA13633/VIT_01s0010g01480, intron 12 of 16)	VIT_01s0010g01460	42749	Pentatricopeptide (PPR) repeat-containing protein
6,61	Distal Intergenic	VIT_05s0062g00020	-82081	F-box family protein
6,47	Promoter (1-2kb)	VIT_19s0014g02860	-1377	Cellulase
6,45	Exon (mRNA14093/VIT_15s0048g02390, exon 5 of 13)	VIT_15s0048g02390	3914	Wall-associated receptor kinase-like 14 *
6,36	Promoter (<=1kb)	VIT_10s0003g02790	0	Double-stranded DNA-binding protein
6	Promoter (<=1kb)	VIT_07s0005g05670	-6	Alba, DNA binding
5,29	Promoter (1-2kb)	VIT_17s0000g08970	-1075	Proline-rich family protein
5,15	Promoter (1-2kb)	VIT_12s0059g00870	-1229	MAPKKK15
5,09	Promoter (<=1kb)	VIT_04s0044g00710	-248	UTP--glucose-1-phosphate uridylyltransferase *
4,47	Distal Intergenic	VIT_11s0052g01840	-4551	Diacylglycerol kinase
4,26	Distal Intergenic	VIT_03s0091g01010	-8277	LIM domain protein WLIM1
4,21	Exon (mRNA19274/VIT_08s0007g08420, exon 1 of 2)	VIT_08s0007g08420	1173	R protein MLA10
4,01	Distal Intergenic	VIT_02s0154g00400	7950	Scarecrow transcription factor 6 (SCL6)
3,75	Distal Intergenic	VIT_03s0091g01010	-8419	LIM domain protein WLIM1
3,74	Promoter (<=1kb)	VIT_05s0020g02820	0	Ubiquitin activating enzyme E1c (ECR1)
3,48	Promoter (<=1kb)	VIT_02s0087g00790	0	F-box domain containing protein *
3,25	Promoter (<=1kb)	VIT_01s0011g05060	-451	Major latex-like protein 34
3,25	Distal Intergenic	VIT_18s0001g05250	-2048	Dehydration Responsive Element-Binding Transcription Factor (VvDREB27)
2,95	Promoter (1-2kb)	VIT_05s0029g00070	-1685	Calcium-binding protein CML
2,86	Downstream (1-2kb)	VIT_08s0007g07930	4919	Clavata1 receptor kinase (CLV1)
2,38	Distal Intergenic	VIT_17s0053g00990	-9034	Expansin (VvEXPA18) *
2,33	Promoter (1-2kb)	VIT_08s0007g00560	-1964	ftsh3 (FtsH protease 3)
2,21	Exon (mRNA699/VIT_05s0020g03440, exon 3 of 4)	VIT_05s0020g03440	1468	Photosystem II 11 kDa protein PSB27
2,17	Promoter (1-2kb)	VIT_15s0048g02900	-1359	CYP78A3p
2,12	Promoter (<=1kb)	VIT_08s0040g03290	-84	Mini-chromosome maintenance protein MCM8
2,01	Distal Intergenic	VIT_19s0014g01460	-2931	Ubiquitin-conjugating enzyme E2 N

Table 46: VviNAC38 direct target genes. The asterisk (*) refers to the transition markers.

VviNAC39

The DAP-seq assay reported 5870 binding sites (peaks), which were reduced to 4682 after the FC>5 filtering; with this stringent filter used, the dataset loss was a

little bit higher, about the 20% (**Fig. 58**). The distribution of peaks revealed that 20% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 1% were in 5′ untranslated regions (UTRs), 7% were located in exons, 15% were located within introns, 2% were located in 3′ UTRs, 5% were located in the 3 kb downstream region, and 30% were intergenic.

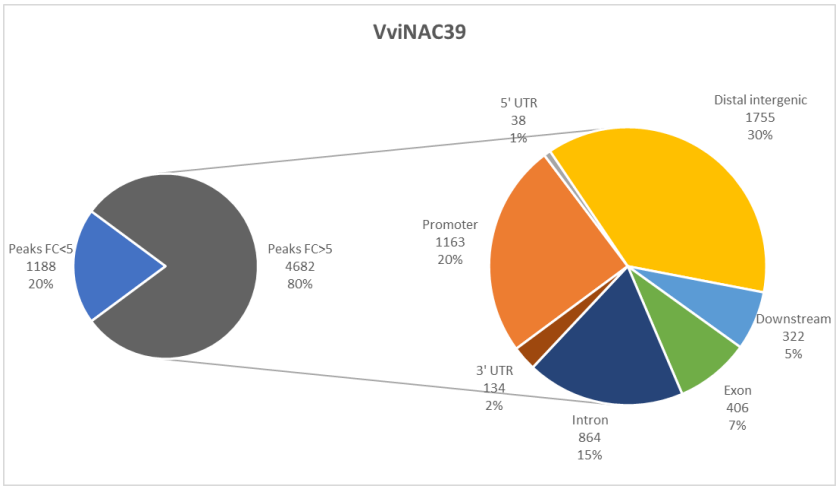


Figure 58: Double pie chart of the VviNAC39 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Two major binding motifs (CAA[A/T]TTG) were identified with strong significance (**Fig. 59**) but the phylogenetic footprints do not correlate with other known NAC.

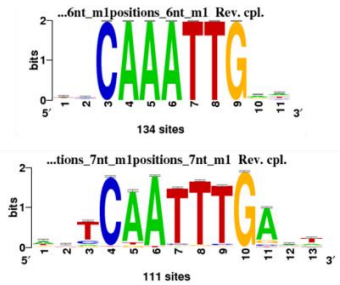


Figure 59: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC39 DAP-seq results. Two top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 21% of the final analyzed binding sites, which belongs to the ‘promoter’ and ‘5’UTR’ genic regions, for a total of 1201

sites. This percentage is low compared to the average number of DAP-seq sites found in literature.

After the elimination of all the 'no hit' and 'unknown' elements, 993 binding sites were obtained for a total of 915 unique genes regulated by VviNAC39.

A GO enrichment analysis was performed on this sub-dataset, revealing a predominance of genes related to oxidation-reduction processes, response to jasmonic acid, biological regulation, carboxylic acid metabolic processes, different biosynthetic processes (also fatty acids), carbon utilization, transport and localization (**Fig. 60**).

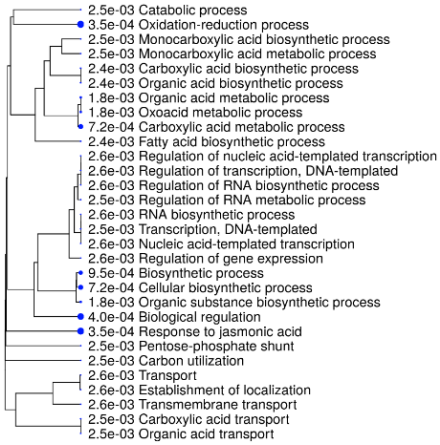


Figure 60: GO enrichment analysis of the VviNAC39 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 47**).

The most represented gene was *3-METHYL-2-OXOBUTANOATE DEHYDROGENASE* (VIT_18s0001g14980), which is present in the DAP-seq dataset of almost all the selected VviNAC TFs but not much information is available concerning its specific role in the plant development.

Moreover, ethylene- and auxin-related genes were found: *ERF/AP2 GENE FAMILY VvERF054* (VIT_16s0050g02400) and *AUXIN-RESPONSIVE SAUR11* (VIT_02s0154g00010). Interestingly, the already mentioned *MYB DOMAIN PROTEIN 4 VvMybC2-L1* (VIT_01s0011g04760) was also present in the list.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
999	Promoter (1-2kb)	VIT_18s0001g14980	-1612	3-methyl-2-oxobutanoate dehydrogenase
305,44	Promoter (<=1kb)	VIT_02s0025g02390	-613	Oligopeptide transporter OPT7
284,87	Promoter (<=1kb)	VIT_11s0016g04270	-797	Hydrolase, alpha/beta fold
260,94	Promoter (<=1kb)	VIT_01s0010g03780	-887	Histidine kinase (AHK3)
240,92	Promoter (<=1kb)	VIT_10s0116g01730	-170	Soluble starch synthase 3, chloroplast precursor
238,33	Promoter (<=1kb)	VIT_17s0000g08450	-727	Carbonic anhydrase chloroplast
205,89	Promoter (1-2kb)	VIT_00s0225g00090	-1298	Copper amine oxidase
202,78	Promoter (1-2kb)	VIT_01s0011g03020	-1443	HAK5 (High affinity K+ transporter 5)
182,08	Promoter (1-2kb)	VIT_16s0050g02400	-1308	ERF/AP2 Gene Family (VvERF054)
180,61	Promoter (<=1kb)	VIT_08s0058g01100	-227	Armadillo/beta-catenin repeat / U-box domain-containing protein
178,85	Promoter (1-2kb)	VIT_00s0394g00020	-1917	Pentatricopeptide (PPR) repeat-containing protein
163,9	Promoter (<=1kb)	VIT_06s0004g07280	-116	UDP-glucuronosyl/UDP-glucosyltransferase
155,69	Promoter (<=1kb)	VIT_01s0011g04760	-91	myb domain protein 4 (VvMybC2-L1)
154,96	Promoter (1-2kb)	VIT_12s0028g00340	-1210	Protein kinase
153,69	Promoter (<=1kb)	VIT_13s0067g03060	-860	Methionyl-tRNA synthetase
146,6	Promoter (1-2kb)	VIT_10s0003g00870	-1233	TCP family transcription factor 4
145,76	Promoter (1-2kb)	VIT_14s0068g00520	-1963	F-box domain containing protein
144,65	Promoter (<=1kb)	VIT_17s0000g08460	-540	Carbonic anhydrase, chloroplast precursor
131,38	Promoter (<=1kb)	VIT_01s0026g02430	-900	Calcineurin B
126,44	Promoter (1-2kb)	VIT_02s0154g00010	-1743	Auxin-responsive SAUR11

Table 47: Top 20 (q-value based) represented direct target genes of VviNAC39.

Afterwards, the list of the VviNAC39 targets was used to find all the possible *VviNACs* targets of the TF. 4 *VviNACs* target genes were found (**Table 48**): *VviNAC12* (VIT_01s0011g02990), *VviNAC15* (VIT_18s0001g01820), *VviNAC23* (VIT_04s0023g03110) and *VviNAC73* (VIT_14s0068g01490).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
8,43	Promoter (1-2kb)	VIT_14s0068g01490	-1161	NAC domain-containing protein (VvNAC73)
8,17	Promoter (1-2kb)	VIT_04s0023g03110	-1126	NAC domain-containing protein (VvNAC23)
3,84	Promoter (<=1kb)	VIT_18s0001g01820	-46	NAC domain-containing protein (VvNAC15)
2,04	Promoter (<=1kb)	VIT_01s0011g02990	0	NAC domain-containing protein (VvNAC12)

Table 48: *Vvi*NAC39 DAP-seq *VviNACs* targets genes.

Then, the previously reported *VviNAC39* transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 8 correlated binding sites (7 unique genes; **Table 49**); none of them were present between the top 20 reported genes.

Only two genes were found up regulated: the *ABC TRANSPORTER VvPDR16 - VvABCG46* (VIT_09s0002g05590) and the *UBIQUITIN-CONJUGATING ENZYME E2 VARIANT* (VIT_19s0090g00080).

ABC transporters function as ATP-dependent pumps but also as ion channels and channel regulators; the substrates of these transporters include sugars, lipids, heavy metal chelates, polysaccharides, alkaloids, steroids, inorganic acids and glutathione conjugates (Theodoulou, 2000). Whereas ubiquitin-conjugating (UBC) E2 enzyme plays crucial roles in plant growth and development; indeed, a recent study has demonstrated that most *VvUBCs* are involved in ripening and post-harvest stage in

grapevine and feature functional roles in grape organs (Gao *et al*., 2017).

The other five genes resulted down regulated by *Vvi*NAC39. Between them a *CA2+-TRANSPORTING ATPase TYPE 2 ISOFORM 8* (VIT_14s0030g02110), a *CHUP1* (VIT_08s0007g04330), which is essential for chloroplast anchorage to the plasma membrane and chloroplast movement (Oikawa *et al*., 2008) and a *GLYCOSYL HYDROLASE FAMILY 17 PROTEIN* (VIT_11s0016g00220) were found. Moreover, two genes were related to pathogenesis mechanisms: *PATHOGENESIS-RELATED PROTEIN 1 PRECURSOR PRP 1* (VIT_11s0052g01620) and *PECTATE LYASE 2* (VIT_19s0015g00510).

A recent study has also demonstrated that another pectate lyase (VIT_17s0000g09810) was found down regulated by grape powdery mildew and methyl salicylate treatments (Toth *et al*., 2016).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
67,46	Promoter (<=1kb)	VIT_08s0007g04330	-115	CHUP1 (chloroplast unusual positioning 1)	-1,80
18,97	Promoter (1-2kb)	VIT_09s0002g05590	-1402	ABC Transporter (VvPDR16 - VvABCG46)	2,97
8,69	Promoter (1-2kb)	VIT_09s0002g05590	-1918	ABC Transporter (VvPDR16 - VvABCG46)	2,97
8,17	Promoter (<=1kb)	VIT_19s0015g00510	-325	Pectate lyase 2	-7,23
8,11	Promoter (<=1kb)	VIT_19s0090g00080	-404	Ubiquitin-conjugating enzyme E2 variant	1,67
3,65	Promoter (<=1kb)	VIT_14s0030g02110	-815	Ca2+-transporting ATPase type 2 isoform 8	-1,79
2,86	Promoter (<=1kb)	VIT_11s0016g00220	-308	Glycosyl hydrolase family 17 protein	-2,20
2,64	Promoter (1-2kb)	VIT_11s0052g01620	-1082	Pathogenesis-related protein 1 precursor (PRP 1)	-2,69

Table 49: VviNAC39 DAP-seq targets genes which have a match in the *VviNAC39* transient over expression dataset.

VviNAC60

The DAP-seq assay reported 7489 binding sites (peaks), which were reduced to 6571 after the FC>5 filtering; a 12% of the peaks was lost (**Fig. 61**). The distribution of peaks revealed that 27% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 1% were in 5′ untranslated regions (UTRs), 9% were located in exons, 13% were located within introns, 2% were located in 3′ UTRs, 8% were located in the 3 kb downstream region, and 28% were intergenic.

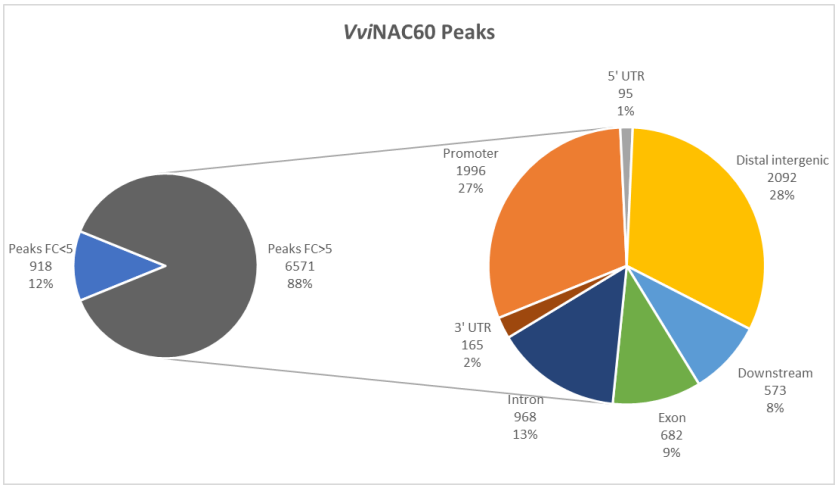


Figure 61: Double pie chart of the VviNAC60 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

Two major binding motifs (AC[G/A][T/C]GT) were identified with strong significance (**Fig. 62**) and the phylogenetic footprints correlate with ANAC055, a transcription activator that regulates jasmonic acid-induced expression of defense genes (Bu *et al*., 2008).

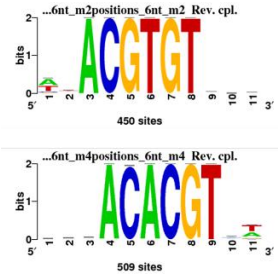


Figure 62: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC60 DAP-seq results. Three top-ranking binding motifs identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was focused only on the 28% of the final analysed binding sites, which belongs to the 'promoter' and '5'UTR' genic regions, for a total of 2091 sites.

After the elimination of all the 'no hit' and 'unknown' elements, 1688 binding sites were obtained for a total of 1511 unique genes regulated by VviNAC60.

A GO enrichment analysis was performed on this sub-dataset, revealing a

predominance of genes related to regulation of cellular, biological and metabolic processes, aromatic compound biosynthetic processes categories, and revealing possible actions concerning transmembrane transport and protein modifications (**Fig. 63**).

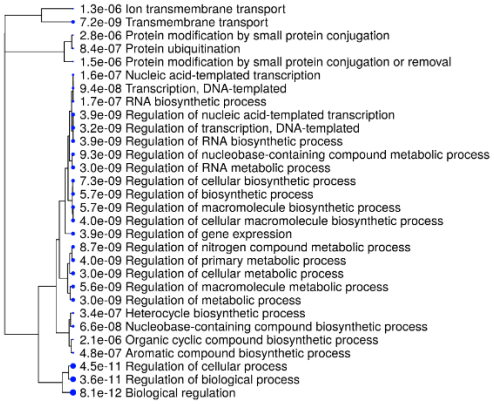


Figure 63: GO enrichment analysis of the VviNAC60 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

Between the top 20 (q-value based) promoter and 5'UTR located binding sites some interesting genes were found (**Table 50**).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
999	Promoter (1-2kb)	VIT_18s0001g14980	-1609	3-methyl-2-oxobutanoate dehydrogenase
242,9	Promoter (<=1kb)	VIT_01s0011g04760	-88	myb domain protein 4 (VvMybC2-L1)
242,78	Promoter (<=1kb)	VIT_17s0000g01280	-75	**WRKY Transcription Factor (VvWRKY52)**
216,51	Promoter (<=1kb)	VIT_17s0000g08450	-733	Carbonic anhydrase chloroplast
181,24	Promoter (<=1kb)	VIT_05s0062g00700	-157	UDP-glucose:flavonoid 7-O-glucosyltransferase
178,68	Promoter (1-2kb)	VIT_13s0067g00600	-1193	Arginine-tRNA-protein transferase 1
178,4	Promoter (<=1kb)	VIT_05s0020g02310	0	Pyruvate,orthophosphate dikinase
165,09	5' UTR	VIT_10s0003g01800	101	Zinc finger (FYVE type)
162,67	Promoter (1-2kb)	VIT_05s0062g01120	-1262	PIN1
155,9	Promoter (1-2kb)	VIT_18s0041g02010	-1882	12-oxophytodienoate reductase 1
152,37	Promoter (<=1kb)	VIT_15s0021g01150	-568	Calcium-dependent protein kinase-related
144,42	Promoter (<=1kb)	VIT_12s0142g00610	-7	Citrate synthase, glyoxysomal precursor
143	Promoter (<=1kb)	VIT_07s0255g00110	-147	WD40
142,63	Promoter (<=1kb)	VIT_06s0004g07930	0	Nucleotidyltransferase family
137,08	Promoter (<=1kb)	VIT_19s0177g00070	-494	Polyubiquitin (UBQ14)
125,17	Promoter (<=1kb)	VIT_04s0008g03430	0	Exostosin family
123,51	Promoter (<=1kb)	VIT_18s0001g08300	0	Tubulin alpha-6 chain
115,1	Promoter (<=1kb)	VIT_15s0048g01740	-355	Growth-regulating factor 9
114,52	Promoter (1-2kb)	VIT_07s0005g02250	-1782	ABC transporter G member 21
114,29	Promoter (<=1kb)	VIT_09s0002g01470	-771	4-phosphopantetheinyl transferase sfp

Table 50: Top 20 (q-value based) represented direct target genes of VviNAC60. In bold are reported the *switch* genes.

The most represented gene (q-value based) was, once again, the *3-METHYL-2-OXOBUTANOATE DEHYDROGENASE* (VIT_18s0001g14980) but not much information is available concerning its specific role in the plant development; however, this is an interesting gene as it was found in almost all the DAP-seq datasets of the studied VviNACs.

The *VvWRKY52* (VIT_17s0000g01280), a red berry *switch* gene which has been shown to play roles in biotic stress responses (Wang *et al*., 2017 and Wang *et al*., 2018), was also present. Moreover, returned the *MYB DOMAIN PROTEIN 4 VvMYBC2-L1* (VIT_01s0011g04760), the *ARGININE_tRNA-PROTEIN TRANSFERASE 1 ATE1* (VIT_13s0067g00600), the *PIN1* (VIT_05s0062g01120) and the *GROWTH-REGULATING FACTOR 9* (VIT_15s0048g01740).
Another *UDP-GLUCOSE:FLAVONOID 7-O-GLUCOSYLTRANSFERASE* (VIT_05s0062g00700) was also found; these class of enzymes has a role in UV protection, pathogen defense and coloration (Kim *et al*., 2006)

Afterwards, the list of the VviNAC60 targets was used to find all the possible *VviNACs* targets of the TF. 6 *VviNACs* target genes were found (**Table 51**); interestingly, *VviNAC05* (VIT_17s0000g06400), which was bounded in two different sites by the TF, and *VviNAC61* (VIT_08s0007g07640) were also found in the list.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation
59,84	Promoter (<=1kb)	VIT_08s0007g07640	0	NAC domain-containing protein (VvNAC61)
33,15	Promoter (1-2kb)	VIT_17s0000g06400	-1862	NAC domain-containing protein (VvNAC05)
18,08	Promoter (<=1kb)	VIT_17s0000g06400	0	NAC domain-containing protein (VvNAC05)
13,32	Promoter (1-2kb)	VIT_14s0068g01490	-1137	NAC domain-containing protein (VvNAC73)
5,76	Promoter (<=1kb)	VIT_12s0028g03050	-414	NAC domain-containing protein (VvNAC34)
5,48	Promoter (<=1kb)	VIT_04s0008g06550	0	NAC domain-containing protein (VvNAC66)
2,01	Promoter (1-2kb)	VIT_08s0040g02110	-1326	NAC domain-containing protein (VvNAC45)

Table 51: VviNAC60 DAP-seq *VviNACs* targets genes.

Then, the previously reported (D'Incà, 2017) *VviNAC60* stable and transient over expression results (FC>|1.5|) were crossed with the DAP-seq promoter and 5'UTR sub-dataset, revealing 196 (170 unique genes) and 34 (25 unique genes) correlated binding sites, respectively (**Table 52-53** and **Table 54-55**).
Only two genes were found in common between the two different over expression experiments and, in both cases, resulted up regulated: *ORGANIC CATION/CARNITINE TRANSPORTER4* (VIT_19s0014g04790) and *CELLULOSE SYNTHASE CSLG2* (VIT_05s0020g05060).

Between the up regulated genes found in the stable over expression of *VviNAC60*, three were also present in the top 20 DAP-seq most represented (**Table 52**): *3-*

METHYL-2-OXOBUTANOATE DEHYDROGENASE (VIT_18s0001g14980), *CITRATE SYNTHASE, GLYOXYSOMAL PRECURSOR* (VIT_12s0142g00610) and a *NUCLEOTIDYLTRANSFERASE FAMILY* (VIT_06s0004g07930).
Moreover, a *MYB FAMILY* (VIT_09s0054g01620) and the *VvWRKY16* (VIT_06s0004g07500) were up regulated.
Interestingly many genes related to hormones were present: four auxin-related genes, *AUXIN-RESPONSIVE SAUR* (VIT_15s0048g00530 and VIT_01s0146g00180), *ARF* (VIT_00s0404g00040), and *AUXIN RESPONSE FACTOR 10* (VIT_13s0019g04380); three ethylene-related genes, *ERF/AP2 GENE FAMILY VvERF006, DEHYDRATION RESPONSIVE ELEMENT-BINDING TRANSCRIPTION FACTOR VvDREB28* (VIT_18s0001g10150), *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 9* (VIT_12s0028g03270), *ERF/AP2 GENE FAMILY VvERF017, DEHYDRATION RESPONSIVE ELEMENT-BINDING TRANSCRIPTION FACTOR VvDREB30* (VIT_18s0089g01030); and a *GIBBERELLIN RECEPTOR GID1L2* (VIT_07s0104g00930).
A marker of the second transition, and a berry *switch* gene, was also identified with the DAP assay as up regulated: *LATE EMBRYOGENIS ABUNDANT PROTEIN 5* (VIT_07s0005g00660). The Late Embryogenesis Abundant (LEA) protein gene family is associated with salt and drought stress tolerance in many plant species and is well known that abiotic stresses have the potential to negatively affect both yield and berry quality in grapes (Ibrahime *et al*., 2019).

Regarding the DAP-seq genes which were also found down regulated in the *VviNAC60* stable over expression (**Table 53**), the *VviNAC34* (VIT_12s0028g03050) and a *BASIC HELIX-LOOP-HELIX bHLH FAMILY* (VIT_08s0058g00960) were present. Moreover, the most down regulated gene was a *PAPAIN CYSTEINE PROTEINASE* (VIT_18s0001g13380); this class of genes is known to play important functions in multiple processes of plant growth including seed germination, leaf senescence, programmed cell death, abiotic stress response and plant immunity (Liu *et al*., 2018).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Stable OE
999	Promoter (1-2kb)	VIT_18s0001g14980	-1609	3-methyl-2-oxobutanoate dehydrogenase	3,09
144,42	Promoter (<=1kb)	VIT_12s0142g00610	-7	Citrate synthase, glyoxysomal precursor	1,59
142,63	Promoter (<=1kb)	VIT_06s0004g07930	0	Nucleotidyltransferase family	2,82
85,5	Promoter (1-2kb)	VIT_18s0001g09420	-1845	Progesterone 5-beta-reductase	1,57
79,94	Promoter (1-2kb)	VIT_05s0020g05060	-1053	Cellulose synthase CSLG2	2,17
71,69	Promoter (<=1kb)	VIT_11s0149g00280	-793	Chitinase A	3,12
64,43	Promoter (<=1kb)	VIT_18s0001g07400	-317	Carbonic anhydrase, chloroplast precursor	2,11
58,06	Promoter (<=1kb)	VIT_07s0104g00860	-195	WD40	1,55
55,11	Promoter (<=1kb)	VIT_04s0044g00220	0	Monooxygenase	1,95
52,2	Promoter (<=1kb)	VIT_01s0011g00760	-868	Beta-glucosidase	7,82
48,91	Promoter (<=1kb)	VIT_01s0146g00180	0	Auxin responsive SAUR protein	2,41
47,53	Promoter (<=1kb)	VIT_09s0054g01620	0	myb family	1,59
46,86	Promoter (<=1kb)	VIT_06s0004g04590	-91	Epsin N-terminal homology (ENTH) domain-containing	6,49
41,26	Promoter (1-2kb)	VIT_13s0019g04620	-1931	OTU cysteine protease	1,58
41,01	Promoter (<=1kb)	VIT_00s0304g00030	-937	VQ motif-containing protein	2,69
35,96	Promoter (1-2kb)	VIT_05s0020g05060	-1084	Cellulose synthase CSLG2	2,17
33,95	Promoter (1-2kb)	VIT_09s0002g03410	-1582	Phytochrome-associated protein 1 (PAP1)	2,12
32,73	Promoter (<=1kb)	VIT_05s0062g00980	-14	Aldo/keto reductase AKR	1,80
30,18	Promoter (<=1kb)	VIT_07s0005g04890	0	**Glutathione S-transferase 25 GSTU7**	3,82
29,03	Promoter (<=1kb)	VIT_18s0001g08430	-350	Branched-chain-amino-acid aminotransferase 2, chloroplast (Atbcat-2) *	11,12
28,78	Promoter (1-2kb)	VIT_07s0104g00930	-1733	Gibberellin receptor GID1L2	3,07
24,63	Promoter (<=1kb)	VIT_05s0049g01980	0	3-isopropylmalate dehydratase large subunit 2	9,61
22,26	Promoter (<=1kb)	VIT_02s0025g00880	-571	BTB/POZ domain-containing protein POB1	1,83
21,75	Promoter (1-2kb)	VIT_13s0019g04620	-1994	OTU cysteine protease	1,58
21,63	Promoter (<=1kb)	VIT_06s0004g04010	-97	Exocyst subunit EXO70 H7	2,45
17,94	Promoter (<=1kb)	VIT_18s0001g15010	-913	F-box and leucine-rich repeat protein 1	2,19
16,19	Promoter (<=1kb)	VIT_00s0179g00260	-282	Calcium-transporting ATPase 12 ACA12	1,96
15,77	Promoter (<=1kb)	VIT_07s0005g00660	-538	**Late embryogenis abundant protein 5 ***	6,76
15,55	Promoter (<=1kb)	VIT_01s0011g05560	-822	TIFY gene family (VvJAZI)	1,94
13,88	Promoter (1-2kb)	VIT_18s0041g00730	-1076	Peptide chain release factor eRF subunit 1	1,82
13,32	Promoter (<=1kb)	VIT_02s0025g00880	-536	BTB/POZ domain-containing protein POB1	1,83
13,24	Promoter (1-2kb)	VIT_13s0019g04380	-1343	Auxin response factor 10	1,57
13,06	Promoter (<=1kb)	VIT_09s0002g08670	-67	Acetylornithine aminotransferase	1,52
13,05	5' UTR	VIT_00s0404g00040	113	ARF GTPase activator, ARF-GAP Domain 5	1,68
12,56	Promoter (<=1kb)	VIT_18s0001g10150	-433	ERF/AP2 Gene Family (VvERF006),Dehydration Responsive Element-Binding Transcription Factor (VvDREB28)	1,90
11,61	Promoter (1-2kb)	VIT_05s0020g01800	-1704	Zinc finger (C3HC4-type ring finger)	1,84
11,55	Promoter (<=1kb)	VIT_18s0001g06310	-56	SnRK2-8	2,12
10,75	Promoter (<=1kb)	VIT_14s0083g00440	-477	PHD finger transcription factor	1,59
10,23	Promoter (<=1kb)	VIT_19s0015g00710	-386	Cellulose synthase CSLE1	2,34
10,05	Promoter (<=1kb)	VIT_10s0116g00960	-709	Transcription factor jumonji (jmjC) domain-containing protein	1,55
9,95	5' UTR	VIT_00s0404g00040	17	ARF GTPase activator, ARF-GAP Domain 5	1,68
9,76	Promoter (<=1kb)	VIT_17s0000g05420	-793	AAA-type ATPase family	3,18
8,97	Promoter (<=1kb)	VIT_00s0203g00100	-102	AarF domain-containing kinase ABC1	1,57
8,95	Promoter (<=1kb)	VIT_18s0001g06310	-4	SnRK2-8	2,12
8,48	Promoter (<=1kb)	VIT_17s0000g05420	0	AAA-type ATPase family	3,18
8,41	Promoter (<=1kb)	VIT_00s0181g00260	-935	High-level expression of sugar-inducible like 1	1,50
8,36	Promoter (<=1kb)	VIT_09s0002g00270	-717	R protein disease resistance protein	1,56
8,32	Promoter (1-2kb)	VIT_13s0047g00010	-1981	ZIFL2 (zinc induced facilitator-like 1)	1,74
8,23	Promoter (<=1kb)	VIT_05s0051g00730	-43	Zinc finger (C3HC4-type ring finger)	1,75
8,15	Promoter (<=1kb)	VIT_09s0002g01590	-256	Nuclear transcription factor Y subunit A-8	2,15
8,12	Promoter (<=1kb)	VIT_18s0001g13450	-588	SLAH1 (SLAC1 homologue 1)	3,50
7,75	Promoter (1-2kb)	VIT_14s0060g01970	-1879	F-box domain containing protein	1,74
7,62	Promoter (1-2kb)	VIT_12s0028g00930	-1278	Glutathione S-transferase (VvGST3)	1,81
7,61	Promoter (1-2kb)	VIT_09s0002g02680	-1887	Early flowering 3	2,00
7,41	Promoter (<=1kb)	VIT_01s0127g00590	0	Protein disulfide isomerase	2,75
7,41	5' UTR	VIT_01s0026g00280	290	Trehalose 6-phosphate synthase	1,51
7,16	Promoter (<=1kb)	VIT_08s0040g02180	-126	Mlo3	3,28
7,02	Promoter (<=1kb)	VIT_18s0001g00360	-154	Dehydrin (VvDHN2)	1,86
6,91	Promoter (<=1kb)	VIT_04s0008g06330	-157	TPR1 (topless-related 1)	2,08
6,85	Promoter (<=1kb)	VIT_11s0016g00710	-95	TIFY gene family (VvJAZ9)	2,55
6,85	Promoter (<=1kb)	VIT_19s0014g04600	-985	S-locus protein kinase	1,79
6,83	Promoter (1-2kb)	VIT_14s0066g01240	-1949	L-aspartate oxidase	2,91
6,78	Promoter (<=1kb)	VIT_04s0008g03770	0	Aspartate aminotransferase P1	2,14
6,77	Promoter (1-2kb)	VIT_07s0005g00860	-1673	U3 snoRNP-associated helicase Ecm16	1,60
6,67	Promoter (1-2kb)	VIT_17s0000g00830	-1879	Nodulin MtN3 family	6,09
6,61	Promoter (1-2kb)	VIT_08s0058g01130	-1108	WNK5 (Arabidopsis WNK kinase 5)	3,15
6,58	Promoter (<=1kb)	VIT_18s0001g00360	-371	Dehydrin (VvDHN2)	1,86
6,07	5' UTR	VIT_11s0016g04490	444	IAA16	1,66
6,06	Promoter (<=1kb)	VIT_07s0005g00660	-590	**Late embryogenis abundant protein 5 ***	6,76
5,95	Promoter (<=1kb)	VIT_10s0003g01500	-816	Zinc finger (C3HC4-type ring finger)	2,63
5,83	Promoter (<=1kb)	VIT_15s0048g00530	-644	Auxin-responsive SAUR11	2,85
5,54	Promoter (<=1kb)	VIT_09s0002g00700	-979	Dormancy/auxin associated protein	4,73
5,16	Promoter (<=1kb)	VIT_13s0019g01980	-10	Aspartic Protease (VvAP32)	2,77
4,92	Promoter (<=1kb)	VIT_08s0040g00040	-233	Ubiquitin-conjugating enzyme E2 D/E	1,50
4,88	Promoter (<=1kb)	VIT_17s0000g07790	-146	N-hydroxythioamide S-beta-glucosyltransferase	3,22
4,78	Promoter (1-2kb)	VIT_03s0091g00690	-1874	Salt tolerance zinc finger	1,66
4,64	Promoter (1-2kb)	VIT_17s0000g00830	-1169	Nodulin MtN3 family	6,09
4,55	Promoter (<=1kb)	VIT_09s0002g02940	-5	Myo-inositol oxygenase 1	1,59
4,52	Promoter (<=1kb)	VIT_13s0019g02200	-525	Protein phosphatase 2CA AHG3 PP2CA (VvPP2C-3)	1,65
4,41	Promoter (<=1kb)	VIT_13s0019g02200	-377	Protein phosphatase 2CA AHG3 PP2CA (VvPP2C-3)	1,65
4,3	Promoter (<=1kb)	VIT_06s0004g07500	-580	WRKY Transcription Factor (VvWRKY16)	2,27
4,1	Promoter (<=1kb)	VIT_01s0011g06460	-54	Deoxymugineic acid synthase	2,86
4,01	Promoter (<=1kb)	VIT_12s0028g00930	0	Glutathione S-transferase (VvGST3)	1,81
3,94	Promoter (<=1kb)	VIT_06s0009g01630	-412	Cc-nbs-lrr resistance protein	1,52
3,75	Promoter (<=1kb)	VIT_05s0020g05060	-954	Cellulose synthase CSLG2	2,17
3,72	Promoter (<=1kb)	VIT_04s0044g00110	-315	High-mobility group B 2	1,86
3,71	Promoter (<=1kb)	VIT_05s0020g01800	-687	Zinc finger (C3HC4-type ring finger)	1,84
3,71	Promoter (<=1kb)	VIT_09s0002g09280	0	Cysteine peptidase	1,74
3,58	Promoter (<=1kb)	VIT_18s0001g07980	-422	CBL-interacting protein kinase 8 (CIPK8)	1,59
3,54	Promoter (1-2kb)	VIT_10s0003g01390	-1512	Cupin, RmlC-type	2,21
3,49	Promoter (<=1kb)	VIT_03s0063g00370	-684	Nitrite reductase	3,38
3,11	Promoter (<=1kb)	VIT_16s0098g00290	-644	GLT1 (NADH-dependent glutamate synthase 1 gene)	1,89
3,05	Promoter (1-2kb)	VIT_08s0007g04470	-1484	Vacuolar protein sorting 37C	1,64
3,01	Promoter (<=1kb)	VIT_12s0028g03270	-79	Ethylene-responsive transcription factor 9	2,02
3	Promoter (1-2kb)	VIT_19s0014g00470	-1470	RKF1 (receptor-like kinase in flowers 1)	1,53
2,88	Promoter (1-2kb)	VIT_19s0090g01870	-1850	ABC Transporter (VvMRP26 - VvABCC26)	2,08
2,88	Promoter (<=1kb)	VIT_08s0007g02200	0	High mobility group protein B1	1,70
2,83	Promoter (<=1kb)	VIT_19s0090g01530	-794	Pirin	2,14
2,83	Promoter (<=1kb)	VIT_00s0424g00020	-154	Receptor serine/threonine kinase	1,96
2,82	5' UTR	VIT_07s0104g00830	34	Sugar transporter ERD6-like 7 *	1,79
2,66	Promoter (<=1kb)	VIT_18s0001g07630	-401	NADPH-cytochrome P450 oxydoreductase isoform 1	1,75
2,63	Promoter (1-2kb)	VIT_18s0089g01030	-1430	ERF/AP2 Gene Family (VvERF017),Dehydration Responsive Element-Binding Transcription Factor (VvDREB30)	4,04
2,63	Promoter (1-2kb)	VIT_11s0118g00480	-1644	Leucine-rich repeat	1,75
2,63	Promoter (<=1kb)	VIT_09s0002g02940	-269	Myo-inositol oxygenase 1	1,59
2,62	Promoter (1-2kb)	VIT_01s0146g00180	-1983	Auxin responsive SAUR protein	2,41
2,43	Promoter (<=1kb)	VIT_04s0044g00110	-194	High-mobility group B 2	1,86
2,22	Promoter (<=1kb)	VIT_19s0090g01170	-194	UPF0041	3,29
2,22	Promoter (1-2kb)	VIT_19s0014g04790	-1503	**Organic cation/carnitine transporter4**	2,94
2,22	Promoter (<=1kb)	VIT_15s0048g00530	-749	Auxin-responsive SAUR11	2,85
2,22	Promoter (<=1kb)	VIT_13s0147g00110	0	ferredoxin	2,00
2,21	5' UTR	VIT_05s0077g01140	104	Basic Leucine Zipper Transcription Factor (VvbZIP14)	2,83
2,21	Promoter (<=1kb)	VIT_02s0012g02650	-834	Purple acid phosphatase 2 PAP2	2,44
2,21	Promoter (1-2kb)	VIT_00s0179g00260	-1425	Calcium-transporting ATPase 12 ACA12	1,96
2,21	Promoter (<=1kb)	VIT_04s0044g01620	0	RGLG1 (ring Domain LIGASE1)	1,88
2,15	Promoter (<=1kb)	VIT_14s0030g00570	-233	Copine BON3 (BONZAI 3)	1,82

Table 52: VviNAC60 DAP-seq targets genes which have a match in the *VviNAC60* stable over expression dataset and resulted up regulated. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Stable OE
48,47	Promoter (<=1kb)	VIT_02s0025g02390	-587	Oligopeptide transporter OPT7	-2,37
48,47	Promoter (<=1kb)	VIT_09s0002g06970	-150	**Palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, chloroplast**	-2,00
31,22	Promoter (<=1kb)	VIT_01s0150g00480	-853	Protein binding protein	-2,16
27,56	Promoter (1-2kb)	VIT_07s0031g01600	-1342	Shikimate kinase	-1,64
25,41	Promoter (1-2kb)	VIT_10s0003g03690	-1593	Beta-1,3-glucanase precursor	-4,43
22,91	Promoter (<=1kb)	VIT_15s0021g02280	-456	Matrix metalloproteinase	-2,88
22,51	Promoter (<=1kb)	VIT_04s0069g00860	-352	Sarcosine oxidase *	-5,81
21,46	Promoter (<=1kb)	VIT_06s0004g06730	-422	Microsomal omega-3 fatty acid desaturase	-16,75
21,2	Promoter (<=1kb)	VIT_01s0026g02500	-167	Amino acid transport protein	-5,98
19,77	Promoter (1-2kb)	VIT_08s0040g02470	-1035	Inorganic pyrophosphatase	-1,56
17,34	Promoter (<=1kb)	VIT_17s0000g05470	-3	Nodulin	-1,69
17,28	Promoter (<=1kb)	VIT_05s0020g04210	-661	Sulfate adenylyltransferase 3	-2,64
16,46	Promoter (<=1kb)	VIT_00s0227g00140	0	MLO1	-1,66
14,96	Promoter (<=1kb)	VIT_18s0001g12990	-881	Anthranilate N-benzoyltransferase protein 1	-3,27
13,03	Promoter (1-2kb)	VIT_02s0025g04720	-1484	Leucoanthocyanidin dioxygenase (VvLDOX) [Vitis vinifera]	-4,08
12,61	Promoter (<=1kb)	VIT_06s0004g02380	0	Cinnamyl alcohol dehydrogenase	-1,78
12,49	Promoter (1-2kb)	VIT_08s0058g00960	-1898	basic helix-loop-helix (bHLH) family	-2,62
10,57	Promoter (1-2kb)	VIT_17s0000g04810	-1963	Auxin-independent growth promoter	-1,75
10,34	Promoter (<=1kb)	VIT_06s0004g00720	-256	Glucan endo-1,3-beta-glucosidase 4 precursor	-1,98
9,98	Promoter (1-2kb)	VIT_01s0011g05600	-1986	Receptor-like protein kinase	-3,05
9,69	Promoter (1-2kb)	VIT_02s0025g04720	-1399	Leucoanthocyanidin dioxygenase (VvLDOX) [Vitis vinifera]	-4,08
9,69	Promoter (<=1kb)	VIT_01s0026g01480	-47	Adenosine/AMP deaminase active site	-1,70
8,9	Promoter (<=1kb)	VIT_10s0003g00330	-881	HSL1 (HAESA-like 1)	-2,31
8,9	Promoter (<=1kb)	VIT_16s0022g00540	-190	Glycerol 3-phosphate permease	-1,90
8,66	Promoter (<=1kb)	VIT_18s0117g00130	0	Pi starvation-induced protein *	-1,60
7,9	Promoter (<=1kb)	VIT_19s0090g00830	0	Aspartic Protease (VvAP45) *	-3,28
7,9	Promoter (1-2kb)	VIT_14s0068g00920	-1576	chalcone synthase 2 (CHS2)	-2,82
7,35	Promoter (1-2kb)	VIT_07s0104g00540	-1850	CYCP1;1; CYCP1;1 *	-1,86
6,83	Promoter (<=1kb)	VIT_14s0068g00920	-749	chalcone synthase 2 (CHS2)	-2,82
6,55	Promoter (<=1kb)	VIT_04s0008g01120	-709	Glutaredoxin	-1,82
6,38	Promoter (<=1kb)	VIT_06s0004g01890	-778	Cu2+-exporting ATPase HMA5 (heavy metal ATPase 5)	-2,12
5,76	Promoter (<=1kb)	VIT_12s0028g03050	-414	NAC domain-containing protein (VvNAC34)	-2,51
5,49	Promoter (1-2kb)	VIT_00s0445g00010	-1276	Strubbelig receptor family 1	-1,64
5,36	Promoter (<=1kb)	VIT_01s0011g05600	0	Receptor-like protein kinase	-3,05
5,36	Promoter (<=1kb)	VIT_12s0059g01560	-131	Protein disulfide-isomerase A6	-2,19
5,15	Promoter (1-2kb)	VIT_05s0049g00880	-1481	Activating signal cointegrator	-1,86
5,12	Promoter (<=1kb)	VIT_18s0001g10040	-570	LRX1 (leucine-rich repeat/extensin 1)	-2,41
5,04	Promoter (<=1kb)	VIT_03s0091g00630	0	YGGT family protein	-2,04
5,04	Promoter (<=1kb)	VIT_06s0004g06930	-173	Zinc finger (C3HC4-type ring finger)	-1,99
5,01	Promoter (<=1kb)	VIT_11s0016g04630	-938	GAI protein.	-2,77
4,92	Promoter (1-2kb)	VIT_06s0004g01050	-1215	Calcineurin phosphoesterase	-7,08
4,86	Promoter (<=1kb)	VIT_05s0051g00660	-797	Octicosapeptide/Phox/Bem1p; Protein kinase	-2,58
4,71	Promoter (<=1kb)	VIT_12s0059g01560	-343	Protein disulfide-isomerase A6	-2,19
4,71	Promoter (<=1kb)	VIT_17s0000g08080	-251	Armadillo/beta-catenin repeat protein / U-box domain-containing protein	-1,96
4,44	Promoter (1-2kb)	VIT_06s0004g05850	-1708	Zinc finger (C3HC4-type ring finger)	-1,53
4,17	Promoter (<=1kb)	VIT_01s0011g03910	-95	Protein phosphatase 2C	-1,63
4,1	Promoter (<=1kb)	VIT_19s0015g01760	-201	Photosystem I reaction center subunit V (PSAG)	-2,13
4,09	Promoter (<=1kb)	VIT_06s0004g03810	-36	Kinesin motor protein *	-2,12
4,06	Promoter (<=1kb)	VIT_07s0005g00010	-95	Glutathione S-transferase 8 GSTF8	-2,24
4,06	Promoter (<=1kb)	VIT_18s0001g09460	-423	GASA5	-1,64
4,04	Promoter (<=1kb)	VIT_14s0068g01540	-74	PBS1 (avrPphB susceptible 1)	-1,51
3,88	Promoter (1-2kb)	VIT_08s0007g02000	-1938	Glycerol-3-phosphate acyltransferase 8	-3,26
3,72	Promoter (1-2kb)	VIT_11s0103g00550	-1927	Subtilisin-like serine protease 3	-1,92
3,71	Promoter (<=1kb)	VIT_10s0003g01490	-476	NIK3 (NSP- interacting kinase 3)	-1,81
3,66	Promoter (<=1kb)	VIT_18s0001g13380	-431	Papain cysteine proteinase isoform I	-19,77
3,49	Promoter (<=1kb)	VIT_14s0066g02020	-160	Proton-dependent oligopeptide transport (POT) family protein	-5,63
3,45	Promoter (<=1kb)	VIT_07s0104g00350	-139	Circadian clock coupling factor ZGT	-1,80
3,11	Promoter (1-2kb)	VIT_08s0007g03060	-1583	Beta-fructofuranosidase	-2,09
3,11	Promoter (<=1kb)	VIT_10s0003g05420	-239	S-2-hydroxy-acid oxidase, peroxisomal	-2,06
3,05	Promoter (<=1kb)	VIT_18s0001g10580	-684	Glycosyl transferase family 8 protein	-2,49
3,05	Promoter (1-2kb)	VIT_17s0000g08290	-1344	Dof zinc finger protein DOF5.6	-2,49
2,74	Promoter (1-2kb)	VIT_04s0008g01150	-1766	Beta-fructosidase (BFRUCT1)	-3,58
2,72	Promoter (<=1kb)	VIT_17s0000g08080	-400	Armadillo/beta-catenin repeat protein / U-box domain-containing protein	-1,96
2,7	Promoter (<=1kb)	VIT_06s0004g00030	0	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase	-1,92
2,66	Promoter (1-2kb)	VIT_18s0001g04680	-1042	RPG related protein 1 RR1	-1,75
2,62	Promoter (<=1kb)	VIT_15s0021g00050	-758	Eceriferum 1 (CER1 protein) Sterol desaturase	-4,69
2,62	Promoter (1-2kb)	VIT_04s0069g00780	-1613	Cellulose synthase CSLC05 *	-2,80
2,54	Promoter (<=1kb)	VIT_05s0020g01980	-106	Acyl carrier protein, mitochondrial 3	-4,54
2,49	Promoter (<=1kb)	VIT_05s0020g04100	-712	Dymeclin	-1,77
2,43	Promoter (<=1kb)	VIT_04s0069g00860	-431	Sarcosine oxidase *	-5,81
2,43	Promoter (<=1kb)	VIT_00s0179g00150	-946	Heat shock transcription factor A6B	-2,95
2,43	Promoter (<=1kb)	VIT_04s0023g01800	-13	**Maf, septum formation protein ***	-1,62
2,42	Promoter (<=1kb)	VIT_18s0089g01410	-293	Purine permease 4 PUP4	-1,63
2,22	Promoter (<=1kb)	VIT_11s0103g00370	-949	Cd2+-exporting ATPase HMA2 (Heavy metal ATPase 2)	-2,34
2,22	Promoter (<=1kb)	VIT_16s0050g02520	0	RNA polymerase sigma subunit SigE (sigE)	-2,08
2,22	Promoter (1-2kb)	VIT_12s0059g00670	-1917	NADP-dependent oxidoreductase	-1,80
2,21	Promoter (1-2kb)	VIT_05s0051g00050	-1769	Myosin-like protein XIB	-2,99
2,21	Promoter (1-2kb)	VIT_05s0077g02330	-1555	Transducin protein	-2,61
2,01	Promoter (1-2kb)	VIT_04s0008g01150	-1913	Beta-fructosidase (BFRUCT1)	-3,58
2,01	Promoter (<=1kb)	VIT_01s0137g00560	0	**CYP71B34**	-2,12
2,01	Promoter (<=1kb)	VIT_03s0063g00390	0	Lag one homologue 2	-2,05

Table 53: VviNAC60 DAP-seq targets genes which have a match in the *VviNAC60* stable over expression dataset and resulted down regulated. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

Looking at the DAP-seq correlation with the *VviNAC60* transient over expression, between the up regulated genes (**Table 54**) a *CELLULOSE SYNTHASE CSLG2* (VIT_05s0020g05060) was found; this is a gene related to the pectin metabolism

genes and is associated with the inner pollen grain wall or intine (Fasoli *et al*., 2012). Low auxin levels are required to trigger the onset of ripening but, at the same time, high auxin levels at pre-veraison stages are required for the induction of genes involved in the ripening inception (Zenoni *et al*., 2017). The regulation of auxin levels is associated with the conjugation of indole acetic acid by the indol-3-acetate beta-glucosyltransferase, which allows an increase in the conjugated form of auxin after veraison. Interestingly, an *INDOLE-3-ACETATE BETA-GLUCOSYLTRANSFERASE* (VIT_06s0004g07230) was found between the *Vvi*NAC60 targets and up regulated by *VviNAC60* transient over expression.

The *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 1* (VIT_02s0025g04660), with three *Vvi*NAC60 binding sites in its promoter region, was also found up regulated; it is involved in chlorophyll and photosystem degradation.

Between the DAP-found *VviNAC60* transient over expression down regulated genes (**Table 55**), no particularly relevant gene was present.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
79,94	Promoter (1-2kb)	VIT_05s0020g05060	-1053	Cellulose synthase CSLG2	2,07
71,32	Promoter (<=1kb)	VIT_18s0001g11740	-375	Ring zinc finger ariadne protein ARI2	1,57
35,96	Promoter (1-2kb)	VIT_05s0020g05060	-1084	Cellulose synthase CSLG2	2,07
31,94	Promoter (<=1kb)	VIT_18s0001g09910	-102	L-asparaginase	2,17
29,09	Promoter (<=1kb)	VIT_06s0004g07230	0	Indole-3-acetate beta-glucosyltransferase	3,45
21,46	Promoter (1-2kb)	VIT_02s0025g04660	-1062	Senescence-inducible chloroplast stay-green protein 1	5,74
17,24	Promoter (<=1kb)	VIT_18s0001g12040	-185	Coniferyl-alcohol glucosyltransferase	1,73
16,11	Promoter (1-2kb)	VIT_18s0075g00550	-1310	RuvB DNA helicase protein	1,98
15	Promoter (<=1kb)	VIT_02s0025g04660	-129	Senescence-inducible chloroplast stay-green protein 1	5,74
12,02	Promoter (1-2kb)	VIT_08s0007g02100	-1896	Alpha-1,4-glycosyltransferase	1,85
11,28	Promoter (1-2kb)	VIT_10s0003g03190	-1612	RNA recognition motif (RRM)-containing	1,62
10,92	Promoter (<=1kb)	VIT_10s0003g05690	-884	Ribulose bisphosphate carboxylase, large chain	1,88
9,17	5' UTR	VIT_18s0001g14710	237	Ketol-acid reductoisomerase precursor	1,61
6,06	Promoter (<=1kb)	VIT_16s0050g00950	-606	Scarecrow-like transcription factor 8 (SCL8)	1,82
5,04	Promoter (<=1kb)	VIT_10s0003g05690	0	Ribulose bisphosphate carboxylase, large chain	1,88
4,8	Promoter (<=1kb)	VIT_02s0025g04660	-174	Senescence-inducible chloroplast stay-green protein 1	5,74
4,65	Promoter (1-2kb)	VIT_15s0046g00490	-1346	Wax synthase	3,12
4,18	5' UTR	VIT_18s0001g14710	437	Ketol-acid reductoisomerase precursor	1,61
3,82	Promoter (<=1kb)	VIT_13s0156g00590	-34	S-receptor kinase	2,23
3,75	Promoter (<=1kb)	VIT_05s0020g05060	-954	Cellulose synthase CSLG2	2,07
2,74	5' UTR	VIT_16s0050g00950	137	Scarecrow-like transcription factor 8 (SCL8)	1,82
2,65	Promoter (<=1kb)	VIT_15s0046g03380	-932	Thiol protease	1,62
2,43	Promoter (<=1kb)	VIT_08s0007g02100	-136	Alpha-1,4-glycosyltransferase	1,85
2,42	Promoter (<=1kb)	VIT_05s0077g00280	-32	Beta-amylase	1,56
2,22	Promoter (1-2kb)	VIT_19s0014g04790	-1503	**Organic cation/carnitine transporter4**	1,55

Table 54: VviNAC60 DAP-seq targets genes which have a match in the *VviNAC60* transient over expression dataset and resulted up regulated. In bold are reported the *switch* genes and the asterisk (*) refers to the transition markers.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
50,7	Promoter (<=1kb)	VIT_19s0014g03370	0	Methyltransferase type 11	-1,68
9,43	Promoter (<=1kb)	VIT_14s0068g01570	-405	Glutaredoxin-like	-1,99
7,1	Promoter (<=1kb)	VIT_10s0003g04270	-76	BCL2 binding anthogene	-1,70
4,32	Promoter (<=1kb)	VIT_10s0003g04270	-22	BCL2 binding anthogene	-1,70
3,71	Promoter (<=1kb)	VIT_02s0154g00080	-114	Multi-copper oxidase (SKU5)	-2,29
3,64	Promoter (1-2kb)	VIT_05s0077g00620	-1296	O-acetyltransferase	-1,54
3,58	Promoter (1-2kb)	VIT_16s0050g01610	-1530	UDP-glycosyltransferase 88A4	-1,77
3,27	5' UTR	VIT_05s0094g01140	1817	Allyl alcohol dehydrogenase	-2,46
2,21	5' UTR	VIT_01s0010g02910	3862	ATP binding protein	-2,09

Table 55: VviNAC60 DAP-seq targets genes which have a match in the *VviNAC60* transient over expression dataset and resulted down regulated.

VviNAC61

The DAP-seq assay reported only 76 binding sites (peaks), which were reduced to 73 after the FC>5 filtering; the loss was around the 4% (**Fig. 64**).

The distribution of peaks revealed that 30% were located within the promoter regions (up to 2 kb upstream from a transcription start site), 3% were in 5′ untranslated regions (UTRs), 13% were located in exons, 5% were located within introns, 3% were located in 3′ UTRs, 12% were located in the 3 kb downstream region, and 30% were intergenic.

The number of regulatory sites was very low but the percentage of binding sites in the promoter and 5' UTR regions was in line, even a little bit higher, with literature.

Considering the good results obtained in the transient over expression of the gene, the DAP-seq assay on VviNAC61 is going to be repeated as soon as possible to validate the findings of the transient over expression and improve the knowledges on the post-harvest (withering) processes (Zenoni *et al*., 2016).

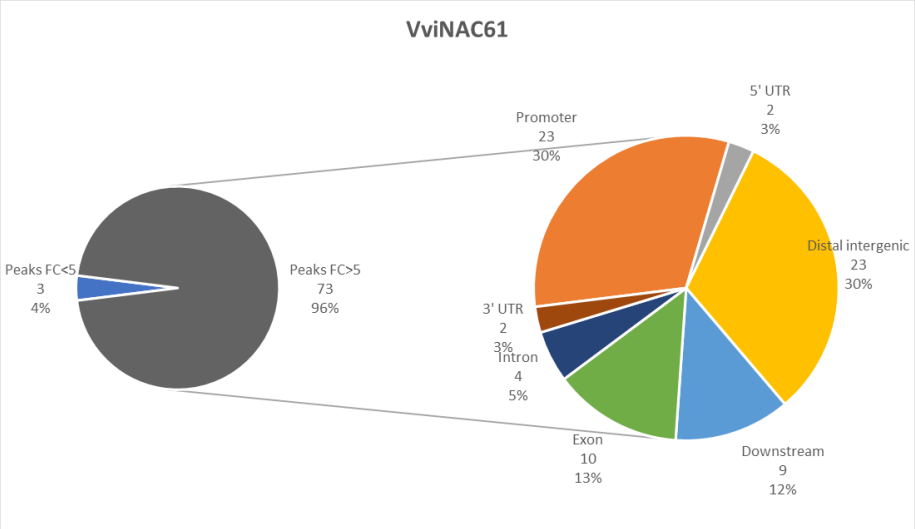


Figure 64: Double pie chart of the VviNAC61 DAP-seq results. The pie on the left represents the total binding sites (peaks) obtained, divided between the FC>5 and FC<5 (compared to the GST-Halo negative control peaks). The pie on the right represents the subdivision into the different genic region of the selected filtered peaks.

A principal binding motif (TTGCGTGT) was identified (**Fig. 65**) and the phylogenetic footprints correlate with ANAC058, for which not much information is available.

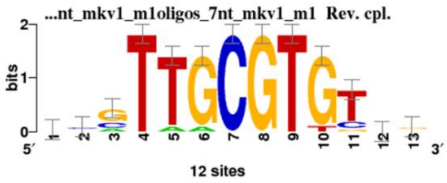


Figure 65: RSAT Plants NGS ChIP-Seq peak motifs analysis on the VviNAC61 DAP-seq results. Top-ranking binding motif identified in the DAP-seq FC>5 filtered dataset, based on the detection of overrepresented oligonucleotides (oligo analysis). The overall height of each letter stack indicates the sequence conservation at that position, and the height of symbols within the stack reflects the relative frequency of the corresponding nucleic acid at that position.

The attention was not focused only on the 33% (25 sites) of the final analyzed binding sites, as was done for almost all the VviNACs TF.

All the DAP-seq dataset was taken in consideration but the 'no hit' and 'unknown' elements were eliminated as usual.

59 binding sites were obtained for a total of 59 unique genes regulated by VviNAC61.

A GO enrichment analysis was performed, revealing a predominance of genes related to the regulation of metabolic processes, aromatic compounds biosynthetic processes and response to jasmonic acid (**Fig. 66**).

Then, the previously reported *VviNAC61* transient over expression results (FC>|1.5|) were crossed with the DAP-seq dataset, revealing only 3 correlated binding sites (**Table 56**): the up regulated *DISEASE RESISTANCE PROTEIN NBS-LRR CLASS* (VIT_19s0027g01630), which TF binding site is in the 'promoter' region, and *EMB2454 EMBRYO DEFECTIVE 2454* (VIT_17s0000g05480), and the down regulated *FRUCTOSE-1,6-BISPHOSPHATASE, CYTOSOLIC* (VIT_18s0072g00770). Always in the 'promoter' region, the *VviWRKY14* (VIT_05s0077g00730), which is known to be highly expressed in berries and rachis during or following veraison (Wang *et al*., 2014), and two ethylene-related genes, *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 9* (VIT_12s0028g03270) and *ERF/AP2 GENE FAMILY VvERF058* (VIT_05s0077g01860), were found.

Interestingly, also a *LACCASE* (VIT_18s0122g00520) was found between the VviNAC61 target genes; unfortunately, the TF binding site was revealed in the 'distal intergenic' region at -16831 bp from the TSS and is difficult to validate with other methods the real interaction.

Unfortunately, no *VviNACs* target genes were found. However, these first findings

are a good result and the new DAP-seq assay will certainly unravel the VviNAC61 regulatory network.

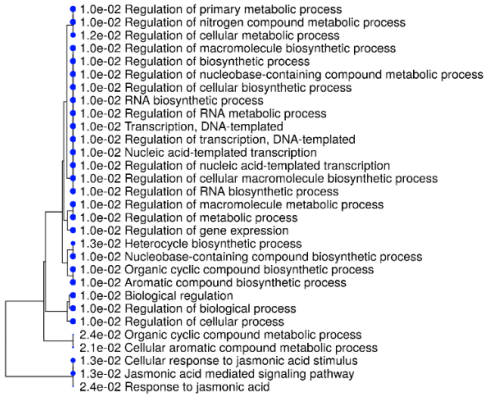


Figure 66: GO enrichment analysis of the VviNAC61 targets found to be regulated in the promoter and 5'UTR regions. The analysis was performed on ShinyGO.

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	Transient OE
5,57	Exon (mRNA27258/VIT_04s0008g02380, exon 1 of 2)	VIT_04s0008g02380	819	Kelch repeat-containing F-box protein	
4,29	Distal Intergenic	VIT_08s0105g00230	-13948	BLH1 (embryo sac development arrest 29)	
4,06	Downstream (1-2kb)	VIT_17s0000g04850	1832	OBF binding protein 4	
4,05	Distal Intergenic	VIT_18s0086g00180	-147831	MATE efflux family protein	
4,04	Distal Intergenic	VIT_14s0081g00520	-12785	ERF12	
3,59	Promoter (1-2kb)	VIT_07s0191g00050	-1874	Peroxidase 17 *	
3,52	Promoter (<=1kb)	VIT_12s0028g03270	-100	Ethylene-responsive transcription factor 9	
3,52	Distal Intergenic	VIT_06s0009g00580	-5284	Receptor protein kinase	
3,51	Distal Intergenic	VIT_10s0003g02010	-3315	RKF1 (receptor-like kinase in flowers 1)	
3,43	Promoter (1-2kb)	VIT_12s0059g00870	-1273	MAPKKK15	
3,31	Exon (mRNA11075/VIT_14s0036g00870, exon 1 of 1)	VIT_14s0036g00870	653	Acyl-CoA ligase	
3,08	Promoter (<=1kb)	VIT_19s0015g00930	-272	Hydroxyproline-rich glycoprotein	
3	Distal Intergenic	VIT_19s0015g01930	2462	LAS (lateral suppressor)	
2,85	Downstream (2-3kb)	VIT_15s0048g01320	4494	Gibberellin 20-oxidase	
2,65	Intron (mRNA11877/VIT_18s0041g02430, intron 2 of 9)	VIT_18s0041g02430	3416	abscisic aldehyde oxidase (AAO3) (VvAAO3)	
2,65	Exon (mRNA14409/VIT_15s0046g01600, exon 1 of 2)	VIT_15s0046g01600	23	Acidic endochitinase (CHIB1)	
2,64	Exon (mRNA13719/VIT_17s0000g08720, exon 6 of 7)	VIT_17s0000g08720	2549	RKF2 (receptor-like serine/threonine kinase 2)	
2,63	Distal Intergenic	VIT_12s0134g00160	-2133	Xyloglucan endotransglycosylase/hydrolase 16	
2,51	Promoter (1-2kb)	VIT_17s0000g08950	-1089	D111/G-patch	
2,51	Downstream (1-2kb)	VIT_19s0015g00770	6363	Protein kinase G11A	
2,51	Distal Intergenic	VIT_01s0011g05560	10260	TIFY gene family (VvJAZI)	
2,49	Exon (mRNA2054/VIT_11s0037g00580, exon 2 of 2)	VIT_11s0037g00580	1010	Anthranilate N-hydroxycinnamoyl/benzoyltransferase	
2,47	Distal Intergenic	VIT_01s0026g00740	-7622	Nucleobase-ascorbate transporter 6 (NAT6)	
2,36	Promoter (<=1kb)	VIT_06s0004g03900	-540	Calcium-transporting ATPase 1, endoplasmic reticulum-type ECA1	
2,35	Distal Intergenic	VIT_13s0158g00370	6396	RNA recognition motif (RRM)-containing CCCH	
2,35	Distal Intergenic	VIT_04s0044g00870	-3866	U-box domain containing protein	
2,31	Promoter (<=1kb)	VIT_04s0044g00710	-161	UTP--glucose-1-phosphate uridylyltransferase *	
2,31	Downstream (2-3kb)	VIT_07s0130g00460	2972	SWIB complex BAF60b domain-containing protein	
2,31	Downstream (<1kb)	VIT_08s0007g08320	3417	Pentatricopeptide (PPR) repeat-containing	
2,3	Promoter (<=1kb)	VIT_10s0042g01150	-6	ARGONAUTE 2 (AGO2)	
2,3	Promoter (<=1kb)	VIT_11s0016g01130	-360	MTD1	
2,3	Intron (mRNA20379/VIT_08s0007g08270, intron 2 of 2)	VIT_08s0007g08280	1991	Remorin	
2,29	Promoter (<=1kb)	VIT_17s0000g05480	-519	EMB2454 (embryo defective 2454)	1,57
2,29	Promoter (<=1kb)	VIT_04s0008g04370	-903	Nucleic acid binding	
2,29	Exon (mRNA21353/VIT_13s0064g00390, exon 7 of 10)	VIT_13s0064g00390	4340	Polyamine oxidase	
2,29	Distal Intergenic	VIT_19s0014g03000	21567	Leucine-rich repeat-containing protein 40	
2,28	Distal Intergenic	VIT_08s0040g02900	-4051	Aluminum sensitive 3	
2,26	Exon (mRNA29767/VIT_06s0009g02110, exon 1 of 2)	VIT_06s0009g02110	714	Kelch repeat-containing F-box family protein	
2,21	Promoter (<=1kb)	VIT_18s0001g13270	0	Papain cysteine peptidase XBCP3	
2,21	Promoter (<=1kb)	VIT_06s0061g01190	-134	SWI/SNF matrix-associated regulator of chromatin sbfamily A mber 3 2	
2,15	Exon (mRNA14074/VIT_05s0020g03310, exon 3 of 4)	VIT_05s0020g03310	1399	Diamine oxidase	
2,15	Distal Intergenic	VIT_08s0040g02730	-3066	Exocyst subunit EXO70 H4	
2,14	Promoter (1-2kb)	VIT_10s0003g02820	-1128	O-acetyltransferase	
2,14	Promoter (<=1kb)	VIT_05s0077g01860	-204	ERF/AP2 Gene Family (VvERF058)	
2,13	Downstream (1-2kb)	VIT_18s0072g00770	6002	fructose-1,6-bisphosphatase, cytosolic	-1,62
2,13	Distal Intergenic	VIT_17s0000g08150	6259	basic helix-loop-helix (bHLH) family	
2,13	Distal Intergenic	VIT_05s0051g00580	-10927	Inosine triphosphate pyrophosphatase	
2,12	Promoter (1-2kb)	VIT_04s0023g01230	-1203	T-complex protein 1 subunit delta	
2,12	Promoter (<=1kb)	VIT_05s0062g01000	-989	Aldo/keto reductase *	
2,12	Intron (mRNA26650/VIT_00s0324g00050, intron 1 of 2)	VIT_00s0324g00050	4476	UDP-glucose glucosyltransferase	
2,12	Intron (mRNA19400/VIT_05s0102g00360, intron 5 of 11)	VIT_05s0102g00360	8794	On-specific serine/threonine protein kinase	
2,12	Distal Intergenic	VIT_19s0027g01630	15308	Disease resistance protein (NBS-LRR class)	1,65
2,11	Promoter (<=1kb)	VIT_13s0067g01920	0	Exportin 7	
2,11	Distal Intergenic	VIT_18s0122g00520	-16831	Laccase	
2,07	Downstream (2-3kb)	VIT_01s0026g02030	3322	basic helix-loop-helix (bHLH) family	
2,06	Promoter (<=1kb)	VIT_08s0040g02210	-861	Lectin protein kinase	
2,06	Distal Intergenic	VIT_18s0001g00840	-14933	Syringolide-induced protein 14-1-1	
2,06	5' UTR	VIT_06s0004g06240	328	Zinc finger (C2H2 type) family	
2,05	Promoter (1-2kb)	VIT_05s0077g00730	-1909	WRKY Transcription Factor (VvWRKY14)	

Table 56: VviNAC61 DAP-seq targets genes and *VviNAC61* transient over expression fold-change values. The asterisk (*) refers to the transition markers.

VviNACs regulation of the *Vitis vinifera* genes

After all the individual analyses on all the 14 above described VviNACs, with the aim to try understanding the general regulative role of the selected VviNAC family members on the grapevine transcriptome, a global analysis of all the possible interactions obtained with the DAP-seq assays was performed.

All the annotated *V. vinifera* genes were listed and, once eliminated all the 'no hit' and 'unknown', the most represented genes between all the DAP-seq results were identified. In this case all the binding sites in all the possible genic regions were taken into consideration, not only the promoter and 5'UTR one.

Ideally, in this way a general observation concerning the mostly regulated (by these TFs) classes of genes should be possible.

13713 genes resulted as direct targets of at least one of the 14 selected VviNACs.

In **Figure 67** are reported the numbers of genes regulated by a different number of TFs.

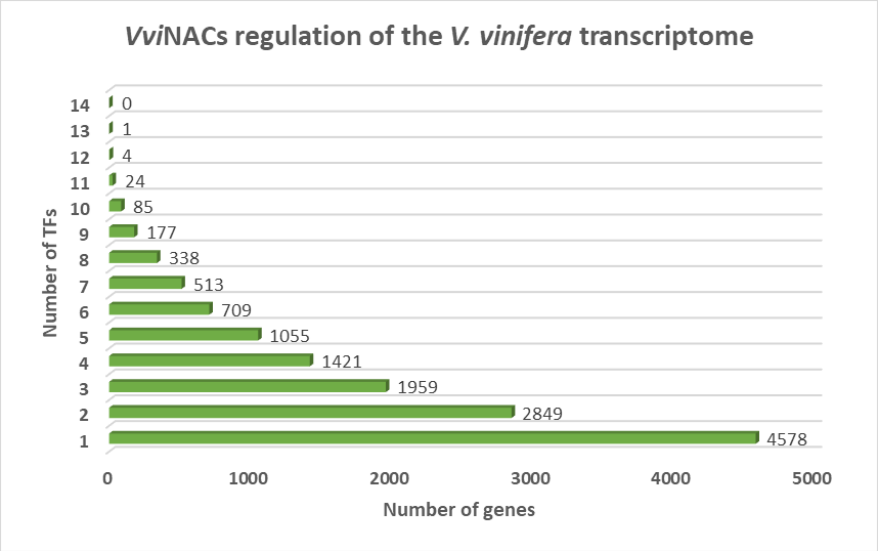


Figure 67: Bar chart reporting the number of annotated *V. vinifera* genes regulated by a different number of the selected VviNACs.

4578 genes were specifically regulated by only one the previously described TFs and only one gene, *UTP--GLUCOSE-1-PHOSPHATE URIDYLYLTRANSFERASE* (VIT_04s0044g00710), was regulated by 13 of the 14 *Vvi*NACs; the only TF which did not regulate this gene was VviNAC08.

Not much information is available in literature concerning this gene, excluding the

fact that it encodes for an enzyme obviously related to the carbohydrate metabolism. For this reason, the Atlas (Fasoli *et al*., 2012) was again investigated to see if this gene could have an interesting expression profile. Considering its role in the sugar metabolism, a specific focus was done on the berry and seed tissues at the different developmental stages (**Fig. 68**).

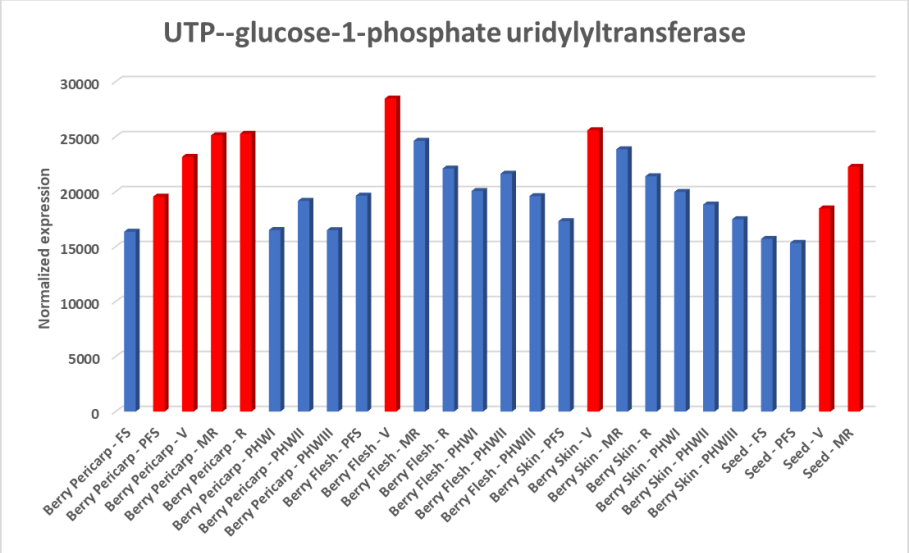


Figure 68: Bar chart reporting the expression profile in the Atlas (Fasoli *et al*., 2012) of the *UTP-GLUCOSE-1-PHOSPHATE URIDYLYLTRANSFERASE*. Description of ATLAS abbreviations (54 developmental stages): **Berry Pericarp** – FS =fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post- harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III**;** **Berry Skin** – PFS = post-fruit set; – V = véraison; – MR = mid-ripening; – R = ripening; – PHWI = post-harvest withering I; – PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Berry Flesh** – PFS = post-fruit set; – V = véraison; – MR = mid- ripening; – R = ripening; – PHWI = post-harvest withering I; –PHWII = post-harvest withering II; – PHWIII = post-harvest withering III; **Seed** – FS = fruit set; – PFS = post-fruit set; – V = véraison; – MR = mid-ripening.

The gene was always abundantly expressed in all the organs at all the developmental stages. However, a significant increase was interestingly noticed in the berry pericarp starting from the post fruit set to the ripening, in the berry flesh and skin at veraison and in the seeds at veraison and mid ripening.

Between the genes regulated by 12 TFs, the *3-METHYL-2-OXOBUTANOATE DEHYDROGENASE* (VIT_18s0001g14980) was found, as expected from the single TF DAP-seq analyses in which it was very often present also in the first positions (q-value based).

Moreover, a *BLH1 EMBRYO SAC DEVELOPMENT ARREST 29*

(VIT_08s0105g00230) and an *EXPANSIN VvEXPA18* (VIT_17s0053g00990), important for the plant cell wall growth (Marowa *et al*., 2016), were also regulated by 12 TFs.

Interestingly, an auxin- and an ethylene-related gene (VIT_00s0184g00110 and VIT_18s0072g00260), the *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 1* (VIT_02s0025g04660), a *MYB* gene (VIT_15s0046g02260) and two *LACCASES* (VIT_18s0122g00520 and VIT_18s0122g00690) resulted regulated by 11 VviNACs.

Three *WRKY* genes, *VviWRKY14* (VIT_05s0077g00730), *VviWRKY43* (VIT_14s0068g01770) and *VviWRKY52* (VIT_17s0000g01280), two *MYB* genes (VIT_12s0028g00980 and VIT_01s0011g04760) and the *VviNAC01* (VIT_01s0146g00280) resulted direct targets of 10 *Vvi*NACs.

The presence of *VviNAC01* in this group is very intriguing as it resulted the TF with more *VviNACs* interactions: 28 direct interactions with another *VviNAC* promoter/5'UTR region for a total of 23 *Vvi*NACs regulated by it and showing also its autoregulation.

VviNAC61 (VIT_08s0007g07640) is the second *VviNAC* most regulated gene with 9 *Vvi*NACs TF interactions.

Considering the very interesting information that this list could give to define the regulative role of the 14 selected VviNACs, in the future a more detailed analysis will be done.

VviNAC60 and ChIP-seq

Considering that the DAP-seq technique gives a global view of all the possible binding sites of a specific transcription factor, without taking into consideration the tissue and developmental stage specificity of regulation, ChIP-seq data were also obtained for *Vvi*NAC60. The aim was to find direct target of the TF which are related to the berry ripening specific regulation.

The analyses were performed, always using cv Shiraz fruiting cuttings (**Fig. 69**), on berries before veraison ('Green berry'), characterized by a very low expression of *VviNAC60* and used as a sort of negative control, and berries after veraison ('Veraison berry'), characterized by a high expression of the gene. Given the

absence of *VviNAC60* expression in young leaves, these were used as another and more powerful negative controls ('Young leaf').

Figure 69: Picture of the fruiting cuttings of cv Shiraz and details of the three tissues used in the ChIP-seq experiment: berries before veraison, characterized by a very low expression of *VviNAC60*, and berries after veraison, characterized by a high expression of the NAC candidate; given the absence of *VviNAC60* expression in young leaves, these were used as negative control.

The ChIP-Seq protocol provided, after the harvesting, the plant tissue fixation using formaldehyde to crosslink TFs–DNA interactions (**Fig. 70**).

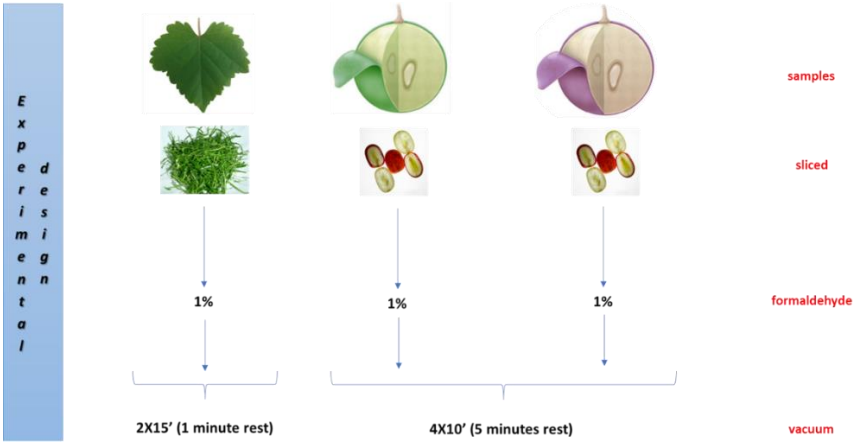


Figure 70: Experimental design of the ChIP-seq assay; the three different tissues harvested, sliced and the TF was crosslinked to the chromatin using 1% formaldehyde under different vacuum condition (depending on the type of tissue).

Then, the isolation of nuclei and the shearing of chromatin were performed. The subsequent sonication permitted to obtain most of the DNA fragments at 200 bp length, the perfect size for the sequencing step. After the precipitation of all the insoluble material, the immunoprecipitation step with the specific antibody against VviNAC60 was performed and, once the antibody–TF/DNA complexes were isolated, the DNA was released by reverse crosslinking and purified.

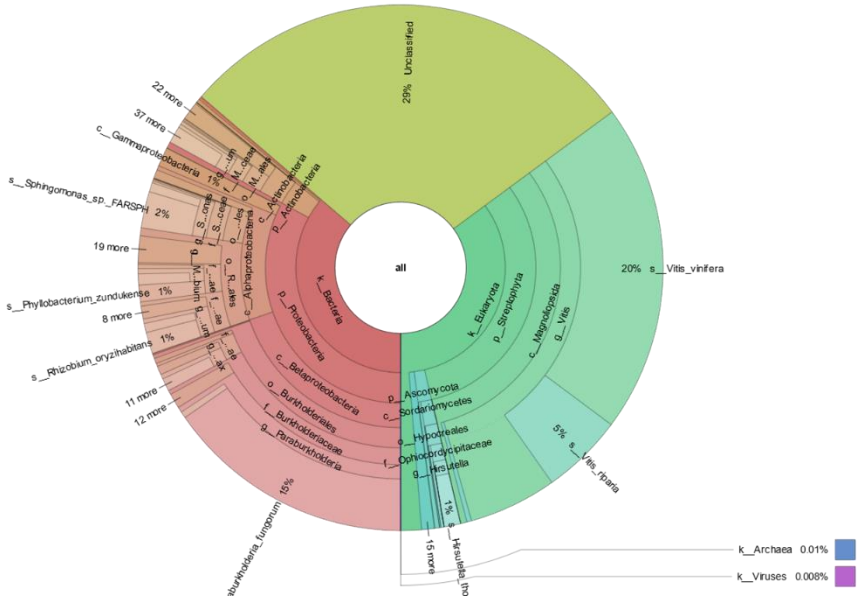
Once being able to obtain enough immunoprecipitated material, especially from the 'Veraison berry' samples (considering the huge difficulties in extracting genomic DNA from the berry tissues at this particular stage of development), the resulting DNA fragments were sequenced.
Looking at each of the three sample percentages of alignment on the grape genome (**Fig. 71**), the 'Young leaf' was in line with the ChIP-seq literature (31,26%); unfortunately, this did not happen for the berry samples for which a 19,73% ('Green berry') and a 5,29% ('Veraison berry') were obtained.

The ChIP-seq experiment was performed again on the 'Veraison berry' sample, increasing of 3 times the chromatin concentration, to obtain a higher correspondence and a more accurate dataset.
Instead, the 'Green berry' results was not repeated as the alignment percentage was not too far from the average 25% found in literature and the developmental stage of the berry tissue was not the main protagonist of our investigation.
Unfortunately, with the second sequencing run nothing changed and a very low alignment was obtained again. For this reason, a specific bioinformatic analysis was applied to all the ChIP-seq samples, in order to understand to which organism the alien sequenced DNA belonged to.

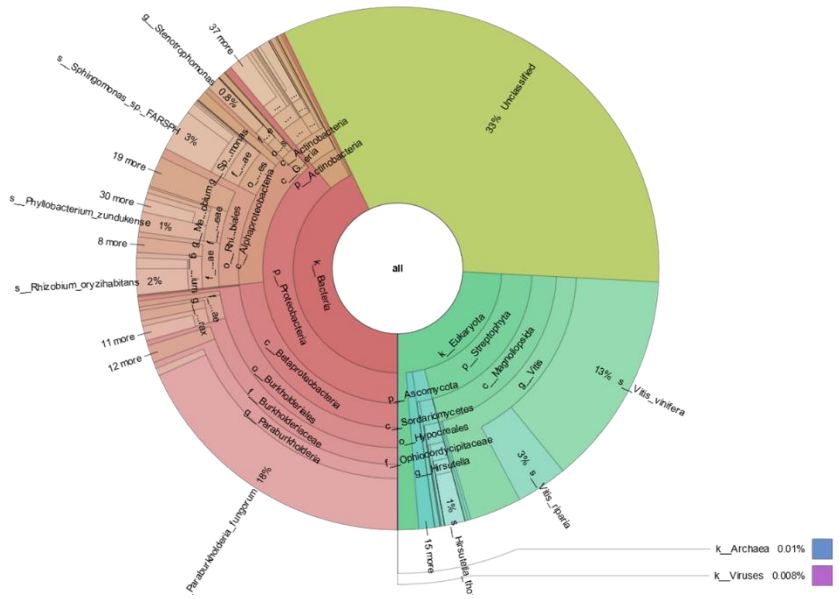
All 'input' samples (the not immunoprecipitated chromatin of each sample), used as a control, perfectly aligned on the *Vitis vinifera* genome (>89%). On the contrary, most of the sequences of the immunoprecipitated samples belonged to microorganisms that usually live on the leaves and grape surface (**Fig. 71a-c**). For some unknown reasons, during the immuno-precipitation steps this DNA was selected and enriched.
The organisms were the same in all the samples and the changes concerned only the numerical amount of the unwanted contamination, which drastically increase in the berry tissues, in particular in the almost mature one.
If the percentage of the different microorganisms was the same in all the samples, one microorganism, the most enriched one, showed differences.
Indeed, the only one that increase between the different tissues and developmental

stages was the *Paraburkholderia fungorum*. This plant probiotic bacterium has never been studied in grapevine; however, a recent study correlates its presence to improvements in growth, yield and content of antioxidants in strawberry fruit (Rahman *et al*., 2018). Moreover, plant probiotic bacteria are generally known to naturally occur in plant and enhance the growth of the host, sometimes suppressing diseases and providing beneficial effects (Rahman *et al*., 2018).

a)



b)



c)

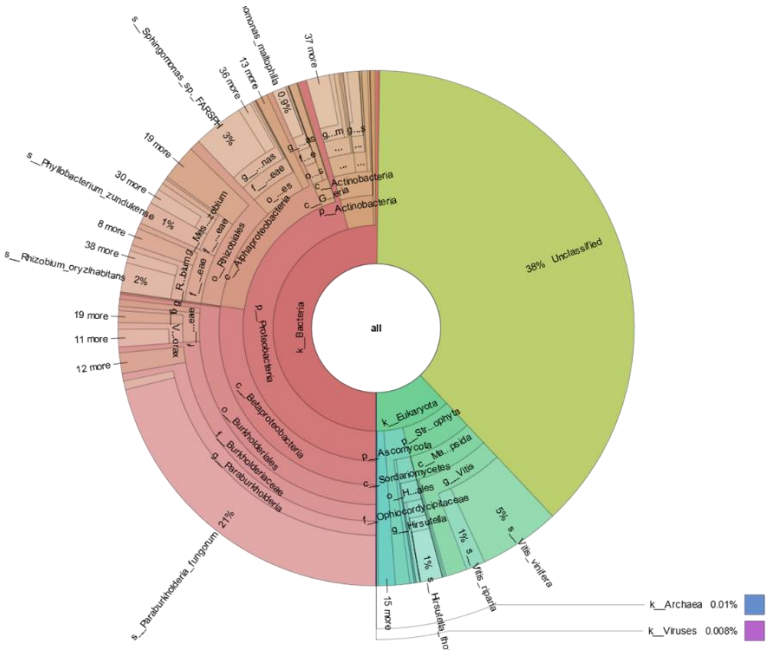


Figure 71: Pie charts of **a)** the 'Young leaf', **b)** the 'Green berry' and **c)** the 'Veraison berry' immunoprecipitated DNA, showing the bacteria DNA enrichment accidentally obtained in the ChIP-seq assay.

However, putting all the sequencing results together and despite the larger amount of alien enriched DNA, the highest number of genes were found in the 'Veraison berry' dataset (**Fig. 72**). This was very comforting, especially considering the low amount of analyzable grape DNA of these samples, and this evidence shows the good transcription factor recognition by the antibody and confirms the highest *VviNAC60* expression in the berries after veraison.

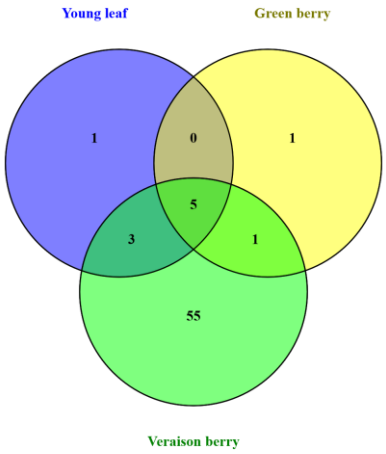


Fig. 72: Venn diagram of the genes found in the ChIP-seq analyzed samples.

Nine genes were found for the 'Young leaf' sample (**Table 57**), only one of which resulted leaf specific (VIT_05s0020g02930), and 3 genes were in common with the 'Veraison sample' (VIT_12s0178g00200, VIT_08s0007g02780 and VIT_13s0074g00400). Seven genes were found in the 'Green berry' sample (**Table 57**): only one was specific of this sample (VIT_15s0107g00120) and another one was in common with 'Veraison berry' sample (VIT_00s0366g00010). In addition to the genes in common with the other two samples, which were already reported above, the 'Veraison berry' sample had other 55 specific genes (**Table 57**). Five genes (**Table 57**) were found in common between all the samples (VIT_06s0004g03090, VIT_13s0101g00230, VIT_13s0101g00220, VIT_13s0064g00830 and VIT_18s0041g01900).

Sample	Gene ID	Functional annotation	Young leaf	Green berry	Veraison berry
Young leaf	VIT_06s0004g03090	Syntaxin of plants SYP7	*	*	*
Young leaf	VIT_13s0101g00230	Unknown	*	*	*
Young leaf	VIT_13s0101g00220	Ribosomal RNA 16S	*	*	*
Young leaf	VIT_13s0064g00830	Disease resistance protein RGA2 (RGA2-blb)	*	*	*
Young leaf	VIT_12s0178g00200	Actin beta/gamma 1	*		*
Young leaf	VIT_18s0041g01900	Elongation factor 1-gamma	*	*	*
Young leaf	VIT_08s0007g02780	Asp/Glu racemase	*		*
Young leaf	VIT_13s0074g00400	PTL (PETAL LOSS)	*		*
Young leaf	VIT_05s0020g02930	DNA-directed RNA polymerase III subunit C3	*		
Green berry	VIT_06s0004g03090	Syntaxin of plants SYP7	*	*	*
Green berry	VIT_13s0101g00230	Unknown	*	*	*
Green berry	VIT_00s0366g00010	No hit		*	*
Green berry	VIT_13s0101g00220	Ribosomal RNA 16S	*	*	*
Green berry	VIT_15s0107g00120	putative MADS-box Short Vegetal Phase 3 (VviSVP3)		*	
Green berry	VIT_18s0041g01900	Elongation factor 1-gamma	*	*	*
Green berry	VIT_13s0064g00830	Disease resistance protein RGA2 (RGA2-blb)	*	*	*
Veraison berry	VIT_06s0004g03090	Syntaxin of plants SYP7	*	*	*
Veraison berry	VIT_13s0101g00230	Unknown	*	*	*
Veraison berry	VIT_13s0101g00220	Ribosomal RNA 16S	*	*	*
Veraison berry	VIT_13s0074g00400	PTL (PETAL LOSS)	*		*
Veraison berry	VIT_08s0007g02780	Asp/Glu racemase	*		*
Veraison berry	VIT_18s0041g01900	Elongation factor 1-gamma	*	*	*
Veraison berry	VIT_13s0064g00830	Disease resistance protein RGA2 (RGA2-blb)	*	*	*
Veraison berry	VIT_13s0106g00500	RNA recognition motif (RRM)-containing protein			*
Veraison berry	VIT_00s1355g00020	Expansin-like B1			*
Veraison berry	VIT_16s0148g00230	Receptor-like kinase ARK1AS			*
Veraison berry	VIT_09s0054g01070	Receptor-like protein kinase PRK1			*
Veraison berry	VIT_11s0037g01330	No hit			*
Veraison berry	VIT_08s0040g02070	fasciclin arabinogalactan-protein (FLA11)			*
Veraison berry	VIT_12s0178g00200	Actin beta/gamma 1	*		*
Veraison berry	VIT_15s0107g00090	Gag-pol polyprotein			*
Veraison berry	VIT_00s0599g00010	No hit			*
Veraison berry	VIT_00s0125g00020	Reverse transcriptase			*
Veraison berry	VIT_08s0032g00780	Calcium Dependent Protein Kinase (VvCPK8)			*
Veraison berry	VIT_08s0105g00140	Bromo-adjacenty (BAH) domain-containing protein			*
Veraison berry	VIT_14s0006g00980	DNA mismatch repair protein Mlh1			*
Veraison berry	VIT_13s0067g03430	ARR9 typeA			*
Veraison berry	VIT_12s0121g00060	R protein MLA10			*
Veraison berry	VIT_18s0041g01130	RNA binding			*
Veraison berry	VIT_00s0125g00060	Unknown			*
Veraison berry	VIT_00s0685g00020	No hit			*
Veraison berry	VIT_01s0113g00430	Mitotic checkpoint protein BUB3			*
Veraison berry	VIT_19s0014g01720	Sodium/calcium exchanger family protein			*
Veraison berry	VIT_04s0044g01360	Unknown protein			*
Veraison berry	VIT_07s0130g00140	Thioesterase family			*
Veraison berry	VIT_06s0004g07650	Taxadien-5-alpha-ol-O-acetyltransferase			*
Veraison berry	VIT_08s0032g01110	Axial regulator YABBY2			*
Veraison berry	VIT_08s0007g09040	DnaJ homolog, subfamily A, member 5			*
Veraison berry	VIT_09s0002g07250	No hit			*
Veraison berry	VIT_00s0131g00320	Annexin ANN3			*
Veraison berry	VIT_07s0151g00380	No hit			*
Veraison berry	VIT_00s1328g00020	Phytosulfokines PSK4			*
Veraison berry	VIT_15s0045g01140	Heavy-metal-associated domain-containing protein			*
Veraison berry	VIT_02s0025g02680	Growth-regulating factor 5			*
Veraison berry	VIT_00s0226g00020	RabGAP/TBC domain-containing protein			*
Veraison berry	VIT_06s0004g02170	Histidinol-phosphate aminotransferase			*
Veraison berry	VIT_12s0059g01110	Tubulin beta-8			*
Veraison berry	VIT_00s0366g00010	No hit		*	*
Veraison berry	VIT_12s0034g01260	R protein L6			*
Veraison berry	VIT_10s0003g04250	Glutathione S-transferase GSTU22			*
Veraison berry	VIT_01s0150g00640	Unknown protein			*
Veraison berry	VIT_16s0013g01320	Ser/Thr receptor-like kinase1			*
Veraison berry	VIT_16s0013g00160	Pectinesterase PPME1			*
Veraison berry	VIT_12s0028g03340	Protein binding / zinc ion binding			*
Veraison berry	VIT_14s0036g01060	Unknown protein			*
Veraison berry	VIT_13s0019g04450	ATSYTA/NTMC2T1.1/NTMC2TYPE1.1/SYTA			*
Veraison berry	VIT_12s0034g00490	No hit			*
Veraison berry	VIT_04s0069g00470	No hit			*
Veraison berry	VIT_00s0169g00110	No hit			*
Veraison berry	VIT_14s0108g00530	No hit			*
Veraison berry	VIT_11s0149g00070	Ribosomal protein S6			*
Veraison berry	VIT_19s0093g00050	R protein PRF disease resistance protein			*
Veraison berry	VIT_10s0003g02400	SRG1 (senescence-related gene 1) oxidoreductase			*
Veraison berry	VIT_09s0096g00830	R protein disease resistance protein			*
Veraison berry	VIT_04s0043g00490	No hit			*
Veraison berry	VIT_07s0005g06010	FAD linked oxidase, N-terminal			*
Veraison berry	VIT_14s0006g01240	Protein kinase beta-1 subunit 5'-AMP-activated			*
Veraison berry	VIT_16s0050g00820	EMB1691 (embryo defective 1691)			*
Veraison berry	VIT_13s0106g00470	RNA-binding protein 45 (RBP45)			*
Veraison berry	VIT_10s0003g05770	Short-chain dehydrogenase/reductase (SDR)			*

Table 57: VviNAC60 ChIP-seq target genes.

Only the small 'Veraison berry' dataset was further analyzed and, once removed all the 'no hit' and 'unknown' genes and all the genes in common with the other two samples, a list of 46 binding sites was obtained (**Table 58**).
Only four genes were found to be regulated in the 'promoter' region: *SODIUM/CALCIUM EXCHANGER FAMILY PROTEIN* (VIT_19s0014g01720), *HEAVY-METAL-ASSOCIATED DOMAIN-CONTAINING PROTEIN* (VIT_15s0045g01140), R protein L6 (VIT_12s0034g01260) and *PROTEIN BINDING / ZINC ION BINDING* (VIT_12s0028g03340). Three genes were found regulated in the 3'UTR region, 21 in the distal intergenic region, 7 in the downstream region, 2 in the exons and 9 in the introns. Moreover, this list was crossed with the already described DAP-seq and over expressions dataset to see if some correlation could be found (**Table 58**).

Three genes were also found up regulated by the *VviNAC60* stable over expression: *PROTEIN BINDING / ZINC ION BINDING* (VIT_12s0028g03340), which *Vvi*NAC60 binding site was found in the promoter, *PHYTOSULFOKINES PSK4* (VIT_00s1328g00020) and *RECEPTOR-LIKE KINASE ARK1AS* (VIT_16s0148g00230), which harbors two binding sites in the intron. Only one gene found correlation with the *VviNAC60* transient over expression: *RNA-BINDING PROTEIN 45 RBP45* (VIT_13s0106g00470), which regulation is related to the binding of VviNAC60 in the distal intergenic region.

Crossing the VviNAC60 DAP-seq and the ChIP-seq datasets, only one gene was found in common; unfortunately, this gene reveals the TF binding site in two different genic regions, in an exon (745 bp from to the TSS) in the ChIP-seq dataset and in the promoter (-946 bp from to the TSS) in the DAP-seq one. However, this gene, *SRG1- SENESCENCE-RELATED GENE 1 OXIDOREDUCTASE* (VIT_10s0003g02400), seems to be very interesting as it is related to senescence and plant immunity (Cui *et al*., 2018).

q-value	Genic region	Gene ID	Distance to TSS	Functional annotation	DAP-seq	Stable OE	Transient OE
999,00	Intron (mRNA383/VIT_06s0004g03090, intron 2 of 8)	VIT_06s0004g03090	1736	Syntaxin of plants SYP7			
256,1	Intron (mRNA383/VIT_06s0004g03090, intron 2 of 8)	VIT_06s0004g03090	1734	Syntaxin of plants SYP7			
78,09	3' UTR	VIT_13s0101g00220	735	Ribosomal RNA 16S			
50,62	Distal Intergenic	VIT_13s0074g00400	19535	PTL (PETAL LOSS)			
37,83	3' UTR	VIT_13s0101g00220	736	Ribosomal RNA 16S			
32,71	Intron (mRNA3531/VIT_13s0106g00500, intron 5 of 9)	VIT_13s0106g00500	3249	RNA recognition motif (RRM)-containing protein			
30,34	Intron (mRNA23493/VIT_16s0148g00230, intron 1 of 2)	VIT_16s0148g00230	5412	Receptor-like kinase ARK1AS		1,50	
30,18	3' UTR	VIT_09s0054g01070	2193	Receptor-like protein kinase PRK1			
16,37	Downstream (<1kb)	VIT_00s0125g00020	1047	Reverse transcriptase			
15,09	Distal Intergenic	VIT_08s0032g00780	13152	Calcium Dependent Protein Kinase (VvCPK8)			
14,97	Distal Intergenic	VIT_08s0105g00140	7371	Bromo-adjacenty (BAH) domain-containing protein			
14,96	Downstream (<1kb)	VIT_14s0006g00980	1019	DNA mismatch repair protein Mlh1			
12,21	Exon (mRNA25235/VIT_13s0067g03430, exon 5 of 5)	VIT_13s0067g03430	939	ARR9 typeA			
12,02	Distal Intergenic	VIT_12s0121g00060	21966	R protein MLA10			
10,92	Distal Intergenic	VIT_18s0041g01130	-33000	RNA binding			
9,79	Distal Intergenic	VIT_08s0105g00140	11152	Bromo-adjacenty (BAH) domain-containing protein			
9,51	Promoter (<=1kb)	VIT_19s0014g01720	-466	Sodium/calcium exchanger family protein			
8,70	Downstream (<1kb)	VIT_07s0130g00140	3720	Thioesterase family			
8,32	Downstream (1-2kb)	VIT_06s0004g07650	3040	Taxadien-5-alpha-ol-O-acetyltransferase			
7,40	Intron (mRNA10573/VIT_08s0032g01110, intron 2 of 6)	VIT_08s0032g01110	1954	Axial regulator YABBY2			
7,27	Distal Intergenic	VIT_08s0007g09040	-4318	DnaJ homolog, subfamily A, member 5			
6,51	Intron (mRNA3531/VIT_13s0106g00500, intron 5 of 9)	VIT_13s0106g00500	3252	RNA recognition motif (RRM)-containing protein			
6,19	Distal Intergenic	VIT_13s0074g00400	19533	PTL (PETAL LOSS)			
6,14	Downstream (1-2kb)	VIT_06s0004g07650	3041	Taxadien-5-alpha-ol-O-acetyltransferase			
5,79	Distal Intergenic	VIT_00s0131g00320	25581	Annexin ANN3			
4,75	Distal Intergenic	VIT_00s1328g00020	-9983	Phytosulfokines PSK4		1,53	
4,38	Promoter (<=1kb)	VIT_15s0045g01140	-252	Heavy-metal-associated domain-containing protein			
4,21	Downstream (1-2kb)	VIT_02s0025g02680	2504	Growth-regulating factor 5			
3,77	Distal Intergenic	VIT_06s0004g02170	-5454	Histidinol-phosphate aminotransferase			
3,39	Distal Intergenic	VIT_12s0059g01110	-5619	Tubulin beta-8			
3,34	Intron (mRNA23493/VIT_16s0148g00230, intron 1 of 2)	VIT_16s0148g00230	5430	Receptor-like kinase ARK1AS		1,50	
3,12	Promoter (1-2kb)	VIT_12s0034g01260	-1022	R protein L6			
3,11	Distal Intergenic	VIT_10s0003g04250	17969	Glutathione S-transferase GSTU22			
2,86	Distal Intergenic	VIT_16s0013g00160	46887	Pectinesterase PPME1			
2,85	Promoter (1-2kb)	VIT_12s0028g03340	-1383	Protein binding / zinc ion binding		1,73	
2,76	Downstream (1-2kb)	VIT_02s0025g02680	2490	Growth-regulating factor 5			
2,65	Distal Intergenic	VIT_13s0019g04450	-12560	ATSYTA/NTMC2T1.1/NTMC2TYPE1.1/SYTA			
2,42	Distal Intergenic	VIT_19s0093g00050	-56392	R protein PRF disease resistance protein			
2,42	Intron (mRNA7571/VIT_11s0149g00070, intron 2 of 4)	VIT_11s0149g00070	8771	Ribosomal protein S6			
2,41	Exon (mRNA27904/VIT_10s0003g02400, exon 3 of 5)	VIT_10s0003g02400	745	SRG1 (senescence-related gene 1) oxidoreductase	Promoter (-946)		
2,40	Distal Intergenic	VIT_09s0096g00830	-28086	R protein disease resistance protein			
2,27	Distal Intergenic	VIT_07s0005g06010	-41522	FAD linked oxidase, N-terminal			
2,18	Distal Intergenic	VIT_16s0050g00820	-4733	EMB1691 (embryo defective 1691)			
2,18	Distal Intergenic	VIT_14s0006g01240	-23558	Protein kinase beta-1 subunit 5'-AMP-activated			
2,17	Distal Intergenic	VIT_13s0106g00470	16599	RNA-binding protein 45 (RBP45)			1,76
2,17	Intron (mRNA10357/VIT_10s0003g05770, intron 1 of 5)	VIT_10s0003g05770	366	Short-chain dehydrogenase/reductase (SDR)			

Table 58: *Vvi*NAC60 'Veraison berry' specific ChIP-seq target genes.

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4. THE VviNACs REGULATION NETWORK

4.1 INTRODUCTION

Advance in the *in vivo* and *in vitro* techniques is helping in the determination of TF-binding sites, slowly bringing to the deciphering of the transcriptional regulatory code (Franco-Zorrilla *et al*., 2014). NAC proteins are among the largest plant specific TF family and are implicated in the regulation of transcriptional reprogramming associated with diverse developmental processes (Kim *et al*., 2016), which face a wide range of environmental stresses. Indeed, NACs TFs are known to have a regulative role in the temporal expression of maturation-associated genes (in particular during leaf aging/senescence), but many members of the NAC family also have roles in the regulation of multiple stress responses (Balazadeh *et al*., 2010). Most of the NAC TFs functionally characterized have been shown to be transcriptional activator but a few other NAC proteins are also transcriptional repressors (Yuan *et al*., 2019). Indeed, many *NACs* show expression changes during leaf aging in *Arabidopsis* and genetic studies identified many NACs as positive and negative regulators of leaf senescence (Kim *et al*., 2018). This duality may be achieved by recruiting or interacting with different transcriptional partners (Yuan *et al*., 2019). Many TFs interact with other proteins, including other types of TFs, to regulate the expression of their target genes or enhance their binding capacity to the *cis* elements in promoters of their target genes (Yuan *et al*., 2019). Auto- and cross-regulation of members within the same TF family forms one of the control nodes of the growth signalling pathways; it is common that multiple members within the same family share related and redundant roles in mediating their downstream signalling pathways, thereby cross-regulating each other at the transcriptional level (Ng *et al.*, 2018). For example, the three closely related WRKY TFs (WRKY18, WRKY40 and WRKY60) were found to cross-regulate each other in mediating the ABA signalling (Ng *et al.*, 2018). Moreover, the NACs are reported to regulate their own and each other transcription, forming a complex regulatory web and providing a finely tuned control system (Kim *et al*., 2018); ANAC013 and ANAC017 were found to be involved in the mitochondrial retrograde regulation under oxidative stress and a cis-regulatory element can be

found at their promoters, suggesting that a positive feedback regulation enhances their transcripts and targets expression under stresses (Ng *et al.*, 2018).

A recent study demonstrated that nine distantly related NAC TFs were able to bind the same core sequence, though with different affinities; moreover, in line with this result, another paper has shown that several other NAC TFs bind the typical NAC binding core but with considerable sequence differences in the flanking bases (Lindemose *et al*., 2014). Thus, as seen in bHLH and homeodomain proteins where few amino acids can play a critical role in the definition of DNA specificities for single TFs, the flanking bases next to the core of NAC binding sites in promoters may determine the binding specificities and fine-tune affinity for different NAC TFs *in vivo* (Lindemose *et al*., 2014). The conformational changes that typically occur in the conserved DNA-binding domain of a TF can influence the dimerization of TFs and DNA binding (Kang et al., 2018). Dimerization of DNA-binding domains is common and can function in modulating the DNA-binding specificity (Müller, 2001). Gel filtration studies on the ANAC NAC domain have shown that it forms mostly dimers in solution (Olsen *et al.*, 2004). The structure determination of the N-terminal domain of ANAC shows that the NAC domain presents a twisted antiparallel β-sheet sandwiched between two helices, suggesting that this domain mediates dimerization of the NAC proteins through conserved interactions and that the DNA binding through the NAC dimer face is rich in positive charges (Ernst *et al*., 2004). Indeed, NAC proteins were shown to bind DNA as dimers in an EMSA experiment with different-sized NAC proteins and a NAC domain truncation, rendering the domain unable to dimerize, also prevented DNA binding (Olsen et al., 2005). It is plausible that promoters showing palindromic dimer sites could be differentially regulated by combinations of NAC homo- and hetero-dimers thus expanding on the range of signals recognized (Lindemose *et al*., 2014). Homo- and hetero-dimer interaction among the previously mentioned WRKY18, WRKY40 and WRKY60 were found to alter their DNA binding activities (Ng *et al.*, 2018). Features of transcriptional autoregulation and inter-regulation through homo-dimerization and hetero-dimerization among the NAC TFs have highlighted the huge network complexity in which they operate, even though the relevance of NAC dimers *in vivo* has not yet been established (Kim *et al*., 2016).

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4.2 MATERIAL AND METHODS

Promoting region isolation and cloning

The promoting sequences were amplified from *Vitis vinifera* cv Shiraz leaf gDNA using KAPA HiFi DNA polymerase (KAPA Biosystems, Wilmington, MA, USA) and the primers listed in **Table 59**.

The PCR products were directionally cloned into the Gateway^{R} entry vector pENTR/D-TOPO (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and then transferred by site-specific LR Gateway^{R} recombination into the binary reporter vector pPGWL7.0 (https://gateway.psb.ugent.be/vector/show/pPGWL7/search/index/transcriptional_reporters, Laboratory of Plant System Biology, Ghent University).

The *VviNACs* ORF were previously amplified from a *V. vinifera* cv Shiraz and cv Corvina berry cDNA and cloned into the binary overexpression vector pK7WG2.0.

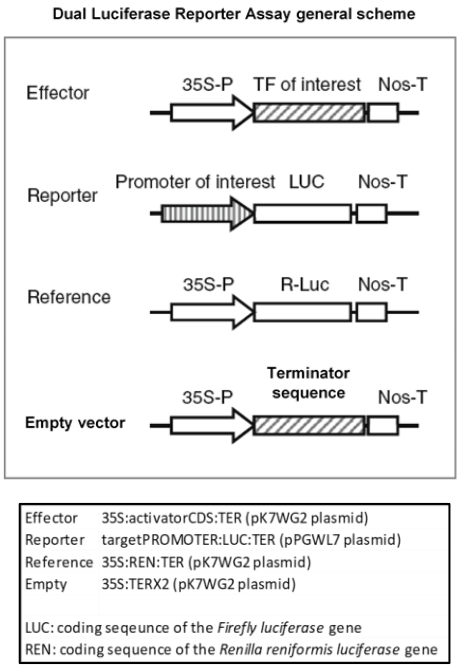
Gene ID	Functional annotation	Direction	Primer	T_a (°C)	Lenght (bp)
VIT_01s0146g00280	VviNAC01	For	5'-CACCCTGTTGAGTGGGAACAGCAA -3'	52	922
		Rev	5'- CGCTAAAAAAATCCTATCAACTCT -3'		
VIT_17s0000g06400	VviNAC05	For	5'-CACCTTGCTTTTGCTATCCGTCCT -3'	51	2142
		Rev	5'-CTGATCATCTTCCACACTAAGT -3'		
VIT_18s0001g02300	VviNAC08	For	5'-CACCTCCCAACCATAGCTTACCTGTC -3'	55	1024
		Rev	5'- ATTAGCTTCTCTCTCCTTCCTCTC -3'		
VIT_12s0028g03050	VviNAC34	For	5'- CACCGGGTGCCTAAAATCCCCTGT -3'	55	1014
		Rev	5'- TTCTCTAGTTTTCCTCTAATGCCCA -3'		
VIT_10s0003g00350	VviNAC37	For	5'- CACCCTTATTCCGCACCTCGGTCA -3'	55	1049
		Rev	5'- CCCTGTTGTTGAGCCTCTATG -3'		
VIT_08s0007g07640	VviNAC61	For	5'- CACCAATCTCTCTGAAAGCGGGGC -3'	55	1051
		Rev	5'- AAGGTCATCACCTTGATCGGC -3'		

Table 59: Primers list for the isolation of the promoting sequences used in the Dual Luciferase Reporter Assay.

Agrobacterium tumefaciens C5851 transformation

The pPGWL7.0 cloned constructs were inserted into *A. tumefaciens* strain C58C1 by electroporation (Bio-Rad electroporation instrument, 25 μF, 200 Ω and 2.5 kV) and the transformed colonies were selected by strain antibiotic resistance (tetracycline) and construct antibiotic resistance (streptomycin and spectinomycin).

Dual-Luciferase^{R} Reporter Assay System (Promega)



- Obtain a 0.2 (OD_{600nm}) resuspension in MMA buffer for each transformed bacteria strain.

  The MMA buffer must be fresh and is composed of:

  - MES 0.01 M pH 5.6
  - MgCl2 0.01 M
  - Acetosyringone 0.01 M

- Incubate 2 hours at room temperature with slow shaking.
- Mix the MMA solution containing the *Agrobacterium* harboring vector of interest basing on your test.

  Proportion among effector, reporter and reference vectors has been determined according to the Dual Luciferase Reporter Assay System technical manual (Promega).

  For example, to the transactivation of the *geneA* by the *geneB* your mix will be the follow:

  $Mix_{control}$: 8 ml of the Reporter vector (*geneA*promoter:LUC)

  1.7 ml of the Reference

  8 ml of the Empty vector

Mix_{test}: 8 ml of the Reporter vector (*geneA*promoter:LUC)
1.7 ml of the Reference
8 ml of the Effector vector (35S:*geneB*)

- For each prepared MMA mix, using a 1ml syringe without the needle, infiltrate three 5 weeks old *N. Benthamiana* plants (biological replicates) on the lower side of 3 leaves.
- 72 hours after infection, collect three fresh leaf disks (1cm diameter disks, technical replicates) from the 3rd leaf of each plant in separate eppy tubes.
- Ground the leaf disks with 250 ml of 1X Passive Lysis Buffer (Promega).
- Centrifuge the lysate for 1 minute at 12.000 g to allow leaf debris to settle.
- Dilute (1:10) the clear supernatant.
- Put 10 µl of each sample diluted supernatant in a white 96-wells plate.

Detect Firefly and *R. reniformis* luminescence following manufactures instruction (Promega) and using a Tecan Infinite ® M200 PLEX instrument with double injectors.

- Inject in each well 40 µl of Luciferase Assay Reagent (LARII, Promega).
- Shake the plate and measure firefly luciferase activity for 2 second.
- Inject 40 µl of 1X Stop & GLO Reagent.
- Shake the plate and measure renilla luciferase activity for 2 second.
- LUC values are analysed relative to the REN value and normalized against the control (empty effector vector).

4.3 RESULTS

The main goal of this chapter is the validation of the interactions between a specific VviNAC and some identified *VviNAC* target genes. Indeed, many of the selected TFs binding sites were found within the promoter regions of other *VviNAC* family members; the number of binding sites and regulated genes for each selected transcription factor is reported in **Figure 73**.

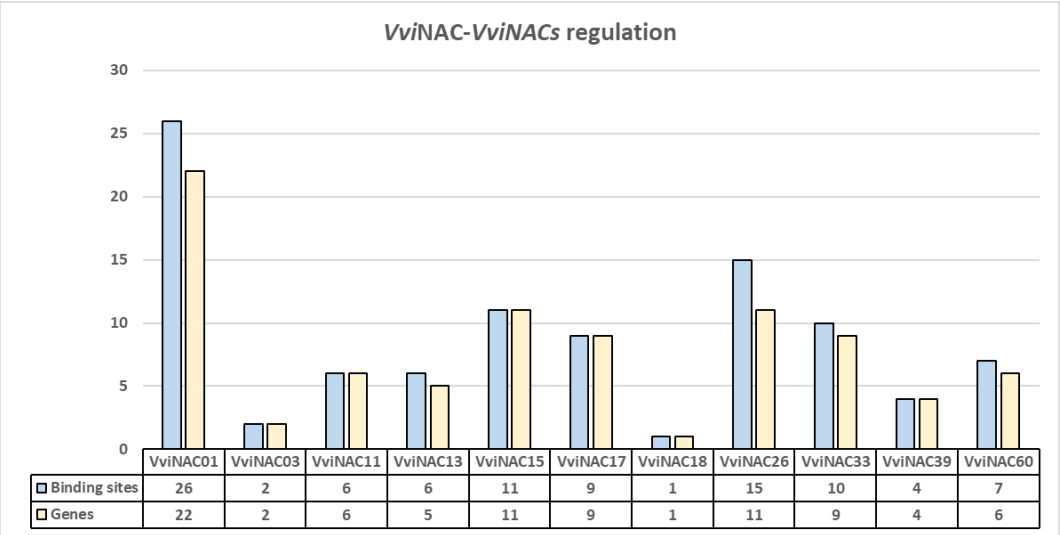


Figure 73: Bar chart reporting the number of interactions between each of the selected VviNAC TFs and the promoting region of *VviNAC* genes. Moreover, the chart reports also the number of *VviNAC* genes directly regulated by each transcription factors.

A total of 33 regulated *VviNAC* target genes were found among all the DAP-seq results (**Table 60**). The final aim is the validation of all these interactions; however, the promoter isolation is a quite long procedure and, for this reason, not all the found interactions were tested in time for this thesis.

The decision was to start isolating and testing the activation/repression of the most represented targets between all the DAP-seq datasets; on the base of this consideration, a ranking (**Table 60**), from the most represented to the least, of the different *VviNAC* targets founds in all the DAP-seq assays results allowed to select 5 candidates, which promoters were isolated (**Appendix D**) and tested in a dual luciferase assay: *VviNAC05* (VIT_17s0000g06400), *VviNAC08* (VIT_18s0001g02300), *VviNAC34* (VIT_12s0028g03050), *VviNAC37* (VIT_10s0003g00350) and *VviNAC61* (VIT_08s0007g07640).

Moreover, *VviNAC01* (VIT_01s0146g00280) promoter was also taken into consideration because it seems to be regulated by VviNAC01 itself; considering the high number of *VviNAC* genes regulated by this TF, it was interesting to see if it could have a positive or negative role on its own regulation.

Dual Luciferase Reporter Assay		TFs											
		VviNAC01	VviNAC03	VviNAC11	VviNAC13	VviNAC15	VviNAC17	VviNAC18	VviNAC26	VviNAC33	VviNAC39	VviNAC60	Somma
Targets	VviNAC05	*	*				*	*	*	*		*	7
	VviNAC34	*		*	*	*			*	*		*	7
	VviNAC61	*		*		*	*		*	*		*	7
	VviNAC73	*	*				*		*		*	*	6
	VviNAC19	*		*	*	*				*			5
	VviNAC20	*			*	*	*			*			5
	VviNAC37	*		*	*	*				*			5
	VviNAC08	*					*		*	*			4
	VviNAC67	*				*	*		*				4
	VviNAC39	*				*			*				3
	VviNAC47	*		*			*						3
	VviNAC53	*				*	*						3
	VviNAC21	*			*								2
	VviNAC23								*		*		2
	VviNAC45			*								*	2
	VviNAC58						*		*				2
	VviNAC63	*								*			2
	VviNAC66								*			*	2
	VviNAC01	*											1
	VviNAC07									*			1
	VviNAC12										*		1
	VviNAC15										*		1
	VviNAC17	*											1
	VviNAC25	*											1
	VviNAC29	*											1
	VviNAC40								*				1
	VviNAC51					*							1
	VviNAC54					*							1
	VviNAC56	*											1
	VviNAC62	*											1
	VviNAC64	*											1
	VviNAC70	*											1
	VviNAC71					*							1

Table 60: Ranking list of all the *VviNAC* genes regulated by at least one of the selected TFs. The purple-colored targets on the left are the one chosen to be tested in the DLRA.

The Dual Luciferase Reporter Assay (DLRA) is a powerful technique used for the validation of the interaction between a specific transcription factor and the promoter of a gene; moreover, the assay is quantitative and the analysis permits to identify the investigated TF as an activator or a repressor.

Moreover, considering the binding of more than one selected VviNAC TFs on some *VviNACs* promoters, the investigation of a possible correlation between the binding site positions revealed with the DAP-seq and the fluorescence quantitative value of the DLRA, could be useful to understand a little bit more about the different action that the TFs could have on a specific gene.

It is obvious that the understanding of the fine regulatory mechanisms of a gene can hardly be explained on the base of a simply binding site observation, especially in

this specific case in which all the selected TFs present a very similar expression profile in Atlas dataset; however, an attempt has been done.

VviNAC01 was validated as repressor of its own transcription; this was very interesting because a sort of negative feedback regulation on its own transcription could be assumed (**Fig. 74**).

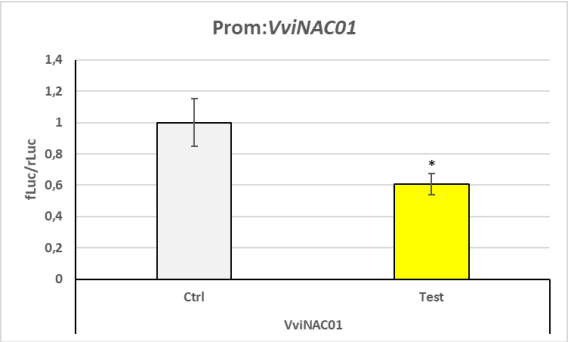


Figure 74: *VviNAC01* promoter activations tested by Dual Luciferase Reporter Assays in infiltrated *Nicotiana benthamiana* leaves. The activity of these promoters was tested in the presence and absence of the 35S:*VviNAC01* effector vector. LUC values are reported relative to the REN value and normalized against the control (empty effector vector). LUC values represent the mean of three biological replicates (three technical replicates each) ± standard deviation (SD). Asterisks (*) indicate significant differences in promoter activation compared with the control (t-test; $p < 0.05$).

VviNAC05 was repressed by the action of VviNAC01, VviNAC03 and VviNAC17; instead, it resulted activated by VviNAC18, VviNAC26, VviNAC33 and VviNAC60 (**Fig. 75**). VviNAC03 and VviNAC18 are both NOR orthologues and the finding that they both regulate the same gene but in an opposite way is very intriguing.

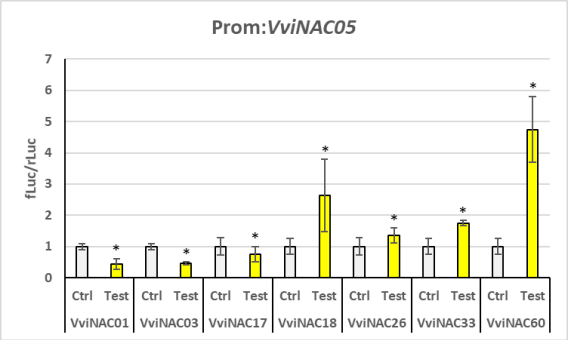


Figure 75: *VviNAC05* promoter activations tested by Dual Luciferase Reporter Assays in infiltrated *Nicotiana benthamiana* leaves. The activity of these promoters was tested in the presence and absence of 7 different 35S:*VviNAC* effector vectors. LUC values are reported relative to the REN value and normalized against the control (empty effector vector). LUC values represent the mean of three biological replicates (three technical replicates each) ± standard deviation (SD). Asterisks (*) indicate significant differences in promoter activation compared with the control (t-test; $p < 0.05$).

Concerning the *VviNAC05* promoter region, it was clear that the binding of VviNAC17 (-1911) and VviNAC03 (-1859) are specific and always brings to a repression of the gene expression; the other interactions revealed a very close or identical position of the binding sites (**Table 61**). VviNAC01 (repressor), VviNAC18 (activator), VviNAC26 (activator), VviNAC33 (activator) and VviNAC60 (activator) bind the promoter of the gene at 0 bp from the transcription start site (TSS), suggesting a very fine stage- or organ- or tissue- specific, and maybe competitive, role; VviNAC26 (activator) and VviNAC60 (activator) bind very close to each other, at -1867 and -1862 bp from the TSS, respectively, suggesting a possible enhanced activation with the heterodimerization of the two TFs.

Interaction elements		Region of interaction		Over expressions		DAP-seq	DLRA	
TFs	Direct regulated genes	Genic region	Distance to TSS	Stable	Transient	q-value	fLuc/rLuc	p<0.05
VviNAC01	VviNAC05	Promoter (<=1kb)	0			12,84	-2,30	*
VviNAC03	VviNAC05	Promoter (1-2kb)	-1859			6,66	-2,20	*
VviNAC17	VviNAC05	Promoter (1-2kb)	-1911			853	-1,33	*
VviNAC18	VviNAC05	Promoter (<=1kb)	0			2,89	2,62	*
VviNAC26	VviNAC05	Promoter (1-2kb)	-1867			1976	1,35	*
VviNAC26	VviNAC05	Promoter (<=1kb)	0			1295	1,35	*
VviNAC33	VviNAC05	Promoter (<=1kb)	0			2,24	1,75	*
VviNAC60	VviNAC05	Promoter (1-2kb)	-1862			33,15	4,74	*

Table 61: List of all the possible TFs binding sites on the *VviNAC05* promoter, correlated with the over expressions, the DAP-seq q-values and the DLRA fluorescence results. The yellow highlighted interactions indicate the same position on the promoting region of the different TFs and the green one the possible heterodimers elements and sites.

VviNAC08 was activated by VviNAC01 and VviNAC33 but its expression resulted suppressed by the action of VviNAC17 and VviNAC26 (**Fig. 76**).

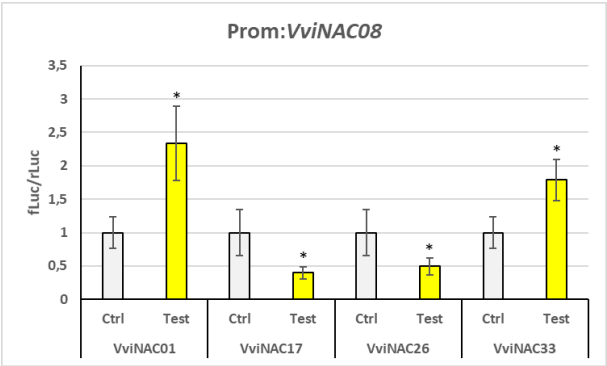


Figure 76: *VviNAC08* promoter activations tested by Dual Luciferase Reporter Assays in infiltrated *Nicotiana benthamiana* leaves. The activity of these promoters was tested in the presence and absence of 4 different 35S:*VviNAC* effector vectors. LUC values are reported relative to the REN value and normalized against the control (empty effector vector). LUC values represent the mean of three biological replicates (three technical replicates each) ± standard deviation (SD). Asterisks (*) indicate significant differences in promoter activation compared with the control (t-test; $p < 0.05$).

VviNAC08 resulted activated by VviNAC01 and VviNAC33, which present very close binding sites, at -327 and -320 bp from TSS respectively, and suggesting a possible interaction between the two TFs (**Table 62**). The suppression of the expression of the gene was, instead, regulated by two other TFs (VviNAC17 and VviNAC26), which presented the same identical binding position at -29 bp from the TSS and introducing the idea of a very fine differentiation in the regulation mechanism of this gene; moreover, VviNAC26 also presented a specific repression binding site at -341 bp from the TSS (**Table 62**). Moreover, the two repressors of the *VviNAC08* expression belong to the same clade (III) in the phylogenetic tree (**Fig. 19**, **Introduction** of **Chapter 2**) and the activator belongs to another clade (V). VviNAC01, which also activated *VviNAC08* expression, belongs to the same clade of the repressors but, in the previous phylogenetic tree (Wang et al, 2013) it was present in another different clade (V).

Interaction elements		Region of interaction		Over expressions		DAP-seq	DLRA	
TFs	Direct regulated genes	Genic region	Distance to TSS	Stable	Transient	q-value	fLuc/rLuc	p<0.05
VviNAC01	VviNAC08	Promoter (<=1kb)	-327			2,4	2,33	*
VviNAC17	VviNAC08	Promoter (<=1kb)	-29			204	-2,49	*
VviNAC26	VviNAC08	Promoter (<=1kb)	-341			636	-2,03	*
VviNAC26	VviNAC08	Promoter (<=1kb)	-29			352	-2,03	*
VviNAC33	VviNAC08	Promoter (<=1kb)	-320			3,4	1,79	*

Table 62: List of all the possible TFs binding sites on the *VviNAC08* promoter, correlated with the over expressions, the DAP-seq q-values and the DLRA fluorescence results. The yellow highlighted interactions indicate the same position on the promoting region of the different TFs and the green one the possible heterodimers elements and sites.

VviNAC34 expression resulted positively controlled by VviNAC11, which reported the highest induction of the gene with about a 16-fold activation, VviNAC13, VviNAC15, VviNAC33 and VviNAC60; whereas, it was negatively regulated by VviNAC26 (**Fig. 77**). Unfortunately, its regulation by VviNAC01 was not validated and this finding suggests the need of a co-factor (or heterodimerization complex with another VviNAC TF) for the activation/repression of this specific gene.

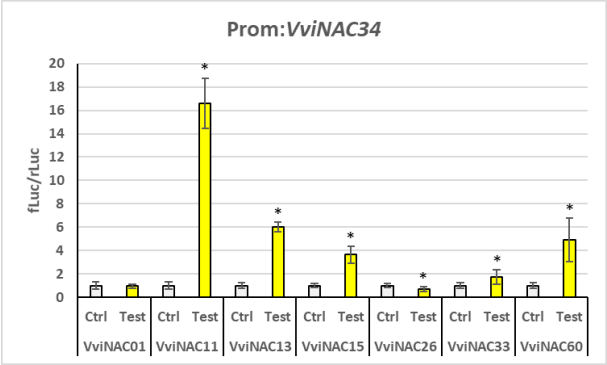


Figure 77: *VviNAC34* promoter activations tested by Dual Luciferase Reporter Assays in infiltrated *Nicotiana benthamiana* leaves. The activity of these promoters was tested in the presence and absence of 7 different 35S:*VviNAC* effector vectors. LUC values are reported relative to the REN value and normalized against the control (empty effector vector). LUC values represent the mean of three biological replicates (three technical replicates each) ± standard deviation (SD). Asterisks (*) indicate significant differences in promoter activation compared with the control (t-test; $p < 0.05$).

VviNAC34 presented a specific binding site for VviNAC26 (-390 bp from the TSS), which brings to the suppression of the gene expression; all the other TFs activate the expression of *VviNAC34* (**Table 63**). VviNAC60 presents a specific binding sites at -414 bp from the TSS, whereas the other four TFs binding sites suggest possible heterodimerization or, being maybe too close, a fine dynamic regulatory mechanism; indeed, VviNAC11 and VviNAC33 present the binding at -353 and -354 bp from the TSS, and VviNAC13 and VviNAC15 at -200 and -203 bp, respectively (**Table 63**).

Even though the interaction of VviNAC01 with the gene were not validated, it is interesting to notice that *VviNAC34* presented a VviNAC01 specific binding site at -229 bp from the TSS and another site at -353 bp from the TSS, the same position found for VviNAC11 and VviNAC33 (**Table 63**).

Interaction elements		Region of interaction		Over expressions		DAP-seq	DLRA	
TFs	Direct regulated genes	Genic region	Distance to TSS	Stable	Transient	q-value	fLuc/rLuc	p<0.05
VviNAC01	VviNAC34	Promoter (<=1kb)	-353			21,71	-1,05	
VviNAC01	VviNAC34	Promoter (<=1kb)	-229			9,34	-1,05	
VviNAC11	VviNAC34	Promoter (<=1kb)	-353			3,26	16,61	*
VviNAC13	VviNAC34	Promoter (<=1kb)	-200			6,76	6,03	*
VviNAC15	VviNAC34	Promoter (<=1kb)	-203			17,55	3,67	*
VviNAC26	VviNAC34	Promoter (<=1kb)	-390			348	-1,39	*
VviNAC33	VviNAC34	Promoter (<=1kb)	-354			5,72	1,73	*
VviNAC60	VviNAC34	Promoter (<=1kb)	-414	-2,51		5,76	4,93	*

Table 63: List of all the possible TFs binding sites on the *VviNAC34* promoter, correlated with the over expressions, the DAP-seq q-values and the DLRA fluorescence results. The green and light-blue highlighted interactions indicate two possible different heterodimers elements and sites; the grey color indicates the not validated interactions.

VviNAC37 regulation resulted to be always in favor of its activation and mediated by VviNAC11, VviNAC13 and VviNAC33 (**Fig. 78**).

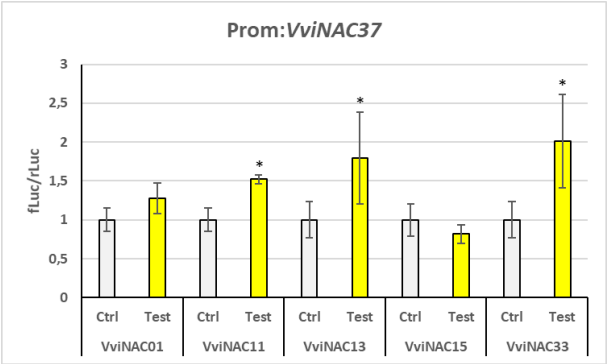


Figure 78: *VviNAC37* promoter activations tested by Dual Luciferase Reporter Assays in infiltrated *Nicotiana benthamiana* leaves. The activity of these promoters was tested in the presence and absence of 5 different 35S:*VviNAC* effector vectors. LUC values are reported relative to the REN value and normalized against the control (empty effector vector). LUC values represent the mean of three biological replicates (three technical replicates each) ± standard deviation (SD). Asterisks (*) indicate significant differences in promoter activation compared with the control (t-test; $p < 0.05$).

VviNAC37 resulted always activated by three TFs: VviNAC11, VviNAC13 and VviNAC33. All present the same binding site position at 0 (or -1) bp from the TSS; however, a VviNAC33 specific activation binding site was found at -899 bp from the TSS (**Table 64**).

Moreover, the two not validated interactions regarding VviNAC01 and VviNAC15 present a very close binding site on the *VviNAC37* promoter (0 bp and -3 bp from the TSS, respectively), suggesting an heterodimerization possibility between the two TFs (**Table 64**).

Interaction elements		Region of interaction		Over expressions		DAP-seq	DLRA	
TFs	Direct regulated genes	Genic region	Distance to TSS	Stable	Transient	q-value	fLuc/rLuc	p<0.05
VviNAC01	VviNAC37	Promoter (<=1kb)	0			15,38	1,28	
VviNAC01	VviNAC37	Promoter (<=1kb)	-905			13,63	1,28	
VviNAC01	VviNAC37	Promoter (<=1kb)	-272			2,63	1,28	
VviNAC11	VviNAC37	Promoter (<=1kb)	0			2,75	1,52	*
VviNAC13	VviNAC37	Promoter (<=1kb)	0			9,64	1,79	*
VviNAC13	VviNAC37	Promoter (<=1kb)	-926			2,3	1,79	*
VviNAC15	VviNAC37	Promoter (<=1kb)	-3			7,52	-1,23	
VviNAC33	VviNAC37	Promoter (<=1kb)	-899			5,72	2,01	*
VviNAC33	VviNAC37	Promoter (<=1kb)	-1			3,97	2,01	*

Table 64: List of all the possible TFs binding sites on the *VviNAC37* promoter, correlated with the over expressions, the DAP-seq q-values and the DLRA fluorescence results. The yellow highlighted interactions indicate the same position on the promoting region of the different TFs and the grey color indicates the not validated interactions.

VviNAC61 resulted activated by VviNAC11, VviNAC33 and VviNAC60, whereas was repressed by the action of VviNAC17 and VviNAC26 (**Fig. 79**).

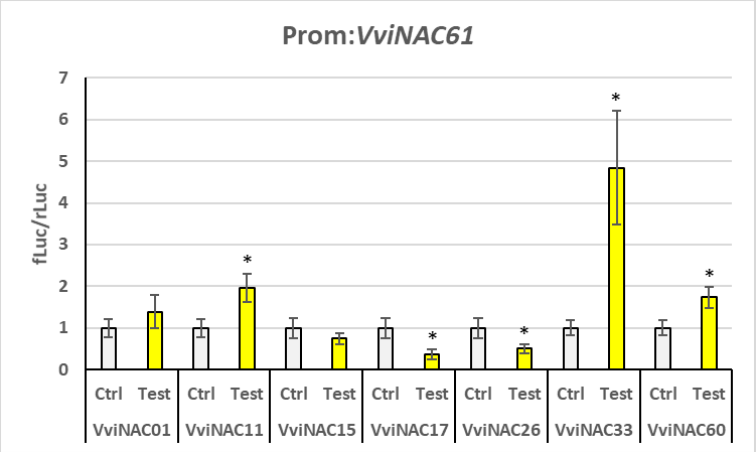


Figure 79: *VviNAC61* promoter activations tested by Dual Luciferase Reporter Assays in infiltrated *Nicotiana benthamiana* leaves. The activity of these promoters was tested in the presence and absence of 7 different 35S:*VviNAC* effector vectors. LUC values are reported relative to the REN value and normalized against the control (empty effector vector). LUC values represent the mean of three biological replicates (three technical replicates each) ± standard deviation (SD). Asterisks (*) indicate significant differences in promoter activation compared with the control (t-test; $p < 0.05$).

All the transcription factors that regulate this gene present the binding site at 0 bp from the TSS (**Table 65**): VviNAC11, VviNAC33 and VviNAC60 act as activators, whereas VviNAC17 and VviNAC26 as repressors. This evidence could be interpreted in different ways, but the most likely hypothesis is that the activators and repressors probably fine regulate the gene in different developmental stages, whereas the activation or repression mechanisms are orchestrated by the different TFs in different organs or tissues. Moreover, both the not validate interactions present the binding sites at 0 bp from the TSS as all the other TFs.

Interaction elements		Region of interaction		Over expressions		DAP-seq	DLRA	
TFs	Direct regulated genes	Genic region	Distance to TSS	Stable	Transient	q-value	fLuc/rLuc	p<0.05
VviNAC01	VviNAC61	Promoter (<=1kb)	0			27,26	1,38	
VviNAC11	VviNAC61	Promoter (<=1kb)	0			2,55	1,96	*
VviNAC15	VviNAC61	Promoter (<=1kb)	0		1,56	25,45	-1,35	
VviNAC17	VviNAC61	Promoter (<=1kb)	0			1869	-2,74	*
VviNAC26	VviNAC61	Promoter (<=1kb)	0			1855	-2,01	*
VviNAC33	VviNAC61	Promoter (<=1kb)	0			3,78	4,84	*
VviNAC60	VviNAC61	Promoter (<=1kb)	0			59,84	1,73	*

Table 65: List of all the possible TFs binding sites on the *VviNAC61* promoter, correlated with the over expressions, the DAP-seq q-values and the DLRA fluorescence results. The yellow highlighted interactions indicate the same position on the promoting region of the different TFs and the grey colour indicates the not validated interactions.

VviNAC11, VviNAC13, VviNAC33 and VviNAC60 appeared to be only

regulators of other *VviNAC* genes, without being present as target themselves (**Appendix E**); they are all *switch* genes, and this evidence highlights once more the importance of these TFs in the regulation of the developmental processes as principal actors.

Moreover, only two interaction presented a match between the over expression and the DLRA results: VviNAC15-*VviNAC61* (**Table 65**) and VviNAC60-*VviNAC34* (**Table 63**). Unfortunately, it was not possible to define a good correlation between the experiments; the repression role of VviNAC15 on *VviNAC61*, validated by the luciferase assay, did not reflect the up regulation of the gene in the transient over expression, and the role of VviNAC60 as an activator of *VviNAC34* was also not reconfirmed with the over expression of the TF, where the gene was down regulated.

At the end of all the analyses, a hierarchical network of all the validated regulations was constructed (**Fig. 80**). The transcription factors hierarchy, considering that many other VviNAC TFs - *VviNAC* targets interactions need to be validated, was given only considering the number of regulated *VviNAC* genes. In **Appendix E**, the entire possible VviNAC-*VviNACs* network, only DAP-seq results based, shows the enormous complexity of the developmental regulatory mechanisms putatively orchestrated by the *Vvi*NAC family.

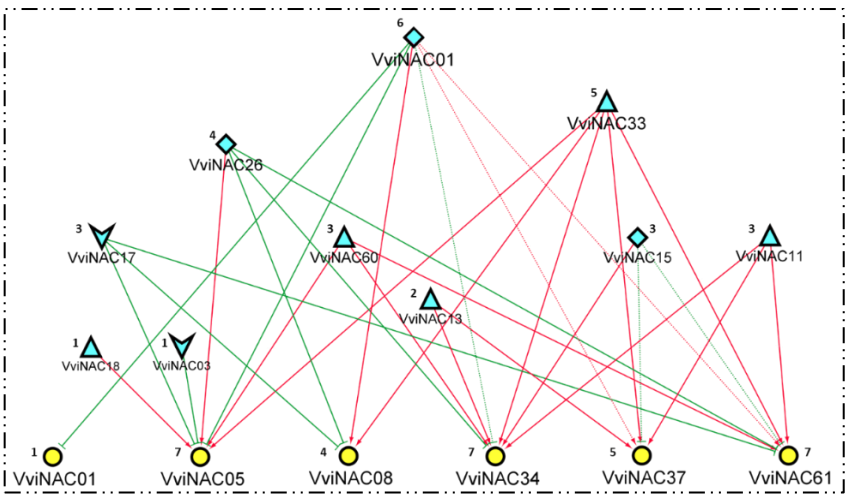


Figure 80: Hierarchical representation of all the validated VviNAC-*VviNACs* interactions. The TFs are highlighted in light-blue and their role as activators, repressors or double-action regulators is represented with a triangle, an arrow and a square, respectively. All the targets are represented as yellow circles. Next to each elements of the hierarchy the number of interactions is reported. The red → arrows indicate the activation of the related target and the green T arrows show the repression of the targets. The continuous lines indicate the validated interactions ($p<0.05$) and the dashed lines indicate the not significant interactions.

5. DISCUSSION

The current global warming contest is assuming a notable attention due to its effects on agriculture (Webb *et al*., 2007). In particular, the grapevine cultivation is highly temperature sensitive and the maintenance of a stable high-quality grape production is a fundamental point for the wide wine industry (Spayd *et al*., 2002; Marais *et al*., 2001; Haselgrove 2000; Hannah *et al*., 2013).

Increase knowledges on the regulation of the developmental process occurring in the plant, in particular concerning the berry ripening, are required to limit the damage caused by the profound climate changes (Pearce and Coombe 2004; Jones and Davis 2000). Studying the transcriptome regulation will help to get a comprehensive picture of the molecular mechanisms of the maturation shift in grapevine which will have an important direct involvement in the management of the vineyard.

The grapevine development is a long and very complex process which is affected by many exogenous and endogenous factors (Duchêne *et al*., 2010; Jones and Davis, 2000; Coombe and McCarthy, 2000; Martinez-Esteso *et al*., 2013; Kennedy *et al*., 2002). The grapevine global gene expression atlas (Fasoli *et al*., 2012) showed the transcriptional shift during the vegetative-to-mature transition in most of grapevine organs and tissues; afterwards, the main actors of this shift, called *switch* genes, were identified with an integrated network analysis (Palumbo *et al*., 2014). Some *switch* genes were found in common between the Atlas and a specific berry dataset (Massonnet *et al*., 2017), suggesting their specific role in the berry ripening. Interestingly, most of these *switch* genes were transcription factors and some of them were identified as markers of the first transition of the onset of berry ripening (Fasoli *et al*., 2018).

Among the *switches*, particular interest was placed on the *NAC* (*NAM/ATAF/CUC*) genes, which belongs to a large plant-specific transcription factors family, involved in many processes such as plant growth and development, response to abiotic and biotic stress, flower and fruit development, hormone signaling and regulation of secondary metabolic processes (Hendelman *et al*., 2013; Raman *et al*., 2008; Fabi *et al*., 2012; Wang *et al*., 2013; Sun *et al*., 2012). Indeed, the identification and characterization of some *VviNAC* genes involved in the grapevine (Wang *et al*., 2013) maturation, in particular in the berry ripening, was the first step to fully

understand the regulation mechanisms underlying these developmental processes. The investigation started by D'Incà (2017) from a group of five *VviNACs*, four *switch* genes (*VviNAC11*, *VviNAC13*, *VviNAC33* and *VviNAC60*) and a *NOR* (Giovannoni, 2004; Giovannoni *et al.*, 1995) orthologue (*VviNAC03)*. Transient over expression assay combined with microarray analyses were already performed on these genes. Moreover, *VviNAC33* and *VviNAC60* were also stably over expressed and the transgenic grapevines were molecularly (microarray) and phenotypically characterized: *VviNAC33* overexpressing leaves showed a yellowing effect due to a chlorophyll breakdown, *VviNAC60* overexpressing plants showed a slightly plant growth and an earlier stem lignification in comparison to the same-age control plant.

Afterwards, on the base of Atlas (Fasoli *et al*., 2012) expression profiles similarity to the one of *VviNAC60*, of the distance to *VviNAC60* in the phylogenetic tree construct upon all the *VviNAC* family (Wang *et al*., 2013) and of some recent RNA-seq transcriptomic analysis performed on grapes (Fasoli *et al*., 2018), other interesting *VviNAC* genes were identified and isolated: *VviNAC01, VviNAC08, VviNAC15, VviNAC17, VviNAC18, VviNAC26, VviNAC38, VviNAC39* and *VviNAC61*. The cv Shiraz isolated coding sequences of the selected *VviNACs* showed a very high similarity to the one of the cv Pinot noir reference genome, reporting only a few SNPs which brought to aminoacidic changes.

After the isolation and cloning, the transient over expressions of the selected *VviNAC* genes were performed directly in grapevine to gain information about the impact of the over expression of these genes on the leaf transcriptome and to have some hints about the possible targets of these TFs. Microarray analyses of the transcriptomes were carried out to complete what was already started with *VviNAC03, VviNAC11, VviNAC13, VviNAC33* and *VviNAC60* (D'Incà, 2017). For these last two genes, the transient over expression results were also compared with the available stable one. The over expression datasets are very useful to highlight the activation/repression role of a specific TF on a metabolic pathway but, at the same time, are very difficult to interpret in the correct way concerning the direct regulation of a specific gene. Moreover, must be considered that the results of the

different approaches (stable and transient) are very different since the transient over expression focus on the primary transcriptomic changes and the stable on the secondary derived metabolisms or developmental processes alterations.

To avoid possible 'false positive' target genes in the direct regulation interpretation of a specific TF, the DAP-seq (Bartlett *et al*., 2017) on the cv Shiraz genome was performed for all the selected VviNACs.
DAP-seq is a fast, high-throughput, inexpensive, and easily scaled method that permits to obtain a cistrome map of a TF (O'Malley *et al*., 2016); indeed, the DAP-seq permits to detect all the possible binding sites of transcription factor, losing the chromatin-structure-associated and tissue specificity regulation variables. It couples *in vitro* translated and affinity-purified TFs with next-generation sequencing of a genomic DNA library to discover all the possible protein binding sites (O'Malley *et al*., 2016; Bartlett *et al*., 2017); moreover, DNA libraries are constructed using native genomic DNA preserving tissue-specific cytosine methylations that are known to impact TF binding. This represents the first *Vitis vinifera* DAP-seq attempt and some optimization were necessary to assure the good yield of the experiment.

The success of the assay is strongly gDNA libraries quality dependent; for this reason, many protocols were tested to be able to obtain a high amount of very pure genomic DNA (Bartlett *et al*., 2017). At first three different samples were considered as tests of the DAP-seq experiments, young leaves, green berries and berries at veraison; the starting idea was to try to reveal some possible differences in the binding of the TFs due to cytosine methylation, which could be tissue and developmental stage related (Bartlett *et al*., 2017). Unfortunately, no protocol tested on the berries at veraison permitted to extract enough genomic DNA to proceed with the library preparation; this was probably due to the high quantity of phenolic compounds present in that type of tissue. Concerning the other two samples (young leaves and green berries), the reported protocol (**Material and methods**, **Chapter 3**) worked on both, with much higher yields on the leaf tissue, and genomic DNA libraries were efficiently prepared. However, considering the not enough remarkable differences (noticed in the final DAP-seq results) between the two

different tissues and the higher difficulties in obtaining the genomic DNA from the green berries, only the leaf results are reported in this thesis.

The optimization steps highlighted the fact that the use of Nanodrop to quantify the plant genomic DNA for the library preparation is not recommended as minor impurities in the purified genomic DNA sample can make the quantification inaccurate (Bartlett *et al*., 2017); for this reason, the suggestion is to use the Qubit. Concentrations of the grapevine DNA libraries after the adapter ligation usually range from around 125–180 ng/μL (recovering about 50–70% of the original genomic DNA amount), which is a very high yield compared to the literature reported one (Bartlett *et al*., 2017; Galli *et al*., 2018). Moreover, the amount of genomic DNA library used in a DAP-seq experiment is highly dependent on genome size (Bartlett *et al*., 2017); for a relatively small genome, as the *A. thaliana* one, 100 ng are sufficient, but with larger genomes (as the *Vitis vinifera* one) 500-1000 ng produce better results. Indeed, 500 ng and, in same cases, 1000 ng of genomic DNA library were used for all the TFs in this study. However, to establish an optimal genomic DNA input amount, it is useful to perform DNA library titration using positive control proteins; it must be considered that too small quantities of genomic DNA would not allow the detection of all the possible TF binding sites and that, on the contrary, an excessive quantity of genomic DNA could lead to many false positives (Bartlett *et al*., 2017).

Moreover, a good expression of the tagged protein is crucial for a successful experiment; for this reason, a Western blot analysis could be performed to verify the TF expression before the DAP part (Bartlett *et al*., 2017). In this type of assay the *in vitro* translation is generally used because, even if it is expensive, it has worked for many TF family. It was noticed that the good translation of the proteins is also family-related; indeed, some TF families showed a higher percentage of success in the DAP-seq assay than others. Concerning the NAC family, generally it works well in this type of assay and the literature findings (Bartlett *et al*., 2017) reflected the results of this thesis where only three TFs did not work properly, reaching an 80% of successfully translated TFs. However, a standard *E. coli* protein expression and purification can also be performed in this step (Galli *et al*., 2018).

Finally, the final concentrations of DAP-seq samples can vary, but the typical amounts range from 10–50 ng/μL (Bartlett *et al*., 2017). To assess whether a particular DAP-seq experiment worked well, the DNA must be sequenced and evaluated for the presence of binding peaks. The target number of sequencing reads will depend on the size of the genome; for *Vitis vinifera* samples (~ 500 Mb genome size), 3,5 million reads per DAP-seq sample were sequenced. This value was decided in proportion to the 10 million reads generally considered for the maize genome, which is enormously larger than the grapevine one (Galli *et al*., 2018).
Even if it represents an easy and fast method, the DAP-seq technique is also subject to some limitations; the causes are generally dependent on the TF-specific DNA binding properties related to the *in vitro* expression and to the absence of co-factors or other protein partners (Bartlett *et al*., 2017). It is also important to consider that the DAP-seq technique is a powerful but very dispersive way to identify the targets of a transcription factor; performing this assay it was possible to obtain all the possible binding sites of the selected VviNACs on the grapevine genome, without highlighting the tissue or time specific role of these TFs. This means that all the possible actions of each VviNAC family member are written inside the DAP datasets but different keys to read them are required in relation to temporal, organ and tissue dynamics. Appears clear that, to fully understand the role of the selected TFs, a huge work will be required and that the transversality between different biotechnological and bioinformatic approaches is needed.

By comparing transcriptomic analyses, obtained from transient and stable grapevine transformation, with the DAP-seq datasets, the identification of some target genes commonly and consistently modulated was possible.
Indeed, many *switch* genes (many of which were transcription factors) often returned in the different DAP-seq datasets and some of them also have a positive correlation in the over expression one; between the most represented TFs, many Zinc-fingers (Takatsuji, 1998; Ciftci-Yilmaz and Mittler, 2008), NAC (Wang *et al*., 2013; Sun *et al*., 2011; Le Henanff *et al*., 2013;), WRKY (Vannozzi *et al*., 2018; Amato *et al*., 2017; Wang *et al*., 2014; Amato *et al*., 2019), MYB (Matus *et al*., 2010; Holl *et al*., 2013; Vannozzi *et al*., 2018; Matus *et al*., 2017; Amato *et al*., 2019; Koyama *et al*., 2014; Czemmel *et al*., 2012) and bHLH (Xu *et al*., 2014;

Matus et al., 2010) constantly appeared. Moreover, a lot of first and second transition markers were also present in the DAP-seq results (Fasoli *et al*., 2018); knowing that these genes are responsible of the primary metabolic pathways shutdown, this evidence emphasized the important role of the selected *VviNAC* genes in the regulation of the grapevine development, activating the maturation processes and repressing the vegetative growth, and, considering that they were selected for their high expression in the berry tissues, confirmed their mainly action on the berry ripening regulation. Moreover, some interesting features concerning the regulatory role of each selected VviNAC TF were found. For some TFs, a putative role was strongly highligthed, for others this thesis represents a good starting point in the elucidation of more complex regulation mechanisms.

VviNAC01, which in the Atlas resulted co-express with many genes, confirmed its important role in the ethylene pathways showing a lot of *ERF/AP2* (Xie *et al*., 2019; Feng *et al*., 2020; Sun *et al*., 2016) up regulated genes as results of its transient over expression and revealing many ethylene-related genes also between the DAP-seq targets; unfortunately, the ethylene role in grapevine has not been well defined yet. Moreover, also the phylogenetic footprints correlate with ANAC092, considered the key positive regulator of leaf senescence, involved in a delicately balanced feed-forward loop that promotes ethylene-mediated chlorophyll degradation (Kim et al., 2009). VviNAC03, one of the closest NOR orthologue, did not reveal a well-defined identity; however, its role seems to be plant growth related and probably activates the glycosylation of flavonoids, which is important for the defense responses and for the berries color. VviNAC08 DAP-seq results highlighted a very low number of direct targets (data validated with multiple DAP-seq assay on this specific TF) and none of them was found in the over expression dataset where, however, a possible role of this TF in the gibberellin-related and circadian mechanisms was noticed. VviNAC11 seems to be related to the control of the auxin pathways and, even if with some mixed results, in promoting the stop of the lateral organs growth and the chlorophylls degradation. VviNAC13 revealed almost no correlation between the over expression and the DAP-seq results; for this reason, the interpretation of its putative role is difficult. However, the GO enrichment of the DAP-seq dataset highlighted a probable action in the lignin and

phenylpropanoid metabolic processes. VviNAC15 revealed many TF between its regulated targets and, considering that this evidence perfectly matched with the over expression results where all the TF genes were up regulated, this suggests its fundamental role in the regulation mechanisms orchestration. VviNAC17 appeared to be a regulator of the jasmonic acid-induced gene expression; this hypothesis was also supported by the correlation of this TF with ANAC055 (known to regulate jasmonic acid-induced expression of defense genes) in the binding motif analysed. VviNAC18 analyses reported its role in the chlorophyll degradation. Indeed, the *SGR1* and *SGR2* where found as targets, respectively up and down regulated by the over expression of the TF, strongly suggesting its action in stopping the SGR2 production (which negatively regulates chlorophylls degradation interfering with SGR1) and directly activating the SGR1 production (Park *et al*., 2007; Sakuraba *et al*., 2014). VviNAC26 matching results between the DAP-seq and the over expression presented many genes related to the sugars biosynthesis, which is important in the leaves and berries maturation processes, and in the anthocyanin synthesis. VviNAC33 was the most studied TF; indeed, a paper has been recently accepted (New Phytologist) and its major role in terminating photosynthetic activity and organ growth, as part of a regulatory network governing the vegetative-to-mature phase transition, was already well established. Unfortunately, VviNAC38 DAP-seq resulted not very informative and *VviNAC38* over expression was not performed; the functional analyses on this TF will be done again as soon as possible. VviNAC39 resulted to up regulate the transport of many metabolites (mostly sugars and lipids) and the ubiquitin-conjugating, which was demonstrated to be involved in ripening and post-harvest stage in grapevine (Gao et al., 2017). VviNAC61 revealed a predominant role in the regulation of the metabolic processes, focused on the aromatic compounds biosynthesis. This TF was already known to have a role in the withering processes (Zenoni *et al*., 2016) and the transient over expression results strongly confirmed this evidence. Unfortunately, the DAP-seq assay did not result very informative and is going to be performed again to complete the information available for this very interesting TF.

VviNAC60 was already identified as the master regulator of the berry ripening; for this reason, a more in-depth analysis was performed on this TF. The DAP-seq

results, combined again with the stable and transient over expression dataset, revealed a lot of hormones related up regulated genes which clearly showed the pro-maturation effects of the VviNAC60 action; whereas the many transcription factors found in its lists resulted regulated by the TF in both positive (when related to secondary metabolic processes activation) and negative (when related to the vegetative growth) ways. This was very interesting because the important and major role of this transcription factor in the grapevine maturation processes was again highlighted. Considering that the DAP-seq technique gives a global view of all the possible binding sites of a specific transcription factor, without taking into consideration the tissue and developmental stage specificity of regulation, ChIP-seq data were also obtained for *Vvi*NAC60. The aim was to find direct target of the TF which are related to the berry ripening specific regulation.

ChIP-seq represents a powerful way to study TF-DNA interactions *in vivo* (Kaufmann *et al*., 2010; Bowler *et al*., 2004); this methodology has produced numerous applications in studying the composition and dynamics of chromatin landscapes and TF binding, as well as assessing the interplay between different factors in gene regulation under different conditions and developmental phases (Kidder *et al*., 2011; Kaufmann *et al*., 2010; Lin *et al*., 2012; Martel *et al*., 2011). ChIP-seq approach can be used to study DNA methylation, chromatin structure/histone modifications, and the cooperative binding of TFs.

As for the DAP-seq, this also represent the first *Vitis vinifera* ChIP-seq attempt. Indeed, genome-wide applications of the ChIP-seq methodology in the plant field are still relatively scarce. This delay is partly due to technical difficulties in ChIP sample preparation related to the complexity of plant tissues for the presence of rigid cell walls, high levels of cellulose and lignin and large vacuoles (Kaufmann *et al*., 2010). The establishment of an efficient ChIP-seq protocols for plant systems (especially for grapevine) is hard and many optimizations were required as different types of tissues were sampled (Bowler *et al*., 2004); indeed, the chromatin extraction from the berry samples was hard and requires many little precautions to reach a good yield.

The ChIP assay consists in the TF-DNA crosslinking by formaldehyde tissues vacuum infiltration, the isolation of chromatin from the extracted nuclei, the

chromatin fragmentation, the immunoprecipitation of the TF-DNA complexes with a specific antibody, the reverse crosslinking for the recovery of DNA and the final identification of associated DNA sequences through sequencing (Kaufmann *et al*., 2010).

The formaldehyde percentage is an important parameter that needs to be taken into consideration (Schmiedeberg *et al*., 2009). It is essential that the samples are treated with a suitable volume of formaldehyde to assure the stable bond between the protein and the DNA: if the formaldehyde concentration is too low or the incubation time is too short, not enough crosslinked material will be produced; on the other hand, a formaldehyde concentration that is too high or an incubation time that is too long also reduces recovery, reflecting the insoluble complexes formation or masking the epitopes recognition by the antibody (Kaufmann *et al*., 2010; Schmiedeberg *et al*., 2009; Solomon *et al*., 1985).

Sonication is the most variable step in the process and will vary greatly depending on the cell type, the quantity of cells, the sonication volume, the crosslinking degree and the specifics of the sonicator used (Kaufmann *et al*., 2010; Bortz *et al*., 2011). Sonication is important for the solubilization and shearing of the chromatin; under sonication brings to a loss of resolution of the binding sites and over sonication can lead to protein denaturation. The optimal chromatin fragments length ranges between 150 and 300 bp (Kaufmann *et al*., 2010).

The quality of antibodies used for ChIP-seq is one of the most important factors that contribute to the quality of the generated data (Park, 2009; Kidder *et al*., 2011). Antibodies that offer high sensitivity and specificity are necessary for ChIP-seq because they allow the detection of enrichment peaks without substantial background noise (Kaufmann *et al*., 2010; Busby *et al*., 2016); indeed, the specificity of an antibody should be directly addressed by immunoblot analysis of the protein of interest. It is also important to consider the potential cross-reactivity of antibodies with closely related family members. The heterogeneity of the antibody should also be considered in the selection of an antibody; monoclonal antibodies, which recognize a single epitope on an antigen, may be a good choice for diminishing background noise in ChIP studies but, however, the use of monoclonal antibodies may result in a lower signal if the epitope is masked by

surrounding chromatin components or if the protein is part of a larger protein complex (Busby *et al*., 2016; Kaufmann *et al*., 2010). The polyclonal antibodies represent the best solution as they offer the flexibility of multiple epitopes recognition (Kaufmann *et al*., 2010; Busby *et al*., 2016).
In this thesis the specificity analyses of the used polyclonal antibody are not reported; however, many Western blot analyses were carried out before using the specific anti-VviNAC60 polyclonal antibody. The obtained results were useful to assure the antibody specific recognition of the TF of interest and be confident of the right fragment selection in the immunoprecipitation step.

The ChIP-seq results were very hard to obtain because of the above-described technical issues that appeared during the setting up of the experiment; however, the efforts brought to a few but interesting observations. Even if the percentages of alignment on the grape genome of the immunoprecipitated chromatin fragments were, for the berry samples, very low (the lowest is the 5,29% of the berries at veraison), the highest number of VviNAC60 regulated genes was found in the veraison berries sample, the main subject of the investigation, where indeed the TF is more expressed.
This result matched with the Atlas (Fasoli *et al*., 2012) transcriptomic findings despite the higher number of contaminants present in that sample; indeed, the most of the immunoprecipitated sequences belonged to microorganisms that usually live on the fruit and leaves surface (Menendez and Garcia-Fraile, 2017). The organisms were the same in all the samples and the percentage of the different microorganisms was almost the same; what increased the contamination in different sequenced samples was the DNA of only one microorganism, the *Paraburkholderia fungorum*. Searching in the literature, this plant probiotic bacterium was found correlated with fruit growth improvements (Rahman *et al*., 2018; Menendez and Garcia-Fraile, 2017); this finding made of this enriched, unexpected and unwanted bacterial DNA a real interesting observation, useful for further studies.
The small ‘Veraison berry’ dataset was analyzed and 46 binding sites were obtained. Only four genes found correlations with the *VviNAC60* stable and transient over expression DEGs and only one of these, the *PROTEIN BINDING/ ZINC ION BINDING* (VIT_12s0028g03340), resulted up regulated by the action of

the TF in promoter region; unfortunately, almost nothing is present in literature concerning this gene. Moreover, this list of the ChIP-seq found gene was crossed with the DAP-seq one but, again, only one gene matched. Unfortunately, this gene, *SRG1- SENESCENCE-RELATED GENE 1 OXIDOREDUCTASE* (VIT_10s0003g02400), revealed the TF binding site in two different genic regions, in an exon in the ChIP-seq dataset and in the promoter in the DAP-seq one. However, this gene, seems to be very interesting as it is related to senescence, a maturation process. Senescence brings to a huge macromolecular catabolism which dismantles the chloroplasts and decreases the photosynthetic capacity of the leaves; moreover, a recent study provided evidence that a delayed leaves senescence enhances carbon assimilation that, ultimately, increased the number of fleshy fruits and their total soluble solid content (Lira *et al*., 2017). Indeed, it was found that one way to delay senescence initiation is the relation of key TFs involved in triggering this process, such as the NAC (Lira *et al*., 2017). For this reason, the fact that VviNAC60 directly regulates *SRG1* only in the veraison berry tissue once again gives strength to the role of this master regulator in the berry ripening and encourages new optimizations on this promising first ChIP-seq attempt.

After many technical and biological considerations, we do not think that the ChIP-seq results were due to the non-specificity antibody issue; probably, the probiotic microorganisms are simply more abundant on berries at veraison (Menendez and Garcia-Fraile, 2017) and this tissue- and developmental stage-specific characteristic is reflected in the sequencing results. However, a solution is needed to avoid the unwanted material selection and to obtain more accurate ChIP-seq results; in the future, a further setting-up of the ChIP-seq protocol will be carried out to better clean the starting harvest tissues, to find a way to increase the chromatin extraction yield from the berries and to avoid the selection and enrichment of the contaminants (for example trying to use a different preclearing method).

After all the individual analyses on all the 14 above described VviNACs, with the aim to try understanding the general regulative role of the selected VviNAC family members on the grapevine transcriptome, a global analysis of all the possible interactions obtained with the DAP-seq assays was performed. The *UTP GLUCOSE-1-PHOSPHATE URIDYLYLTRANSFERASE* (VIT_04s0044g00710),

resulted regulated by 13 of the 14 *Vvi*NACs. Not much information is available in literature concerning this gene, excluding the fact that it encodes for an enzyme obviously related to the carbohydrate metabolism; however, looking at the Atlas (Fasoli *et al*., 2012), the gene showed a significant increase in its expression in the berry pericarp starting from the post fruit set to the ripening, in the berry flesh and skin at veraison and in the seeds at veraison and mid ripening. Considering the role of the *UTP GLUCOSE-1-PHOSPHATE URIDYLYLTRANSFERASE* in the sugar metabolism and the interesting results obtained with its expression profile investigation, this gene could really represent an important target in the VviNAC-mediated berry development.

As said previously in this chapter, many *VviNAC* genes were found as targets of the selected TFs; to assess if these genes were for sure direct targets of the VviNACs regulation, another experimental approach, the Dual Luciferase Reporter Assay (DLRA), was used. The DLRA is a powerful technique used for the validation of the interaction between a specific transcription factor and the promoter of a gene using a fluorescent reporter; moreover, the assay is quantitative and the analysis permits to identify the investigated TFs as activators or repressors (Sherf *et al*., 1996). Considering the binding of more than one selected VviNAC TFs on some *VviNAC* promoters, the fluorescence quantitative value of the DLRA, correlated with the q-value of the DAP-seq and the FC of the over expression, could also be useful to understand which TF has the predominant regulative role on a gene.

The decision of starting from the identification of a possible mechanism of regulation between VviNACs comes from the idea that the understanding of the connection between the members of this family could facilitate the further interpretation of a wider regulation network, especially being conscious of the high expression similarity between the selected TFs. Indeed, many genes were found as common targets of more than one TF and the specific single VviNAC-related genes most of the times belonged to biological and metabolic processes regulation functional categories, which have a fundamental but very generic role in the development scenario.

The final aim is, of course, to validate all of them; however, the promoters isolation and cloning are long and difficult steps considering the possible differences

between the reference genome (Pinot Noir) and the selected cultivar for this thesis (Shiraz), the A and T enrichment generally present in the promoting regions which makes specific primers design hard and the possible similarity between the promoters of genes belonging to the same family. For all these reasons, the decision was to start testing the activation/repression effects of some selected TFs on five of the most represented *VviNAC* genes between all the DAP-seq datasets: *VviNAC05*, *VviNAC34* and *VviNAC61* were found to be regulated by seven of the 14 TFs; *VviNAC37* by five of them and *VviNAC08* by four. Moreover, *VviNAC01* was also taken into consideration because it resulted directly regulated by VviNAC01 itself. It was very interesting to find *VviNAC61* between the most DAP-seq represented regulated genes; this TF was already known to have a role in the withering processes (Zenoni *et al*., 2016) and the transient over expression results confirm this evidence. The fact that it seems to be regulated by such a high number of VviNAC TFs could be related to its role in secondary metabolic processes, which are relevant in the last stages of the development and in the post harvesting phase and could place it downstream of the regulatory network of the NAC TFs. Moreover, VviNAC61 presented a very low number of binding sites in its DAP-seq dataset, with no interactions found with the promoters of other *VviNACs*. Looking at the DEGs, *VviNAC61* over expression regulated 1135 gene, supporting the hypothesis of its role in the downstream processes of development.

The DLRA was performed on *N. benthamiana* leaves; this choice has the limit that, working on a different plant species, no hypothetical grapevine co-factor is available and, knowing that VviNACs often form homodimers in their regulatory action, some activation/repression could be lost. This consideration must be done when correlating the over expression results with the fluorescence quantification: all the over expressions were performed in the plant of interest (*V. vinifera*) and all the other VviNACs are also present and could interact, bringing to the regulation of a gene. However, the DLRA gives the possibility to test the action on a gene of more TFs together, permitting to identify possible heterodimerizations; this part has not been performed yet but will be a crucial point of the VviNACs investigation.

The results obtained in this thesis showed that VviNAC01 can act both as an activator and a repressor; indeed, it directly repressed *VviNAC05* expression,

whereas activated the *VviNAC08* one. Moreover, VviNAC01 was validated as repressor of its own transcription; this was very interesting because, also considering the high number of genes regulated by this TF, a sort of negative feedback regulation on its own transcription could be assumed. No significant fluorescence values where obtained, in comparison to the control, concerning the other three DAP-seq identified regulated genes (*VviNAC34*, *VviNAC37* and *VviNAC61*); probably a partner is needed in these specific cases. VviNAC03 was confirmed in its action on the *VviNAC05* expression and results a repressor of this gene. VviNAC11 acted always as an activator in all the validated interactions and directly regulates *VviNAC34*, *VviNAC37* and *VviNAC61*. Moreover, the *VviNAC34* expression activation was the highest revealed, with a 16-fold induction compared to the control. VviNAC13, which also acted always as an activator, results to regulate *VviNAC34* and *VviNAC37* expression. VviNAC15 presented a binding site on three of the six selected *VviNAC* genes (*VviNAC34*, *VviNAC37* and *VviNAC61*) but only one interaction was significantly validated; indeed, it results an activator of *VviNAC34*. Unfortunately, this demonstrated *VviNAC61* repression by VviNAC15 did not reflect the transient over expression findings, where the gene was found between the up regulated DEGs. VviNAC17 acted always as a repressor and significantly down regulates *VviNAC05*, *VviNAC08* and *VviNAC61* expression. VviNAC18 resulted a direct activator of *VviNAC05*. Worth notices that VviNAC18 and VviNAC03 are both NOR orthologues (Giovannoni *et al*., 1995; Giovannoni *et al*., 2004) and present very similar expression profiles; these knowledges, together with the fact that in the DAP-seq results they presented two totally different binding position on *VviNAC05*, indicate that they do not compete and suggests that they could probably be partners of other TFs which activate or repress the gene in relation to different dynamics. VviNAC26 acts both as an activator and a repressor; indeed, it positively regulates the expression of *VviNAC05* and directly down regulates the *VviNAC08*, *VviNAC34* and *VviNAC61* expression. VviNAC33 was found as a direct activator of *VviNAC05*, *VviNAC08*, *VviNAC34*, *VviNAC37* and *VviNAC61* expression. The other master regulator VviNAC60 was also found to only direct up regulate the expression of the DAP-seq identified target; indeed, high fluorescence values were induced when it regulated *VviNAC05*, *VviNAC34* and

VviNAC61. Unfortunately, the activation acted by VviNAC60 on *VviNAC34* found no confirmation in the *VviNAC60* stable over expression results, where the gene was down regulated.

Not looking only at the single validated activations/repressions and at the specific found binding sites, it was very interesting to see that more TFs could bind to the same *VviNAC* promoter region, sometimes exactly at the same site, other times very close to each other. The presence of more than one transcription factor binding site in the promoter region of a gene could highlight the ability of the TFs to form homo- or hetero- dimers (Wehner *et al*., 2011; Mitsuda and Ohme-Takagi, 2009). Generally, these complexes contribute to increase the regulatory effect of a gene in comparison to the result of a single TF binding on the DNA; indeed, it is plausible that the presence of more binding sites brings to a synergic regulation of the gene expression (Malhotra and Sowdhamini, 2014; Lai *et al*., 2019). Considering the highly similar expression profiles of the selected TFs and the fact that sometimes more than two TFs binds very close to each other on the same promoter, it is hard to confidently hypothesize an organ or tissue dependent fine regulation mechanism or to identify only one specific heterodimerization complex; moreover, even in the case of great diversity among the regulators, it would still be difficult to identify the biological causes of possible interactions between TFs. Whether more than one transcription factor binding sites on the promoter of a target gene increase the gene regulation is dependent on several factors (such as proximity), most of them still unclarified; the transcription factor binding attribution to a specific associated gene regulation is an ongoing issue which is mainly bioinformatically studied and does not present many biological validations (Spadafore *et al*., 2017; Tian *et al*., 2020). Thanks to the genome editing technology, it would be possible to individually mutate each binding site maintaining its real biological context, identifying in this way the specific binding position-related role of a TF; unfortunately, this is not trivial, especially considering the well know difficulty of working with a not model crop species such as grapevine.

Concerning the DLRA not matching results with the DEGs values, must be considered that the over expressions have helped in the definition of a more specific role of these TFs but the limits of this type of technique are many and the

interpretation of the results is sometimes hard. Only a few cases (for example VviNAC61 and VviNAC01, the most evident) exhibited a clear regulative role from the DAP-seq results and the DEGs analysis correlation; looking at the stable *VviNACs* over expression, probably what was in most of the cases seen is the long-term action of the TF regulation, on the other way, the transient over expression can give a more instant picture of the transcriptome regulation but the results are highly infiltration-dependent. Moreover, in both the over expressions must be considered the fact that, performing the experiments in grapevine, all the possible co-factors of the TF were available and the identified DEGs could have been positively or negatively regulated by a combination of different TF; as said before, the ability of the NAC TFs to form homodimers is, indeed, well known. It was also very interesting to see that the *switch* master regulators *VviNAC33* and *VviNAC60*, together with the *NOR* orthologues *VviNAC03* and *VviNAC18*, and with other two Atlas switch TFs *VviNAC11* and *VviNAC13*, did not present regulation by any VviNACs. Probably they are placed at the very beginning of the development regulatory network induced by the VviNAC family and orchestrate all the possible mechanisms of control.

Considering all the ongoing climate changes (Hannah *et al*., 2013; Webb et al., 2007; Spayd *et al*., 2002), in particular the continuous temperature increasing that is altering the maturation process, that could modify the physiological characteristics of grape, its final quality and consequently the wine one, the development of different strategies to prevent these negative effects is fundamental. Indeed, the study and the interpretation of the molecular mechanisms controlling the onset of berry ripening could provide a solution to the all the current agronomical problems. This thesis represents another little step in the definition of a clear regulatory network of the grapevine maturation processes, particularly focused on the VviNAC TFs and their direct regulated target genes.
In the future, many other putative target genes (and not also between the *VviNAC* family) will be investigated to obtain more information about the roles of all the *VviNACs*, but these preliminary results clearly highlight the crucial roles of the VviNAC transcription factors in the regulation of grapevine development through an inter-family network.

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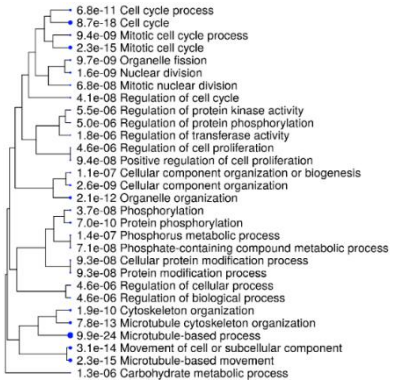
APPENDIX

Appendix A: A) *VviNAC01*, B) *VviNAC08*, C) *VviNAC15*, D) *VviNAC17*, E) *VviNAC18*, F) *VviNAC26*, G) *VviNAC38*, H) *VviNAC39* and I) *VviNAC61* co-expressing genes GO enrichment analyses using ShinyGO.

A

B



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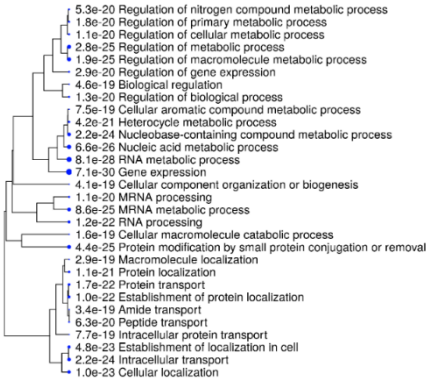

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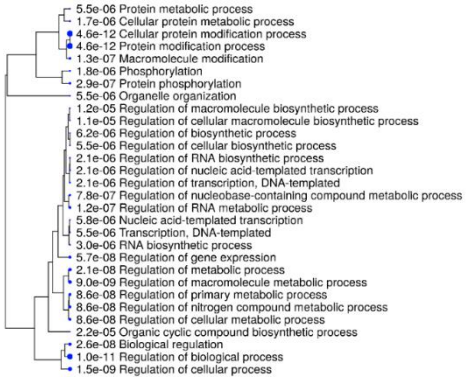

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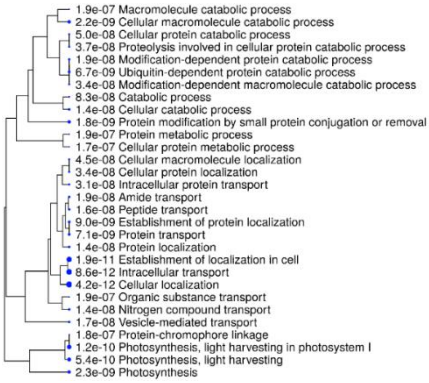

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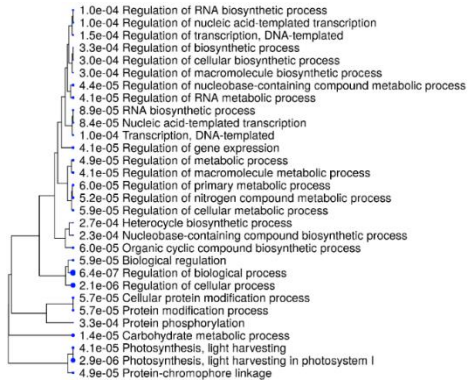

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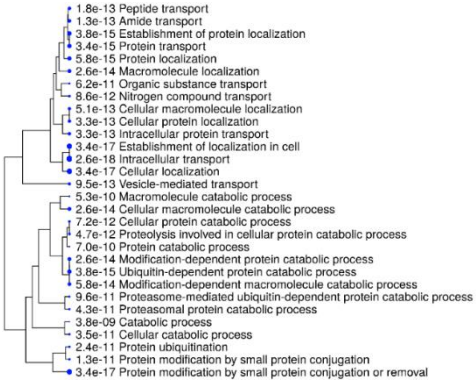
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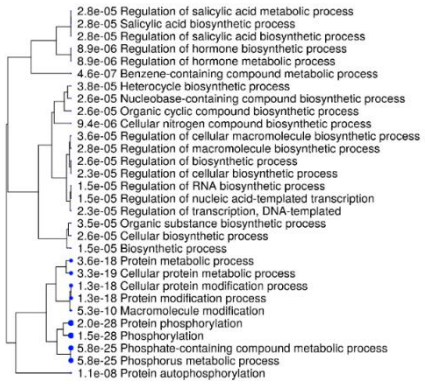
1.3e-13 Amide transport
3.8e-15 Establishment of protein localization
3.4e-15 Protein transport
5.8e-15 Protein localization
2.6e-14 Macromolecule localization
6.2e-11 Organic substance transport
8.6e-12 Nitrogen compound transport
5.1e-13 Cellular macromolecule localization
3.3e-13 Cellular protein localization
3.3e-13 Intracellular protein transport
3.4e-17 Establishment of localization in cell
2.6e-18 Intracellular transport
3.4e-17 Cellular localization
9.5e-13 Vesicle-mediated transport
5.3e-10 Macromolecule catabolic process
2.6e-14 Cellular macromolecule catabolic process
7.2e-12 Cellular protein catabolic process
4.7e-12 Proteolysis involved in cellular protein catabolic process
7.0e-10 Protein catabolic process
2.6e-14 Modification-dependent protein catabolic process
3.8e-15 Ubiquitin-dependent protein catabolic process
5.8e-14 Modification-dependent macromolecule catabolic process
9.6e-11 Proteasome-mediated ubiquitin-dependent protein catabolic process
4.3e-11 Proteasomal protein catabolic process
3.8e-09 Catabolic process
3.5e-11 Cellular catabolic process
2.4e-11 Protein ubiquitination
1.3e-11 Protein modification by small protein conjugation
3.4e-17 Protein modification by small protein conjugation or removal

H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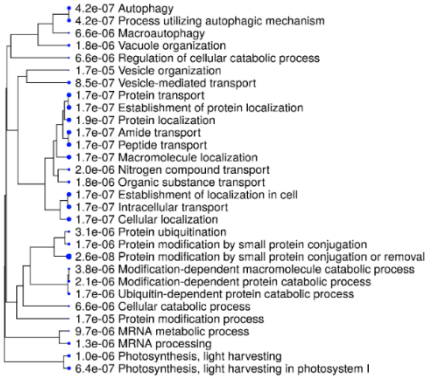
2.8e-05 Salicylic acid biosynthetic process
2.8e-05 Regulation of salicylic acid biosynthetic process
8.9e-06 Regulation of hormone biosynthetic process
8.9e-06 Regulation of hormone metabolic process
4.6e-07 Benzene-containing compound metabolic process
3.8e-05 Heterocycle biosynthetic process
2.6e-05 Nucleobase-containing compound biosynthetic process
2.6e-05 Organic cyclic compound biosynthetic process
9.4e-06 Cellular nitrogen compound biosynthetic process
3.6e-05 Regulation of cellular macromolecule biosynthetic process
2.8e-05 Regulation of macromolecule biosynthetic process
2.6e-05 Regulation of biosynthetic process
2.3e-05 Regulation of cellular biosynthetic process
1.5e-05 Regulation of RNA biosynthetic process
1.5e-05 Regulation of nucleic acid-templated transcription
2.3e-05 Regulation of transcription, DNA-templated
3.5e-05 Organic substance biosynthetic process
2.6e-05 Cellular biosynthetic process
1.5e-05 Biosynthetic process
3.6e-18 Protein metabolic process
3.3e-19 Cellular protein metabolic process
1.3e-18 Cellular protein modification process
1.3e-18 Protein modification process
5.3e-10 Macromolecule modification
2.0e-28 Protein phosphorylation
1.5e-28 Phosphorylation
5.8e-25 Phosphate-containing compound metabolic process
5.8e-25 Phosphorus metabolic process
1.1e-08 Protein autophosphorylation

I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4.2e-07 Process utilizing autophagic mechanism
6.6e-06 Macroautophagy
1.8e-06 Vacuole organization
6.6e-06 Regulation of cellular catabolic process
1.7e-05 Vesicle organization
8.5e-07 Vesicle-mediated transport
1.7e-07 Protein transport
1.7e-07 Establishment of protein localization
1.9e-07 Protein localization
1.7e-07 Amide transport
1.7e-07 Peptide transport
1.7e-07 Macromolecule localization
2.0e-06 Nitrogen compound transport
1.8e-06 Organic substance transport
1.7e-07 Establishment of localization in cell
1.7e-07 Intracellular transport
1.7e-07 Cellular localization
3.1e-06 Protein ubiquitination
1.7e-06 Protein modification by small protein conjugation
2.6e-08 Protein modification by small protein conjugation or removal
3.8e-06 Modification-dependent macromolecule catabolic process
2.1e-06 Modification-dependent protein catabolic process
1.7e-06 Ubiquitin-dependent protein catabolic process
6.6e-06 Cellular catabolic process
1.7e-05 Protein modification process
9.7e-06 MRNA metabolic process
1.3e-06 MRNA processing
1.0e-06 Photosynthesis, light harvesting
6.4e-07 Photosynthesis, light harvesting in photosystem I

Appendix B: Alignments of A) *VviNAC01*, B) *VviNAC08*, C) *VviNAC15*, D) *VviNAC17*, E) *VviNAC18*, F) *VviNAC26*, G) *VviNAC38*, H) *VviNAC39* and I) *VviNAC61* nucleotide sequences from Shiraz and Pinot Noir cultivars.

A

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VviNAC01_Shiraz         ATGGAGAAGCTCAATTTTGTGAAGAATGGCGTCCTCAGATTGCCTCCTGGCTTCCGCTTC
VviNAC01_Reference      ATGGAGAAGCTCAATTTTGTGAAGAATGGCGTCCTCAGATTGCCTCCTGGCTTCCGCTTC
                        ************************************************************

VviNAC01_Shiraz         CATCCTACTGATGAAGAGCTGGTGGTGCAGTACTTGAAGCGCAAGGCCTATTCTTGCCCC
VviNAC01_Reference      CATCCTACTGATGAAGAGCTGGTGGTGCAGTACTTGAAGCGCAAGGCCTATTCTTGCCCC
                        ************************************************************

VviNAC01_Shiraz         CTGCCCGCCTCCATCATCCCTGAGGTCGATGTCTGCAAGGCTGATCCTTGGGATTTGCCA
VviNAC01_Reference      CTGCCCGCCTCCATCATCCCTGAGGTCGATGTCTGCAAGGCTGATCCTTGGGATTTGCCA
                        ************************************************************

VviNAC01_Shiraz         GGTGATTTGGAGCAGGAGAGGTACTTCTTCAGCACCAGGGAGGCCAAGTACCCGAATGGA
VviNAC01_Reference      GGTGATTTGGAGCAGGAGAGGTACTTCTTCAGCACCAGGGAGGCCAAGTACCCGAATGGA
                        ************************************************************

VviNAC01_Shiraz         AACCGGTCGAATAGAGCCACGGTTTCGGGTTACTGGAAGGCAACTGGAATTGACAAGCAA
VviNAC01_Reference      AACCGGTCGAATAGAGCCACGGTTTCGGGTTACTGGAAGGCAACTGGAATTGACAAGCAA
                        ************************************************************

VviNAC01_Shiraz         ATTGTAGCTTCCAAGGGGAACCAGGTTGTGGGGATGAAGAAAACTTTGGTTTTTTATAGA
VviNAC01_Reference      ATTGTAGCTTCCAAGGGGAACCAGGTTGTGGGGATGAAGAAAACTTTGGTTTTTTATAGA
                        ************************************************************

VviNAC01_Shiraz         GGAAAGCCTCCACATGGGTCGAGAACCGATTGGATCATGCACGAATACCGGCTTGTTGGT
VviNAC01_Reference      GGAAAGCCTCCACATGGGTCGAGAACCGATTGGATCATGCACGAATACCGGCTTGTTGGT
                        ************************************************************

VviNAC01_Shiraz         GCTGAAACCACCCCACAGAGAAAGAGCTCGACAACTCAGAGCTCAATGGCGCAAGCGGAG
VviNAC01_Reference      GCTGAAACCACCCCACAGAGAAAGAGCTCGACAACTCAGAGCTCAATGGCGCAAGCGGAG
                        ************************************************************

VviNAC01_Shiraz         AACTGGGTGTTGTGCCGCATATTCCTGAAGAAACGGGGCACGAAAAATGATGAGGAGATG
VviNAC01_Reference      AACTGGGTGTTGTGCCGCATATTCCTGAAGAAACGGGGCACGAAAAATGATGAGGAGATG
                        ************************************************************

VviNAC01_Shiraz         ATGCAAACCAACAATGAGAACCGGGTGGTTCAGAAGTTGAGGAGCAGCAGGCCTGTTTTC
VviNAC01_Reference      ATGCAAACCAACAATGAGAACCGGGTGGTTCAGAAGTTGAGGAGCAGCAGGCCTGTTTTC
                        ************************************************************

VviNAC01_Shiraz         TATGATTTCTTGACAAGGGACAGGGATGATACTAATCTTGCTTCTTCCAATTCTTCGGGT
VviNAC01_Reference      TATGATTTCTTGACAAGGGGCAGGGATGATACTAATCTTGCTTCTTCCAATTCTTCGGGT
                        ******************* ****************************************

VviNAC01_Shiraz         TCAAGTGGGATCACAGAGGTGTCGAATACCGAATCAGAAGAACACGAAGAAAGCAGTAGT
VviNAC01_Reference      TCAAGTGGGATCACAGAGGTGTCGAATACCGAATCAGAAGAACACGAAGAAAGCAGTAGT
                        ************************************************************

VviNAC01_Shiraz         TGCAATAGTTTCTCGCCTTTCAGAAGAAAACCATAA
VviNAC01_Reference      TGCAATAGTTTCTCGCCTTTCAGAAGAAAACCATAA
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B

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VviNAC08_Shiraz         ATGACAGCGGAGTTGCAGTTACCTCCAGGCTTCAGGTTCCATCCGACGGATGAGGAGCTT
VviNAC08_Reference      ATGACAGCGGAGTTGCAGTTACCTCCAGGCTTCAGGTTCCATCCGACGGATGAGGAGCTT
                        ************************************************************

VviNAC08_Shiraz         GTGATGCACTATCTGTGCCGTAAATGTGCATCGCAATCGATCGCCGTGCCGATCATTGCC
VviNAC08_Reference      GTGATGCACTATCTGTGCCGTAAATGTGCATCGCAATCGATCGCCGTGCCGATCATTGCC
                        ************************************************************

VviNAC08_Shiraz         GAAATTGATCTCTACAAATTCGATCCCTGGCAGCTTCCTGAGATGGCCTTGTACGGAGAG
VviNAC08_Reference      GAAATTGATCTCTACAAATTCGATCCCTGGCAGCTTCCTGAGATGGCCTTGTACGGAGAG
                        ************************************************************

VviNAC08_Shiraz         AAAGAGTGGTACTTCTTTTCGCCGAGAGATCGGAAATATCCGAACGGTTCAAGGCCGAAC
VviNAC08_Reference      AAAGAGTGGTACTTCTTTTCGCCGAGAGATCGGAAATATCCGAACGGTTCAAGGCCGAAC
                        ************************************************************

VviNAC08_Shiraz         CGGGCAGCGGGAACAGGGTACTGGAAGGCCACCGGAGCGGATAAGCCTATTGGGCATCCG
VviNAC08_Reference      CGGGCAGCGGGAACAGGGTACTGGAAGGCCACCGGAGCGGATAAGCCTATTGGGCATCCG
                        ************************************************************

VviNAC08_Shiraz         AAACCGGTTGGGATTAAGAAGGCTTTGGTTTTTTATGCCGGAAAAGCCCCCAGGGGAGAG
VviNAC08_Reference      AAGCCGGTTGGGATTAAGAAGGCTTTGGTTTTTTATGCCGGAAAAGCCCCCAGGGGAGAG
                        ** ************************************************************

VviNAC08_Shiraz         AAGACAAATTGGATTATGCATGAATACCGGCTGGCAGACGTGGACCGGTCGGCTCGCAAG
VviNAC08_Reference      AAGACAAATTGGATTATGCATGAATACCGGCTGGCAGACGTGGACCGGTCGGCTCGCAAG
                        ************************************************************

VviNAC08_Shiraz         AAGAATAATAGCTTAAGGTTGGACGATTGGGTTCTGTGCCGCATATACAACAAGAAGGGC
VviNAC08_Reference      AAGAATAATAGCTTAAGGTTGGACGATTGGGTTCTGTGCCGCATATACAACAAGAAGGGC
                        ************************************************************

VviNAC08_Shiraz         ATTGTCGAGAAACAACACACCGCTGCCCGGAAATCAGATTGCTCCGATGTTGAGGATCAA
VviNAC08_Reference      ATTGTCGAGAAACAACACACCGCTGCCCGGAAATCAGATTGCTCCGATGTTGAGGATCAA
                        ************************************************************

VviNAC08_Shiraz         AAGCCTGGACCTCTTGCTCTAAGCAGGAAGGTAGGTGCGATGCCTCCACCTCCGCCGCCG
VviNAC08_Reference      AAGCCTGGACCTCTTGCTCTAAGCAGGAAGGTAGGTGCGATGCCTCCACCTCCGCCGCCA
                        ************************************************************

VviNAC08_Shiraz         TCGTCCTCTACGGCACCAACTGCGACAGCGGCACTGGACGATTTGGTGTACTTCGACTCA
VviNAC08_Reference      TCGTCCTCTACGGCACCAACTGCGACAGCGGCACTGGACGATTTGGTGTACTTCGACTCA
                        ************************************************************

VviNAC08_Shiraz         TCGGATTCGGTGCCGCGCCTCCACACCGACTCGAGCTGTTCGGAGCACGTGGTGTCGCCG
VviNAC08_Reference      TCGGATTCGGTGCCGCGCCTCCACACCGACTCGAGCTGTTCGGAGCACGTGGTGTCGCCG
                        ************************************************************

VviNAC08_Shiraz         GAGTTCACGTGCGAGAGGGAGGTGCAGAGCGAGCCCAAGTGGAAGGAGTGGGAAAATCCC
VviNAC08_Reference      GAGTTCACGTGCGAGAGGGAGGTGCAGAGCGAGCCCAAGTGGAAGGAGTGGGAAAATCCC
                        ************************************************************

VviNAC08_Shiraz         ATGGACTTTTCGTACAATTACATGGATGCCACAGTTGACAACGCATTTTTGTCTCAGTTC
VviNAC08_Reference      ATGGACTTTTCGTACAATTACATGGATGCCACAGTTGACAACGCATTTTTGTCTCAGTTC
                        ************************************************************

VviNAC08_Shiraz         CCGAATAATCAGATGTCGCCATTGCAGGACATGTTCATGTACCTGCAGAAGCCCTTCTGA
VviNAC08_Reference      CCGAATAATCAGATGTCGCCATTGCAGGACATGTTCATGTACCTGCAGAAGCCCTTCTGA
                        ************************************************************
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C

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VviNAC15_Shiraz         ATGAAGGTGACAGTGGGTGATTCGTCGCCGTGCTTTGACGGAGACGAAAAGTCTGCGTGG
VviNAC15_Reference      ATGAAGGTGACAGTGGGTGATTCGTCGCCGTGCTTTGACGGAGACGAAAAGTCTGCGTGG
                        ************************************************************

VviNAC15_Shiraz         CCACCCGGGTTCCGATTCCATCCCACCGATGAAGAGCTGGTTCTGTATTATCTGAAGAAG
VviNAC15_Reference      CCACCCGGGTTCCGATTCCATCCCACCGATGAAGAGCTGGTTCTGTATTATCTGAAGAAG
                        ************************************************************

VviNAC15_Shiraz         AAGATCTGCCGGCGACGGCTGAAGCTCGATATCATCGCCGAGGTCGATGTCTACAAGTGG
VviNAC15_Reference      AAGATCTGCCGGCGACGGCTGAAGCTCGATATCATCGCCGAGGTCGATGTCTACAAGTGG
                        ************************************************************

VviNAC15_Shiraz         GACCCCGAGGATTTGCCTGGGCTATCTAAATTGAAGACAGGAGATAGGCAATGGTTCTTT
VviNAC15_Reference      GACCCCGAGGATTTGCCTGGACTATCTAAATTGAAGACAGGAGATAGGCAATGGTTCTTT
                        ******************* ************************************

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VviNAC15_Reference      TTTAGCCCCAGAGACAGGAAGTACCCTAATGGAGCTAGGTCTAATAGGGCAACCAGGCAT
                        ************************************************************

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                        ************************************************************

VviNAC15_Shiraz         GTGAAGAAGACCTTGGTTTTCTATAAAGGCCGTGCTCCAAGTAAAGAGCGCACAGACTGG
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                        ************************************************************

VviNAC15_Shiraz         GTGATGCATGAGTATACAATGGATGAAGAGGAGCTCAAGAGATGCCCGAATGTGCAGGAT
VviNAC15_Reference      GTGATGCATGAGTATACAATGGATGAAGAGGAGCTCAAGAGATGCCCGAATGTGCAGGAT
                        ************************************************************

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VviNAC15_Reference      TATTATGCACTTTATAAGGTCTTCAAGAAGAGTGGACCTGGTCCCAAAAATGGTGAGCAA
                        ************************************************************

VviNAC15_Shiraz         TACGGGGCTCCATTTAAAGAAGAGGAATGGGCTGACGAAGATGACCTAGATGTTAGTAAC
VviNAC15_Reference      TACGGGGCTCCATTTAAAGAAGAGGAATGGGCTGACGAAGATGACCTAGATGTTAGTAAC
                        ************************************************************

VviNAC15_Shiraz         TACTCTGTTGAAGAGACTCCTCCAGAGCAGTTGAATGGCGTTATTTCTGTCAATAATTCC
VviNAC15_Reference      TACTCTGTTGAAGAGACTCCTCCAGAGCAGTTGAATGGCGTTATTTCTGTCAATAATTCC
                        ************************************************************

VviNAC15_Shiraz         AAACCTAATGGGCAAGACTGTCAAGCAGATGCTTGGGATGACATCTGGAAAGGACTTGCA
VviNAC15_Reference      AAACCTAATGGGCAAGACTGTCAAGCAGATGCTTGGGATGACATCTGGAAAGGACTTGCA
                        ************************************************************

VviNAC15_Shiraz         GAAGCACCTCCAGTTGTTCCTCTGCGTGTTGATGATTATGTTAATCTACTAGCTCAGGTT
VviNAC15_Reference      GAAGCACCTCCAGTTGTTCCTCTGCGTGTTGATGATTATGTTAATCTACTAGCTCAGGTT
                        ************************************************************

VviNAC15_Shiraz         ATTGGTGAAGAAGAAGCTCAAACTCCTTTGGTGGATTCATCACTCAATGGAGCTTTCGTT
VviNAC15_Reference      ATTGGTGAAGAAGAAGCTCAAACTCCTTTGGTGGATTCATCACTCAATGGAGCTTTCGTT
                        ************************************************************

VviNAC15_Shiraz         GCTGATCCAATAAGCACAGTATTAACCCCTACCTCTCAGCAGTATGCTGTGCCAGAGAAC
VviNAC15_Reference      GCTGATCCAATAAGCACAGTATTAACCCCTACCTCTCAGCAGTATGCTGTGCCAGAGAAC
                        ************************************************************

VviNAC15_Shiraz         GTTGAGTTTACACAATCAGCCTCCTCTCAGTTGCAATTGCACGAGGCACCTGAGGTCACA
VviNAC15_Reference      GTTGAGTTTACACAATCAGCCTCCTCTCAGTTGCAATTGCACGAGGCACCTGAGGTCACA
                        ************************************************************

VviNAC15_Shiraz         TCTGCTCCTAACATTAGTGAGCAGGAACGTGGATTAAGTGAGGAGGACTTTCTAGAAATG
VviNAC15_Reference      TCTGCTCCTAACATTAGTGAGCAGGAACGTGGATTAAGTGAGGAGGACTTTCTAGAAATG
                        ************************************************************

VviNAC15_Shiraz         GATGATCTCCTTGGTCCAGAACCCATTCCTCAAAACTATGAAAAAACTGAGGAGAACTTG
VviNAC15_Reference      GATGATCTCCTTGGTCCAGAACCCATTCCTCAAAACTATGAAAAAACTGAGGAGAACTTG
                        ************************************************************

VviNAC15_Shiraz         CAGTTTGAAGACGATGGATTGAGCATGCTTGACCTGTACCATGATGCAGCCATGTTTCTT
VviNAC15_Reference      CAGTTTGAAGGCGATGGATTGAGCATACTTGACCTGTACCATGATGCAGCCATGTTTCTT
                        ********** *************** ********************************

VviNAC15_Shiraz         CGTGACATTGGCCCTATTGATCAAGGAACGGTTCCGCATCCATATTTGAATACCATTGAG
VviNAC15_Reference      CGTGACATTGGCCCTATTGATCAAGGAACGGTTCCGCATCCATATTTGAATACCATTGAG
                        ************************************************************

VviNAC15_Shiraz         AATGAGATGGTGAACCAGTTGAATTACCAGCTGCAGCCCCATTCTGTTGGTGCAGATCAG
VviNAC15_Reference      AATGAGATGGTGAACCAGTTGAATTACCAGCTGCAGCCCCATTCTGTTGGTGCAGATCAG
                        ************************************************************

VviNAC15_Shiraz         ATTAGTGGTCAGCTGTGGACACTCGATCAAAGTGTCTGTACCTCAGCAGAATCTATTCAG
VviNAC15_Reference      ATTAGTGGTCAGCTGTGGACACTCGATCAAAGTGTCTGTACCTCAGCAGAATCTATTCAG
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VviNAC15_Shiraz         GGGATCATTGGGCAGCCAACCTCAGGTGTCGTATATGCCAGCAGTTCTACAAATGTTCCC
VviNAC15_Reference      GGGATCATTGGGCAGCCAACCTCAGGTGTTGTATATGCCAGCAGTTCTACAAATGTTCCC
                        *************************** ****************************

VviNAC15_Shiraz         ACCGAAGGAAATCAAAACATGAATGGCGAAGGGGGTAACGGTGCAGGGAACCGATTCACT
VviNAC15_Reference      ACCGAAGGAAATCAAAACATGAATGGCGAAGGGGGTAACGGTGCAGGGAACCGATTCACT
                        ************************************************************

VviNAC15_Shiraz         TCTGCTCTATGGTCCTTTGTGGAGTCAATACCTACCACACCTGCATCAGCTTCAGAAAAT
VviNAC15_Reference      TCTGCTCTATGGTCCTTTGTGGAGTCAATACCTACCACACCTGCATCAGCTTCAGAAAAT
                        ************************************************************

VviNAC15_Shiraz         GCGTTGGTAAATCGGGCATTGGTGAGAATGTCTAGCTTTAGTAGGATGAGAATGAATGCA
VviNAC15_Reference      GCGTTGGTAAATCGGGCATTGGTGAGAATGTCTAGCTTTAGTAGGATGAGAATGAATGCA
                        ************************************************************

VviNAC15_Shiraz         TTGAACACAAATGCAGGTAATGGAGGTGCAGCCACATGGAAGGGAGGTATAAATAAGGGG
VviNAC15_Reference      TTGAACACAAATGCAGGTAATGGAGGTGCAGCCACATGGAAGGGAGGTATAAATAAGGGG
                        ************************************************************

VviNAC15_Shiraz         GGATTCATCATTCTTTCAGTTATTGGAGCACTGATAGCTATATTCTGGGTCCTAATGCTA
VviNAC15_Reference      GGATTCATCATTCTTTCAGTTATTGGAGCACTGATAGCTATATTCTGGGTCCTAATGCTA
                        ************************************************************

VviNAC15_Shiraz         GGACCTGTGAAGATGTTAGGAAGATGCCTCCCCTCATGA
VviNAC15_Reference      GGACCTGTGAAGATGTTAGGAAGATGCCTCCCCTCATGA
                        ***************************************
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D

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VviNAC17_Shiraz         ATGGGTGTACCGGAGACTGACCCGCTTTCACAGCTTAGTTTGCCGCCTGGGTTCCGATTT
VviNAC17_Reference      ATGGGTGTACCGGAGACTGACCCGCTTTCACAGCTTAGTTTGCCGCCTGGGTTCCGATTT
                        ************************************************************

VviNAC17_Shiraz         TATCCCACCGATGAGGAGCTTCTGGTGCAGTATCTCTGCCGGAAAGTGGCCGGACAGGGG
VviNAC17_Reference      TATCCCACCGATGAGGAGCTTCTGGTGCAGTATCTCTGCCGGAAAGTGGCCGGACAGGGG
                        ************************************************************

VviNAC17_Shiraz         TTTTCATTGGAGATAATTGGCGAAATCGATCTGTACAAGTTTGACCCATGGGTTCTTCCC
VviNAC17_Reference      TTTTCATTGGAGATAATTGGCGAAATCGATCTGTACAAGTTTGACCCATGGGTTCTTCCC
                        ************************************************************

VviNAC17_Shiraz         AGTAAAGCTATATTTGGAGAGAAAGAGTGGTACTTTTTCAGTCCCAGAGATCGGAAGTAC
VviNAC17_Reference      AGTAAAGCTATATTTGGAGAGAAAGAGTGGTACTTTTTCAGTCCCAGAGATCGGAAGTAC
                        ************************************************************

VviNAC17_Shiraz         CCAAATGGGTCCAGACCCAATAGGGTTGCTGGGTCTGGGTATTGGAAGGCCACCGGAACT
VviNAC17_Reference      CCAAATGGGTCCAGACCCAATAGGGTTGCTGGGTCTGGGTATTGGAAGGCCACCGGAACT
                        ************************************************************

VviNAC17_Shiraz         GATAAGGTGATTACCACCGAGGGCCGGAAAGTTGGCATCAAGAAAGCTCTGGTGTTTTAC
VviNAC17_Reference      GATAAGGTGATTACCACCGAGGGCCGGAAAGTTGGCATCAAGAAAGCTCTGGTGTTTTAC
                        ************************************************************

VviNAC17_Shiraz         GTCGGCAAAGCTCCAAAAGGAACCAAAACTAATTGGATCATGCATGAGTACAGACTCCTA
VviNAC17_Reference      GTCGGCAAAGCTCCAAAAGGAACCAAAACTAATTGGATCATGCATGAGTACAGACTCCTA
                        ************************************************************

VviNAC17_Shiraz         GAAAATTCGAGGAAAAATGGAAGCTCCAAGTTGGATGATTGGGTTCTGTGCCGAATTTAC
VviNAC17_Reference      GAAAATTCGAGGAAAAATGGAAGCTCCAAGTTGGATGATTGGGTTCTGTGCCGAATTTAC
                        ************************************************************

VviNAC17_Shiraz         AAGAAGAATTCCAACTCTTCGAAACCCATAGCAGCTGTACTTCCCAGCAAAGCGCACAGC
VviNAC17_Reference      AAGAAGAATTCCAACTCTTCGAAACCCATAGCAGCTGTACTTCCCAGCAAAGCGCACAGC
                        ************************************************************

VviNAC17_Shiraz         AACGGCTCGTCATCGTCATCGTCGTCCCACCTCGACGACGTCCTGGAGTCGCTGCCGGAG
VviNAC17_Reference      AACGGCTCGTCATCGTCATCGTCGTCCCACCTCGACGACGTCCTGGAGTCGCTGCCGGAG
                        ************************************************************

VviNAC17_Shiraz         ATCGATGACAGGTTCTTTTCTCCCAATCGGATGAATTCTCTGAGAGTTTCACAGCCGGAC
VviNAC17_Reference      ATCGATGACAGGTTCTTTTCTCCTAATCGGATGAATTCTCTGAGAGTTTCACAGCCGGAC
                        ********************** ***********************************

VviNAC17_Shiraz         GAGAAAGTCAACTTCCATAACCTGGGCTCGGGCAACTTCGACTGGGCCACTCTAGCAGGC
VviNAC17_Reference      GAGAAAGTCAACTTCCATAACCTGGGCTCGGGCAACTTCGACTGGGCCACTCTAGCAGGC
                        ************************************************************

VviNAC17_Shiraz         GTCTCCTCCTTGCAGGAGTTGGTCTCCGGCGTCCAATCCCACGCCCAGCCTCCCGCAGCT
VviNAC17_Reference      GTCTCCTCCTTGCAGGAGTTGGTCTCCGGCGTCCAATCCCACGCCCAGCCTCCCGCAGCT
                        ************************************************************

VviNAC17_Shiraz         GTCAACAACAGCAACGAAATGTACGTTCCGTCACTGCCGCCGCTAATCCAAGCCGAAGAA
VviNAC17_Reference      GTCAACAACAGCAACGAAATGTACGTTCCGTCACTGCCGCCGCTAATCCAAGCCGAAGAA
                        ************************************************************

VviNAC17_Shiraz         GAAGTCCAGAGCGGACTCAGAACCCAGAGAGTCGACCCAGTAATGAACCAAGGGTTCTTC
VviNAC17_Reference      GAAGTCCAGAGCGGACTCAGAACCCAGAGAGTCGACCCAGTAATGAACCAAGGGTTCTTC
                        ************************************************************

VviNAC17_Shiraz         CCGCAGAACTCGAACGCGTTCAGTCAGAGTTTCTCTAACTCACTCGACCCGTTCGGGTTT
VviNAC17_Reference      CCGCAGAACTCGAACGCGTTCAGTCAGAGTTTCTCTAACTCACTCGACCCGTTCGGGTTT
                        ************************************************************

VviNAC17_Shiraz         CGGTACCCGACCCAACCTAGCGGATTTGGATATAGGCAGTAA
VviNAC17_Reference      CGGTACCCGACCCAACCTAGTGGATTTGGATATAGGCAGTAA
                        ******************* **********************
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E

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VviNAC18_Shiraz         ATGGAGAGCACCGATTCATCTTCGGGCTCGCCGCAGCCGCAGCTTCCACCGGGGTTCCGC
VviNAC18_Reference      ATGGAGAGCACCGATTCATCTTCGGGCTCGCCGCAGCCGCAGCTTCCACCGGGGTTCCGC
                        ************************************************************

VviNAC18_Shiraz         TTTCACCCCACCGATGAAGAGTTAGTAGTTCACTATCTGAAGAAGAAGGCCTCATCTGCT
VviNAC18_Reference      TTTCACCCCACCGATGAAGAGTTAGTAGTTCACTATCTGAAGAAGAAGGCCTCATCTGCT
                        ************************************************************

VviNAC18_Shiraz         CCTCTTCCGGTCGCCATAATCGCAGAGGTTGATCTCTATAAGTTTGATCCCTGGGAACTC
VviNAC18_Reference      CCTCTTCCGGTCGCCATAATCGCAGAGGTTGATCTCTATAAGTTTGATCCCTGGGAACTC
                        ************************************************************

VviNAC18_Shiraz         CCTGCTAAGGCCAGTTTTGGAGAACAGGAATGGTACTTCTTCAGCCCTAGAGACCGGAAG
VviNAC18_Reference      CCCGCTAAGGCCAGTTTTGGAGAACAGGAATGGTACTTCTTCAGCCCTAGAGACCGGAAG
                        ** ************************************************************

VviNAC18_Shiraz         TACCCAAACGGGGCTCGGCCTAATCGAGCGGCGACTTCTGGTTATTGGAAGGCGACGGGG
VviNAC18_Reference      TACCCAAACGGGGCTCGGCCTAATCGAGCGGCGACTTCTGGTTATTGGAAGGCGACGGGG
                        ************************************************************

VviNAC18_Shiraz         ACGGACAAGCCGGTGTTGACCTCTGGGGGTACTCAGAAGGTGGGTGTGAAGAAGGCGCTG
VviNAC18_Reference      ACGGACAAGCCGGTGTTGACCTCTGGGGGTACTCAGAAGGTGGGTGTGAAGAAGGCGCTG
                        ************************************************************

VviNAC18_Shiraz         GTTTTCTATGGAGGGAAGCCCCCAAAGGGGATCAAAACCAATTGGATCATGCATGAGTAC
VviNAC18_Reference      GTTTTCTATGGAGGGAAGCCCCCAAAGGGGATCAAAACCAATTGGATCATGCATGAGTAC
                        ************************************************************

VviNAC18_Shiraz         AGGCTTGCTGACAACAAGGTTAACACAAAGCCTCCCGGATGCGATATGGGCAACAAGAAG
VviNAC18_Reference      AGGCTTGCTGACAACAAGGTTAACACAAAGCCTCCCGGATGCGATATGGGCAACAAGAAG
                        ************************************************************

VviNAC18_Shiraz         AACTCTTTGCGGCTTGATGATTGGGTGCTATGTCGAATCTACAAGAAGAACAACACGCAT
VviNAC18_Reference      AACTCTTTGCGGCTTGATGATTGGGTGCTATGTCGAATCTACAAGAAGAACAACACGCAT
                        ************************************************************

VviNAC18_Shiraz         AGAACGCTGGATCCTGACAAGGATGACTCCATGGACGACATGCTTGGGCCGGTGCCGAGC
VviNAC18_Reference      AGAACGCTGGATCCTGACAAGGATGACTCCATGGACGACATGCTTGGGCCGGTGCCGACC
                        ************************************************************ *

VviNAC18_Shiraz         TCGATCTCGATGGGGCAACAAAGCCTGAAACTGCAGTTCCCAAAGGTCCCAAACTACAGT
VviNAC18_Reference      TCGATCTCGATGGGGCAACAAAGCCTGAAACTGCAGTTCCCAAAGGTCCCAAACTACAGT
                        ************************************************************

VviNAC18_Shiraz         GCATTGCTAGAAAATGAGCAGAGCCTGTTTGAAGGGATGATAAACAGCGATGGTATCAAC
VviNAC18_Reference      GCATTGCTAGAAAATGAGCAGAGCCTGTTTGAAGGGATGATAAACAGCGATGGTATCAAC
                        ************************************************************

VviNAC18_Shiraz         AGTTCCGGCACTATTTCTCAGCTGGCCTGCTCAAGCTCAAAGCCGGATCACCTCTCCTTG
VviNAC18_Reference      AGTTCCGGCACTATTTCTCAGCTGGCCTGCTCAAGCTCAAAGCCGGATCACCTCTCCTTG
                        ************************************************************

VviNAC18_Shiraz         GTTGCAGCCACAACATCAAGCATCCTCCCTCTGAAGCGAACGCTCCCTTCTCTATACTGG
VviNAC18_Reference      GTTGCAGCCACAACATCAAGCATCCTCCCTCTGAAGCGAACGCTCCCTTCTCTATACTGG
                        ************************************************************

VviNAC18_Shiraz         AACGACGATGATACTGCCGGTCCTTCCACGACAAAGAGGTTCCAAGCCGAAAACACAGAT
VviNAC18_Reference      AACGACGATGATACTGCCGGTCCTTCCACGACAAAGAGGTTCCAAGCCGAAAACACAGAT
                        ************************************************************

VviNAC18_Shiraz         GGAAACATTGGTAGAACAACTGATGGGAACAATTCCATCGCCACTCTGCTCAGCCAGCTT
VviNAC18_Reference      GGAAACATTGGTAGAACAACTGATGGGAACAATTCCATCGCCACTCTGCTCAGCCAGCTT
                        ************************************************************

VviNAC18_Shiraz         CCGCAGACTCCTTCATTGCACCAACAGTCAATGCTGGGGTCTCTTGGCGAAGGCGTTTTC
VviNAC18_Reference      CCGCAGACTCCTTCATTGCACCAACAGTCAATGCTGGGGTCTCTTGGCGAAGGCGTTTTC
                        ************************************************************

VviNAC18_Shiraz         CGGCAACCCTTTCAACTCCCTGGCATGAATTGGTATGCTTAG
VviNAC18_Reference      CGGCAACCCTTTCAACTCCCTGGCATGAATTGGTATGCTTAG
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F

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VviNAC26_Shiraz         ATGGATGGAAAAGGCAGCTCTCAACTTCCTCCCGGTTTTAGATTCCACCCCACCGACGAG
VviNAC26_Reference      ATGGATGGAAAAGGCAGCTCTCAACTTCCTCCCGGTTTTAGATTCCACCCCACCGACGAG
                        ************************************************************

VviNAC26_Shiraz         GAACTCATCATGTATTACCTCAAAAACCAAGCCACTTCCAAGCCATGCCCCGTATCCATT
VviNAC26_Reference      GAACTCATCATGTATTACCTCAAAAACCAAGCCACTTCCAAGCCATGCCCCGTATCCATT
                        ************************************************************

VviNAC26_Shiraz         ATCCCCGAAGTGGATATCTACAAATTCGAGCCTTGGGAATTGCCTGAGAAGGCGGAATTT
VviNAC26_Reference      ATCCCCGAAGTGGATATCTACAAATTCGAGCCTTGGGAATTGCCTGAGAAGGCGGAATTT
                        ************************************************************

VviNAC26_Shiraz         GGAGAAAATGAGTGGTATTTCTTTAGCCCGCGTGACCGTAAGTATCCCAATGGGGCTAGA
VviNAC26_Reference      GGAGAAAATGAGTGGTATTTCTTTAGCCCGCGTGACCGTAAGTATCCCAATGGGGCTAGA
                        ************************************************************

VviNAC26_Shiraz         CCCAACCGAGCTACAGTGTCCGGCTACTGGAAAGCCACAGGGACAGACAAGGCAATCTAC
VviNAC26_Reference      CCCAACCGAGCTACAGTGTCCGGCTACTGGAAAGCCACAGGGACAGACAAGGCAATCTAC
                        ************************************************************

VviNAC26_Shiraz         AGTGGGGCTAAGTATGTGGGGGTGAAAAAGGCTCTTGTGTTCTACAAGGGTAGGCCTCCT
VviNAC26_Reference      AGTGGGGCTAAGTATGTGGGGGTGAAAAAGGCTCTTGTGTTCTACAAGGGTAGGCCTCCT
                        ************************************************************

VviNAC26_Shiraz         AAGGGCATTAAGACCGATTGGATTATGCATGAATATCGCCTTAGTGATTCAAGGCCACGC
VviNAC26_Reference      AAGGGCATTAAGACCGATTGGATTATGCATGAATATCGCCTTAGTGATTCAAGGCCACGC
                        ************************************************************

VviNAC26_Shiraz         CCCAAGAAGCACAATGGTTCCATGAGATTGGATGATTGGGTGCTATGTAGGATCTATAAG
VviNAC26_Reference      CCCAAGAAGCACAATGGTTCCATGAGATTGGATGATTGGGTGCTATGTAGGATCTATAAG
                        ************************************************************

VviNAC26_Shiraz         AAGAAGCATGTGGGGAGAATTTTGGAAGAGAAAGAAGAAAATTTAGGTCCCCAAATACCC
VviNAC26_Reference      AAGAAGCATGTGGGGAGAATTTTGGAAGAGAAAGAAGAAAATTTAGGTCCCCAAATACCC
                        ************************************************************

VviNAC26_Shiraz         GTTACAAATTCAGATGATGGCGGTGAGCAGCACCAAGTGAAATTTCCAAGGACTTTTTCC
VviNAC26_Reference      GTTACAAATTCAGATGATGGCGGTGAGCAGCACCAAGTGAAATTTCCAAGGACTTTTTCC
                        ************************************************************

VviNAC26_Shiraz         CTTGCTCATTTATTGGACATGGAATACTTGGGTCCAATTTCACAACTTCTAGGTGACAAT
VviNAC26_Reference      CTTGCTCATTTATTGGACATGGAATACTTGGGTCCAATTTCACAACTTCTAGGTGACAAT
                        ************************************************************

VviNAC26_Shiraz         TCATACCATTCAGCCTTTGATTTCCAAGGCACCATAAGCAATATTGCCGGAACCGACCCC
VviNAC26_Reference      TCATACCATTCAGCCTTTGATTTCCAAGGCACCATAAGCAATATTGCCGGAACCGACCCC
                        ************************************************************

VviNAC26_Shiraz         CCTGGCGTGGACAAATTCGAGTTATTCCAACTGCCATGCCAATACAACGATTCAACCAAG
VviNAC26_Reference      CCTGGCGTGGACAAATTCGAGTTATTCCAACTGCCATGCCAATACAACGATTCAACCAAG
                        ************************************************************

VviNAC26_Shiraz         TTCCAAGTGAATCAGAATCACATTCCGAACCAGCCTCTATTTGTGAACCCAGTGTATGAA
VviNAC26_Reference      TTCCAAGTGAATCAGAATCACATTCCGAACCAGCCTCTATTTGTGAACCCAGTGTATGAA
                        ************************************************************

VviNAC26_Shiraz         TTTCAGTGA
VviNAC26_Reference      TTTCAGTGA
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G

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VviNAC38_Shiraz         ATGATGGGGAAGGGTTCCAAGAGCAATTGCAAGTCGGCGTCCCACAAGATGTTCAAGGAC
VviNAC38_Reference      ATGATGGGGAAGGGTTCCAAGAGCAATTGCAAGTCGGCGTCCCACAAGATGTTCAAGGAC
                        ************************************************************

VviNAC38_Shiraz         AAGGCCAAGAACCGTGTTGATGATCTACAGGGGATGTTCACGGATCTGCAGTCTGCGAGG
VviNAC38_Reference      AAGGCCAAGAACCGTGTTGATGATCTACAGGGGATGTTCACGGATCTGCAGTCTGCGAGG
                        ************************************************************

VviNAC38_Shiraz         AAGGAGAGCCGATCGGTGGATGTCGCGGTCCTTGAGGAGCAGCTTCATCAGATGCTTCGT
VviNAC38_Reference      AAGGAGAGCCGATCGGTGGATGTCGCGGTCCTTGAGGAGCAGCTTCATCAGATGCTTCGT
                        ************************************************************

VviNAC38_Shiraz         GAGTGGAAAGCCGAACTCAACGAACCCTCTCCCGCATCTTCTCTACAAGGCGGTAGTCTG
VviNAC38_Reference      GAGTGGAAAGCCGAACTCAACGAACCCTCTCCCGCATCTTCTCTACAAGGCGGTAGTCTG
                        ************************************************************

VviNAC38_Shiraz         GGGTCGTTTTCCTCGGACATTTGCCGGCTGTTGCAGCTTTGTGAAGAGGAGGACGATGCC
VviNAC38_Reference      GGGTCGTTTTCCTCGGACATTTGCCGGCTGTTGCAGCTTTGTGAAGAGGAGGACGATGCC
                        ************************************************************

VviNAC38_Shiraz         ACTAGTGCGTTAGCTGATGGGGCAGTGCCCAAACCTGAGCCTGATGCTCAAGGCCATCAA
VviNAC38_Reference      ACTAGTGCGTTAGCTGATGGGGCAGTGCCCAAACCTGAGCCTGATGCTCAAGGCCATCAA
                        ************************************************************

VviNAC38_Shiraz         ATTGGAGCTAGTGTGCTTTTCCAAGAGCGCTATAATAAGGGCCCACAGGAGCATGGCTTT
VviNAC38_Reference      ATTGGAGCTAGTGTGCTTTTCCAAGAGCGCTATAATAAGGGCCCACAGGAGCATGGCTTT
                        ************************************************************

VviNAC38_Shiraz         CAATTGGTGGATCAATGCAAAATTTCTCCTTCAGGTGCTCACAATATGGGAGTTCACAAT
VviNAC38_Reference      CAATTGGTGGATCAATGCAAAATTTCTCCTTCAGGTGCTCACAATATGGGAGTTCACAAT
                        ************************************************************

VviNAC38_Shiraz         TTGGAAGGAGCTACTCAGTTGGACTATCGTCAGTTTGATTTGCAACAAGACTTTGAGCAA
VviNAC38_Reference      TTGGAAGGAGCTACTCAGTTGGACTATCGTCAGTTTGATTTGCAACAAGACTTTGAGCAA
                        ************************************************************

VviNAC38_Shiraz         AACTTCTTTGCTGGTTATGATGGGACTGGTCTGTGTGGAGAGGATGCTATGCCTCATATT
VviNAC38_Reference      AACTTCTTTGCTGGTTATGATGGGACTGGTCTGTGTGGAGAGGATGCTATGCCTCATATT
                        ************************************************************

VviNAC38_Shiraz         TCTAGCTTTTTGCCAAGTATTTGCCTTCCACCTTCTGCATTCTTGGGGCCAAAATGTGCA
VviNAC38_Reference      TCTAGCTTTTTGCCAAGTATTTGCCTTCCACCTTCTGCATTCTTGGGGCCAAAATGTGCA
                        ************************************************************

VviNAC38_Shiraz         CTCTGGGATTGCCCCAGGCCAGCTCAAGGGATGGATTGGTGTCAAAACTATTGCAGCAGC
VviNAC38_Reference      CTCTGGGATTGCCCCAGGCCAGCTCAAGGGATGGATTGGTGTCAAAACTATTGCAGCAGC
                        ************************************************************

VviNAC38_Shiraz         TTTCATGCTACTCTGGCATTGAGTGAAGGCCCTCCTGGTATGACCCCAGTTCTACGACCT
VviNAC38_Reference      TTTCATGCTACTCTGGCATTGAGTGAAGGCCCTCCTGGTATGACCCCAGTTCTACGACCT
                        ************************************************************

VviNAC38_Shiraz         GGGGGCATTGGCCTGAAGGATGGTCTGCTTTTTGCTGCTCTTAGTGCAAAGGTGCAAGGA
VviNAC38_Reference      GGGGGCATAGGCCTGAAGGATGGTCTGCTTTTTGCTGCTCTTAGTGCAAAGGTGCAAGGA
                        ******** ***************************************************

VviNAC38_Shiraz         AAAGATGTTGGTATCCCAGAATGTGAAGGGGCTGCAACTGCAAAATCCCCATGGAATGCT
VviNAC38_Reference      AAAGATGTTGGTATCCCAGAATGTGAAGGGGCTGCAACTGCAAAATCCCCATGGAATGCT
                        ************************************************************

VviNAC38_Shiraz         CCTGAGCTATTTGATCTGTCAGTTCTTGAGGGTGAAACAATTAGGGAATGGCTTTTTTTT
VviNAC38_Reference      CCTGAGCTATTTGATCTGTCAGTTCTTGAGGGTGAAACAATTAGGGAATGGCTTTTTTTT
                        ************************************************************

VviNAC38_Shiraz         GACAAGCCTCGAAGAGCATTTGAGAGTGGAAATAGAAAGCAGAGGTCATTGCCAGATTAT
VviNAC38_Reference      GACAAGCCTCGAAGAGCATTTGAGAGTGGAAATAGAAAGCAGAGGTCATTGCCAGATTAT
                        ************************************************************

VviNAC38_Shiraz         AGTGGGCGTGGTTGGCATGAGTCAAGGAAGCAAGTGATGAATGAATATGGGGGACTGAAA
VviNAC38_Reference      AGTGGGCGTGGTTGGCATGAGTCAAGGAAGCAAGTGATGAATGAATATGGGGGACTGAAA
                        ************************************************************

VviNAC38_Shiraz         AGATCTTACTACATGGATCCGCAACCTCTGAACCATTTTGAGTGGCACCTTTATGAATAT
VviNAC38_Reference      AGATCTTACTACATGGATCCGCAACCTCTGAACCATTTTGAGTGGCACCTTTATGAATAT
                        ************************************************************

VviNAC38_Shiraz         GAAATCAGTAAGTGTGATGCTTGTGCCTTGTATAGGTTGGAACTGAAGCTTGTTGATGGG
VviNAC38_Reference      GAAATCAGTAAGTGTGATGCTTGTGCCTTGTATAGGTTGGAACTGAAGCTTGTTGATGGG
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VviNAC38_Shiraz         AAGAAGAGTTCCAAAGCAAAAGCAACAACTGATTCAGTGGCTGATCTGCAGAAGCAGATG
VviNAC38_Reference      AAGAAGA-------------------------------------------------------
                        *******

VviNAC38_Shiraz         GGAAGGCTTACTGCTGAGTTTCCCTTCACCATGATGGGGAAGGGTTCCAAAGCAAAAGCA
VviNAC38_Reference      -------------------------------------------GTTCCAAAGCAAAAGCA
                                                                   *****************

VviNAC38_Shiraz         ACAACTGATTCAGTGGCTGATCTGCAGAAGCAGATGGGAAGGCTTACTGCTGAGTTTCCC
VviNAC38_Reference      ACAACTGATTCAGTGGCTGATCTGCAGAAGCAGATGGGAAGGCTTACTGCTGAGTTTCCC
                        ************************************************************

VviNAC38_Shiraz         TTAGATAACAAGCGCTCTGTTAAAGGAAGGACAAAAATTAATATGAAGGATGGTGTTGGA
VviNAC38_Reference      TTAGATAACAAGCGCTCTGTTAAAGGAAGGACAAAAATTAATATGAAGGATGGTGTTGGA
                        ************************************************************

VviNAC38_Shiraz         GATGTTTATTCTACTCCAAATCGGGTAGGACCTCCAAATCAACAAGGTGATTATGGGGTA
VviNAC38_Reference      GATGTTTATTCTACTCCAAATCGGGTAGGACCTCCAAATCAACAAGGTGATTATGGGGTA
                        ************************************************************

VviNAC38_Shiraz         GGAGGACCTTACGATTATCTTGTGGAGAATTTAGGTGACTATTATTTGACATGA
VviNAC38_Reference      GGAGGACCTTACGATTATCTTGTGGAGAATTTAGGTGACTATTATTTGACATGA
                        ******************************************************
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H

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VviNAC39_Shiraz         ATGATGAGCGGAGATCAGTTGCAGTTGCCGGCTGGTTTCAGATTTCATCCGACGGACGAG
VviNAC39_Reference      ATGATGAGCGGAGATCAGTTGCAGTTGCCGGCTGGTTTCAGATTTCATCCGACGGACGAG
                        ************************************************************

VviNAC39_Shiraz         GAGCTCGTGGTGCATTATCTGGTTCGTAAATGCGCATCACAGAGCATCTCCGTTCCGATT
VviNAC39_Reference      GAGCTCGTGGTGCATTATCTGGTTCGTAAATGCGCATCACAGAGCATCTCCGTTCCGATT
                        ************************************************************

VviNAC39_Shiraz         ATTGCCGAGGTTGATCTCTACAAGTACGATCCATGGCAGCTTCCAGGAATGGCTCTGTAC
VviNAC39_Reference      ATTGCCGAGGTTGATCTCTACAAGTACGATCCATGGCAGCTTCCAGGAATGGCTCTGTAC
                        ************************************************************

VviNAC39_Shiraz         GGTGAAAAGGAGTGGTACTTCTTCTCTCCAAGGGACCGCGAATATCTCAACGGTTCAAGG
VviNAC39_Reference      GGTGAAAAGGAGTGGTACTTCTTCTCTCCAAGGGACCGCAAATATCCCAACGGTTCAAGG
                        ************************************** ****** **************

VviNAC39_Shiraz         CCTAACCGGGCGGCCGGATCCGGCTACTGGAAGGCCACCGGCGCCGATAAGCCCATCGGC
VviNAC39_Reference      CCTAACCGGGCGGCCGGATCCGGCTACTGGAAGGCCACCGGCGCCGATAAGCCCATCGGC
                        ************************************************************

VviNAC39_Shiraz         CGACCCAAGACGGTCGGAATTAAAAAAGCACTCGTATTTTATGCCGGCAAAGCTCCCAGA
VviNAC39_Reference      CGACCCAAGACGGTCGGAATTAAAAAGGCACTCGTATTTTATGCCGGCAAAGCTCCCAGA
                        ************************** *********************************

VviNAC39_Shiraz         GGTGTCAAAACCAATTGGATCATGCACGAGTATCGCCTCGCCAATGTTGATCGATCCGCC
VviNAC39_Reference      GGTGTCAAAACCAATTGGATCATGCACGAGTATCGCCTCGCCAATGTTGATCGATCCGCC
                        ************************************************************

VviNAC39_Shiraz         GGCAAGAAAAACAACTTAAGGCTTGACGATTGGGTCTTATGTCGAATATACAACAAGAAA
VviNAC39_Reference      GGCAAGAAAAACAACTTAAGGCTTGACGATTGGGTCTTATGTCGAATATACAACAAGAAA
                        ************************************************************

VviNAC39_Shiraz         GGCAGCGCCGAGAAACAGCACACCTTTGATCATAAGTCAATGAAATATCCAGAACTTGAA
VviNAC39_Reference      GGCAGCGCCGAGAAACAGCACACCTTTGATCAAAAGTCAATGAAATATCCAGAACTTGAA
                        ********************************* ****************************

VviNAC39_Shiraz         GACCAGAAGCCAAAAATAATATCAACGGTTCAAGATAAAGCAGTACCCTCTTCATTGCCG
VviNAC39_Reference      GACCAGAAGCCAAAAATAATATCAACGGTTCAAAATAAAGTAGTACCCTCTTCATTGCCG
                        ********************************* ****** ********************

VviNAC39_Shiraz         TCCCTACCTCCGATGCCTGCGCCGCCAACAACCAATGACTACCTATACTTCGAGACATCT
VviNAC39_Reference      TCCCTACCTCCAATGCCTGCGCCGCCAACAACCAATGACTACCTATACTTCGAGACATCT
                        *********** ************************************************

VviNAC39_Shiraz         GATTCAGTGCCGAGGTTACACACTCACACAGATTCTAGTGGTTCCGAACAAGTAATGTCA
VviNAC39_Reference      GATTCAGTGCCGAGGTTACACACTCACACAGATTCTAGTGGATCCGAACAAGTAATGTCA
                        ****************************************** ******************

VviNAC39_Shiraz         CCGGAGAAGGAGGTTCAAAGCGAGCCTAAGTGGAATGACTTTGATTTCAGCCTCTATATG
VviNAC39_Reference      CCGGAGAAGGAGGTTCAAAGCGAGCCTAAGTGGAATGACTTTGATTTTAGCCTCTATATG
                        ************************************************ ************

VviNAC39_Shiraz         GATGGTTTTGCCAACGACCCTTTTGCCTCTCAAGCACAGTTTTCGGGAGACTTGTTAACA
VviNAC39_Reference      GATGGTTTTGCCAACGACCCTTTTGCCTCTCAAGCACAGTTTTCGGGAGACTTATTAACA
                        ************************************************** ******

VviNAC39_Shiraz         TCGTCGTGGCAGGACATGTTGATGTTCTCGAACAAGTCATTTTGA
VviNAC39_Reference      TCGTCGTGGCAGGACATGTTGATGTTCTCGAACAAGTCATTTTGA
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I

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VviNAC61_Shiraz         ATGGAAGAGGCTTCACTTGCCGATCAAGGTGATGACCTTATGGATTTGCCCCCAGGTTTT
VviNAC61_Reference      ATGGAAGAGGCTTCACTTGCCGATCAAGGTGATGACCTTATGGATTTGCCCCCAGGTTTT
                        ************************************************************

VviNAC61_Shiraz         AGATTCCACCCTACAGATGAAGAGATCATATCTTATTATCTCACGGAGAGGGTGATGAAT
VviNAC61_Reference      AGATTCCACCCTACAGATGAAGAGATCATATCTTATTATCTCACGGAGAGGGTGATGAAT
                        ************************************************************

VviNAC61_Shiraz         AGTAGCTTCAGTGCAAGAGCTATTGGAGAAGTTGACCTTAACAAGTGTGAACCCTGGGAT
VviNAC61_Reference      AGTAGCTTCAGTGCAAGAGCTATTGGAGAAGTTGACCTTAACAAGTGTGAACCCTGGGAT
                        ************************************************************

VviNAC61_Shiraz         TTACCTAAGAAAGCAAAGATGGGAGAGAAGGAATGGTATTTCTTTTGCCAGAGAGATAGG
VviNAC61_Reference      TTACCTAAGAAAGCAAAGATGGGAGAGAAGGAATGGTATTTCTTTTGCCAGAGAGATAGG
                        ************************************************************

VviNAC61_Shiraz         AAGTATCCTACAGGCATGAGAACGAATCGAGCTACCGAATCTGGATACTGGAAGGCCACT
VviNAC61_Reference      AAGTATCCTACAGGCATGAGAACGAATCGAGCTACCGAATCTGGATACTGGAAGGCCACT
                        ************************************************************

VviNAC61_Shiraz         GGAAAGGATAAAGAGATTTACAAGGGGAAGGGTTGTCTTGTTGGGATGAAGAAGACCCTT
VviNAC61_Reference      GGAAAGGATAAAGAGATTTACAAGGGGAAGGGTTGTCTTGTTGGGATGAAGAAGACCCTT
                        ************************************************************

VviNAC61_Shiraz         GTTTTCTACCGAGGGAGAGCCCCCAAAGGAGAGAAAAGCAATTGGGTCATGCACGAATAC
VviNAC61_Reference      GTTTTCTACCGAGGGAGAGCCCCCAAAGGAGAGAAAAGCAATTGGGTCATGCACGAATAC
                        ************************************************************

VviNAC61_Shiraz         AGACTGGAAGGCAAATTCTCATATTACAACCTCCCCAAAGCCGCAAAGGATGAATGGGTC
VviNAC61_Reference      AGACTGGAAGGCAAATTCTCATATTACAACCTCCCCAAAGCCGCAAAGGATGAATGGGTC
                        ************************************************************

VviNAC61_Shiraz         GTCTGTAGGGTATTCCACAAGAGCGCGGGGATCAAACGAAGTCTTATTCCTGCGCCGATA
VviNAC61_Reference      GTCTGTAGGGTATTCCACAAGAGCGCGGGGATCAAACGAAGTCTTATTCCTGCGCCGATA
                        ************************************************************

VviNAC61_Shiraz         AGGATGAACTCTTTTGGGGATGATCTCTTGGACTGTTCTTCTCTACCACCTCTTATTGAC
VviNAC61_Reference      AGGATGAACTCTTTTGGGGATGATCTCTTGGACTGTTCTTCTCTACCACCTCTTATTGAC
                        ************************************************************

VviNAC61_Shiraz         CCTCCTTATTCCAACCCCAACAAAAATGATTCCTGCTTTACAAATGGTGAAGATGAATTC
VviNAC61_Reference      CCTCCTTATTCCAACCCCAACAAAAATGATTCCTGCTTTACAAATGGTGAAGATGAATTC
                        ************************************************************

VviNAC61_Shiraz         AAGGGATCATCAACCAGATTTTCAGATGGAAACCACCACTACCCTTACTTCTCCCCCACC
VviNAC61_Reference      AAGGGATCATCAACCAGATTTTCAGATGGAAACCACCACTACCCTTACTTCTCCCCCACC
                        ************************************************************

VviNAC61_Shiraz         ATAAACGGTCATCAACTGCAAAAGCAACAAGATCAGAAGAGCTTTCTTCTATCCCCCAAT
VviNAC61_Reference      ATAAACGGTCATCAACTGCAAAAGCAACAAGATCAGAAGAGCTTTCTTCTATCCCCCAAT
                        ************************************************************

VviNAC61_Shiraz         AACGTGCACACCACCTCCAACTACCAAGCCACCCTAAGCAACCCTACACCAAACTCCATT
VviNAC61_Reference      AACGTGCACACCACCTCCAACTACCAAGCCACCCTAAGCAACCCTACACCAAACTCCATT
                        ************************************************************

VviNAC61_Shiraz         TTCTACCCCCAAGTTTCTCCCTCGAATCCTCTTTTCCCCTTCCAAGCATCCCCAACTCCT
VviNAC61_Reference      TTCTACCCCCAAGTTTCTCCCTCGAATCCTCTTTTCCCCTTCCAAGCATCCCCAACTCCT
                        ************************************************************

VviNAC61_Shiraz         AGTTATTCACACTGGCAGATGGGTAGTTCATTTCCAAGCTTTCCGGGCTCTGGCTTCAAA
VviNAC61_Reference      AGTTATTCACACTGGCAGATGGGTAGTTCATTTCCAAGCTTTCCGGGCTCTGGCTTCAAA
                        ************************************************************

VviNAC61_Shiraz         GGTAGTGATCCGACCAGTACCGTAAGAGCCCAGACATCTGGCGTACAGAGGCAATGCAAG
VviNAC61_Reference      GGTAGTGATCCGACCAGTACCGTAAGAGCCCAGACATCTGGCGTACAGAGGCAATGCAAG
                        ************************************************************

VviNAC61_Shiraz         GTGGAGCAGTTCTCATCCAACCAGTCTATGGTCAGCCTCTCGCAGGACACCGGACTCAGC
VviNAC61_Reference      GTGGAGCAGTTCTCATCCAACCAGTCTATGGTCAGCCTCTCGCAGGACACCGGACTCAGC
                        ************************************************************

VviNAC61_Shiraz         ACTGACATGAACACGGAGATATCCTCTGTGCTTTCAAAGCATCAAGACACAGGAAACAAC
VviNAC61_Reference      ACTGACATGAACACGGAGATATCCTCTGTGCTTTCAAAGCATCAAGACACAGGAAACAAC
                        ************************************************************

VviNAC61_Shiraz         AGGTCTTACGAAGATCTTCAAGGTCCATCGGTCGGCCAAATCGGAGATTTGGATTATTTA
VviNAC61_Reference      AGGTCTTACGAAGATCTTCAAGGTCCATCGGTCGGCCAAATCGGAGATTTGGATTATTTA
                        ************************************************************

VviNAC61_Shiraz         CTCGACTACTGA
VviNAC61_Reference      CTCGACTACTGA
                        ************
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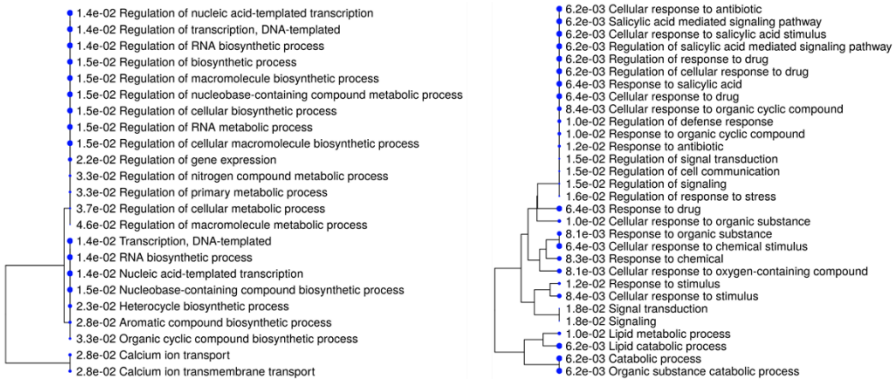
Appendix C: GO enrichment analysis tree of the A) *VviNAC01*, B) *VviNAC08*, C) *VviNAC15*, D) *VviNAC17*, E) *VviNAC18*, F) *VviNAC26*, G) *VviNAC39* and H) *VviNAC61* over expression DEGs, divided into up-regulated (on the left) and down- regulated (on the right).

A



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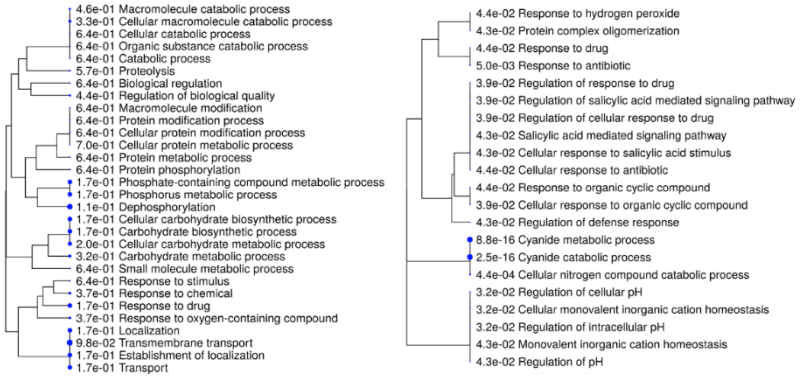

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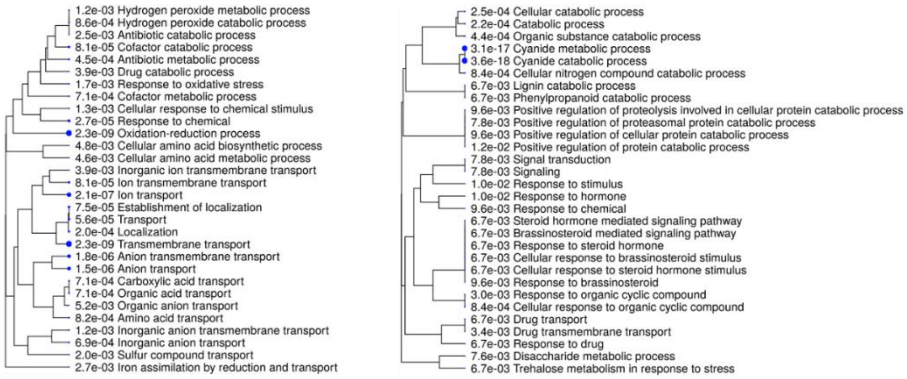

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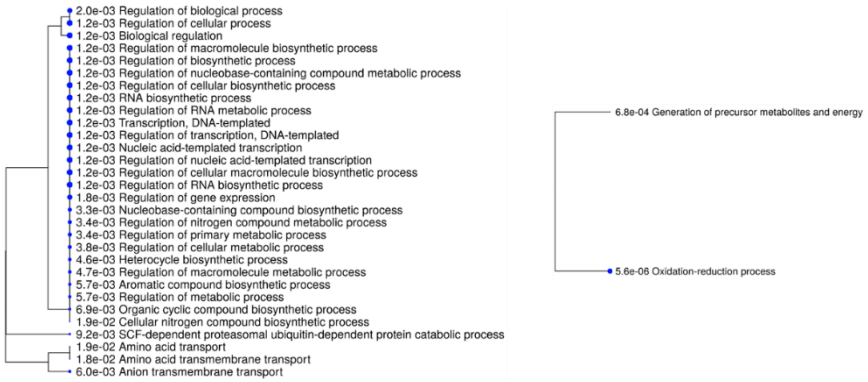
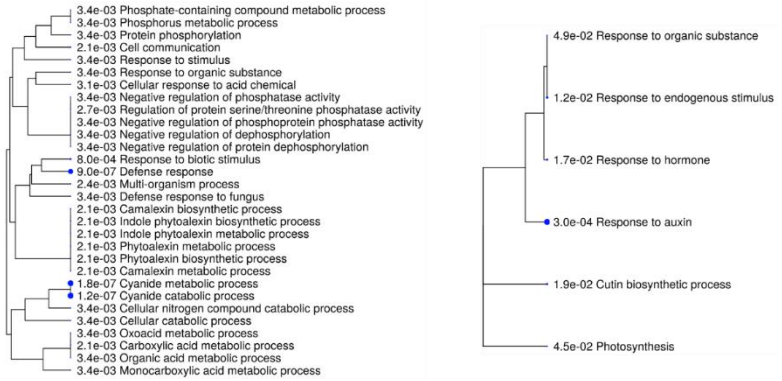
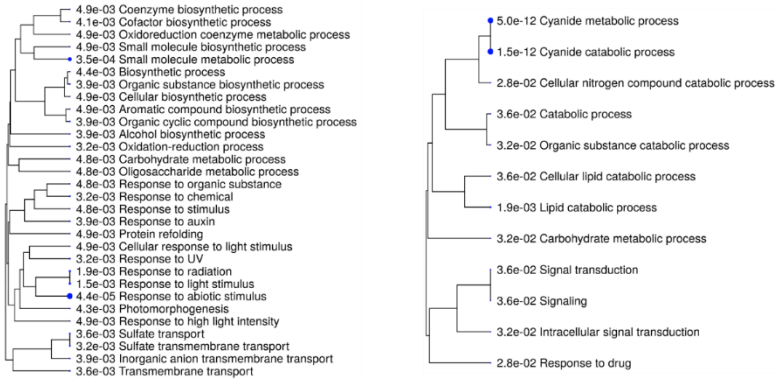

2.0e-03 Regulation of biological process
1.2e-03 Regulation of cellular process
1.2e-03 Biological regulation
1.2e-03 Regulation of macromolecule biosynthetic process
1.2e-03 Regulation of biosynthetic process
1.2e-03 Regulation of nucleobase-containing compound metabolic process
1.2e-03 Regulation of cellular biosynthetic process
1.2e-03 RNA biosynthetic process
1.2e-03 Regulation of RNA metabolic process
1.2e-03 Transcription, DNA-templated
1.2e-03 Regulation of transcription, DNA-templated
1.2e-03 Nucleic acid-templated transcription
1.2e-03 Regulation of nucleic acid-templated transcription
1.2e-03 Regulation of cellular macromolecule biosynthetic process
1.2e-03 Regulation of RNA biosynthetic process
1.8e-03 Regulation of gene expression
3.3e-03 Nucleobase-containing compound biosynthetic process
3.4e-03 Regulation of nitrogen compound metabolic process
3.4e-03 Regulation of primary metabolic process
3.8e-03 Regulation of cellular metabolic process
4.6e-03 Heterocycle biosynthetic process
4.7e-03 Regulation of macromolecule metabolic process
5.7e-03 Aromatic compound biosynthetic process
5.7e-03 Regulation of metabolic process
6.9e-03 Organic cyclic compound biosynthetic process
1.9e-02 Cellular nitrogen compound biosynthetic process
9.2e-03 SCF-dependent proteasomal ubiquitin-dependent protein catabolic process
1.9e-02 Amino acid transport
1.8e-02 Amino acid transmembrane transport
6.0e-03 Anion transmembrane transport
6.8e-04 Generation of precursor metabolites and energy
5.6e-06 Oxidation-reduction process

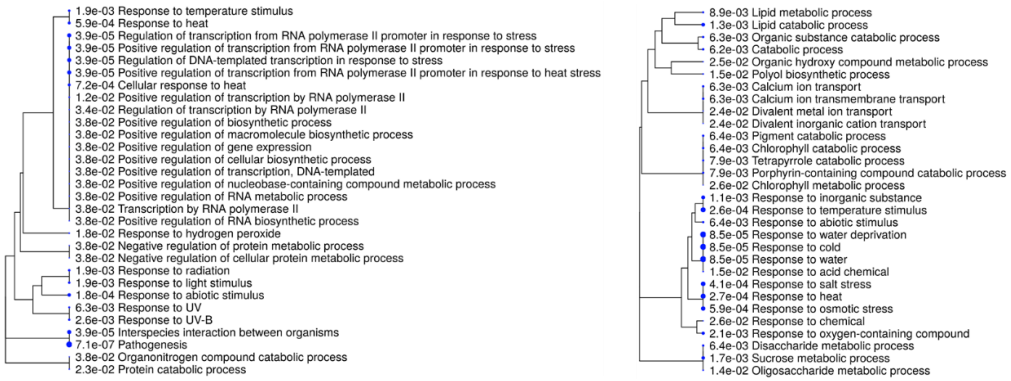
E


3.4e-03 Phosphate-containing compound metabolic process
3.4e-03 Phosphorus metabolic process
3.4e-03 Protein phosphorylation
2.1e-03 Cell communication
3.4e-03 Response to stimulus
3.4e-03 Response to organic substance
3.1e-03 Cellular response to acid chemical
3.4e-03 Negative regulation of phosphatase activity
2.7e-03 Regulation of protein serine/threonine phosphatase activity
3.4e-03 Negative regulation of phosphoprotein phosphatase activity
3.4e-03 Negative regulation of dephosphorylation
3.4e-03 Negative regulation of protein dephosphorylation
8.0e-04 Response to biotic stimulus
9.0e-07 Defense response
2.4e-03 Multi-organism process
3.4e-03 Defense response to fungus
2.1e-03 Camalexin biosynthetic process
2.1e-03 Indole phytoalexin biosynthetic process
2.1e-03 Indole phytoalexin metabolic process
2.1e-03 Phytoalexin metabolic process
2.1e-03 Phytoalexin biosynthetic process
2.1e-03 Camalexin metabolic process
1.8e-07 Cyanide metabolic process
1.2e-07 Cyanide catabolic process
3.4e-03 Cellular nitrogen compound catabolic process
3.4e-03 Cellular catabolic process
3.4e-03 Oxoacid metabolic process
2.1e-03 Carboxylic acid metabolic process
3.4e-03 Organic acid metabolic process
3.4e-03 Monocarboxylic acid metabolic process
4.9e-02 Response to organic substance
1.2e-02 Response to endogenous stimulus
1.7e-02 Response to hormone
3.0e-04 Response to auxin
1.9e-02 Cutin biosynthetic process
4.5e-02 Photosynthesis

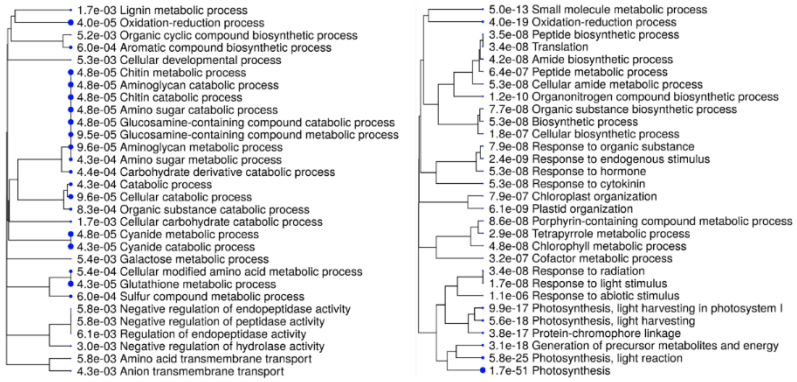
F


4.9e-03 Coenzyme biosynthetic process
4.1e-03 Cofactor biosynthetic process
4.9e-03 Oxidoreduction coenzyme metabolic process
4.9e-03 Small molecule biosynthetic process
3.5e-04 Small molecule metabolic process
4.4e-03 Biosynthetic process
3.9e-03 Organic substance biosynthetic process
4.9e-03 Cellular biosynthetic process
4.9e-03 Aromatic compound biosynthetic process
3.9e-03 Organic cyclic compound biosynthetic process
3.9e-03 Alcohol biosynthetic process
3.2e-03 Oxidation-reduction process
4.8e-03 Carbohydrate metabolic process
4.8e-03 Oligosaccharide metabolic process
4.8e-03 Response to organic substance
3.2e-03 Response to chemical
4.8e-03 Response to stimulus
3.9e-03 Response to auxin
4.9e-03 Protein refolding
4.9e-03 Cellular response to light stimulus
3.2e-03 Response to UV
1.9e-03 Response to radiation
1.5e-03 Response to light stimulus
4.4e-05 Response to abiotic stimulus
4.3e-03 Photomorphogenesis
4.9e-03 Response to high light intensity
3.6e-03 Sulfate transport
3.2e-03 Sulfate transmembrane transport
3.9e-03 Inorganic anion transmembrane transport
3.6e-03 Transmembrane transport
5.0e-12 Cyanide metabolic process
1.5e-12 Cyanide catabolic process
2.8e-02 Cellular nitrogen compound catabolic process
3.6e-02 Catabolic process
3.2e-02 Organic substance catabolic process
3.6e-02 Cellular lipid catabolic process
1.9e-03 Lipid catabolic process
3.2e-02 Carbohydrate metabolic process
3.6e-02 Signal transduction
3.6e-02 Signaling
3.2e-02 Intracellular signal transduction
2.8e-02 Response to drug

G


1.9e-03 Response to temperature stimulus
5.9e-04 Response to heat
3.9e-05 Regulation of transcription from RNA polymerase II promoter in response to stress
3.9e-05 Positive regulation of transcription from RNA polymerase II promoter in response to stress
3.9e-05 Regulation of DNA-templated transcription in response to stress
3.9e-05 Positive regulation of transcription from RNA polymerase II promoter in response to heat stress
7.2e-04 Cellular response to heat
1.2e-02 Positive regulation of transcription by RNA polymerase II
3.4e-02 Regulation of transcription by RNA polymerase II
3.8e-02 Positive regulation of biosynthetic process
3.8e-02 Positive regulation of macromolecule biosynthetic process
3.8e-02 Positive regulation of gene expression
3.8e-02 Positive regulation of cellular biosynthetic process
3.8e-02 Positive regulation of transcription, DNA-templated
3.8e-02 Positive regulation of nucleobase-containing compound metabolic process
3.8e-02 Positive regulation of RNA metabolic process
3.8e-02 Transcription by RNA polymerase II
3.8e-02 Positive regulation of RNA biosynthetic process
1.8e-02 Response to hydrogen peroxide
3.8e-02 Negative regulation of protein metabolic process
3.8e-02 Negative regulation of cellular protein metabolic process
1.9e-03 Response to radiation
1.9e-03 Response to light stimulus
1.8e-04 Response to abiotic stimulus
6.3e-03 Response to UV
2.6e-03 Response to UV-B
3.9e-05 Interspecies interaction between organisms
7.1e-07 Pathogenesis
3.8e-02 Organonitrogen compound catabolic process
2.3e-02 Protein catabolic process
8.9e-03 Lipid metabolic process
1.3e-03 Lipid catabolic process
6.3e-03 Organic substance catabolic process
6.2e-03 Catabolic process
2.5e-02 Organic hydroxy compound metabolic process
1.5e-02 Polyol biosynthetic process
6.3e-03 Calcium ion transport
6.3e-03 Calcium ion transmembrane transport
2.4e-02 Divalent metal ion transport
2.4e-02 Divalent inorganic cation transport
6.4e-03 Pigment catabolic process
6.4e-03 Chlorophyll catabolic process
7.9e-03 Tetrapyrrole catabolic process
7.9e-03 Porphyrin-containing compound catabolic process
2.6e-02 Chlorophyll metabolic process
1.1e-03 Response to inorganic substance
2.6e-04 Response to temperature stimulus
6.4e-03 Response to abiotic stimulus
8.5e-05 Response to water deprivation
8.5e-05 Response to cold
8.5e-05 Response to water
1.5e-02 Response to acid chemical
4.1e-04 Response to salt stress
2.7e-04 Response to heat
5.9e-04 Response to osmotic stress
2.6e-02 Response to chemical
2.1e-03 Response to oxygen-containing compound
6.4e-03 Disaccharide metabolic process
1.7e-03 Sucrose metabolic process
1.4e-02 Oligosaccharide metabolic process

H


1.7e-03 Lignin metabolic process
4.0e-05 Oxidation-reduction process
5.2e-03 Organic cyclic compound biosynthetic process
6.0e-04 Aromatic compound biosynthetic process
5.3e-03 Cellular developmental process
4.8e-05 Chitin metabolic process
4.8e-05 Aminoglycan catabolic process
4.8e-05 Chitin catabolic process
4.8e-05 Amino sugar catabolic process
4.8e-05 Glucosamine-containing compound catabolic process
9.5e-05 Glucosamine-containing compound metabolic process
9.6e-05 Aminoglycan metabolic process
4.3e-04 Amino sugar metabolic process
4.4e-04 Carbohydrate derivative catabolic process
4.3e-04 Catabolic process
9.6e-05 Cellular catabolic process
8.3e-04 Organic substance catabolic process
1.7e-03 Cellular carbohydrate catabolic process
4.8e-05 Cyanide metabolic process
4.3e-05 Cyanide catabolic process
5.4e-03 Galactose metabolic process
5.4e-04 Cellular modified amino acid metabolic process
4.3e-05 Glutathione metabolic process
6.0e-04 Sulfur compound metabolic process
5.8e-03 Negative regulation of endopeptidase activity
5.8e-03 Negative regulation of peptidase activity
6.1e-03 Regulation of endopeptidase activity
3.0e-03 Negative regulation of hydrolase activity
5.8e-03 Amino acid transmembrane transport
4.3e-03 Anion transmembrane transport
5.0e-13 Small molecule metabolic process
4.0e-19 Oxidation-reduction process
3.5e-08 Peptide biosynthetic process
3.4e-08 Translation
4.2e-08 Amide biosynthetic process
6.4e-07 Peptide metabolic process
5.3e-08 Cellular amide metabolic process
1.2e-10 Organonitrogen compound biosynthetic process
7.7e-08 Organic substance biosynthetic process
5.3e-08 Biosynthetic process
1.8e-07 Cellular biosynthetic process
7.9e-08 Response to organic substance
2.4e-09 Response to endogenous stimulus
5.3e-08 Response to hormone
5.3e-08 Response to cytokinin
7.9e-07 Chloroplast organization
6.1e-09 Plastid organization
8.6e-08 Porphyrin-containing compound metabolic process
2.9e-08 Tetrapyrrole metabolic process
4.8e-08 Chlorophyll metabolic process
3.2e-07 Cofactor metabolic process
3.4e-08 Response to radiation
1.7e-08 Response to light stimulus
1.1e-06 Response to abiotic stimulus
9.9e-17 Photosynthesis, light harvesting in photosystem I
5.6e-18 Photosynthesis, light harvesting
3.8e-17 Protein-chromophore linkage
3.1e-18 Generation of precursor metabolites and energy
5.8e-25 Photosynthesis, light reaction
1.7e-51 Photosynthesis

Appendix D: Alignments of A) *VviNAC01*, B) *VviNAC05*, C) *VviNAC08*, D) *VviNAC34*, E) *VviNAC37* and F) *VviNAC61* promoters nucleotide sequences from Shiraz and Pinot Noir cultivars.

A

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PromoterVviNAC01_Pinot      CTGTTGAGTGGGAACAGCAAGTAATCTGAAGGACCCTCGTGACCGTTTTCTGGCAGCGCA
PromoterVviNAC01_Shiraz     CTGTTGAGTGGGAACAGCAAGTAATCTGAAGGACCCTCGTGACCGTTTTCTGGCAGCGCA
                            ************************************************************

PromoterVviNAC01_Pinot      GCATAGAGGCAAAAATATTTCTGTTCCCTCATGCTCAGCATCCACAAAAAAGGAAAAAAG
PromoterVviNAC01_Shiraz     GCATAGAGGCAAAAATATTTCTGTTCCCTCATGCTCAGCATCCACAAAAAAGGAAAAAAG
                            ************************************************************

PromoterVviNAC01_Pinot      GAAAACCAAAAAAAAAAAAAAAAAAGAAAAACCCTAGGGGTAATCATGTACTTTCACATCT
PromoterVviNAC01_Shiraz     GAAAACCAAAAAAAAAAAAAAAAA--AAAAACCCTAGGGGTAATCATGTACTTTCACATCT
                            ********************** ************************************

PromoterVviNAC01_Pinot      CAATTAAGGCACTTTGTGCCCTCCAGCTGTGAGCATGACATAAGGTGACTTCACCCTTAC
PromoterVviNAC01_Shiraz     CAATTAAGGCACTTTGTGCCCTCCAGCTGTGAGCATGACATAAGGTGACTTAACCCTTAC
                            ************************************************* ********

PromoterVviNAC01_Pinot      GAGACTGCGAGGGTGGCAATTTTCTTCGAAATGACTAAAACACCCTTCATTAGGATCCTT
PromoterVviNAC01_Shiraz     GAGACTGCGAGGGTGGCAATTTTCTTCGAAATGACTAAAACACCCTTCATTAGGATCCTT
                            ************************************************************

PromoterVviNAC01_Pinot      GATGACAGAATCGCCCATCCCACCGTCCACAAATTAGACCACATCGCGATTTTATTGGGT
PromoterVviNAC01_Shiraz     GATGACAGAATCGCCCATCCCACCGTCCACAAATTAGACCACATCGCGATTTTATTGGGT
                            ************************************************************

PromoterVviNAC01_Pinot      ACGCACGGTGAGGGAAGAAAGGGTGTTTTGGTCATTTTACAAACCCGACAGGGGATTCGA
PromoterVviNAC01_Shiraz     ACGCACGGTGAGGGAAGAAAGGGTGTTTTGGTCATTTTACAAACCCGACAGGGGATTCGA
                            ************************************************************

PromoterVviNAC01_Pinot      AGGTCCTTTTAGCAGCTTCGGTTATTCCCACACACAAAGGTCGAACACAACTAAGAAACG
PromoterVviNAC01_Shiraz     AGGTCCTTTTAGCAGCTTCGGTTATTCCCACACACAAAGGTCGAACACAACTAAGAAACG
                            ************************************************************

PromoterVviNAC01_Pinot      TTAACTATTTGGAAAGCGGCCGTAATTAATTAATCTCTCTAAACCTTTTGTTAAAATCAT
PromoterVviNAC01_Shiraz     TTAACTATTTGGAAAGCGGCCGTAATTAATTAATCTCTCTAAACCTTTTGTTAAAATCAT
                            ************************************************************

PromoterVviNAC01_Pinot      GATCCGAGGAGAAAATTTGGAAAAGAAGGAAAAGAAAAAAAAGAAAGAGTTTGAGTCTTG
PromoterVviNAC01_Shiraz     GATCCGAGGAGAAAATTTGGAAAAGAAGGAAAAGAAAAAAAAGAAAGAGTTTGAGTCTTG
                            ************************************************************

PromoterVviNAC01_Pinot      TTTACGCCAAAGGGGTCGTCGTATTCTATTCTCCTCCTCTGCGAGACCTTGTCTCGCCGC
PromoterVviNAC01_Shiraz     TTTACGCCAAAGGGGTCGTCGTATTCTATTCTCCTCCTCTGCGAGACCTTGTCTCGCCGC
                            ************************************************************

PromoterVviNAC01_Pinot      ACCATTTATATAAAGAACCCGCACTTTATCTCTCAACCCACACCCCACCTTTCGCCTTTT
PromoterVviNAC01_Shiraz     ACCATTTATATAAAGAACCCGCACTTTATCTCTCAACCCACACCCCACCTTTCGCCTTTT
                            ************************************************************

PromoterVviNAC01_Pinot      CTTTTCTTTCTTTCTTTCTTTACACCAGTCCCCCTCCCTTCCTTTTTCCTCCCTCTCTTT
PromoterVviNAC01_Shiraz     CT----TTTCTTTCTTTCTTTACACCAGTCCCCCTCCCTTCCTTTTTCCTCCCTCTCTTT
                            **    ************************************************************

PromoterVviNAC01_Pinot      TCTTATTCTCTTACTTTGCTGGATTATAAGTACTACCACATATGTCTTCCTTCGAATTGG
PromoterVviNAC01_Shiraz     TCTTATTCTCTTATTTGGCTGGATTATAAGTACTACCACATATGTCTTCCTTCGAATTGG
                            ************************************************************

PromoterVviNAC01_Pinot      ACCTCAATCAGTTATCATTTCTCAACTAGTCTCTTCTTCTTCTCCTTCAGCTTCAGCTCT
PromoterVviNAC01_Shiraz     ACCTCAATCAGTTATCATTTCTCAACTAGTCTCTTCTTCTTCTCCTTCAGCTTCAGCTCT
                            ************************************************************

PromoterVviNAC01_Pinot      AGAGTTGATAGGATTTTTTTAGCG
PromoterVviNAC01_Shiraz     AGAGTTGATAGGATTTTTTTAGCG
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B

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PromoterVviNAC05_Pinot      TTGCTTTTGCTATCCGTCCTACAATTTTATTTTATTTTTTAATTTTTTTAATTTAAATGC
PromoterVviNAC05_Shiraz     TTGCTTTTGCTATCCGTCCTACAATTTTATTTTATTTTTTAATTTTTTTAATTTAAATGC
                            ************************************************************

PromoterVviNAC05_Pinot      ATATCAAAATGACTCAATTACCCTCATCTTAGTTGTATTTACCTCCACCCAATCATTCTA
PromoterVviNAC05_Shiraz     ATATCAAAATGACTCAATTACCCTCATCTTAGTTGTATTTACCTCCACCCAATCATTCTA
                            ************************************************************

PromoterVviNAC05_Pinot      ATTATTTTAAAAATAAAATAAAATAAAAGAATACGAAGAATTAAAAAATATATATATATG
PromoterVviNAC05_Shiraz     ATTATTTTAAAAATAAAATAAAATAAAAGAATACGAAGAATTAAAAAATATATATATATG
                            ************************************************************

PromoterVviNAC05_Pinot      GTATTAATGGTAATATGATTAATCTTTCAACACCATATTTTAAAAACTTGTTTGACTTAT
PromoterVviNAC05_Shiraz     GTATTAATGGTAATATGATTAATCTTTCAACACCATATTTTAAAAACTTGTTTGACTTAT
                            ************************************************************

PromoterVviNAC05_Pinot      TTTATTCAAAATTAAGTTCTAAACAGTCAAATATGATTAAATTAGTATAATTATAATAAA
PromoterVviNAC05_Shiraz     TTTATTCAAAATTAAGTTCTAAACAGTCAAATATGATTAAATTAGTATAATTATAATAAA
                            ************************************************************

PromoterVviNAC05_Pinot      GTTAATCAACATAATTATTAAATGTATTAATATGAATTAAAGTTGTATTGATAAGTTGAT
PromoterVviNAC05_Shiraz     GTTAATCAACATAATTATTAAATGTATTAATATGAATTAAAGTTGTATTGATAAGTTGAT
                            ************************************************************

PromoterVviNAC05_Pinot      TAAAAAATAATCTATTCCTTAAACAATTATGTAATTTGACATAAATTTGATATAAACAAG
PromoterVviNAC05_Shiraz     TAAAAAATAATCTATTCCTTAAACAATTATGTAATTTGACATAAATTTGATATAAACAAG
                            ************************************************************

PromoterVviNAC05_Pinot      GATAAAAAAACACATTAGATTCGAGTAAAAATATCATATTTTCTCACCATTATGTGATGT
PromoterVviNAC05_Shiraz     GATAAAAAAACACATTAGATTCGAGTAAAAATATCATATTTTCTCACCATTATGTGATGT
                            ************************************************************

PromoterVviNAC05_Pinot      TAAAAATGGCATGTCAAGCACAATGAAAAATTTGGGAATAAAGTTGCAAAATACGCAATT
PromoterVviNAC05_Shiraz     TAAAAATGGCATGTCAAGCACAATGAAAAATTTGGGAATAAAGTTGCAAAATACGCAATT
                            ************************************************************

PromoterVviNAC05_Pinot      GGGTCAAATTACTTGGTTACCCTCAACAGAGGGGCACTTGACTTAAATCCAAACGTTTGG
PromoterVviNAC05_Shiraz     GGGTCAAATTACTTGGTTACCCTCAACAGAGGGGCACTTGACTTAAATCCAAACGTTTGG
                            ************************************************************

PromoterVviNAC05_Pinot      AAATTGAGTTTTTTCATGGGTAGTTGGATTTAGGTCGGGGAATTAGAAAATAACGGGAGA
PromoterVviNAC05_Shiraz     AAATTGAGTTTTTTCATGGGTAGTTGGATTTAGGTCGGGGAATTAGAAAATAACGGGAGA
                            ************************************************************

PromoterVviNAC05_Pinot      TTTATTGAACTTGTGAATTGCGGCTTAAATGACTCAATACTTATGGGGTTGATGCCCATA
PromoterVviNAC05_Shiraz     TTTATTGAACTTGTGAATTGCGGCTTAAATGACTCAATACTTATGGGGTTGATGCCCATA
                            ************************************************************

PromoterVviNAC05_Pinot      GTTTGGAAGGAGGATAGAGATGTCCCAAGCAAAAGTGTGACAAGCATTTCGTATGTGGAT
PromoterVviNAC05_Shiraz     GTTTGGAAGGAGGATAGAGATGTCCCAAGCAAAAGTGTGACAAGCATTTCGTATGTGGAT
                            ************************************************************

PromoterVviNAC05_Pinot      GGTTTATTTATTTAAGTCCCATGAAAGTCGTCTGAAAACTTAGGATAAGTTTCATAAAAA
PromoterVviNAC05_Shiraz     GGTTTATTTATTTAAGTCCCATGAAAGTCGTCTGAAAACTTAGGATAAGTTTCATAAAAA
                            ************************************************************

PromoterVviNAC05_Pinot      GGAGAGTCATATTGTATTTTAGCTTAAAGTGATCGGAAAAAGAATAGTGACCTTTTCTGA
PromoterVviNAC05_Shiraz     GGAGAGTCATATTGTATTTTAGCTTAAAGTGATCGGAAAAAGAATAGTGACCTTTTCTGA
                            ************************************************************

PromoterVviNAC05_Pinot      TTTGTTTTTTACCTGGAAATTAGCTGAAAGATCAAAGGTGCATGTTTAGCTTGTGTTTGC
PromoterVviNAC05_Shiraz     TTTGTTTTTTACCTGGAAATTAGCTGAAAGATCAAAGGTGCATGTTTAGCTTGTGTTTGC
                            ************************************************************

PromoterVviNAC05_Pinot      AAGGTGCTCAACTTCCTTCCACACCCAAGGAGATAAATGCCTCTCAACTACAAGTCACTT
PromoterVviNAC05_Shiraz     AAGGTGCTCAACTTCCTTCCACACCCAAGGAGATAAATGCCTCTCAACTACAAGTCACTT
                            ************************************************************

PromoterVviNAC05_Pinot      TATTCCATGTACACCTCCTTTGCAAGAAGGTCAAACCACATTTAATTTGTGAGGACCCCA
PromoterVviNAC05_Shiraz     TATTCCATGTACACCTCCTTTGCAAGAAGGTCAAACCACATTTAATTTGTGAGGACCCCA
                            ************************************************************
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PromoterVviNAC05_Pinot       AAAGATTAACTTGGTGAAAAAAATGAGATGATTTGATTGGTCTTGACCTATTAAACTATT
PromoterVviNAC05_Shiraz      AAAGATTAACTTGGTGAAAAAAATGAGATGATTTGATTGGTCTTGACCTATTAAACTATT
                             ************************************************************

PromoterVviNAC05_Pinot       CAAGATTTATGAGGTTTTGGGCCCTCCAGGGGAGGCCCCTACTTTATCCTAACTCCATGA
PromoterVviNAC05_Shiraz      CAAGATTTATGAGGTTTTGGGCCCTCCAGGGGAGGCCCCTACTTTATCCTAACTCCATGA
                             ************************************************************

PromoterVviNAC05_Pinot       CACCAATGATGGTCAGCTTTCATTTATAACACACCTAAAGCATCTTGCCTTAGATATTTA
PromoterVviNAC05_Shiraz      CACCAATGATGGTCAGCTTTCATTTATAACACACCTAAAGCATCTTGCCTTAGATATTTA
                             ************************************************************

PromoterVviNAC05_Pinot       TTTAGAAAGGGAAGGCCAATAGGGTGATTTTTGGATATCTTAGAGTCGAATAAATGGAAT
PromoterVviNAC05_Shiraz      TTTAGAAAGGGAAGGCCAATAGGGTGATTTTTGGATATCTTAGAGTCGAATAAATGGAAT
                             ************************************************************

PromoterVviNAC05_Pinot       CTTTTTAAGAAGCTAAGATGGGAGCATCTGTAATTCTCTATGCATCCAAACAGATGTTAC
PromoterVviNAC05_Shiraz      CTTTTTAAGAAGCTAAGATGGGAGCATCTGTAATTCTCTATGCATCCAAACAGATGTTAC
                             ************************************************************

PromoterVviNAC05_Pinot       CAGCTGCTGAGATAAACCAGCTTTATTTGGGAATTGCCCATTTCCATTTCTTGGCCAACT
PromoterVviNAC05_Shiraz      CAGCTGCTGAGATAAACCAGCTTTATTTGGGAATTGCCCATTTCCATTTCTTGGCCAACT
                             ************************************************************

PromoterVviNAC05_Pinot       ATTATTACAATGCATGATGGACAATTGTGGTATACTGATGAAACAAGCTGTGAAGTTGAC
PromoterVviNAC05_Shiraz      ATTATTACAATGCATGATGGACAATTGTGGTATACTGATGAAACAAGCTGTGAAGTTGAC
                             ************************************************************

PromoterVviNAC05_Pinot       ACCAAAGGCTTCATGGGTGTTCTTGCTGCAGATGATAATTCTCCTTTTAATTCAGATTTT
PromoterVviNAC05_Shiraz      ACCAAAGGCTTCATGGGTGTTCTTGCTGCAGATGATAATTCTCCTTTTAATTCAGATTTT
                             ************************************************************

PromoterVviNAC05_Pinot       TGTGAAAAAATAAATGGGAATACTTGAATTAGTAAATTGGTAAGTATCAAAATTACATGC
PromoterVviNAC05_Shiraz      TGTGAAAAAATAAATGGGAATACTTGAATTAGTAAATTGGTAAGTATCAAAATTACATGC
                             ************************************************************

PromoterVviNAC05_Pinot       AGAGGGTTGGAGATGAGTTGTCTTTGGTCAAATTTTTGAAGGATAGATTTATGGCAGAAA
PromoterVviNAC05_Shiraz      AGAGGGTTGGAGATGAGTTGTCTTTGGTCAAATTTTTGAAGGATAGATTTATGGCAGAAA
                             ************************************************************

PromoterVviNAC05_Pinot       AGTTAAAGGAGGGAGATGAAACAAACCAACCAAGTTGCCTTACATTCCACGTTTCTCTCT
PromoterVviNAC05_Shiraz      AGTTAAAGGAGGGAGATGAAACAAACCAACCAAGTTGCCTTACATTCCACGTTTCTCTCT
                             ************************************************************

PromoterVviNAC05_Pinot       CTCCAAACACCAGTATTTAGGGGCTTCTGTTCCATGTATTGCTTTTTTCTTAAACACTCT
PromoterVviNAC05_Shiraz      CTCCAAACACCAGTATTTAGGGGCTTCTGTTCCATGTATTGCTTTTTTCTTAAACACTCT
                             ************************************************************

PromoterVviNAC05_Pinot       CCATCTTTCAGGTTTACAGCTTTTAGTTCCATGGCTTCCCTGCTTTTTCTCTCTGCCTTT
PromoterVviNAC05_Shiraz      CCATCTTTCAGGTTTACAGCTTTTAGTTCCATGGCTTCCCTGCTTTTTCTCTCTGCCTTT
                             ************************************************************

PromoterVviNAC05_Pinot       TGTTGCCAAATTTATGGCTTTTCTTCATGCTTTTCCTTCTAGTTCCTAGAAATCTACTTT
PromoterVviNAC05_Shiraz      TGTTGCCAAATTTATGGCTTTTCTTCATGCTTTTCCTTCTAGTTCCTAGAAATCTACTTT
                             ************************************************************

PromoterVviNAC05_Pinot       CTATTTGAATTTTCCCAGGCTTCTCGGTTGGATGTCCTGAATTTAGAAATTTTGGACAGT
PromoterVviNAC05_Shiraz      CTATTTGAATTTTCCCAGGCTTCTCGGTTGGATGTCCTGAATTTAGAAATTTTGGACAGT
                             ************************************************************

PromoterVviNAC05_Pinot       TTGTTTGGTACCCATTTGAAATATAGGGGTCCTAGGGGTCTGGGTGTAGGAATTGAGGTA
PromoterVviNAC05_Shiraz      TTGTTTGGTACCCATTTGAAATATAGGGGTCCTAGGGGTCTGGGTGTAGGAATTGAGGTA
                             ************************************************************

PromoterVviNAC05_Pinot       GAGTAGTGGAGGTTCATAGCTTTTTAATCAGAACCTACTGAGATTTTAGCGGACTTTCAA
PromoterVviNAC05_Shiraz      GAGTAGTGGAGGTTCATAGCTTTTTAATCAGAACCTACTGAGATTTTAGCGGACTTTCAA
                             ************************************************************

PromoterVviNAC05_Pinot       AGAATGGAAAACATTGCTGGACTTAGTGTGGAAGATGATCAG
PromoterVviNAC05_Shiraz      AGAATGGAAAACATTGCTGGACTTAGTGTGGAAGATGATCAG
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C

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PromoterVviNAC08_Pinot      TCCCAACCATAGCTTACCTGTCATTTATTGAGGCCAAACCGTTTTGACGTGTCTAGACGT
PromoterVviNAC08_Shiraz     TCCCAACCATAGCTTACCTGTCATTTATTGAGGCCAAACCGTTTTGACGTGTCTAGACGT
                            ************************************************************

PromoterVviNAC08_Pinot      TTCTTCGCATGATTTTGAGCATTAAATGCTTCCACCATTCTGTAAACCAAGACGGACACG
PromoterVviNAC08_Shiraz     TTCTTCGCATGATTTTGAGCATTAAATGCTTCCACCATTCTGTAAACCAAGACGGACACG
                            ************************************************************

PromoterVviNAC08_Pinot      TGGTATCTACGTGGCCC-TTTTTTTTTTTCTGGTAGTGTCTTATTCTGACAATATTTTAT
PromoterVviNAC08_Shiraz     TGGTATCTACGTGGCCCATTTTTTTTTTTCTGGTAGTGTCTTATTCTGACAATATTTTAT
                            ***************** ******************************************

PromoterVviNAC08_Pinot      CTGCCTGGTTTCTAAC-TTTTTTTTTTTTAAAATACATTAAATTAAATTAAAAGACGACC
PromoterVviNAC08_Shiraz     CTGCCTGGTTTCTAACTTTTTTTTTTTTTTAAATACATTAAATTAAATTAAAAGACGACC
                            *************** ************ ******************************

PromoterVviNAC08_Pinot      TAAACAGGATGTTGGACCATAAGATAAATGAAAACAATCGTTATCAACCCAACCAGTACC
PromoterVviNAC08_Shiraz     TAAACAGGATGTTGGACCATAAGATAAATGAAAACAATCGTTATCAACCCAACCAGTACC
                            ************************************************************

PromoterVviNAC08_Pinot      TCAAATAATTCAATTCAACGTGCACATGAAAATTTGATAAAATCGATTTAAACGCCGTCA
PromoterVviNAC08_Shiraz     TCAAATAATTCAATTCAACGTGCACATGAAAAATTGATAAAATCGATTTAAACGCCGTCA
                            ******************************** ***************************

PromoterVviNAC08_Pinot      TAACCGACAATTGCATTTTTTTCAAACCACAAGGTTGAAAAAAAAAAAAAAAAAAAGGTT
PromoterVviNAC08_Shiraz     TAAGCGACAATTGCATTTTTTTCAAACCACAAGGTTGAAAAAAAAAAAAAAAAAAAGGTT
                            *** ******************************** ***********************

PromoterVviNAC08_Pinot      TTATGAAAAATAAGTCACCGGCGCATATATTTATTTCAATATGGGGCCACAGGAAAACCC
PromoterVviNAC08_Shiraz     TTATGAAAAATAAGTCACCGGCGCATATATTTATTTCAATATGGGGCCACAGGAAAACCC
                            ************************************************************

PromoterVviNAC08_Pinot      AGGTCTAACGTTTCACGACACACGTGAAGGCTCCCACTAACTTGCACCGGCTGACATCAG
PromoterVviNAC08_Shiraz     AGGTCTAACGTTTCACGACACACGTGAAGGCTCCCACTAACTTGCACCGGCTGACATCAG
                            ************************************************************

PromoterVviNAC08_Pinot      CGTCGCACGTGGATGATCCACGATGACGTGGACATTGAATCCACCGTTAAACTGTCGGCT
PromoterVviNAC08_Shiraz     CGTCGCACGTGGATGATCCACGATGACGTGGACATTGAATCCACCGTTAAACTGTCGGCT
                            ************************************************************

PromoterVviNAC08_Pinot      CAGCGACCAAGAGAAAAGAGGAGGAAAACCAAGCGGAGCCGTCGACTGCACACGGTTCCC
PromoterVviNAC08_Shiraz     CAGCGACCAAGAGAAAAGAGGAGGAAAACCAAGCGGAGCCGTCGACTGCACACGGTTCCC
                            ************************************************************

PromoterVviNAC08_Pinot      CATGACAGTTGGTCCCCATATGACTCGCACGTGCGTCCAAAACAGGTGACGCAAGCAGTG
PromoterVviNAC08_Shiraz     CATGACAGTTGGTCCCCATATGACTCGCACGTGCGTCCAAAACAGGTGACGCAAGCAGTG
                            ************************************************************

PromoterVviNAC08_Pinot      ACTAAATCACAGAGTGTGGGCTCTCGACACGCATACACTCACACTTCATAATCCGCCACG
PromoterVviNAC08_Shiraz     ACTAAATCACAGAGTGTGGGCTCTCGACACGCATACACTCACACTTCATAATCCGCCACG
                            ************************************************************

PromoterVviNAC08_Pinot      TGTCCGGCTGCGGTCAGAATGGTGGCTATTCCCCTAGAACAACCGAAGAAATATTATCGC
PromoterVviNAC08_Shiraz     TGTCCGGCTGCGGTCAGAATGGTGGCTATTCCCCTAGAACAACCGAAGAAATATTATCGC
                            ************************************************************

PromoterVviNAC08_Pinot      CCACGTGTATTCTAATAACCACCGTTCCCACCTGCCACGTGTCCTCTTTTTCTCTATAAA
PromoterVviNAC08_Shiraz     CCACGTGTATTCTAATAACCACCGTTCCCATCTGCCACGTGTCCTCTTTTTCTCTATAAA
                            ****************************** ***************************

PromoterVviNAC08_Pinot      TTCCTCGCTCTCCCTTCCGGTCTAAGTTTCAAGCGCCGTCTTGGCTCAGAGCAAAGCCGT
PromoterVviNAC08_Shiraz     TTCCTCGCTCTCCCTTCCGGTCTAAGTTTCAAGCGCCGTCTTGGCTCAGAGCAAAGCCGT
                            ************************************************************

PromoterVviNAC08_Pinot      GAGAGAAGAGAAAAAAAAGGTCAGGGAGAAACATTCATTCAAGAGAGGAAGGAGAGAGAA
PromoterVviNAC08_Shiraz     GAGAGAAGAGAAAAAAAAGGTCAGGGAGAAACATTCATTCAAGAGAGGAAGGAGAGAGAA
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PromoterVviNAC08_Pinot      GCTAAT
PromoterVviNAC08_Shiraz     GCTAAT
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D

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PromoterVviNAC34_Pinot      GGGTGCCTAAAATCCCCTGTCCTATTGCCATCTGTACATATGTTTTAACAAAATTAAAAA
PromoterVviNAC34_Shiraz     GGGTGCCTAAAATCCCCTGTCCTATTGCCATCTGTACATATGTTTTAACAAAATTAAAAA
                            ************************************************************

PromoterVviNAC34_Pinot      AATTGAAATTATTAGTATGTTTAATATTTATAAAAATATAAATATAATTAGAAAATGTAT
PromoterVviNAC34_Shiraz     AATTGAAATTATTAGTATGTTTAATATTTATAAAAATATAAATATAATTAGAAAATGTAT
                            ************************************************************

PromoterVviNAC34_Pinot      ATTCTATTTTTTTTTGAAAAATATAGTAAAAAAACAAAGTGCATACAAAGGACACCTACCT
PromoterVviNAC34_Shiraz     ATTCTA-TTTTTTTTGAAAAATATAGTAAAAAAACAAAGTGCATACAAAGGACACCTACCT
                            ****** ************************************************************

PromoterVviNAC34_Pinot      TTTGCATAACTTCTACACTCAAACCAAATAATATTATACATGTCCTTTCAACTATTGAGC
PromoterVviNAC34_Shiraz     TTTGCATAACTTCTACACTCAAACCAAATAATATTATACATGTCCTTTCAACTATTGAGC
                            ************************************************************

PromoterVviNAC34_Pinot      CATTATTTTCAAATTTTCAATCATAAAGATGTAATAATTTATTTATTTATTTATTTGTTT
PromoterVviNAC34_Shiraz     CATTATTTTCAAATTTTCAATCATAAAGATGTAATAATTTATTTATTTATTTATTTGTTT
                            ************************************************************

PromoterVviNAC34_Pinot      ATTATGAAATGCTTTTGGGGAGAGGTTTCTTGCTTTACATGAGAAGTCTACGTGAAGAAA
PromoterVviNAC34_Shiraz     ATTATGAAATGCTTTTGGGGAGAGGTTTCTTGCTTTACATGAGAAGTCTACGTGAAGAAA
                            ************************************************************

PromoterVviNAC34_Pinot      TAGAATTATGGTTGGGTGCTAAATGTATACAGTAGTTCTTCTTCTTTATGTTTTCTTTTT
PromoterVviNAC34_Shiraz     TAGAATTATGGTTGGGTGCTAAATGTATACAGTAGTTCTTCTTCTTTATGTTTTCTTTTT
                            ************************************************************

PromoterVviNAC34_Pinot      CTGGAGTGTTTATATTTTTAGCTTTATCCACACTCTAGGGCGACATTGGAACCGAAATGA
PromoterVviNAC34_Shiraz     CTGGAGTGTTTATATTTTTAGCTTTATCCACACTCTAGGGCGACATTGGAACCGAAATGA
                            ************************************************************

PromoterVviNAC34_Pinot      ATTCTCTCCCCCTCTCTCTCACTCTCACAGGTTGCCTCTTCTCCACTCAACCCTTTTTAA
PromoterVviNAC34_Shiraz     ATTCTCTCCCCCTCTCTCTCACTCTCACAGGTTGCCTCTTCTCCACTCAACCCTTTTTAA
                            ************************************************************

PromoterVviNAC34_Pinot      TCTTTGGTTGTTTTCATGAAATTCTTAGTGTCTTTGGACCATTTTTAACAAATCAAAGCT
PromoterVviNAC34_Shiraz     TCTTTGGTTGTTTTCATGAAATTCTTAGTGTCTTTGGACCATTTTTAACAAATCAAAGCT
                            ************************************************************

PromoterVviNAC34_Pinot      GTCTTTACACACTGTTAAAAGGACACAGACCGACATTTAGTCTCCTACAAGTATTCATAT
PromoterVviNAC34_Shiraz     GTCTTTACACACTGTTAAAAGGACACAGACCGACATTTAGTCTCCTACAAGTATTCATAT
                            ************************************************************

PromoterVviNAC34_Pinot      ACCAAAACTTGAGATCCAAGCAATCTTCATTTTTTGTTGAATCTCATATCATACTTGTAG
PromoterVviNAC34_Shiraz     ACCAAAACTTGAGATCCAAGCAATCTTCATTTTTTGTTGAATCTCATATCATACTTGTAG
                            ************************************************************

PromoterVviNAC34_Pinot      GGTTTCCTCTCTCTCCATTTTCATATTCTGTAGCCCTCTGAATATATATAGTCATTCAAA
PromoterVviNAC34_Shiraz     GGTTTCCTCTCTCTCCATTTTCATATTCTGTAGCCCTCTGAATATATATAGTCATTCAAA
                            ************************************************************

PromoterVviNAC34_Pinot      GGATCAAAAAAAGTGAAGAGGAATTCAATTCCTTACCAACCCTAATGAAGAACTGGCTTC
PromoterVviNAC34_Shiraz     GGATCAAAAAAAGTGAAGAGGAATTCAATTCCTTACCAACCCTAATGAAGAACTGGCTTC
                            ************************************************************

PromoterVviNAC34_Pinot      ATAGGGTCATGCTTTAGAAGACACATCTTGCTTTTCTTTCTTTTTCTTTTTACCCCTTCC
PromoterVviNAC34_Shiraz     ATAGGGTCATGCTTTAGAAGACACATCTTGCTTTTCTTTCTTTTTCTTTTTACCCCTTCC
                            ************************************************************

PromoterVviNAC34_Pinot      TCTCTGTCCCTCTCTTCTTAGATTTTTTGTTTACTTCTGGAGTTTCTGTAGAGAAGCTAC
PromoterVviNAC34_Shiraz     TCTCTGTCCCTCTCTTCTTAGATTTTTTGTTTACTTCTGGAGTTTCTGTAGAGAAGCTAC
                            ************************************************************

PromoterVviNAC34_Pinot      AAACAATAAACTTAGGGTTCACTGTGGGATGGGCATTAGAGGAAAACTAGAGAA
PromoterVviNAC34_Shiraz     AAACAATAAACTTAGGGTTCACTGTGGGATGGGCATTAGAGGAAAACTAGAGAA
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E

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PromoterVviNAC37_Pinot      CTTATTCCGCACCTCGGTCATGCCATGTTCAGGTAAAGAAAAGTTGTTTTGGCCATGAGA
PromoterVviNAC37_Shiraz     CTTATTCCGCACCTCGGTCATGCCATGTTCAGGTAAAGAAAAGTTGTTTTGGCCATGAGA
                            ************************************************************

PromoterVviNAC37_Pinot      AGTATGTTAGAAATGGTTTAGCCTCACCATAAGAATTAACCATATATATATATATACATA
PromoterVviNAC37_Shiraz     AGTATGTTAGAAATGGTTTAGCCTCACCATAAGAATTAACC----ATATATATATACATA
                            ******************************************    ***************

PromoterVviNAC37_Pinot      TACCCAGTTCAGACAGAGATATATATACACATAGATAGAATGAGAAATCATCCGGACAAA
PromoterVviNAC37_Shiraz     TACCCAGTTCAGACAGAGATATATATACACATAGATAGAATGAGAAATCATCCGGACAAA
                            ************************************************************

PromoterVviNAC37_Pinot      ATCATGTTTCACGTGTTCATGTAATGAAAGTGTTATAATATGATACGAATCATCCAAAAG
PromoterVviNAC37_Shiraz     ATCATGTTTCACGTGTTCATGTAATGAAAGTGTTATAATATGATACGAATCATCCAAAAG
                            ************************************************************

PromoterVviNAC37_Pinot      AAGAAGGAATGGTGCTGATCTCTCA----------GAGAAAGAGGGGATTCCTTGCTTC
PromoterVviNAC37_Shiraz     AAGAAGGAATGGTGCTGATCTCTCAGACATACACACGAGAAAGAGGGGATTCCTTGCTTC
                            ************************  **********************************

PromoterVviNAC37_Pinot      AAATGAGAAGTCTACGTTAAGAAATGGATTAATACATGTAGAGGTTGGTGTAATATCCGT
PromoterVviNAC37_Shiraz     AAATGAGAAGTCTACGTTAAGAAATGGATTAATACATGTAGAGGTTGGTGTAATATCCGT
                            ************************************************************

PromoterVviNAC37_Pinot      TTGTTATTTTCATTTTTTGGTGGGATTGTAAACATACATAAAATTTCACATAGCTTTAAA
PromoterVviNAC37_Shiraz     TTGTTATTTTCATTTTTTGGTGGGATTGTAAACATACATAAAATTTCACATAGCTTTAAA
                            ************************************************************

PromoterVviNAC37_Pinot      GACACTCTAGGGTGACGCTGGAGCTAAAGCCATCTCCCCCTTGTACTGGGGAGGGTGAAA
PromoterVviNAC37_Shiraz     GACACTCTAGGGTGACGCTGGAGCTAAAGCCATCTCCCCCTTGTACTGGGGAGGGTGAAA
                            ************************************************************

PromoterVviNAC37_Pinot      CGTCCCAGGAGAATATCAGCTTGCCCCATCTCCACTCTACCCTTTATGCATTATATAGGT
PromoterVviNAC37_Shiraz     CGTCCCAGGAGAATATCAGCTTGCCCCATCTCCACTCTACCCTTTATGCATTATATAGGT
                            ************************************************************

PromoterVviNAC37_Pinot      TGTTAAATTTAAGGTTAACTTTCTTGAATTACCTTTTCAATAAACCAAAACTC-CTCTTC
PromoterVviNAC37_Shiraz     TGTTAAATTTAAGGTTAACTTTCTTGAATTACCTTTTCAATAAACCAAAACTCTCTCTTC
                            ************************************************** ******

PromoterVviNAC37_Pinot      AAACATAGTAAAGAATGCAACGACCAAAACTGCATTTATATTCTTATACTAGTCAGTATC
PromoterVviNAC37_Shiraz     AAACATAGTAAAGAATGCAACGACCAAAACTGCATTTATATTCTTATACTAGTCAGTATC
                            ************************************************************

PromoterVviNAC37_Pinot      CATACATTAACAAAACCTAACTTGACGTCCAAGCAACCTTTTTTTTATATTTCTAGAAAA
PromoterVviNAC37_Shiraz     CATACATTAACAAAACCTAACTTGACGTCCAAGCAACC-TTTTTTTATATTTCTAGAAAA
                            ************************************ **********************

PromoterVviNAC37_Pinot      TTTTCATTCTCTCCTCATCTTCTTTTATACCCTTTCTCCTCCTCTTCATCTCCCTCTTAG
PromoterVviNAC37_Shiraz     TTTTCATTCTCTCCTCATCTTCTTTTATACCCTTTCTCCTCCTCTTCATCTCCCTCTTAG
                            ************************************************************

PromoterVviNAC37_Pinot      AAAGAAACAAGCTGTAGAGAGGACACAGTTCAAGGAAAAACATAAAGTGAAGGAGAATTA
PromoterVviNAC37_Shiraz     AAAGAAACAAGCTGTAGAGAGGACACAGTTCAAGGAAAAACATAAAGTGAAGGAGAATTA
                            ************************************************************

PromoterVviNAC37_Pinot      ATTCCTTTCCAAACATCAAGAAGAAGAATTGTTTAACGTAAAACATGCTTTAAAAGAGAC
PromoterVviNAC37_Shiraz     ATTCCTTTCCAAACATCAAGAAGAAGAATTGTTTAACGTAAAACATGCTTTAAAAGAGAC
                            ************************************************************

PromoterVviNAC37_Pinot      AAGTTGTTGCTTTGCTTTTCTTTTTTCTTTTTTTCCATTCCAAATTGAATCCTTTTTATT
PromoterVviNAC37_Shiraz     AAGTTGTTGCTTTGCTTTTC-TTTTTCTTTTTTTCCATTCCAAATTGAATCCTTTTTATT
                            ******************* ****************************************

PromoterVviNAC37_Pinot      TTATGTCTGCCTGTTGCTGTAATTCAGTCTATTTTGGAGAAGTGGAGGAGCCAGGAGAGG
PromoterVviNAC37_Shiraz     TTATGTCTGCCTGTTGCTGTAATTCAGTCTATTTTGGAGAAGTGGAGGAGCCAGGAGAGG
                            ************************************************************

PromoterVviNAC37_Pinot      AAATTCCCGTCTGGGATGGGCATAGAGGCTCAACAACAGGG
PromoterVviNAC37_Shiraz     AAATTCCCGTCTGGGATGGGCATAGAGGCTCAACAACAGGG
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F

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PromoterVviNAC61_Pinot      AATCTCTCTGAAAGCGGGGCTGGGCTTTGGAGTTGAAGTTTGAGTTGCCAGCGCGCACCT
PromoterVviNAC61_Shiraz     AATTTCTCTGAAAGCGGGGCTGGGCTTTGGAGTTGAAGTTTGAGTTGCCAGCGCGCACCT
                            *** ************************************************************

PromoterVviNAC61_Pinot      TTTCTCAGTCGGCGCCTCATGTTTACTTTTTTTATTTTTTTTTGAAAAATTTTCCCATATG
PromoterVviNAC61_Shiraz     TTTTTCAGTCGGCGCTTCATGTTTACTTTTTTTATTTTTTTTTGAAAAATTTTCCCATATG
                            *** *********** ********************************************

PromoterVviNAC61_Pinot      CACGTGGGGATTCCCTTTCTCCCACACCCCAACTGGATTTCCTGCAAAATAAAAGATGGG
PromoterVviNAC61_Shiraz     CACGTGGGGATTCCCTTTCTCCCACACCCCAACTGGATTTCCTGCAAAATAAAAGATGGG
                            ************************************************************

PromoterVviNAC61_Pinot      TAAGCTTGATTTTGTGGAAATATGAAAAAAAAAATACAATATACTGGACAAAAAATTCAA
PromoterVviNAC61_Shiraz     TAAGCTTGATTTTGTGGAAATATGAAAAAAAAAATACAATATACTGGACAAAAAATTCAA
                            ************************************************************

PromoterVviNAC61_Pinot      AAAGTTGTAAGAGATTAATATAAATATGAATGTATGTTTGGGAGTATGTCTAATTTGACA
PromoterVviNAC61_Shiraz     AAAGTTGTAAGAGATTAATATAAATATGAATGTATGTTTGGGAGTATGTCTAATTTGACA
                            ************************************************************

PromoterVviNAC61_Pinot      GAACCCTCCAAGTGGCTTCTTCCAGTCATGGATCCATGTTCCTGGAAAGGGACAAGATAT
PromoterVviNAC61_Shiraz     GAACCCTCCAAGTGGCTTCTTCCAGTCATGGATCCATGTTCCTGGAAAGGGACAAGATAT
                            ************************************************************

PromoterVviNAC61_Pinot      TACAAGGAGAAAGCTTAGGAAAATATGGAGGATAATGGGAGTGAAGCTGGGGAGGAGGAT
PromoterVviNAC61_Shiraz     TACAAGGAGAAAGCTTAGGAAAATATGGAGGATAATGGGAGTGAAGCTGGGGAGGAGGAT
                            ************************************************************

PromoterVviNAC61_Pinot      GAGGATGGGGCTGGGGCTTAAGCCAGGAGTGCGCCACTTCGAAATGGATGGATTTGACAT
PromoterVviNAC61_Shiraz     GAGGATGGGGCTGGGGCTTAAGCCAGGAGTGCGCCACTTCGAAATGGATGGATTTGACAT
                            ************************************************************

PromoterVviNAC61_Pinot      TTTCCCAAAGCATTAAAGGGGGGAGTGTTGTTGGTTAGGGATGAGGGATGAGGGATGAAG
PromoterVviNAC61_Shiraz     TTTCCCAAAGCATTAAAGGGGGGAGTGTTGTTGGTTAGGGATGAGGGATGAGGGATGAAG
                            ************************************************************

PromoterVviNAC61_Pinot      GGGGATTAAAGACGAAATCCAAATTCCAAAGTCGTATTTTTAATGTGAAAGGGAGCTGGA
PromoterVviNAC61_Shiraz     GGGGATTAAAGACGAAATCCAAATTCCAAAGTCGTATTTTTAATGTGAAAGGGAGCTGGA
                            ************************************************************

PromoterVviNAC61_Pinot      AACATGGGTCCCAGACCAGCCCCAACATAGAAACCACCTTGTACCATTTCCCTTTTCAAA
PromoterVviNAC61_Shiraz     AACATGGGTCCCAGACCAGCCCCAACATAGAAACCACCTTGTACCATTTCCCTTTTCAAA
                            ************************************************************

PromoterVviNAC61_Pinot      GAGGTAGATAAAGCTAATTAAGAAAATACACAAAAAGGACAAGAGGAAAACCATAAAAAA
PromoterVviNAC61_Shiraz     GAGGTAGATAAAGCTAATTAAGAAAATACACAAAAAGGACAAGAGGAAAACCATAAAAAA
                            ************************************************************

PromoterVviNAC61_Pinot      AAATGATATTGTAAGGCTTCTCGGAGAAGTGCCGTGCAGCGCAAGGATCTTATGACTTGT
PromoterVviNAC61_Shiraz     AAATGATATTGTAAGGCTTCTCGGAGAAGTGCCGTGCAGCGCAAGGATCTTATGACTTGT
                            ************************************************************

PromoterVviNAC61_Pinot      GAGCTGCGGGCGAAGCTCCTCTATTTAAGCTCCTTTTCTTTCCTTTCTTCTACTTTCTTA
PromoterVviNAC61_Shiraz     GAGCTGCGGGCGAAGCTCCTCTATTTAAGCTCCTTTTCTTTCCTTTCTTCTACTTTCTTA
                            ************************************************************

PromoterVviNAC61_Pinot      ACTGCTCTCTTTGAAAACTCCTTCAATACAGGCTCTTCTCTCCCTTGCTTTCTGCTATTG
PromoterVviNAC61_Shiraz     ACTGCTCTCTTTGAAAACTCCTTCAATACAGGCTCTTCTCTCCCTTGCTTTCTGCTATTG
                            ************************************************************

PromoterVviNAC61_Pinot      TTGTTTGTGCGTATTTGGTTTGGAATATTACTTCTTTTCTGTATTCTACATTCGCTTAGA
PromoterVviNAC61_Shiraz     TTGTTTGTGCGTATTTGGTTTGGAATATTACTTCTTTTCTGTATTCTACATTCGCTTAGA
                            ************************************************************

PromoterVviNAC61_Pinot      GCTTTATTTTTTTTTTTAAAGGTAATAATTGATATACAGTAGTGGTGTAACCATGGAAGA
PromoterVviNAC61_Shiraz     GCTTTATTTTTTTTTTTAAAGGTAATAATTGATATACAGTAGTGGTGTAACCATGGAAGA
                            ************************************************************

PromoterVviNAC61_Pinot      GGCTTCACTTGCCGATCAAGGTGATGACCTT
PromoterVviNAC61_Shiraz     GGCTTCACTTGCCGATCAAGGTGATGACCTT
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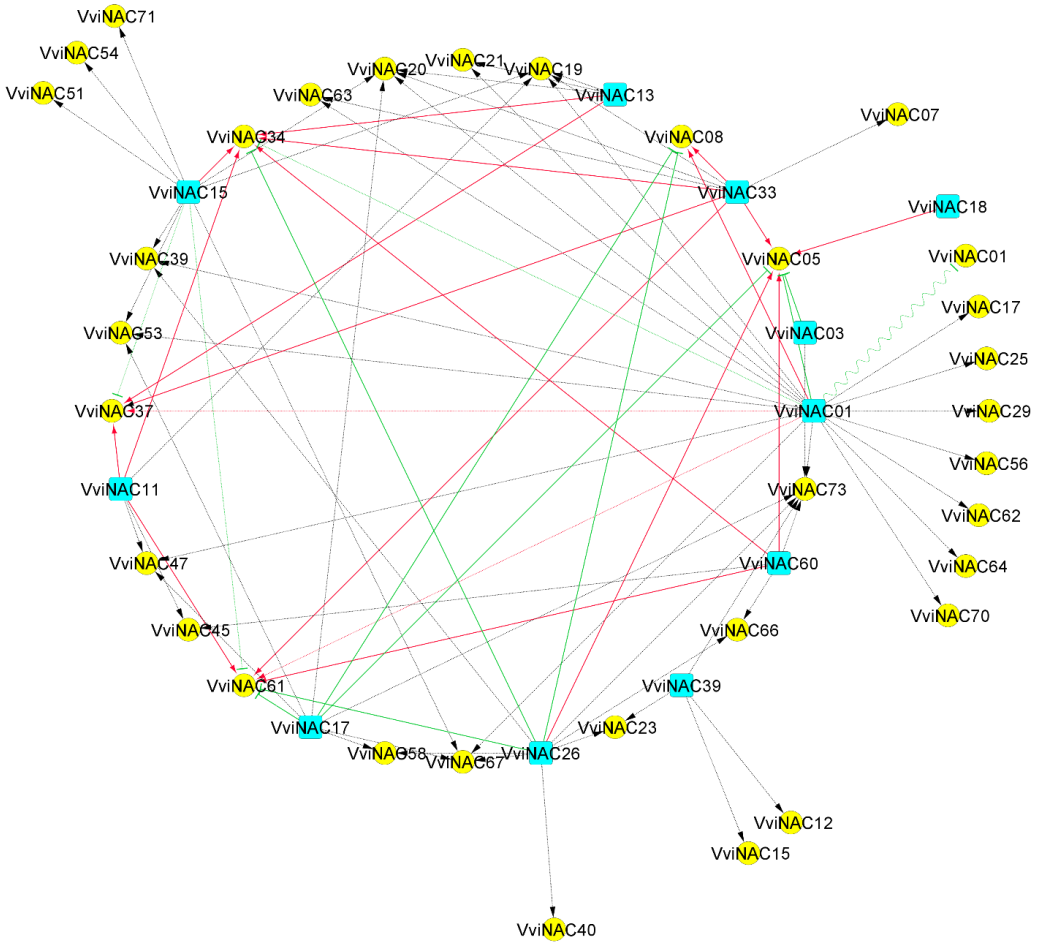
Appendix E: Circular representation of all the *Vvi*NAC-*VviNACs* interactions revealed in the DAP-seq datasets. The TFs are highlighted in light-blue squares and the targets are represented as yellow circles. The red → arrows indicate the activations and the green **T** arrows show the repressions; the curved arrow indicates the autoregulation of *Vvi*NAC01. The continuous lines indicate the validated interactions ($p<0.05$) and the dashed lines indicate the not significant interactions. The image was done with Cytoscape.



Non sono mai stata brava con le parole e proprio per questo non ho mai scritto dei ringraziamenti. La pandemia mi ha tuttavia spinta a tentare; il non poter vedere le persone a me care mi ha fatto capire che la vicinanza e l'affetto vadano talvolta dimostrate anche in maniera diversa…o per lo meno bisogna provarci.
Mi ritrovo quindi a scrivere la parte più difficile della mia tesi, quella non basata su dati scientifici, quella dove solo le mie sensazioni possono essere prese in considerazione. Tre anni sono volati ma non sono stati di certo sempre facili. Le ansie, le preoccupazioni e le difficoltà che derivano dal trovarsi a sviluppare un progetto di ricerca sono molte; ciò che mi ha spinto ad andare avanti è sicuramente la passione verso il mio lavoro ma ha anche dei nomi e delle facce.
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