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MASTER REGULATORS OF THE VEGETATIVE-TO-MATURE ORGAN TRANSITION IN GRAPEVINE: THE ROLE OF NAC TRANSCRIPTION FACTORS

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MASTER REGULATORS OF THE VEGETATIVE-TO-MATURE ORGAN TRANSITION IN GRAPEVINE: THE ROLE OF NAC TRANSCRIPTION FACTORS

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ABSTRACT

Grapevine is the most widely cultivated and economically important fruit crop in the world. Viticulture has been affected by the global warming currently under way over the past few decades (Webb *et al.,* 2007). Improving the genetics of key grapevine functions is needed to keep producing high quality grapes and wine. In this context, a challenging task is to identify master regulators that program the development of grapevine organs and control transition from vegetative-to-mature growth featured by grape berries during the annual plant cycle. This transition, called véraison, is marked by profound biochemical, physiological and transcriptomic modifications that allow vegetative green berries to enter the ripening process. Thanks to an integrated network analysis performed on the grapevine global gene expression atlas and from a large berry transcriptomic data set (Massonnet, 2015; Palumbo *et al.,* 2014; Fasoli *et al.,* 2012) a new category of genes, called 'switch' genes, was identified; they were significantly up-regulated during the developmental shift and inversely correlated with many genes suppressed during the mature growth phase. Among them, plant-specific NAM/ATAF/CUC (NAC) transcription factors represent an interesting gene family due to their key role in the biological processes in plant development and stress responses (Jensen *et al.,* 2014). Five NAC genes were selected for functional characterization as key factor candidates of the major transcriptome reprogramming during grapevine development. *VvNAC11*, *VvNAC13*, *VvNAC33* and *VvNAC60* were identified as 'switch' genes in the above-mentioned analysis whereas VvNAC03 was selected because it is a close homologue of tomato NOR (non-ripening), known for its crucial role in tomato fruit ripening regulation (Giovannoni, 2004; Giovannoni *et al.,* 1995). Firstly, the five transcription factors were transiently over-expressed in *Vitis vinifera* to get an overview of their primary effects on native species. Secondly, we obtained grapevine plants that were stably transformed with *VvNAC33* and *VvNAC60* and

subjected to molecular/phenotypic characterizations. *VvNAC33* seemed to be involved in negative regulation of photosynthesis since over-expressing leaves revealed a chlorophyll breakdown, while *VvNAC60* affected regular plant development, showing a slight growth and earlier stem lignification in comparison to a same-age plant control. These results reflected typical behaviors of plants undergoing ripening and senescence, thus supporting our working hypothesis proposing a crucial role of NACs in the transition from vegetative to mature development in grapevine. In order to identify downstream targets of the NAC transcription factors analyzed in this work, we performed microarray analysis on leaves of transient and stable ectopic expressing plants. We noted that both over-expressions affected a wide range of cellular processes and among the most represented functional categories we found transport, secondary metabolism and transcription factor activity. The identification of *VvMYBA1*, a known grapevine regulator of the anthocyanin biosynthetic pathway (Kobayashi *et al.,* 2002), as *VvNAC60* target suggests a *VvNAC60* role in processes like anthocyanin biosynthesis featured by grape berries at the onset of ripening. Another approach used to clarify NACs roles was to check the ability of *VvNACs* to fulfil the tomato *NOR* function. Preliminary results revealed that *VvNAC03* and *VvNAC60* could partially complement the *nor* mutation in tomato, establishing a partial ripening phenotype in fruits.

Taken together, these findings suggest the ability of the selected *VvNACs* to affect the expression of genes involved in the regulatory network that controls the developmental shift to a mature phase in grapevine. This work has shed some light on the roles of these NACs in grapevine development, but further analysis must be conducted to fully elucidate the molecular machinery in this complex regulation system.

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Chapter 1

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most widely grown fruit crops in the world and enology and viticulture play an important role in the economy of many developed countries (Martinez-Esteso *et al.,* 2013). It is a woody temperate-perennial plant that presents a period of active growth from spring to fall, followed by a rest period in the winter. During dormancy phase organs undergo an acclimation process to tolerate freezing temperatures and to ensure plant survival. This stage extends until budburst the following spring, when the growing season starts (Hellman, 2003). In this season flower develop76ment, fruit development and ripening occur. Grapevine growth can be described by its phenological events, whose understanding is important in determining the ability of a region to produce a crop in that specific climatic regime (Jones and Davis, 2000). Grapevine developmental cycle could be described using three main phenological stages: budbreak, that is the onset of vegetative growth, flowering, during which the fertilisation process leads to the formation of berries and véraison, corresponding to the beginning of the ripening which ends at harvest when sugar content and acidity reach required levels. Maturity is not considered a phenological stage because it is difficult to establish uniform criteria among varieties (Duchêne *et al.,* 2010). The time between these phenological stages varies greatly with grapevine variety, climate, and geographic location (Jones and Davis, 2000).

Physiological, biochemical and molecular characteristics that regulate grapevine growth, development and ripening have been studied intensely to underline the basis that determine fruit and wine quality. One of the most important and characterized processes of this plant is berry development, described as a dynamic and complex process involving a cascade of biochemical changes (Coombe and McCarthy, 2000). This non-climateric fruit exhibits a double sigmoid pattern of growth which is divided into three distinct stages: a green phase and a maturation phase, separated by a lag phase. The first one, which lasts from flowering to about 60 days after that, involves

rapid growth and cell division. Secondary compounds like hydroxycinnamic acids, tannins and aroma compounds are synthetized and organic acids, such as tartrate and malate, accumulate in the vacuole. A lag phase follows during which fruit growth slows and organic acid concentration of the berry reaches its highest level. The second growth stage coincides with the onset of ripening, called véraison, a striking metabolic transition phase characterized by important biochemical and physiological changes, such as softening and coloring of the berry. Some compounds produced during the first period of growth decline, such as malic acid and tannins, leading to a decrease of acidity and astringency. Sugars and pigments begin to accumulate and volatile secondary metabolites that contribute flavor and aroma are synthesized (Martinez-Esteso *et al.,* 2013; Kennedy e*t al.*, 2002).

The high economic impact of grapevine largely depends on the characteristic of its berries. Viticulture sustainability and wine quality are particularly sensitive to these variations caused by the global warming currently under way over the past few decade (Webb *et al.,* 2007). The phenology of grapevine development is predominantly influenced by temperature (Pearce and Coombe 2004; Jones and Davis 2000) and future temperature increases may change the timing of grape ripening with consequent on harvest date and therefore negative impact on grape quality and yield (Spayd *et al.,* 2002; Marais *et al.,* 2001; Haselgrove 2000). Some solutions commonly used nowadays for a sustainable viticulture maintaining quality wine production under global warming are viticultural practices like canopy management (Greer *et al.,* 2010) or irrigation and wine processing such as electrodialysis or acidification. These methods are however not so efficient and only short-term. A more long-term and powerful solution could be shifting of grape growing areas to cooler climate regions (Hanna *et al.,* 2013; van Leeuwen *et al.,* 2013; Ollat *et al.,* 2011), but with dramatic socio-economic consequences, or developing new cultivars better adapted to the climate changes (Ollat *et al.,*

2014). In this context, a better understanding of the knowledge on the genetics of key structural and regulatory gene functions is required.

For the last decade, thanks to the availability of the complete grapevine genome sequence (Jaillon *et al.,* 2007; Velasco *et al.,* 2007) and the expansion of genetic resources and tools, many studies have been carried out to shed light on the mechanisms underlying these profound physiological and biochemical changes. In order to understand the transcriptional organization of the whole plant, a grapevine global gene expression atlas was created (Fasoli *et al.,* 2012); it has been developed on the gene expression profiling of 54 samples representing green and woody tissues and organs at different developmental stages. This study revealed a clear distinction between vegetative/green and mature/woody sample transcriptomes, suggesting a deep shift in global gene expression driving the entire plant into a maturation program. Pollen and leaves undergoing senescence were excluded due to their atypical transcriptomes. Moreover, it has been shown that developmental stages are more important than organ or tissue identity in defining the transcriptome similarity. Recently, a berry transcriptome comparison of ten Italian grapevine varieties collected at four phenological growth stages, two pre- and two post-véraison, showed how the transcriptome reprogramming during plant development occurs at véraison independently on skin color and variety (Massonnet, 2015).

The characterization of genes governing the key processes during developmental growth in grapevine could be interesting and useful for the scientific community and more particularly for future research on grape plant development. A powerful approach to identify these master regulators has been performed by Palumbo *et al.* (2014), that developed an integrated network analysis. By analyzing grapevine gene expression atlas, the samples were divided into two groups: vegetative/green organs and mature organs; 1686 differentially expressed genes (DEGs) between vegetative/green and

mature/woody samples were identified. Among them, 1220 genes were down-regulated, but only 466 were up-regulated during the developmental transition, suggesting that the shift to mature growth mainly involves the suppression of vegetative metabolic processes rather than the activation of mature pathways. With the above-mentioned DEGs a gene co-expression network has been generated in which nodes are genes and a link is present when the Pearson correlation between the expression profiles of two genes is greater than 0.8 in absolute value. Moreover, according to node connectivity in the network, genes with an extremely high level of connectivity were classified as hubs. A clear bimodal profile has been revealed in agreement with the presence of a large group of inversely correlated profiles (Fasoli *et al.,* 2012). By searching for topological properties of the co-expression network, the date/party hub classification system based on the average Pearson correlation coefficients (APCCs) between profile of each hub and its partners was used. Interestingly, the distribution of APCC was trimodal with two peaks corresponding to low APCC (date hubs) and high APCC (party hubs) and a third peak at negative APCC values. This last peak represented a new class of hubs named 'fight-club hubs' characterized by a dominant anti-correlated profile. Moreover, a heat cartography (Figure 1) has been built by assigning a role to each node of the network and a color corresponds to its APCC value; this method revealed that fight-club hubs encompassed a new subset of 113 genes called 'switch' genes. These genes were expressed at low levels in the vegetative/green tissues, while their expression significantly increased in the mature/woody organs. They were likely to act as switches during developmental shift by changing their state from off to on. This behavior suggested that they could play a key role in the regulation of organ transition from immature to mature phase, maybe through the suppression of vegetative pathway. Indeed, they were connected to more than the 50% of genes in the network and all of these neighboring genes, which have an

opposite profile in comparison with that of the 'switch' genes, are associated with vegetative growth. Moreover, they were predominantly connected outside their module.

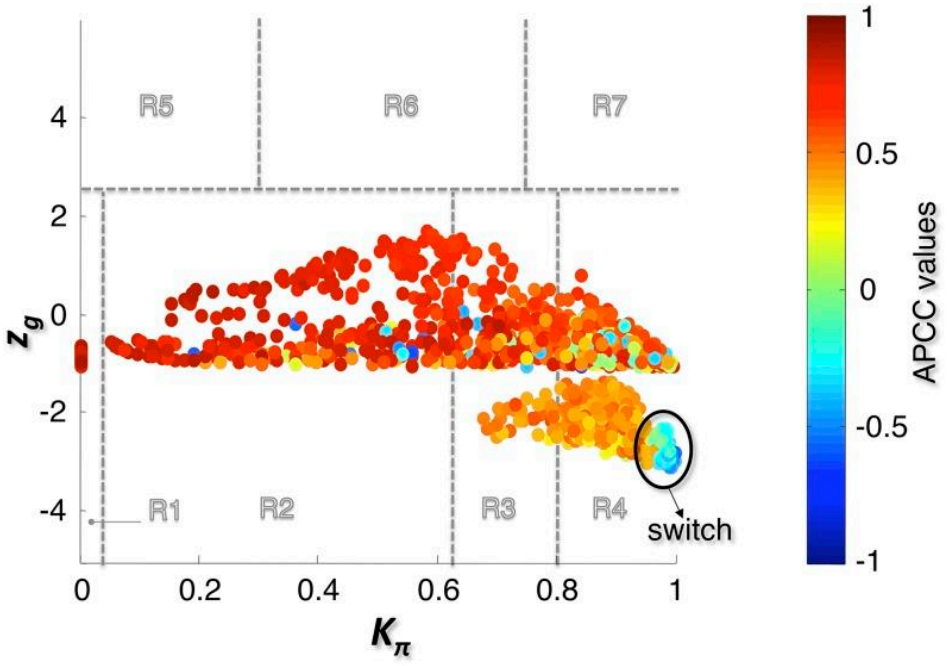

Figure 1: Grapevine Atlas Heat Cartography Map from Palumbo *et al.* (2014). The parameter Z_g represent a normalized measure of intramodule communication and K_π the mode of communication between nodes in different modules. The plane identified by these two parameters is divided into seven regions each defining a specific node role. Each point represents a node in the correlation network and the color of each node is proportional to its average Pearson correlation coefficients APCC value. According to the heat cartography a role has been assigned to each node.

By looking at the functional categories the most represented ones were secondary metabolic process, such as genes involved in carotenoid metabolism and phenylpropanoid metabolism, and the transcription factors, representing the 12% of the total 'switch' genes. Transcription factors naturally have function in regulating developmental processes, so they are expected to be excellent candidates as master regulators of the global transcriptomic reprogramming that marks the deep shift during plant development. Some examples of transcription factors encoded by 'switch' genes were ZFWD2 proteins, involved in the photomorphogenesis regulation (Németh *et al.,* 1998; Torii *et al.,* 1998) and the NAC (NAM, ATAF1/2, and

CUP-SHAPED COTYLEDON2 [CUC2]) family proteins NAC33 and NAC60, having a role in the regulation of vegetative and reproductive development in many plant species (Hendelman *et al.,* 2013; Raman *et al.,* 2008; Fabi *et al.,* 2012), including grapevine (Wang *et al.,* 2013; Sun *et al.,* 2012).

The same approach was used to analyze the above-mentioned berry transcriptomic data sets (Massonnet, 2015), in order to identify 'switch' genes involved in the regulation of the grape berry developmental transition. Once again, the shift to ripe/mature berry is mainly characterized by the suppression of genes involved in vegetative pathway than the induction of genes specific for the mature phase. Transcription factors have been found as significantly overexpressed functional category among the 190 'switch' genes of red berries and the 212 for white berries (data not published) identified. Interestingly, *VvNAC33* and *VvNAC60* were also found as berry 'switch' genes and moreover other two NAC proteins, NAC11 and NAC13, were identified. It is worth mentioning that Palumbo *et al.* (2014) showed the 'switch' genes are also the direct targets of the putative master regulators of developmental shift. Indeed, for example, well-characterized targets of MYB transcription factors were found, such as the enzyme UFGT, known as a key regulator of skin berry color (This *et al.,* 2007; Walker *et al.,* 2007; Lijavetzky *et al.,* 2006; Kobayashi *et al.,* 2004).

By comparing the grapevine atlas and berry data sets a list of 131 shared 'switch' genes was revealed; they could be defined as a core of key metabolic components inducing major transcriptome remodeling during grapevine development. More than half of these common genes may be regulated by microRNAs; an example of this interaction, experimentally validated in grapevine by Sun *et al.* (2012), is NAC33-domain mRNAs as putative target of miR64. The presence of miRNAs in the vegetative tissue could be a possible cause of the low expression of 'switch' genes in the green phase,

demonstrating a fine-tuned regulation of transcriptional shift by a relatively small number of miRNAs, during plant development.

The topological network analysis performed by Palumbo *et al.* (2014) was validated applying it to two tomato transcriptomic data sets. Tomato has proven to be a useful model for fleshy fruit development and ripening and many of the master ripening regulators have already been identified (Karlova *et al.,* 2014; Vrebalov *et al.,* 2002); several of these key genes have been revealed by the analysis.

In conclusion, the integrated network analysis that combine large-scale gene expression data with network topological properties has provide extremely useful to select those putative genes that could play a pivotal role in the developmental phase transition to mature growth in grapevine. It would be interesting to deeply characterize the genes identified by this powerful and original bioinformatic approach.

Outline of the thesis

The goal of this PhD project was the identification and characterization of putative master regulators able to promote the organ phase transition from vegetative to mature growth in grapevine. We focused our attention on some members of NAC transcription factor family; in particular, *VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* were identified as 'switch' genes by Palumbo *et al.* (2014) and *VvNAC03* was selected as one of the closest homologue gene of tomato *nor* (non-ripening).

Chapter 2 reviews the current knowledge about the *NACs* in plants and describes the criteria used to select the five genes. We reported their analysis in term of features of the corresponding encoded protein sequences, expression profiles in different grapevine organs and developmental stages and correlated genes.

In **chapter 3** the functional characterization of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* in grapevine is reported. As first, we performed a grapevine transient transformation of selected *VvNACs* to obtain an overview on their primary effects on plant transcriptome and to select some their target genes. Second, *Vitis vinifera* was stably transformed with *Agrobacterium tumefaciens* to overexpress both atlas 'switch' genes; thanks to a molecular and phenotypic characterization of the obtained grapevine plants we were able to get a comprehensive description of the stably transformed grapevines. The results obtained in this chapter suggested the ability of these genes to affect the expression of genes involved in the organ phase transition and supported our working hypothesis proposing these transcription factors as key regulators of this transcriptome reprogramming during grapevine development.

In **chapter 4** we described the functional complementation on *nor* tomato mutant. Preliminary results revealed that *VvNAC03* and *VvNAC60* could partially complement the *nor* mutation.

In **chapter 5** we summarized the findings that support the involvement of the members of NAC transcription factor family selected in this project in the grapevine organ phase transition to mature growth. Moreover, we suggested how further analysis might be very helpful in trying to elucidate the molecular mechanisms underlying the regulation of the developmental shift.

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Chapter 2

INVOLVEMENT OF NAC GENE FAMILY IN PLANT DEVELOPMENT

1. INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the most widely grown fruit crop in the world with a high economic impact. Its value depends largely on the quality of its berries, whose qualitative characteristics affect the organoleptic properties to the major grapevine products. This non-climacteric fruit needs a relatively long period to reach its ripened state, which involves several dramatic physical and biochemical changes. This complex and dynamic phenomenon has been intensively studied at a transcriptomic level for the last decade (Tornielli *et al.,* 2012). Thanks to the impressive expansion of technologies, significant progresses have been occurred to increase knowledge about the molecular basis underlying these profound events and about agricultural practices or effect of environmental changes on fruit quality and yield.

A recent analysis of the grapevine global atlas (Fasoli *et al.,* 2012) revealed a clear distinction between vegetative/green and mature/woody tissues, reflecting the existence of a deep transcriptome reprogramming that driving the entire plant into a maturation program. Currently, an integrated network analysis has been performed (Palumbo *et al.,* 2014) in order to better understand the overall regulation of this developmental transition from vegetative to mature growth and, therefore, to identify putative master regulators that could control this major regulatory switch. One of the most important insights gained from this original bioinformatic approach was the identification of a category of genes, named 'switch' genes, significantly up-regulated in the mature/woody tissues and inversely correlated with many genes down-regulated during the developmental transition. On the basis on these features, 'switch' genes might represent master regulators of the recently discovered major transcriptome reprogramming occurring during organ phase transition to mature growth. They encoded several transcription factors (TFs) that naturally controlling many vital cellular processes in plants. Among them,

the NAC TFs family, one of the largest in plants, has been functionally implicated in a variety of programs related to plant growth and development. The NAC acronym is derived from three earliest characterized proteins with the so-called NAC domain from petunia NAM (no apical meristem), *Arabidopsis* ATAF (*Arabidopsis* transcription activator factor) and CUC (cuo-shaped cotyledon) (Aida *et al.,* 1997; Souer *et al.,* 1996). Thanks to the availability of public plant genome and EST sequences, an ever-increasing number of putative *NAC* genes have been identified in various plant species, such as 117 in *Arabidopsis,* 151 in rice (Nuruzzaman *et al.,* 2010), 152 in soybean (Le *et al.,* 2011), 180 in apple (Su *et al.,* 2013), 167 in banana (Cenci *et al.,* 2014), 104 in tomato (Su *et al.,* 2015), 74 in grape (Wang *et al.,* 2013) and so on.

The wide studies on model plants, including *Arabidopsis* and rice, revealed the presence of a highly conserved N-terminal 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). This NAC domain is associated with DNA binding, nuclear localization and the formation of homodimers or heterodimers with other NAC proteins (Olsen *et al.,* 2005). On the contrary, the highly divergent C-terminal, which can present a transmembrane motif, has a putative transcriptional positive or negative regulatory function and, sometimes, it might have protein binding activity (Puranik *et al*., 2011).

The NAC TFs undergo intensive post-transcriptional regulation that includes microRNA-mediated cleavage of genes; a typical example could be miRNA164 that shows its affinity for the development-associated and mechanical stress-regulated *NAC* genes (Khraiwesh *et al.,* 2012; Mallory *et al.,* 2004). The NAC TFs are also characterized by a complex post-translational regulation involving ubiquitination, dimerization, phosphorylation or proteolysis (Nakashima *et al.,* 2012; Puranik *et al.,* 2012). This regulatory mechanism could help NAC domain-containing proteins

playing various roles in multiple plant processes. Many of them have been identified as involved in plant abiotic and biotic responses (Mao *et al*. 2012; Nakashima *et al.,* 2012; Christianson *et al.,* 2010; Olsen *et al.,* 2005; Tran *et al.,* 2004). In *Arabidopsis, ANAC019*, *ANAC055* and *ANAC072* were induced by drought and salinity and involved in regulating abscisic acid and jasmonic acid-signaled defense responses; their overproduction also decreased resistance to *Botrytis cinerea* (Jensen *et al.,* 2010; Bu *et al.,* 2008; Fujita *et al.,* 2004). In addition, *ATAF1* and *HvNAC6* homologue in barley have a direct role in regulating basal defense (Jensen *et al.,* 2007) and *StNAC* in potato was involved in the resistance against *Phytophtora infestans* (Collinge and Boller, 2001). Increasing evidences indicate roles for NAC proteins in developmental processes and transcriptional regulatory networks. For example, *CUC2* is implicated in shoot apical meristem development (Nikovics *et al.*, 2006), *AtNAC2* in lateral root development (He *et al.,* 2005) and *ANAC036* in cell division (Kato *et al.,* 2010). Some other NAC TFs 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, a large number of these TFs are associated with senescence processes; a significant role in leaf senescence has so far been demonstrated in *Arabidopsis*, such as the recently discovered *ANAC046* that is a positive regulator of chlorophyll degradation and senescence in *Arabidopsis* leaves (Oda-Yamamizo *et al.,* 2016). Finally, it is worth underlining that in tomato, which has served as the primary model for fleshy fruit development and ripening, members of NAC family have been characterized to be involved in the flower morphogenesis (*NAM2*; Hendelman *et al.,* 2013) or as regulators of the fruit ripening, as *NOR* (Giovannoni, 2004) and *NAC4* (Zhu *et al.,* 2014). Moreover, *ANAC019* and *ANAC078* in *Arabidopsis* and in *BL* in peach regulate anthocyanin biosynthesis (Zhou *et al.,* 2015).

Despite the crucial role played by the NAC family in plants, much research will be required to characterize each NAC gene. As mentioned above, 74 members of *NAC* genes have been identified by a comprehensive analysis of these TF gene family in grapevine (Wang *et al.,* 2013), but the specific biological function of each of them remains unknown. We decided to focus our attention on five NAC family members that might participate in regulating transition from immature to mature phase in grapevine. In this chapter, we describe the five grapevine NAC TFs family members (NAC03, NAC11, NAC13, NAC33 and NAC60) selected for the functional characterization, by studying their expression profiles of the different grapevine organs and developmental stages, by analyzing the NAC domains of related proteins and by performing a co-expression analysis.

2. MATERIALS AND METHODS

2.1 Phylogenetic analysis of grapevine *NACs* and tomato *NOR*

The software MEGA7 (http://www.megasoftware.net/; Kumar *et al.,* 2016) was employed to conduct the phylogenetic analysis. The protein sequences of the grapevine *NAC* and of tomato *NOR* genes were imported into MEGA7 and the multiple alignment was performed using ClustalW in this software. The unrooted phylogenetic trees were constructed using Neighbor-Joining (NJ) method and the bootstrap test was carried out with 1,000 iterations.

2.2 *NACs* expression analysis in grapevine

The expression profiles of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* genes were analyzed in the *Vitis vinifera* (*V. vinifera)* cv. Corvina (clone 48) gene expression atlas of different organs at various developmental stages. Microarray data were obtained from Gene Expression Omnibus website (https://www.ncbi.nlm.nih.gov/geo/) searching for the GSE36128 entry.

The expression profiles of grapevine selected *NACs* genes were also analyzed in a berry specific expression map. Transcriptomic data were obtained by RNAseq performed on whole berry samples collected from 10 different grapevine varieties (Sangiovese, Barbera, Negroamaro, Refosco, Primitivo, Vermentino, Garganega, Glera, Moscato, Passerina) at two pre-véraison (pea and touch), two post-véraison (soft and harvest) developmental stages (Massonnet, 2015). Details about the developmental stages of berries sampled are described in Palumbo *et al.* (2014).

2.3 Isolation and cloning of *VvNACs*

The ORF of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* were amplified by PCR from cDNA of *V. vinifera* cv. Corvina (synthesized from RNA isolated from 200 mg of ground berry skin at post-harvest withering; see Section

2.4, Chapter 3 for RNA extraction and reverse transcription protocol) and HiFi DNA Polymerase (KAPA Biosystems) according to the manufacturer's instruction. Each generated PCR fragment was purified and directionally cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) thanks to the 5'-CACC sequence in the forward primer. After the sequencing, each ORF of interest was transferred into the binary overexpression vector pK7GW2,0 (Laboratory of Plant Systems Biology, PSB; Ghent University, Belgium) by site specific recombination.

Gene specificity	Primer Name	Primers sequence (5'-3')
VvNAC03	NAC03For	CACCATGGACAGCACCGATTCCTC
	NAC03Rev	TCAACCAACTTTGTTTCTTCTTA
VvNAC11	NAC11For	CACCATGAAAGGAAAAAGCAACCCT
	NAC11Rev	TCAGTAATTCCAGAGGCAATC
VvNAC13	NAC13For	CACCATGGGAGGCGCGTCTCTG
	NAC13Rev	TTACATTACAATCACCAAGTTG
VvNAC33	NAC33For	CACCATGGTTGAGTCAAGGTTGCCA
	NAC33Rev	CTAACAATAATGGTTCCAAATGG
VvNAC60	NAC60For	CACCATGGACAACCCGCAATCCAC
	NAC60Rev	TCATCCTTGAAATGGGAAATAAG

Table 2: Transcription factors identified by analyzing global gene expression atlas and large

2.4 NACs domain analysis

The online Multiple Expectation Maximization for Motif Elicitation (MEME) Suite (http://meme-suite.org; Bailey and Elkan, 1994) was used to discover protein motifs with an expected value > 0.05, using any number of repetitions and 10 motifs maximum as search parameters.

2.5 Co-expression analysis

Four different transcriptomic datasets generated in our laboratory were used. The first one was the global gene expression atlas of *V. vinifera* cv. Corvina obtained

by microarray approach (Fasoli *et al.,* 2012); the second one was a berry transcriptome of *V. vinifera* cv. Corvina obtained by microarray approach (Dal Santo *et a*l., 2013); the third one was generated by RNAseq approach on 10 different grapevine Italian varieties, 5 red- and 5 white-skinned, in four berry developmental phases, two pre- and two post-véraison (Massonnet, 2015). The last database was a RNAseq analysis of cv. Cabernet Sauvignon berries during development, sampled every ten days from fruit set to ripening (data not shown). Co-expression analysis was performed on each database using the specific tool named Cor.To (http://www.usadellab.org/cms/index.php?page=corto) with three correlation metrics: Pearson, Spearman and Lasso (Vasilevski *et al.,* 2012). We selected the first ten genes with a positive correlation obtained using each of these metrics and the non-redundant transcripts were selected.

3. RESULTS

3.1 *VvNAC* gene-family member selection

As mentioned in the Introduction, Palumbo *et al.* (2014) identified a set of genes called 'switch' genes by analyzing gene co-expression networks generated from the grapevine global gene expression atlas and from a large berry transcriptomic dataset, including five white and five red berries. These genes are significantly up-regulated during the developmental transition from vegetative to mature and inversely correlated with many genes suppressed during the mature growth phase. They could represent key regulators of transcriptome reprogramming during grapevine organ development, including berry ripening. It was expected to find many TFs among 'switch' genes because naturally they are master regulators of many biological processes like growth, development and adaption to adverse factors. By combining grapevine berry and atlas datasets we compiled a list (Table 2) of 'switch' genes belonging to TFs functional category; we identified the ones present in both transcriptomes and the ones one specific of one of them. We found some basic helix-loop-helix, MYB and zinc finger (C2H2 and C3HC4 type) family members and some lateral organ boundary, WRKY DNA-binding and NAC domain-containing proteins.

We focused our attention on the four members of NAC TFs family: in grapevine, a comprehensive analysis of this family has been conducted by Wang *et al.* (2013), but no more studies have been reported on some members in this plant species. Therefore, we selected *VvNAC33* (VIT_19s0027g00230) and *VvNAC60* (VIT_08s0007g07670) as master regulators of the organ phase transition in the whole plant, since they belong to the global expression atlas 'switch' genes. *VvNAC11* (VIT_14s0108g01070) and *VvNAC13* (VIT_02s0012g01040) were selected as 'switch' genes representing the immature-to-mature transition in grapevine berry development; indeed, they have proved to be 'switch' genes in both red and white berries datasets.

ID_code	Description	A	W	R
VIT_17s0000g00430	basic helix-loop-helix (bHLH) family		*	*
VIT_05s0077g00750	basic helix-loop-helix (bHLH) family		*	
VIT_11s0037g01230	basic helix-loop-helix (bHLH) family		*	
VIT_18s0122g01340	BTB/POZ domain-containing protein	*		
VIT_15s0046g00150	DOF affecting germination 1		*	*
VIT_06s0004g07790	Lateral organ boundaries Domain 15	*	*	*
VIT_03s0091g00670	Lateral organ boundaries protein 38		*	*
VIT_15s0048g00830	LOB domain-containing 18 (Asymmetric leaves 2-like protein 20)	*		
VIT_13s0158g00100	MADS-box agamous-like 15		*	*
VIT_12s0028g00980	myb family	*		
VIT_07s0005g02730	Myb Radialis	*		
VIT_07s0031g01930	myb TKI1 (TSL-KINASE INTERACTING PROTEIN 1)		*	*
VIT_02s0033g00380	Myb VvMYBA1		*	*
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_02s0033g00410	VvMybA1		*	*
VIT_02s0033g00390	VvMybA2		*	*
VIT_02s0033g00450	VvMybA3		*	*
VIT_07s0005g01710	WRKY DNA-binding protein 23		*	*
VIT_12s0059g00880	WRKY DNA-binding protein 6		*	
VIT_17s0000g01280	WRKY DNA-binding protein 75			*
VIT_13s0064g01210	Zf A20 and AN1 domain-containing stress-associated protein 2			*
VIT_13s0047g01130	Zfwd2 protein (ZFWD2)	*		
VIT_10s0071g00580	Zfwd2 protein (ZFWD2)	*		
VIT_00s0203g00210	Zinc finger (B-box type)	*	*	
VIT_00s0347g00031	Zinc finger (B-box type)	*	*	
VIT_12s0059g02510	Zinc finger (B-box type)			*
VIT_06s0061g00760	Zinc finger (C2H2 type) family			*
VIT_06s0004g04180	Zinc finger (C2H2 type) protein (ZAT11)	*		
VIT_18s0001g01060	Zinc finger (C3HC4-type ring finger)	*	*	*
VIT_08s0040g01950	Zinc finger (C3HC4-type ring finger)	*	*	*
VIT_05s0020g04730	Zinc finger (C3HC4-type ring finger)		*	*
VIT_14s0219g00040	Zinc finger (C3HC4-type ring finger)	*		
VIT_12s0028g03860	Zinc finger (C3HC4-type ring finger) protein (RMA1)			*
VIT_03s0091g00260	Zinc finger protein 4		*	*

Table 2: Transcription factors identified by analyzing global gene expression atlas and large berry transcriptome data sets. Shared genes among atlas (A_Fasoli et *al.,* 2012), white berries (W_Massonnet, 2015) and red berries (R_Palumbo *et al.*, 2014) are indicated with an asteriskc (*).

We decided to consider also information obtained by the functional characterization of the tomato *NOR*, a NAC-domain TF whose mutation leads to a non-ripening phenotype (Giovannoni *et al.,* 1995; Tigchelaar *et al.,* 1973). Since *nor* mutant has been classified as one of the key regulators of fruit development and ripening, we looked at the grape *NOR* homologue. A phylogenetic tree was constructed including all the 74 grape NAC and tomato NOR proteins.

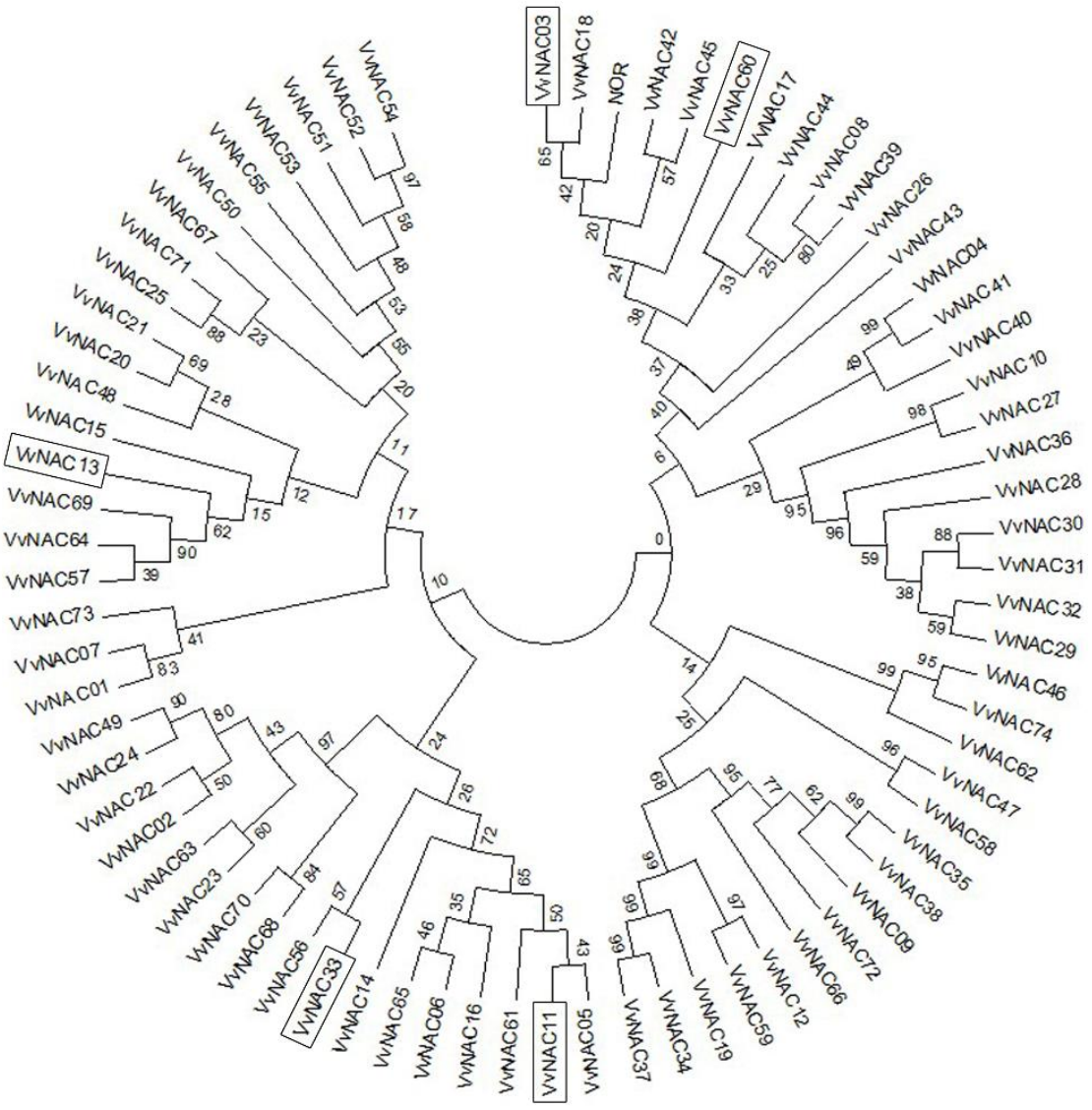


Figure 1: Phylogenetic tree of grapevine *NAC* and tomato *NOR* genes. The analysis was performed using the neighbor-joining method by the Mega version 7 program (Kumar *et al.*, 2016). The numbers next to the nodes are bootstrap values from 1,000 replicates. The five selected NAC TFs are highlighted by open boxes.

As shown in Figure 1, the four selected *NAC* TFs for our analysis were not the closest homologue genes of tomato *NOR*. Therefore, we decided to select one of the two closest *NACs*, *VvNAC18* and *VvNAC03*, on the basis on the expression profile that it was determined by consulting the global gene expression map of *V. vinifera* cv. Corvina (Fasoli *et al.,* 2012). *VvNAC03* expression resulted to be similar to *VvNAC11* one (as showed below), while *VvNAC18* expression did not exactly match the putative role as regulator of berry phase transition (data not shown). Hence, we selected *VvNAC03* (VIT_00s0375g00040) as fifth *NAC* gene to be analyzed.

3.2 *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC6* sequences in *Vitis vinifera* cv 'Corvina'

The full-length coding regions of the five selected *VvNACs* were amplified from grapevine cv. Corvina berry skin cDNA at ripening, directionally cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) and sequenced. *VvNAC03, VvNAC33* and *VvNAC60* contain a 996, 885 and 1005 bp open reading frame encoding a protein of 332, 422 and 366 amino acidic residues with a predicted mass of 36.75, 33.37 and 37.51 kDa and a calculated pI = 9.31, 6.33 and 8.75, respectively. *VvNAC11* and *VvNAC13* genes were composed by a 1263 and 1098 bp coding sequence and encoded a protein of 422 and 366 amino acids, respectively. They had a predicted mass of 47.87 and 41.12 kDa and a calculated pI = 5.52 and 4.78 and 8.75, respectively. Since the last release of the gene prediction V1 and automatic annotation of the 12X sequence assembly of the grape genome, it has been also possible to blast the 'Corvina' sequences against the 'Pinot noir' genome. The analysis of the isolated sequences revealed that the predicted VvNAC11 and VvNAC13 proteins is identical to the sequence from 'Corvina'; for *VvNAC03*, *VvNAC33* and *VvNAC60*, the sequences isolated from the two cultivars share 99% similarity (Supplementary Figure S1).

By further analyzing ‘Corvina’ NAC and tomato NOR proteins by MEME bioinformatic tool, we highlighted that all of them presented the highly conserved NAC domain at N-terminus, divided into five subdomains [A–E; Figure 2).

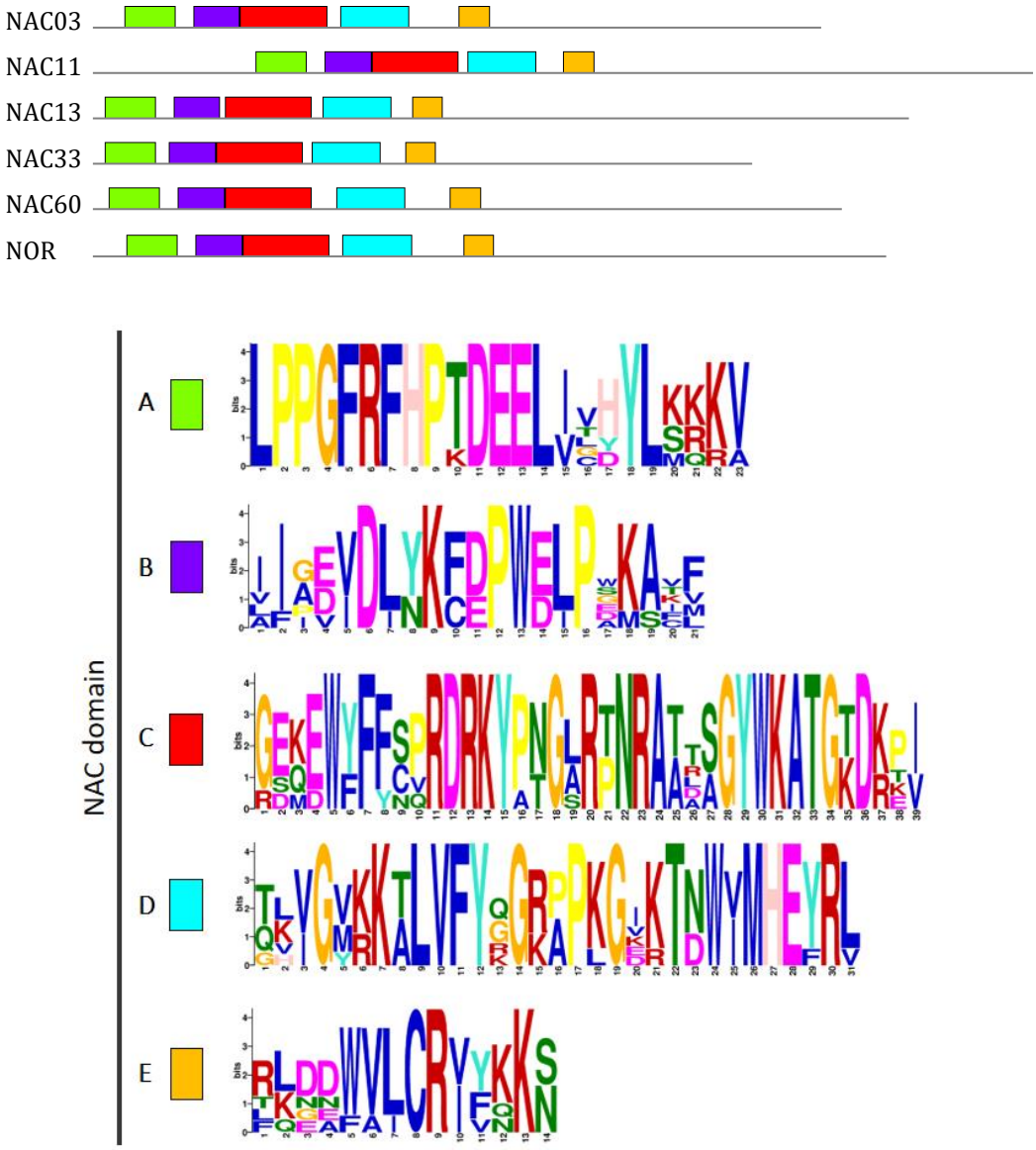


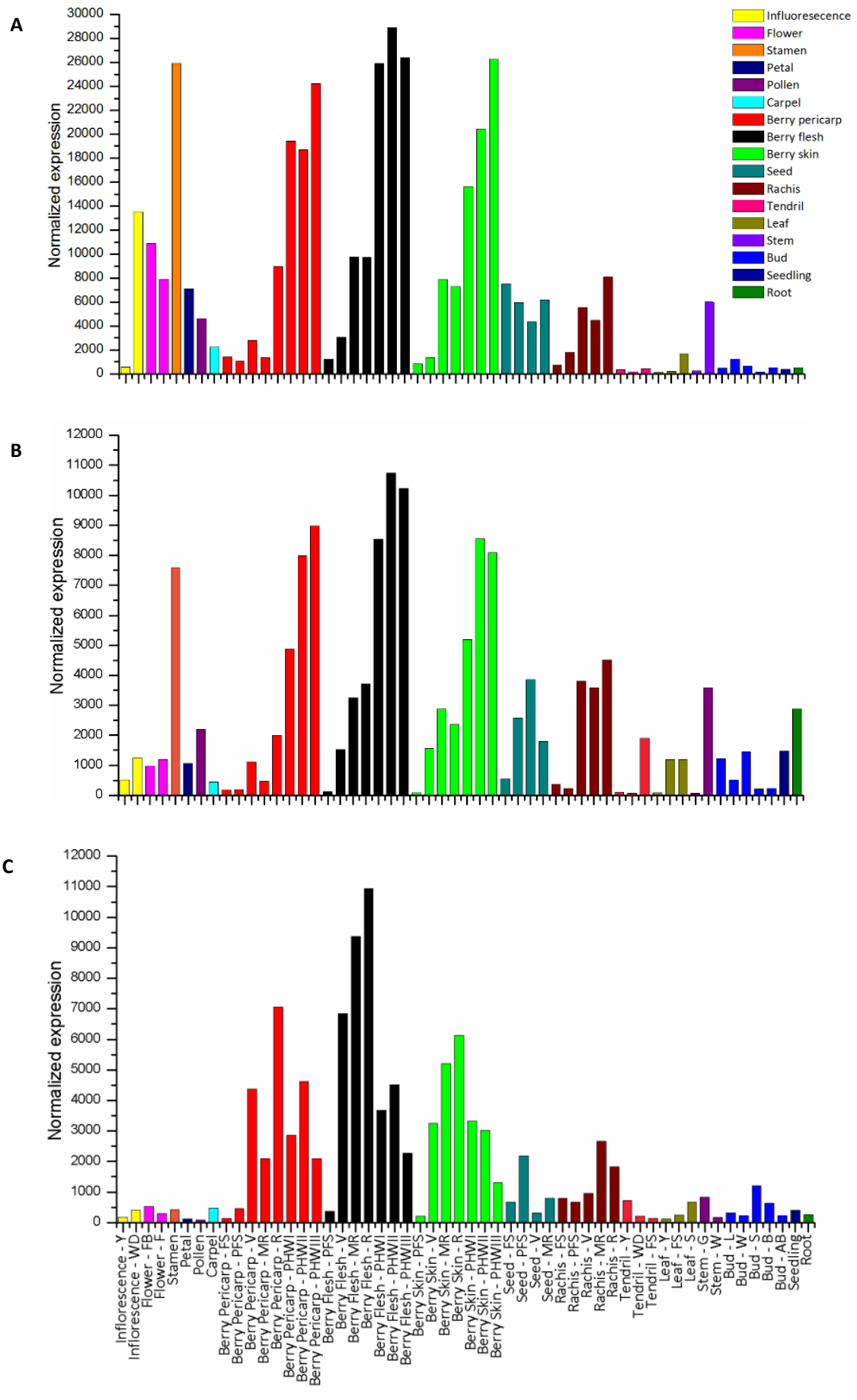
Figure 2: Protein domains organization of NAC03, NAC11, NAC13, NAC33, NAC60 and NOR factors represented by colored boxes identified by MEME Suite. The consensus sequence of the motifs is reported.

Among them, subdomains C and D are conserved and bind to DNA, whereas subdomain A may have an important role in the formation of functional NAC

dimeric proteins. The highly divergent subdomains B and E may confer functional diversity to NAC TFs (Jensen *et al.,* 2008; Ernst *et al.,* 2004).
By analyzing the motifs present in the C-terminus of the NAC TFs proteins, no significant conserved motifs were identified by MEME tool. However, we noted a frequent occurrence of simple amino acid repeats and regions rich in serine and threonine, proline and glutamine, or acidic residues (Supplementary Figure S2).

3.3 Expression profiles of the five selected NAC members

We analyzed genes expression patterns of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* by consulting the global gene expression atlas (Fasoli *et al.,* 2012) and the berry transcriptome dataset (Massonnet, 2015). 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. The expression profile of *VvNAC03* was very similar to *VvNAC11* one, as previously stated. *VvNAC11* and *VvNAC13* were identified as berries 'switch' genes and their expression in all berry tissues appeared, as expected, up-regulated when the ripening phase starts (Figures 3B, 3C). We noted that *VvNAC13,* unlike *VvNAC03* and *VvNAC11*, decreased its expression during post-harvest withering, after an increase in the mature stage (Figure 3C). *VvNAC33* and *VvNAC60*, identified as 'switch' genes in the atlas transcriptome, were expressed at low levels in vegetative/green tissues and up-regulated in the mature/woody phase (Figures 3D, 3E). In detail, their expression levels increased not only in berry tissues, but also consistently in seeds, tendrils, leaves and stems. These observations encouraged an involvement of the selected NACs in driving the regulation of immature-to-mature organ phase transition during grape development.


A
B
C
Normalized expression
Influoresecence
Flower
Stamen
Petal
Pollen
Carpel
Berry pericarp
Berry flesh
Berry skin
Seed
Rachis
Tendril
Leaf
Stem
Bud
Seedling
Root
Inflorescence - Y
Inflorescence - WD
Flower - FB
Flower - F
Stamen
Petal
Pollen
Carpel
Berry Pericarp - FS
Berry Pericarp - PFS
Berry Pericarp - V
Berry Pericarp - MR
Berry Pericarp - R
Berry Pericarp - PHWI
Berry Pericarp - PHWII
Berry Pericarp - PHWIII
Berry Flesh - PFS
Berry Flesh - V
Berry Flesh - MR
Berry Flesh - R
Berry Flesh - PHWI
Berry Flesh - PHWII
Berry Flesh - PHWIII
Berry Skin - PFS
Berry Skin - V
Berry Skin - MR
Berry Skin - R
Berry Skin - PHWI
Berry Skin - PHWII
Berry Skin - PHWIII
Seed - FS
Seed - PFS
Seed - V
Seed - MR
Rachis - FS
Rachis - PFS
Rachis - V
Rachis - MR
Rachis - R
Tendril - Y
Tendril - WD
Tendril - FS
Leaf - Y
Leaf - FS
Leaf - S
Stem - G
Stem - W
Bud - L
Bud - W
Bud - S
Bud - B
Bud - AB
Seedling
Root

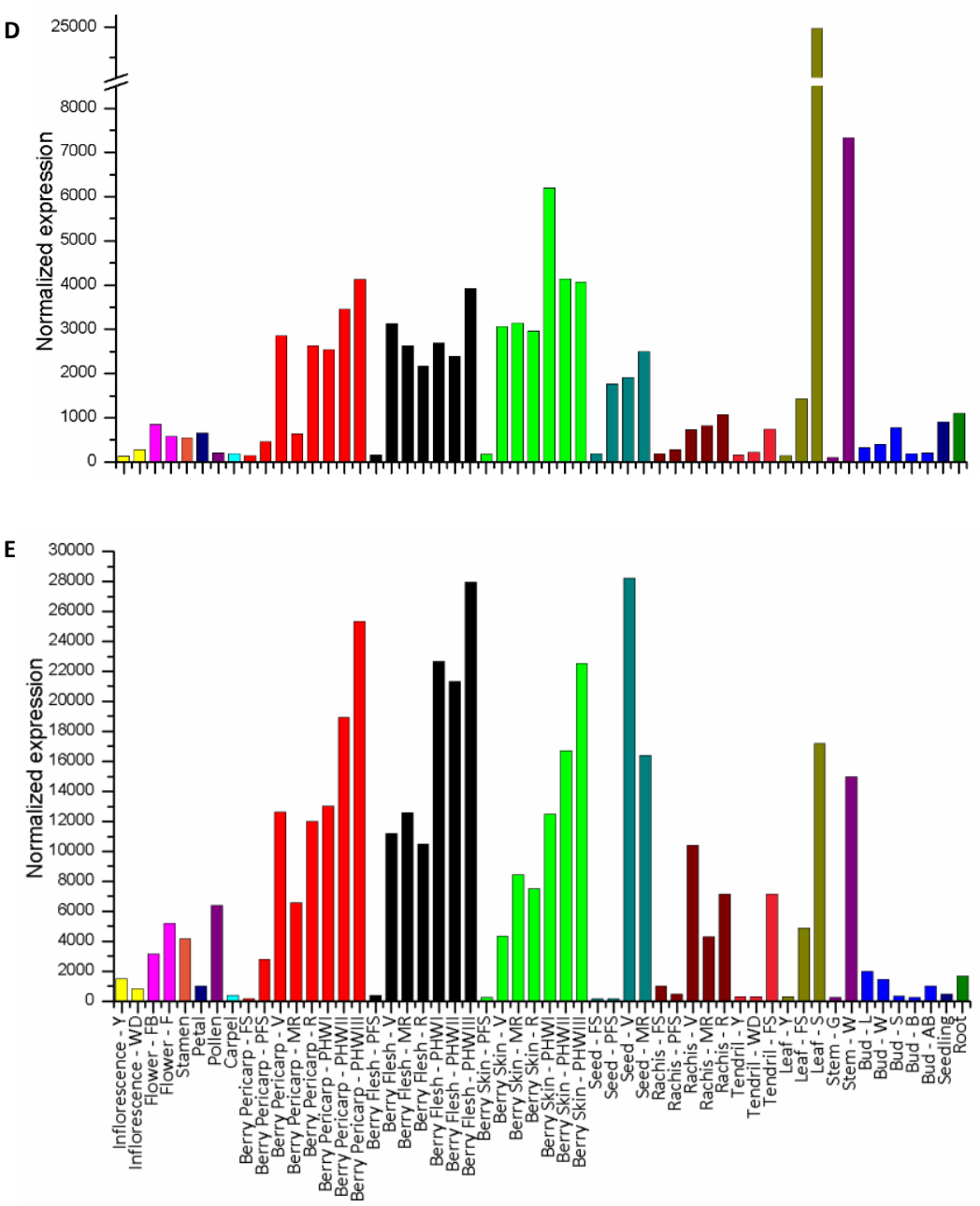
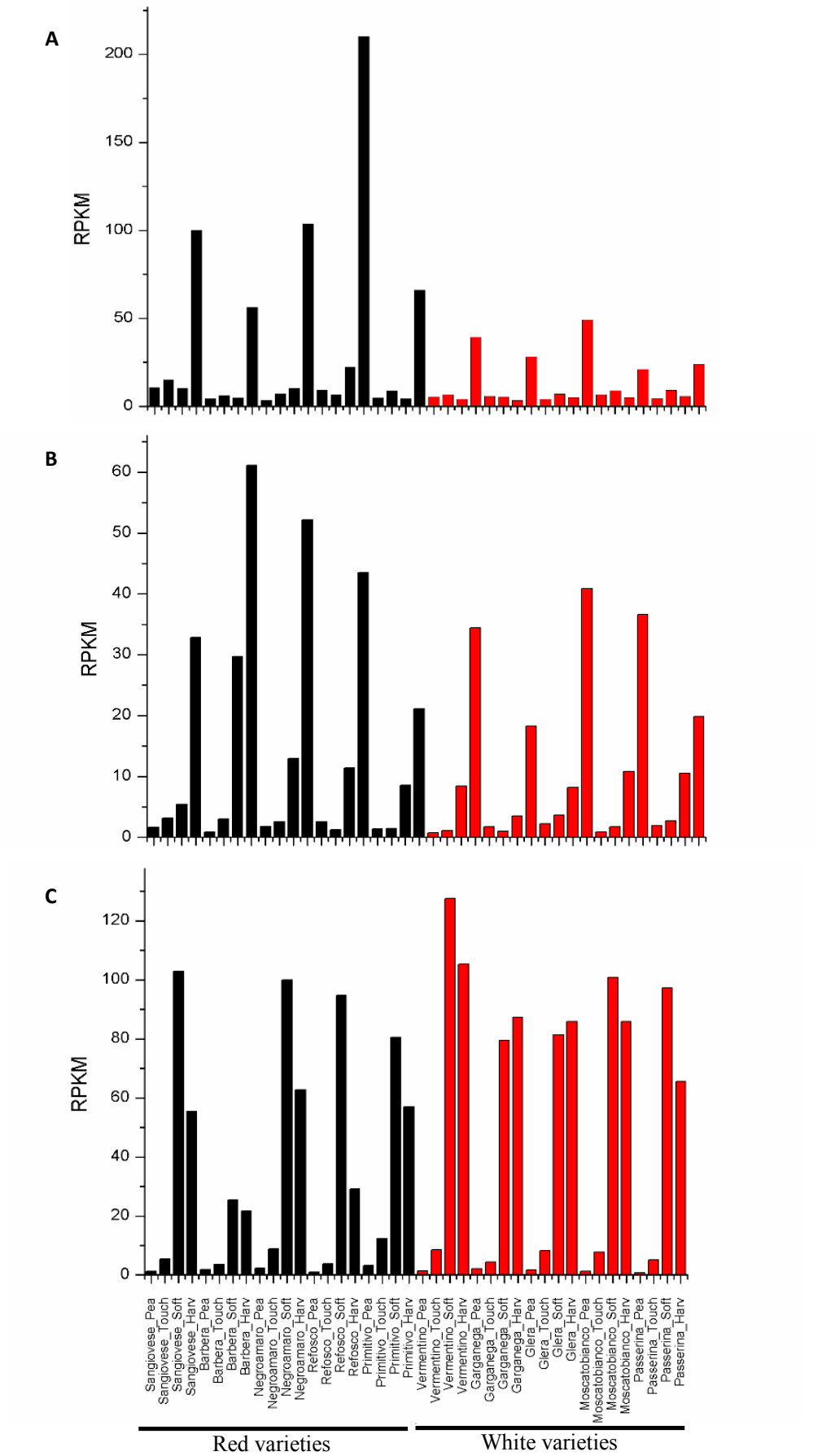


Figure 3: *VvNAC03* (A), *VvNAC11* (B), *VvNAC13* (C), *VvNAC33* (D) and v*NAC60* (E) expression profiles in 54 grape organs at different developmental stages; transcriptomic data were obtained by a global expression map of *Vitis vinifera* cv. Corvina by microarray (Fasoli *et al.,* 2012).

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.

The hypothesis of a putative regulatory role of these TFs in the transcriptome reprogramming has been supported also by analyzing the *VvNAC03, VvNAC11*, *VvNAC13, VvNAC33* and *VvNAC60* expression profiles in a berry specific expression map obtained by RNAseq on whole berry samples collected from 10 different grapevine varieties at various developmental stages (Figure 4; Massonnet, 2015). In all varieties, all five NAC factors showed a higher expression level after véraison, during the ripening phase, supporting their hypothetical participation in the regulation of ripening processes. In particular, we observed that *VvNAC03* and *VvNAC11* showed a higher expression at the last ripening stage (harvest). Albeit less pronounced, also *VvNAC33* followed this trend, showing a higher expression at softening stage in some varieties, such as Garganega and Passerina. On the other hand, *VvNAC13* and *VvNAC60* resulted more expressed at softening stage in almost all varieties, suggesting their involvement in regulation of the berry transition phase earlier than *VvNAC03, VvNAC11* and *VvNAC33*.


A
B
C
RPKM
Sangiovese_Pea
Sangiovese_Touch
Sangiovese_Soft
Sangiovese_Harv
Barbera_Pea
Barbera_Touch
Barbera_Soft
Barbera_Harv
Negroamaro_Pea
Negroamaro_Touch
Negroamaro_Soft
Negroamaro_Harv
Refosco_Pea
Refosco_Touch
Refosco_Soft
Refosco_Harv
Primitivo_Pea
Primitivo_Touch
Primitivo_Soft
Primitivo_Harv
Vermentino_Pea
Vermentino_Touch
Vermentino_Soft
Vermentino_Harv
Garganega_Pea
Garganega_Touch
Garganega_Soft
Garganega_Harv
Glera_Pea
Glera_Touch
Glera_Soft
Glera_Harv
Moscatobianco_Pea
Moscatobianco_Touch
Moscatobianco_Soft
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Passerina_Pea
Passerina_Touch
Passerina_Soft
Passerina_Harv
Red varieties
White varieties

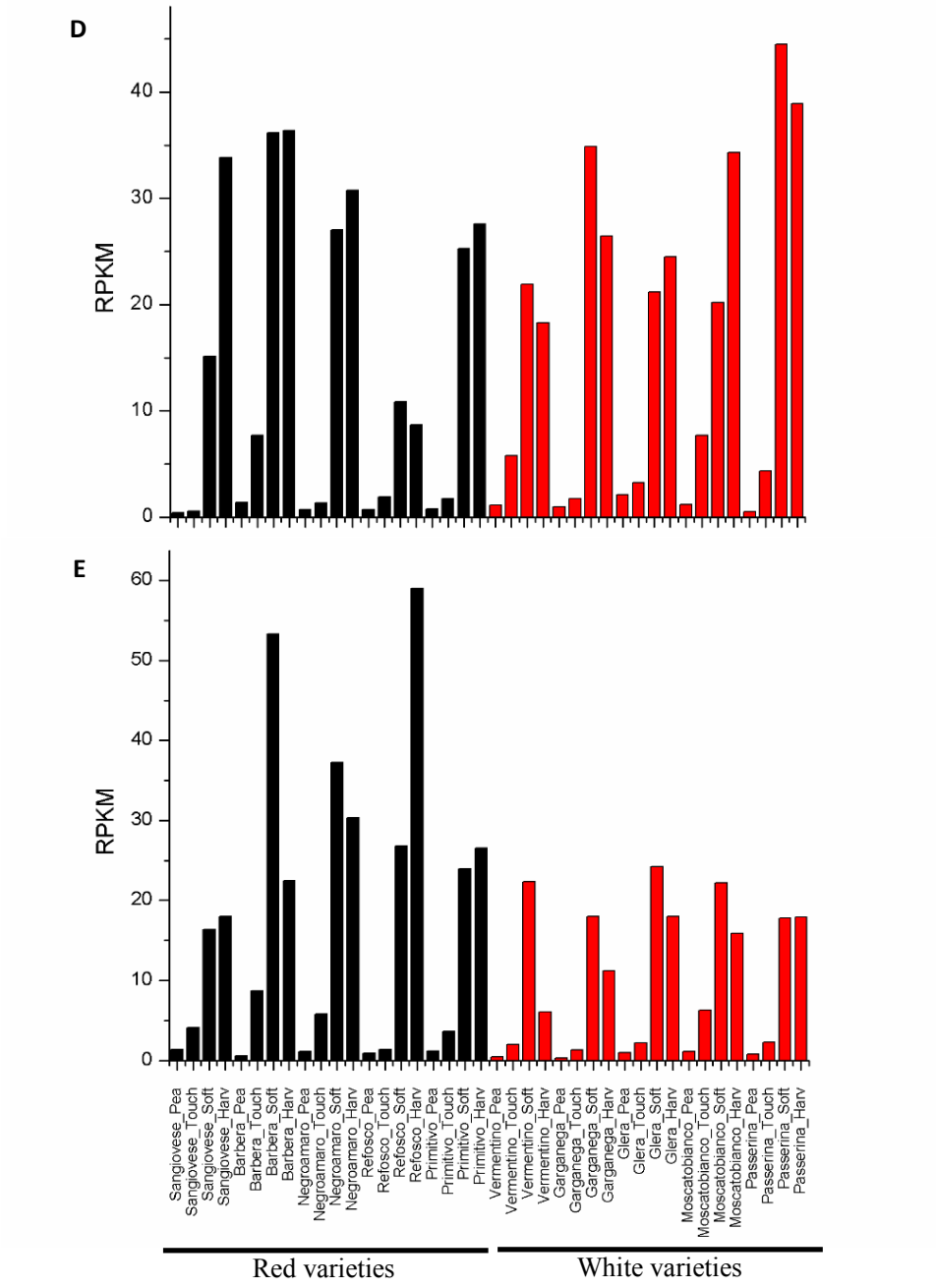


Figure 4: *NAC03* (A), *NAC11* (B), *NAC13* (C), *NAC33* (D) and *NAC60* (E) expression profiles of 10 different grapevine varieties at four developmental stages. Transcriptomic data were obtained by a berry specific expression map obtained by RNAseq (Massonnet, 2015; Palumbo *et al.,* 2014).

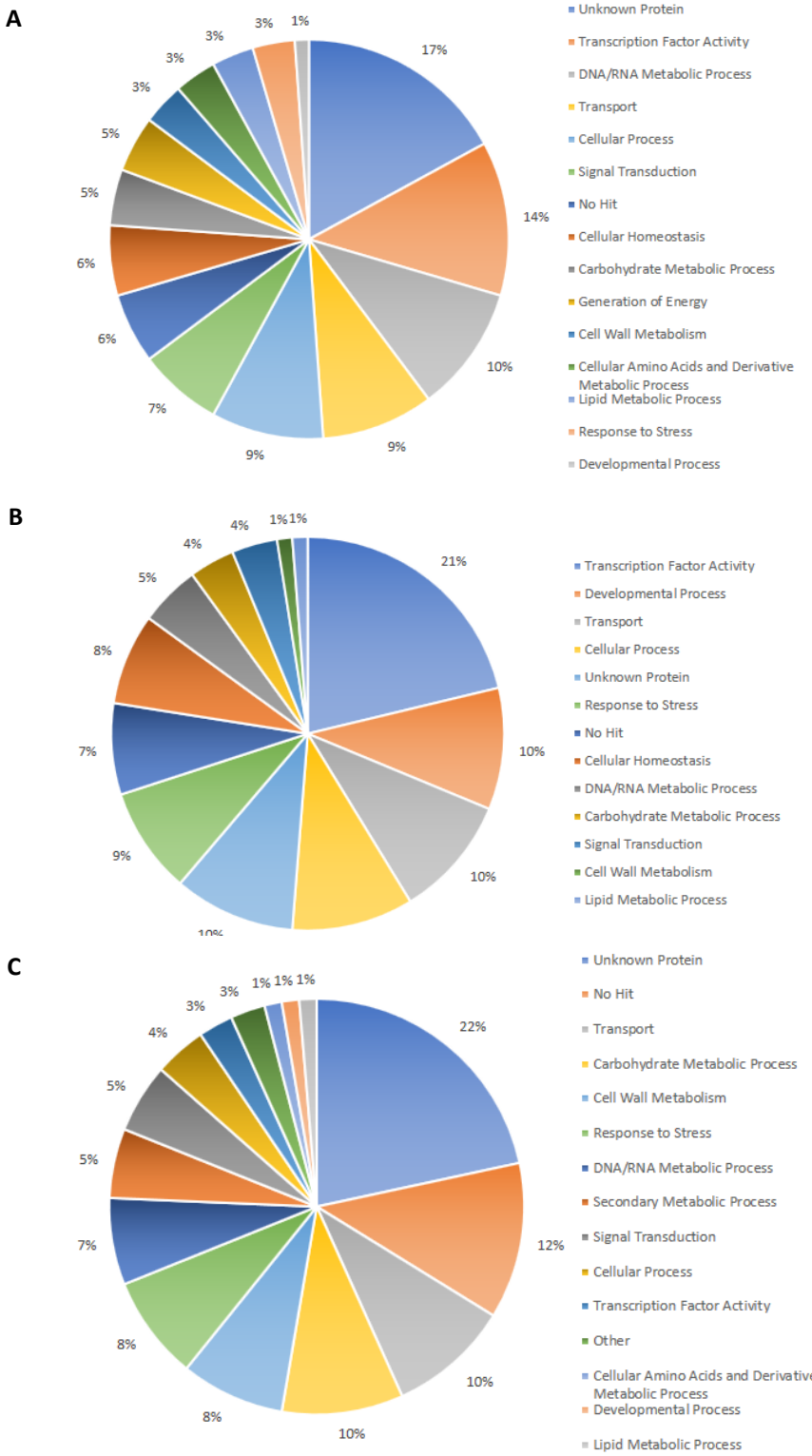
3.4 Co-expression analysis

In order to discover genes co-expressed with the selected *VvNACs* and possibly representing their target or partners, we investigated four different transcriptomic datasets previously obtained in our laboratory, described in

detail in Material and Methods section. Thanks to a specific correlation tool named Cor.To, we selected the more correlated genes with *NAC03*, *NAC11*, *NAC13*, *NAC33* and *NAC60*. In Supplementary Data are indicated five lists with about the first twenty genes for each dataset mostly related to the five *NACs* (Supplementary Figure S3). For each *NAC*, we observed that some gene has been identified at least in two of the four analyzed datasets; for example, *ARMADILLO/BETA-CATENIN REPEAT PROTEIN / U-BOX DOMAIN-CONTAINING PROTEIN* (VIT_04s0043g00840) among *NAC03* correlated genes, *ORGANIC CATION/CARNITINE TRANSPORTER4* (VIT_19s0014g04790) and *POTASSIUM CHANNEL* (VIT_04s0008g04990) among *NAC11* correlated genes, *VvEXPB4* (VIT_15s0021g02700) and *THAUMATIN SCUTL2* (VIT_18s0001g11930) among *NAC13* correlated genes, *PEPTIDOGLYCAN-BINDING LYSM DOMAIN-CONTAINING PROTEIN* (VIT_16s0100g00270) among *NAC33* correlated genes and *4-COUMARATE-COA LIGASE* (VIT_16s0050g00390) among *NAC13* correlated genes. These evidences indicated that this analysis could be a valid approach to get information about the regulatory network in which *NACs* may have a crucial role.

In Figure 5 are reported co-expressed genes distributed them into 18 Gene Ontology functional categories. First of all, we observed that transport and TF activity were present among the most represented functional categories and cellular process and carbohydrate metabolic process were nonetheless well-represented. In particular, we observed that *NAC03, NAC11* and *NAC60* were related to *ORGANIC CATION/CARNITINE TRANSPORTER4* (VIT_19s0014g04790), which is a 'switch' gene, and *NAC11* and *NAC60* to a potassium channel (VIT_04s0008g04990). Moreover, we found that *NAC03* was related to *NAC11* and vice versa and *NAC33* to *NAC60*.

Unfortunately, even after a manually improvement of unknown genes annotation by BLAST, many genes fell in ‘no hit’ and ‘unknown protein’ categories. The lack of more complete and accurate annotation of the grapevine genome is still a critical issue and is the focus of several scientific groups in the ‘grapevine community’.



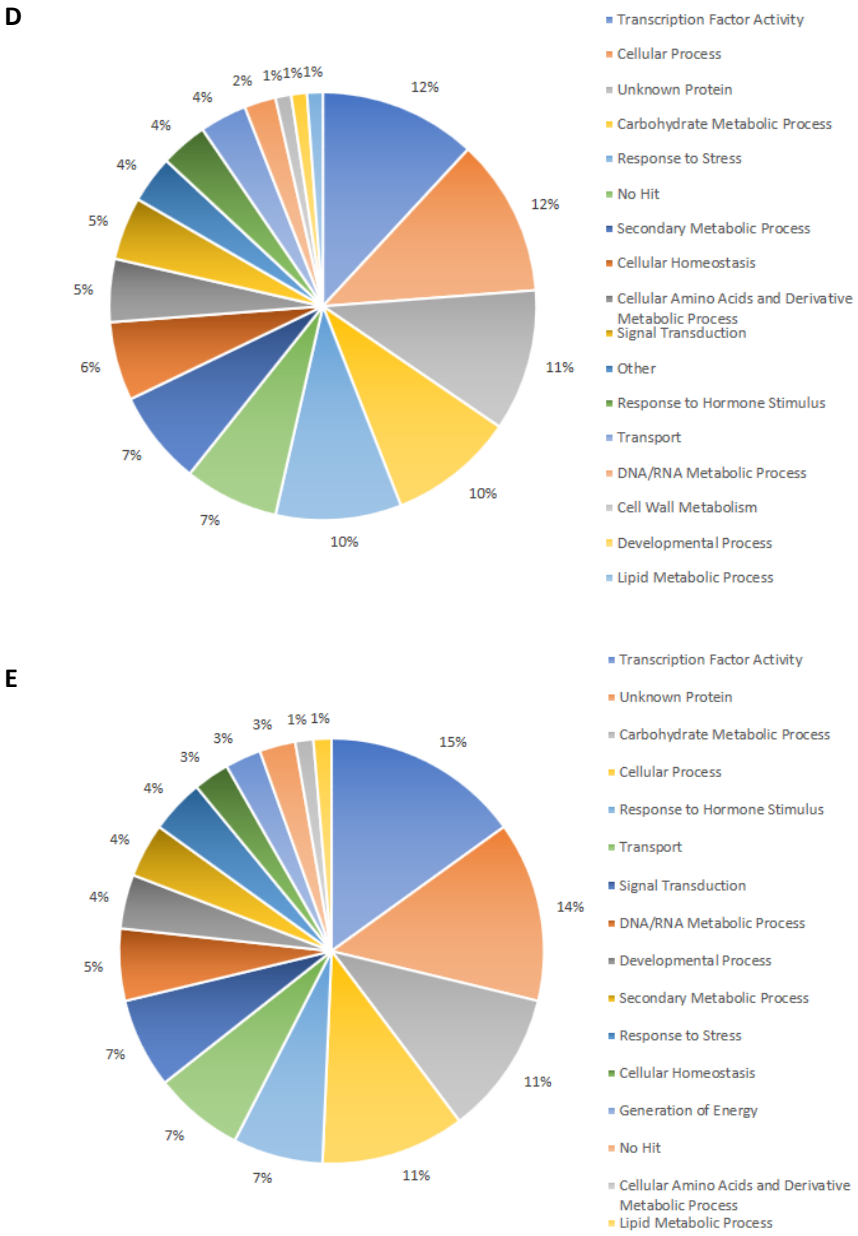


Figure 5: Distribution (%) of genes correlated to *NAC03* (A), *NAC11* (B), *NAC13* (C), *NAC33* (D) and *NAC60* (E) into 18 Gene Ontology functional categories. Co-expression analysis was performed thanks to a specific tool named Cor.To using four datasets as described in the section and three correlation metrics (Pearson, Spearman, Lasso).

4. DISCUSSION

The identification and characterization of genes involved in the direct regulation of grapevine maturation and, particularly, of berry ripening represent an important starting point to deeply understand the mechanisms underlying these processes and to provide a suitable knowledge that could be used to cope the environmental factors that affect the final quality traits in grape berries, such as the global warming.

We started our investigation on a group of genes, called 'switch' genes, identified by an original integrated networking method based on the study of grapevine global gene expression atlas and a grapevine berry transcriptomic dataset (Palumbo *et al.,* 2014). Among this genes category, we decided to focus on NAC TFs because they seem to be attractive candidates as key regulators of the recently discovered largescale transcriptome reprogramming that marks the developmental shift from immature to mature growth (Fasoli *et al.,* 2012). They control both developmental and stress induced processes in plants. In this work, we isolated and characterized five different members of this gene family; *VvNAC33* and *VvNAC60* have been selected as 'switch' genes of the entire plant development, while *VvNAC11* and *VvNAC13* of berry development. Moreover, *VvNAC13* was found to co-localize with the véraison-related metaQTLs identified by analyzing meta-QTL for grapevine phenology associated traits with the aim of providing a genomic overview of the genetic control of grapevine development and berry ripening (not published). This finding further confirms *VvNAC13* key functional involvement along grapevine berry ripening. Thanks to the relevance of tomato as model for fruit development and ripening and the availability of collected tomato-ripening mutants such as *nor*, we considered also *VvNAC03* which resulted to be one of the two closest tomato *NOR* homologues. Our phylogenetic analysis showed that *VvNAC03, VvNAC60* and *NOR* belonged to the same clade, whereas *VvNAC11* and *VvNAC33* to a different one and *VvNAC13* to

an even different one. We are wondering to understand if these differences could reflect an involvement in the control of various processes.

We inspected the global gene expression atlas of cv. Corvina (Fasoli *et al.,* 2012) and the berry specific expression map (Massonnet, 2015; Palumbo *et al.,* 2014) to analyze the expression pattern of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60*.

As expected as 'switch' genes, they were expressed at low level in vegetative/green organs and at higher levels in the mature/woody tissues, suggesting that these genes could be directly involved in the grapevine transcriptomic reprogramming which take place along development and berry ripening. Moreover, these analyses showed that the expression profiles were independent from the skin color.

We isolated the sequences of selected *NACs* from *V. vinifera* cv. Corvina and for three of them, namely *VvNAC03, VvNAC33* and *VvNAC60*, showed 99% similarity between 'Corvina' and 'Pinot noir' genome, confirming the presence of single nucleotide polymorphisms between these two cultivars (Venturini *et al.,* 2013). We observed that any different amino acid was in the domain at N-terminus that are conserved in all NAC TFs (Olsen *et al.,* 2005).

The sequences of the other two NACs, *VvNAC11* and *VvNAC13*, were identical to the reference genome. By looking at the protein level, we found in all five NAC proteins the conserved NAC domain at N-terminus. Commonly, the C-terminal region is more diverged and serves as a potential transcriptional regulatory domain with either activator or repressor function and may occasionally possess protein binding activity (Puranik *et al.,* 2012). In this analysis, we did not find any known protein domains (Ooka *et al.,* 2003), but we verified the presence of simple amino acid repeats and regions rich in serine and threonine, proline and glutamine, or acidic residues (Olsen *et al.,* 2005). As reported in Lui *et al.* (1999), this common feature and the divergence in the NAC protein C-terminus sequences are typical characteristics of plant activation domains.

Furthermore, thanks to a correlation analysis, we extrapolated those genes expressed in a consistent manner with *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* analyzing four different datasets, two of them already published (Dal Santo *et al.,* 2013; Fasoli *et al.,* 2012) and the other two available in our laboratory. Our co-expression analysis revealed a strong relation between *NACs* and transport-related genes. An interesting gene seem to be the *ORGANIC CATION/CARNITINE TRANSPORTER4* (VIT_19s0014g04790) that resulted to be correlated to *VvNAC03, VvNAC11* and *VvNAC60.* We hypothesized that *NACs* could control primary processes, involved in plant maturation, able to activate vacuolar transport or, even if it seems less probable, that the transport processes could act upstream to *NAC* activity. The high number of TFs related to the selected *NACs* supported the existence of a complex regulatory network during plant development. In particular, we found a correlation between *VvNAC03* and *VvNAC11* and we noted that *NAC33* was co-expressed with *VvNAC60*. Moreover, several 'switch' genes - highlighted in bold in the Supplementary Figure S3 - identified by Palumbo *et al.* (2014) emerged from this analysis, indicating that the 'switch' genes could include putative key regulators of the developmental phase transition but also their direct targets. Interestingly, we found that the 'switch' gene *MADS-BOX AGAMOUS-LIKE 15* (VIT_13s0158g00100) was related to *VvNAC03* and the *EXPANSIN* (*VvEXPB4*) (VIT_15s0021g02700) related to *VvNAC13*, which is known to be up-regulated in berry flesh and skin during ripening (Dal Santo *et al.,* 2013). Furthermore, we observed a correlation between *NACs* and secondary metabolic processes, such as *VvNAC33* related to *VvMYBA3* (VIT_02s0033g00450), which has a central role during the transition to berry ripening, and *VvNAC60* to *4-COUMARATE-COA LIGASE* (VIT_16s0050g00390) involved in the phenylpropanoid pathway.

This overall approach could help us to support some results obtained in further functional analyses.

In conclusion, since NAC TFs are able to form multiple protein complexes, they can regulate many cellular processes through a network that integrates multiple biological processes during development and stress response. Much remains to be understood concerning the mechanisms of action and the modes of regulation of *NACs* and, therefore, determining the role of individual NAC proteins represents a daunting task. An important step towards reaching this goal could certainly be overexpressing selected *NAC* candidate genes in model plants or directly in the plant of interest in order to identify the specific *NAC* target genes.

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SUPPLEMENTARY DATA

Supplementary Figure S1: Alignment of VvNAC03 (A), VvNAC33 (B) and VvNAC60 (C) predicted amino acidic sequences from 'Corvina' and 'Pinot Noir' cultivars. Different amino acids are indicated with a dot.

A

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Corvina     MDSTDSSSGSHHPQLPPGFRFHPTDEELVVHYLKRKAASVPLPVTIIADVDLYKFDPWEL
Pinot       MDSTDSSSGSHHPQLPPGFRFHPTDEELVVHYLKRKAASVPLPVTIIADVDLYKFDPWEL
            ************************************************************

Corvina     PSKATFGEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPILTSNGSQKVGVKKAL
Pinot       PSKATFGEQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPILTSNGSQKVGVKKAL
            ************************************************************

Corvina     VFYGGKPPKGIKTNWIMHEYRLIDSTSSNTKPPGADMGNKKGSLRLDDWVLCRIYKKNIS
Pinot       VFYGGKPPKGIKTNWIMHEYRLIDSTSSNTKPPGADMGNKKGSLRLDDWVLCRIYKKNIS
            ************************************************************

Corvina     QRPMERDKEESMEALLASLPASSMTSQQNTRAQTLRSTNFGAVLEHEEISFEGMLTGQNI
Pinot       QRPMERDKEESMEALLASLPASSMTSQQNTRAQTLRSTNFGAVLEHEEISFEGMLTGQSI
            ************************************************************.*

Corvina     QGLQSSSMSQYWNGAGSIGPSSSGKHLHTDHTSGTSTSEMDGNSSFVSLLNQLPQSGTFH
Pinot       QGLQSSSMSQYWNGAGSIGPSSSGKHLHTDHTSGTSTSEMDGNSSFVSLLNQLPQSGTFH
            ************************************************************

Corvina     QNTLLGSLADEEKFNLRNINRKKCLRRNKVG*
Pinot       QNTLLGSLADEEKFNLRNINRKKCLRRNKVG*
            ********************************
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B

```
Corvina     MVESRLPPGFRFHPKDEELICDYLMKKVTSSESSLFIEVDLNKCEPWDIPEMACVGSKDW
Pinot       MVESRLPPGFRFHPKDEELICDYLMKKVTSSESSLFIEVDLNKCEPWDIPEMACVGSKDW
            ************************************************************

Corvina     YFYNQRDRKYATGLRTNRATLSGYWKATGKDRPILSKGTLVGMRKTLVFYQGRAPKGKKT
Pinot       YFYNQRDRKYATGLRTNRATLSGYWKATGKDRPILSKGTLVGMRKTLVFYQGRAPKGKKT
            ************************************************************

Corvina     DWVMHEFRLQGPLTPPAIPSLKEDWVLCRVFNKSRSEAAGKAITSNMGNGYYDNMNMGSS
Pinot       DWVMHEFRLQGPLTPPAIPSLKEDWVLCRVFNKSRSEAAGKAITSNMGNGYYDNMNMGSS
            ************************************************************

Corvina     TLPPLVDSYINFDQTEIKLNEYEQVPCFSDMCSPNPSNLVFPHITHMEPHLLTKTIAPIF
Pinot       TLPPLVDSYINFDQTEIKLNEYEQVPCFSDMCSPNPSNLVFPHITHLEPHLLTKTIAPIF
            ******************************************:*************

Corvina     GGMPDLGTFSCDKMVIKTVLNQLSNVEESPSFGEGSSSESYLSEVALPPIWNHYC*
Pinot       GGMPDLGTFSCDKMVIKTVLNQLSNVEESPSFGEGSSSESYLSEVALPPIWNHYC*
            ************************************************************
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C   Corvina     MDNPQSTLPPGFRFHPTDEELILHYLSKKVTSTPFPVSIIADVDIYKFDPWELPGKAVFG
    Pinot       MDNPQSTLPPGFRFHPTDEELILHYLSKKVTSTPFPVSIIADVDIYKFDPWELPGKAVFG
                ************************************************************

    Corvina     EKEWYFFSPRDRKYPNGLRPNRAAASGYWKATGTDKTIVAASSIGGGGHGHIGVKKALVF
    Pinot       EKEWYFFSPRDRKYPNGLRPNRAAASGYWKATGTDKTIVAASSIGGGGHGHIGVKKALVF
                ************************************************************

    Corvina     YQGRPPKGIKTNWIMHEYRLAQPPNPAINKPPLKLRDASMRLDNWVLCRIYKKSNAVPPA
    Pinot       YQGRPPKGIKTNWIMHEYRLAQPPNPAINKPPLKLRDASMRLDNWVLCRIYKKSNAVPPA
                ************************************************************

    Corvina     TAAAIDDDREQEDSFMEESLKSHPNQSTIQPQKPSSFSNILDVMDSSTLGHLFSDIQYSD
    Pinot       TAAAIDDDREQEDSFMEESLKSHPNQSTIQPQKPSSFSNILDAMDSSTLGHLFSDIQYSD
                ****************************************.*******************

    Corvina     PTGFEPTPPAKYGSLSQSNDILPKLPYWKSVPSMENQLKRQRSSMDGDVPCPSKKLTSSC
    Pinot       PTGFEPTPPAKYGSLGQSNDILPKLPYWKSVPSMENQLKRQRSSMDGDVPCPSKKLTSSC
                **************.*********************************************

    Corvina     TFTTNPNQSDLPQSYFNQSLFNQALLLNPYFPFQG*
    Pinot       TFTTNPNQSDLPQSYFNQSLFNQALLLNPYFPFQG*
                ************************************
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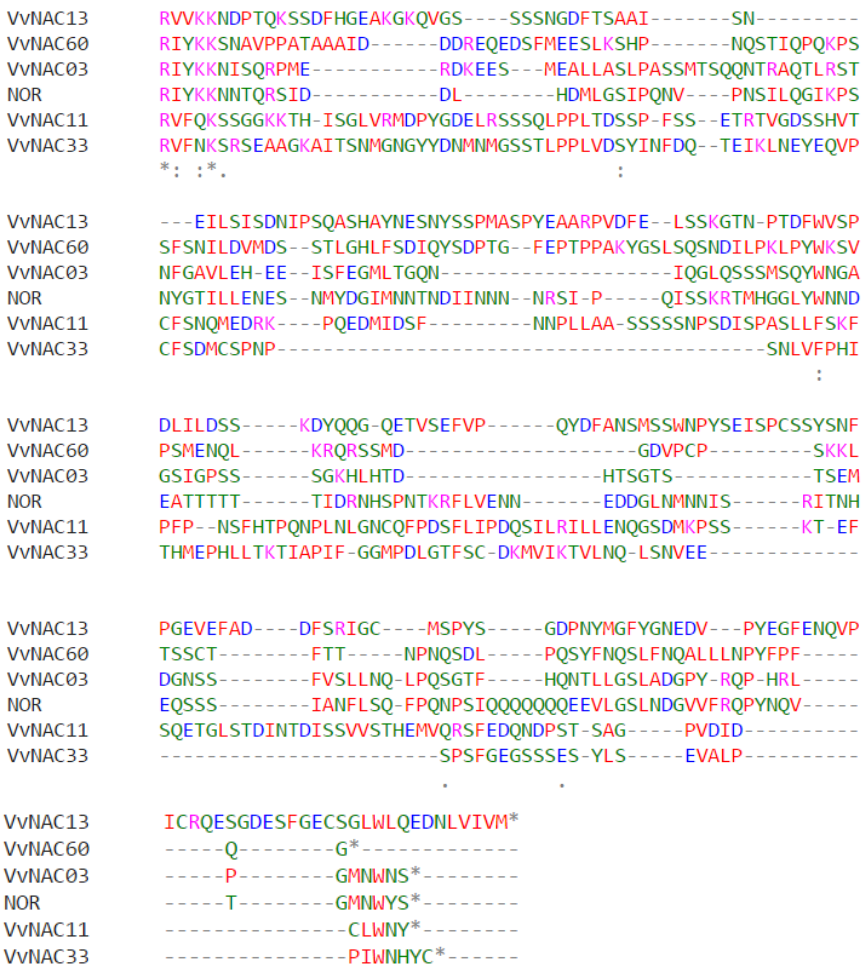
Supplementary Figure S2: Alignment of the VvNAC03, VvNAC11, VvNAC13, VvNAC33, VvNAC60 and NOR protein sequences.

```
VvNAC13     ------------------------------------------------------------
VvNAC60     ------------------------------------------------------------
VvNAC03     ------------------------------------------------------------MD
NOR         ------------------------------------------------------------ME
VvNAC11     MKGKSNPYYRGVEENFGVRVGTEESPSVIFERWDAGFCILFPHSDRYDNKGFREDFEGME
VvNAC33     ------------------------------------------------------------

VvNAC13     --------MGGASLPPGFRFHPTDEELVGYYLKRKVEGQKFELEVIPVIDLYKFDPWELP
VvNAC60     ------MDNPQSTLPPGFRFHPTDEELILHYLSKKVTSTPFPVSIIADVDIYKFDPWELP
VvNAC03     STDSSSG-SHHPQLPPGFRFHPTDEELVVHYLKRKAASVPLPVTIIADVDLYKFDPWELP
NOR         STDSSTGTRHQPQLPPGFRFHPTDEELIVHYLKKRVAGAPIPVDIIGEIDLYKFDPWELP
VvNAC11     KAPDQSKDDEMMELPPGFRFHPTDEELITHYLSQKVLNSGFCAVAIGEVDLNKCEPWDLP
VvNAC33     --------MVESRLPPGFRFHPKDEELICDYLMKKVTSSES--SLFIEVDLNKCEPWDIP
                      *********.****:  ** ::. .       :  :*: * :**::*

VvNAC13     DKSFLPKRDMEWFFFCPRDRKYPNGSRTNRATRAGYWKATGKDRKVVACQ------STVI
VvNAC60     GKAVFG--EKEWYFFSPRDRKYPNGLRPNRAAASGYWKATGTDKTIVAASSIGGGGHGHI
VvNAC03     SKATFG--EQEWYFFSPRDRKYPNGARPNRAATSGYWKATGTDKPILT-----SNGSQKV
NOR         AKAIFG--EQEWFFFSPRDRKYPNGARPNRAATSGYWKATGTDKPVFT-----SGGTQKV
VvNAC11     WKAKMG--EKEWYFFCVRDRKYPTGLRTNRATDAGYWKATGKDKEIYKMK-------TLV
VvNAC33     EMACVG--SKDWYFYNQRDRKYATGLRTNRATLSGYWKATGKDRPILSKG-------TLV
              : .   . :*:*:  ***** .* * ***: :*******.*: :            :

VvNAC13     GYRKTLVFYRGRAPLGDRTDWVMHEYRLCDDPSQG---------------SGFQGAFALC
VvNAC60     GVKKALVFYQGRPPKGIKTNWIMHEYRLAQPPNPAIN----KPPLKLRDASMRLDNWVLC
VvNAC03     GVKKALVFYGGKPPKGIKTNWIMHEYRLIDSTSSNTKPPGA--DMGNKKGSLRLDDWVLC
NOR         GVKKALVFYGGKPPKGVKTNWIMHEYRVVENKTNN-KPLGCDNIVANKKGSLRLDDWVLC
VvNAC11     GMKKTLVFYKGRAPKGEKTNWVMHEYRLEGKHSMYNLP------------KTTKNEWVIC
VvNAC33     GMRKTLVFYQGRAPKGKKTDWVMHEFRLQGPLTPPAIP------------S-LKEDWVLC
            * :*:**** *: * * :*:*:***:*:    .               .     :.:*
```



Supplementary Figure S3: Lists of genes mostly related to *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* obtained by co-expression analysis using the specific tool named Cor.To with three correlation metrics: Pearson, Spearman and Lasso (Vasilevski *et al.,* 2012). Four different transcriptomic datasets were used: the global gene expression atlas of *V. vinifera* cv. Corvina obtained by microarray approach (red_Fasoli *et al.,* 2012); the berry transcriptome of *V. vinifera* cv. Corvina obtained by microarray approach (blue_Dal Santo *et a*l., 2013); the dataset generated by RNAseq approach on 10 different grapevine Italian varieties, 5 red- and 5 white-skinned, in four berry developmental phases, two pre- and two post-véraison (green_ Massonnet, 2015); the dataset generated by RNAseq analysis of cv. Cabernet Sauvignon berries during development, sampled every ten days from fruit set to ripening (violet). In grey those genes identified in more at least two datasets are reported. In bold the 'switch' genes are highlighted.

A

ID_gene	Gene annotation	NAC11	NAC13	NAC33	NAC60
VIT_00S0301G00100	BFN1 (bifunctional nuclease I)	*			
VIT_14S0108G01070	**NAC domain-containing protein (VvNAC11)**				
VIT_04S0023G02200	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase				
VIT_01S0011G00760	Beta-glucosidase				
VIT_01S0011G04680	Glycosyltransferase family 14				
VIT_17S0000G09750	transducin protein	*			
VIT_06S0004G03440	Ulp1 protease	*			
VIT_04S0043G00840	armadillo/beta-catenin repeat protein / U-box domain-containing protein	*			
VIT_00S0125G00190	potassium transporter (KUP1)	*			
VIT_19S0014G00410	PMR5 (powdery mildew resistant 5)				
VIT_19S0014G03300	NAC domain-containing protein (VvNAC18)				
VIT_18S0001G09480	PUMILIO 8 (APUM8)				
VIT_13S0158G00100	**MADS-box AGAMOUS-LIKE 15**				
VIT_14S0219G00110	**Unknown protein**	*			
VIT_19S0090G01350	**aspartyl protease**				
VIT_06S0004G01650	acetyl-CoA carboxylase, biotin carboxylase				
VIT_13S0019G04620	OTU cysteine protease	*			*
VIT_09S0002G06420	**lactoylglutathione lyase**		*	*	
VIT_14S0068G02120	CYP94B3	*			
VIT_02S0025G00810	cation/hydrogen exchanger (CHX18)				
VIT_09S0002G07860	glycerol kinase				
VIT_04S0023G02200	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase				
VIT_16S0022G01690	Band 7 family				
VIT_14S0060G00420	**pyruvate dehydrogenase kinase**	*			
VIT_01S0011G04210	Amino acid permease		*		
VIT_16S0039G00260	pectate lyase				
VIT_02S0033G01170	replication protein RPA 70kDa subunit				
VIT_02S0087G01020	CRK10 (cysteine-rich RLK10)				
VIT_17S0000G05240	nuclear transport factor 2 (NTF2)	*			
VIT_08S0105G00460	Acyl-CoA oxidase ACX4				
VIT_17S0000G08910	Unknown protein				
VIT_05S0020G02280	Unknown protein	*			
VIT_19S0014G04790	**Organic cation/carnitine transporter4**	*			*
VIT_13S0084G00150	choline kinase				
VIT_14S0083G00200	DDT domain-containing protein				
VIT_01S0026G01870	Avr9/Cf-9 induced kinase 1	*			
VIT_14S0083G01170	Mitochondrial substrate carrier family protein				
VIT_11S0052G00640	nitrogen fixation NifU				
VIT_04S0008G04730	Unknown protein				
VIT_04S0008G03770	Aspartate aminotransferase P1				
VIT_07S0031G01330	Alanine:glyoxylate aminotransferase 2				
VIT_19S0090G01710	FRIGIDA-like 2	*			
VIT_11S0037G00040	basic helix-loop-helix (bHLH) family				
VIT_04S0008G02340	exocyst subunit EXO70 protein				
VIT_14S0060G01870	Unknown protein				
VIT_04S0043G00840	armadillo/beta-catenin repeat protein / U-box domain-containing protein	*			
VIT_15S0046G03360	Unknown protein				
VIT_18S0001G13680	Histone H3				
VIT_08S0007G04480	pectinesterase family				
VIT_12S0028G00980	**myb family**				*
VIT_19S0090G00130	Unknown protein				
VIT_08S0032G00950	Unknown protein				
VIT_19S0015G01060	Unknown protein				
VIT_00S0479G00010	Protein kinase 6				
VIT_02S0012G01010	Leucine-rich repeat				
VIT_12S0055G01290	no hit				
VIT_19S0090G00450	Unknown protein				
VIT_07S0005G00170	rubber elongation factor (REF)				
VIT_07S0031G02870	Nucleotidyltransferase				
VIT_10S0003G01120	no hit				
VIT_00S0566G00020	Unknown protein				
VIT_04S0023G00140	Pelota (PEL1)				
VIT_05S0020G04800	Histone H1				
VIT_16S0050G00480	CYP715A1				
VIT_07S0151G00260	Quinone-oxidoreductase, Chloroplast				
VIT_01S0011G02900	no hit				
VIT_04S0043G00840	armadillo/beta-catenin repeat protein / U-box domain-containing protein	*			
VIT_04S0069G00760	Ubiquitin-specific protease 15				
VIT_00S0276G00020	no hit				
VIT_17S0000G09380	WNK kinase 3				
VIT_19S0090G00850	SWAP (Suppressor-of-White-APricot)				
VIT_17S0000G00910	ATPase domain-containing protein				
VIT_07S0005G02190	Zinc finger (C2H2 type) family				
VIT_18S0072G00630	TUBBY LIKE PROTEIN 1 TLP10				
VIT_05S0102G01120	BZIP transcription factor, putative (bZIP69)				
VIT_14S0030G02200	bZIP transcription factor BZO2H1				
VIT_13S0156G00250	Cytidine/deoxycytidylate deaminase				
VIT_19S0015G00160	Agenet domain-containing protein				
VIT_19S0090G01690	WNK3 (Arabidopsis WNK kinase 3)				
VIT_19S0085G01160	Unknown protein				
VIT_18S0001G08140	ENHANCED SILENCING PHENOTYPE 4 ESP4 symplekin				
VIT_16S0050G00790	hydroxyproline-rich glycoprotein				
VIT_07S0005G04560	Unknown protein				
VIT_07S0005G06360	E3 ubiquitin-protein ligase PRT1				
VIT_15S0021G01730	Unknown protein				
VIT_05S0020G00940	DNA helicase SNF2 domain-containing protein				
VIT_11S0016G01420	mechanosensitive ion channel				
VIT_05S0124G00580	Unknown protein				
VIT_11S0052G00170	Armadillo-like helical domain-containing				
VIT_18S0001G08770	CF9				
VIT_06S0004G08330	no hit				

B

ID_gene	Gene annotation	NAC03	NAC13	NAC33	NAC60
VIT_00S0375G00040	NAC domain-containing protein (VvNAC03)				
VIT_12S0034G00960	Unknown				
VIT_07S0104G00140	no hit				
VIT_04S0043G00840	armadillo/beta-catenin repeat protein / U-box domain-containing protein	*			
VIT_06S0004G03440	Ulp1 protease	*			
VIT_00S0285G00050	no hit				
VIT_00S0301G00100	BFN1 (bifunctional nuclease I)	*			
VIT_00S0125G00190	potassium transporter (KUP1)	*			
VIT_17S0000G09030	**disease resistance protein (NBS-LRR class)**				
VIT_17S0000G09750	transducin protein	*			
VIT_14S0030G00400	WD40				*
VIT_19S0014G04790	**Organic cation/carnitine transporter4**	*			*
VIT_08S0040G02160	zinc finger (C3HC4-type RING finger)				
VIT_11S0016G00400	zinc finger (FYVE type)				
VIT_12S0034G02170	SAG18 (Senescence associated gene 18)				
VIT_13S0019G04620	OTU cysteine protease	*			*
VIT_12S0028G03860	**zinc finger (C3HC4-type RING finger) protein (RMA1)**				*
VIT_11S0016G05620	TPR4/WSIP2 (topless-related 4)				
VIT_14S0171G00470	no hit				
VIT_18S0001G10370	S-ribonuclease binding protein SBP1				
VIT_12S0055G00420	bZIP transcription factor				
VIT_19S0014G00860	zinc finger (FYVE type)				
VIT_01S0011G04920	MAPK (MPK9)				
VIT_06S0061G00180	WD40				
VIT_18S0001G00100	auxin-independent growth promoter				
VIT_14S0060G00420	**pyruvate dehydrogenase kinase**	*			
VIT_14S0036G01380	PHR1 (phosphate starvation response 1)				
VIT_10S0003G00350	NAC domain-containing protein (VvNAC37)				
VIT_00S1488G00020	glycogen (starch) synthase				
VIT_17S0000G05240	nuclear transport factor 2 (NTF2)	*			
VIT_01S0010G02480	ankyrin repeat				
VIT_19S0014G04790	**Organic cation/carnitine transporter4**	*			*
VIT_19S0090G01710	FRIGIDA-like 2	*			
VIT_01S0026G01870	Avr9/Cf-9 induced kinase 1	*			
VIT_05S0020G02280	Unknown protein	*			
VIT_14S0030G02310	Auxin-induced protein 22D				
VIT_18S0001G05580	Unknown protein				
VIT_01S0146G00100	ACT domain containing protein (ACR4)				
VIT_07S0151G00770	Kelch repeat-containing F-box protein				
VIT_00S0375G00040	NAC domain-containing protein (VvNAC03)				
VIT_12S0028G03860	**zinc finger (C3HC4-type RING finger) protein (RMA1)**				*
VIT_08S0040G00100	myb family				
VIT_02S0025G04660	senescence-inducible chloroplast stay-green protein 1			*	*
VIT_14S0066G01710	**Leaf senescence protein**				*
VIT_13S0074G00470	OTU cysteine protease				
VIT_09S0002G00750	P-GLYCOPROTEIN 19				
VIT_04S0008G04990	**potassium channel (VvK1.2)**				*
VIT_07S0031G02260	Unknown protein				*
VIT_02S0033G01390	**no hit**				
VIT_05S0049G00120	Unknown protein				
VIT_19S0014G04790	**Organic cation/carnitine transporter4**	*			*
VIT_14S0068G02120	CYP94B3	*			
VIT_11S0016G03810	Unknown protein				
VIT_06S0004G00960	unknown				
VIT_15S0048G01980	COP9 signalosome complex subunit 1				
VIT_17S0053G00980	DnaJ homolog, subfamily C, member 15				
VIT_13S0139G00310	DNA-directed RNA polymerase II subunit E				
VIT_01S0011G03670	**bifunctional nuclease**		*		
VIT_14S0083G00940	**auxin-independent growth promoter**				
VIT_02S0154G00610	Pex19 protein				
VIT_06S0061G00760	**Zinc finger (C2H2 type) family**				
VIT_07S0141G00520	**serine carboxypeptidase 1 precursor**				
VIT_00S0337G00010	salt tolerance protein 2				
VIT_01S0010G01280	no hit				
VIT_06S0004G00960	unknown				
VIT_02S0012G02220	**xyloglucan endotransglucosylase/hydrolase 30**		*		
VIT_09S0018G01370	**STE20/SPS1 proline-alanine-rich protein kinase**				
VIT_19S0014G04790	**Organic cation/carnitine transporter4**	*			*
VIT_05S0020G04010	Unknown protein				
VIT_09S0018G01390	**STE20/SPS1 proline-alanine-rich protein kinase**				
VIT_14S0006G02240	2-hydroxyacyl-CoA lyase 1				
VIT_02S0025G00900	6-phosphogluconate dehydrogenase				
VIT_13S0019G05130	serine carboxypeptidase III				
VIT_00S0347G00030	**zinc finger (B-box type)**				
VIT_04S0008G04990	**potassium channel (VvK1.2)**				*
VIT_14S0219G00110	**Unknown protein**	*			
VIT_11S0052G00730	adagio protein 1				
VIT_08S0032G01210	mal d 1-associated protein				
VIT_06S0004G06510	phosphoesterase				
VIT_01S0011G04370	**Phosphatidylserine synthase 2**				
VIT_04S0008G05770	**CBL-interacting protein kinase 25 (CIPK25)**				
VIT_18S0001G11910	1-acyl-sn-glycerol-3-phosphate acyltransferase 4		*		
VIT_12S0028G03860	**zinc finger (C3HC4-type RING finger) protein (RMA1)**				*
VIT_14S0030G01600	DNA-binding storekeeper protein				
VIT_09S0002G07110	**KEG (keep on going)**				
VIT_14S0219G00040	**zinc finger (C3HC4-type RING finger)**				
VIT_01S0146G00260	**nodulin MtN3**				
VIT_16S0100G00570	**dehydration-responsive protein**		*		*
VIT_00S0203G00210	**zinc finger (B-box type)**				

C

ID_gene	Gene annotation	NAC03	NAC11	NAC33	NAC60
VIT_01S0150G00380	**Unknown protein**				
VIT_14S0066G01140	**Unknown protein**				
VIT_17S0000G06200	MINI ZINC FINGER 1 MIF1				
VIT_05S0049G00040	cellulose synthase CSLG2				
VIT_09S0002G04620	Unknown protein				
VIT_18S0086G00320	no hit				
VIT_18S0086G00680	no hit				
VIT_02S0025G01450	**Unknown protein**				
VIT_06S0004G00380	Unknown protein				
VIT_15S0021G02700	**Expansin (VvEXPB4)**				
VIT_06S0004G07560	**ribosomal protein S29 28S**			*	
VIT_15S0046G00150	**DOF affecting germination 1**				
VIT_10S0116G00570	**Nuclear transport factor 2B**				
VIT_12S0059G00590	Allergenic protein Pt2L4				
VIT_06S0061G00550	**xyloglucan endotransglucosylase/hydrolase 32**			*	
VIT_14S0030G01880	**calmodulin-binding region IQD26**				
VIT_02S0234G00100	Ubiquitinyl hydrolase 1				
VIT_19S0090G00240	**R protein disease resistance protein**				
VIT_01S0011G03670	**bifunctional nuclease**		*		
VIT_18S0001G11930	**Thaumatin SCUTL2**				
VIT_06S0004G07550	**Wound-induced protein WI12**				
VIT_17S0000G02740	no hit				
VIT_00S1466G00020	Unknown protein				
VIT_13S0064G00360	notchless-like protein				
VIT_01S0150G00380	**Unknown protein**				
VIT_17S0000G00850	FRIGIDA-like 2				
VIT_07S0031G01200	RNase L inhibitor protein				
VIT_07S0255G00160	Unknown protein				
VIT_18S0001G11910	1-acyl-sn-glycerol-3-phosphate acyltransferase 4		*		
VIT_14S0066G01140	**Unknown protein**				
VIT_15S0021G02700	**Expansin (VvEXPB4)**				
VIT_04S0023G01010	no hit				
VIT_15S0046G01420	exonuclease RRP41 (RRP41)				
VIT_04S0023G02510	**glycosyl transferase family 1 protein**				
VIT_18S0001G14350	no hit				
VIT_08S0040G00500	Cupin, RmlC-type				
VIT_18S0122G01110	phosphatidylinositolglycan class O (PIG-O)				
VIT_06S0004G00380	Unknown protein				
VIT_07S0129G00020	glycosyl hydrolase family 43 protein				
VIT_05S0077G01980	**Unknown protein**				
VIT_01S0011G03670	**bifunctional nuclease**		*		
VIT_02S0012G02220	**xyloglucan endotransglucosylase/hydrolase 30**		*		
VIT_11S0016G04920	**early nodulin 93**				
VIT_19S0090G01370	**no hit**				
VIT_01S0127G00680	**SRO2 (SIMILAR TO RCD ONE 2)**				*
VIT_12S0034G01970	**cupin**				
VIT_15S0046G00150	**DOF affecting germination 1**				
VIT_02S0012G00500	**invertase/pectin methylesterase inhibitor**				
VIT_05S0094G00340	Chitinase class IV				
VIT_01S0137G00520	**CYP71B35**				
VIT_11S0016G03400	MSS1 (sugar transport protein 13)				
VIT_01S0137G00550	**CYP71B34**				
VIT_14S0066G01600	**NHL repeat-containing protein**				
VIT_13S0156G00330	Unknown				
VIT_04S0008G05620	adenine phosphoribosyltransferase				
VIT_16S0100G00270	**peptidoglycan-binding LysM domain-containing protein**			*	
VIT_00S0188G00150	auxilin				
VIT_08S0007G05210	Amino acid permease				
VIT_07S0031G00660	Unknown protein				
VIT_11S0016G00770	Actin beta/gamma 1				
VIT_08S0056G01240	**no hit**				
VIT_18S0001G11930	**Thaumatin SCUTL2**				
VIT_04S0023G00130	**Unknown protein**				
VIT_01S0127G00680	**SRO2 (SIMILAR TO RCD ONE 2)**				*
VIT_07S0141G00580	glycosyl transferase family 8 protein				
VIT_14S0060G01980	**Unknown protein**				*
VIT_02S0012G02220	**xyloglucan endotransglucosylase/hydrolase 30**		*		
VIT_05S0049G00760	**no hit**				
VIT_09S0002G06420	**lactoylglutathione lyase**	*		*	
VIT_10S0116G00370	**Unknown protein**				
VIT_01S0137G00430	**Cellulase**				
VIT_01S0011G04960	Unknown protein				
VIT_16S0100G00570	**dehydration-responsive protein**		*		*
VIT_06S0004G04920	RAB5-interacting protein isoform a				
VIT_06S0009G03120	**Cytochrome P450, family 79, subfamily A, polypeptide 2**				
VIT_01S0011G04210	Amino acid permease	*			
VIT_14S0068G01400	**UPF0497 family**				
VIT_17S0000G02870	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase				
VIT_11S0118G00490	Octicosapeptide/Phox/Bem1p (PB1) domain-containing protein				
VIT_17S0000G03200	**Unknown protein**				*
VIT_03S0038G03570	**monocopper oxidase SKS5 (SKU5 Similar 5)**				
VIT_19S0015G01850	CLC-F (chloride channel F)				
VIT_06S0004G04490	Unknown protein				

D

ID_gene	Gene annotation	NAC03	NAC11	NAC13	NAC60
VIT_18S0001G08300	**tubulin alpha-6 chain**				
VIT_05S0020G00330	galactinol synthase				
VIT_14S0030G01500	SF21				
VIT_05S0077G00430	galactinol synthase				
VIT_01S0011G06460	Deoxymugineic acid synthase				
VIT_15S0048G01010	2'-hydroxy isoflavone/dihydroflavonol reductase				
VIT_00S2634G00010	receptor kinase RK20-1				
VIT_18S0166G00060	no hit				
VIT_14S0006G01610	PMI2 (plastid movement impaired 2)				
VIT_04S0008G05400	serine hydrolase [Vitis vinifera]				
VIT_11S0118G00580	**unknown**				
VIT_00S0324G00060	**UDP-glycosyltransferase 85A8**				
VIT_18S0001G11060	Calcineurin phosphoesterase				
VIT_08S0105G00440	selenoprotein				
VIT_03S0038G04400	Dehydrin (VvDHN4)				
VIT_08S0007G07670	**NAC domain-containing protein (VvNAC60)**				
VIT_04S0008G04170	Unknown				
VIT_04S0023G01080	**Lys Motif-Type Receptor-Like Kinase LYK4**				
VIT_02S0154G00390	autophagy 8f (APG8f)				
VIT_13S0084G00670	Unknown protein				
VIT_06S0004G06380	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase				
VIT_16S0022G00480	Salt tolerance protein 3				
VIT_02S0025G04660	senescence-inducible chloroplast stay-green protein 1		*		*
VIT_18S0001G00210	Cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG5				
VIT_02S0025G00240	β-carotene hydroxylase (BCH1) (VvBCH1)				
VIT_15S0048G01930	ATPP2-A13				
VIT_11S0016G02980	unknown protein				
VIT_07S0005G00340	phytochrome A-associated F-box protein				
VIT_00S0265G00090	unknown protein				
VIT_04S0008G04020	RD22 [Vitis vinifera]				
VIT_06S0004G06850	MAPKKK14				
VIT_00S0187G00120	Cystatin				
VIT_19S0014G05090	thioredoxin h				
VIT_05S0020G01850	F-box domain containing protein				
VIT_01S0026G02710	NAC domain-containing protein (VvNAC26)				
VIT_19S0014G03290	NAC domain-containing protein (VvNAC17)				*
VIT_13S0019G03760	lateral organ boundaries protein 11				
VIT_17S0000G07520	ribosomal-protein S6 kinase p70				
VIT_18S0001G15730	Dof zinc finger protein DOF3.5				
VIT_08S0007G05570	embryo-abundant protein				
VIT_16S0100G00270	**peptidoglycan-binding LysM domain-containing protein**			*	
VIT_08S0040G03400	Short-chain dehydrogenase/reductase				
VIT_09S0002G03690	Avr9/Cf-9 rapidly elicited protein 146				
VIT_08S0105G00300	Phospholipase C				
VIT_15S0021G00890	RING-H2 zinc finger protein ATL4				
VIT_03S0088G01300	no hit				
VIT_18S0122G01340	**BTB/POZ domain-containing protein**				
VIT_06S0009G03130	Cytochrome P450, family 79, subfamily A, polypeptide 2				
VIT_02S0033G00450	**VvMybA3**				
VIT_09S0002G06420	**lactoylglutathione lyase**	*		*	
VIT_05S0029G00140	ERF/AP2 Gene Family (VvERF005)				
VIT_02S0025G04310	Thaumatin				
VIT_06S0004G03910	**Unknown protein**				
VIT_14S0006G02660	Receptor serine/threonine kinase PR5K-l				
VIT_06S0004G07560	**ribosomal protein S29 28S**			*	
VIT_04S0008G05940	glycoprotein, Mitochondrial				
VIT_07S0005G03050	Deoxyhypusine hydroxylase				
VIT_05S0020G03480	Ribosomal protein L18P/L5E				
VIT_06S0004G04030	copper chaperone				
VIT_01S0011G02310	**Unknown protein**				
VIT_06S0061G01430	flavonoid 1-2 rhamnosyltransferase				
VIT_10S0071G01070	Unknown protein				
VIT_14S0066G01610	zinc finger (C3HC4-type RING finger)				
VIT_01S0011G03480	cinnamoyl CoA reductase				
VIT_02S0025G04300	Thaumatin				
VIT_08S0007G05580	**embryo-abundant protein**				
VIT_02S0025G00780	cation/hydrogen exchanger (CHX18)				
VIT_03S0063G00490	no hit				
VIT_07S0005G01710	**WRKY transcription factor (VvWRKY19)**				
VIT_08S0040G01190	3-oxoacyl-reductase, chloroplast precursor				
VIT_11S0118G00200	**sucrose-phosphate synthase**				
VIT_06S0004G02560	Kiwellin Ripening-related protein grip22				
VIT_16S0100G00270	**peptidoglycan-binding LysM domain-containing protein**			*	
VIT_14S0081G00030	**Pathogenesis-related protein-4 (Chitinase)**				
VIT_19S0014G02450	**ALF5 (Aberrant lateral root formation 5)**				*
VIT_05S0049G00510	**ethylene response factor ERF1**				
VIT_17S0000G00430	**basic helix-loop-helix (bHLH) family**				
VIT_08S0040G02610	**Unknown**				
VIT_06S0061G00550	**xyloglucan endotransglucosylase/hydrolase 32**			*	
VIT_16S0022G01440	transketolase, chloroplast precursor				
VIT_19S0177G00230	**Unknown**				
VIT_02S0012G02810	**CYP76C4**				
VIT_05S0094G00330	Chitinase, class IV [Vitis vinifera]				
VIT_07S0005G00390	Beta Glucosidase 11				
VIT_15S0048G01920	F-box family protein				
VIT_16S0039G00150	Unknown protein				
VIT_02S0025G04310	Thaumatin				

E

ID_gene	Gene annotation	NAC03	NAC11	NAC13	NAC60
VIT_14S0068G01360	GEM-like protein 5				
VIT_01S0127G00680	SRO2 (SIMILAR TO RCD ONE 2)			*	
VIT_16S0050G00390	4-coumarate-CoA ligase				
VIT_08S0007G01150	Unc51-like kinase				
VIT_18S0072G01010	Peptide chain release factor eRF subunit 1				
VIT_06S0004G07790	lateral organ boundaries DOMAIN 15				
VIT_19S0014G04790	Organic cation/carnitine transporter4	*	*		
VIT_13S0019G04620	OTU cysteine protease	*	*		
VIT_12S0028G03580	lectin-receptor like protein kinase 3				
VIT_00S1278G00010	Brassinosteroid insensitive 1-associated receptor kinase 1				
VIT_08S0058G00440	ferritin				
VIT_07S0005G00290	universal stress protein (USP) family protein				
VIT_08S0058G00410	ferritin 1 (FER1)				
VIT_00S0291G00050	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein				
VIT_04S0044G01230	Unknown				
VIT_12S0028G00980	myb family	*			
VIT_01S0011G03660	IMP dehydrogenase/GMP reductase				
VIT_08S0105G00400	GT-1-like transcription factor				
VIT_00S0404G00040	ARF GTPase activator, ARF-GAP Domain 5				
VIT_11S0016G02470	Carrier protein, Mitochondrial				
VIT_01S0146G00410	GEM-like protein 5				
VIT_07S0005G02570	WRKY transcription factor (VvWRKY20)				
VIT_01S0150G00410	Unknown protein				
VIT_01S0011G03110	myb family				
VIT_13S0019G04610	Salt stress-inducible protein kinase				
VIT_14S0060G00650	no hit				
VIT_19S0014G04960	nodulation receptor kinase				
VIT_00S0324G00050	UDP-glucose glucosyltransferase				
VIT_19S0014G02450	ALF5 (Aberrant lateral root formation 5)				*
VIT_06S0004G07500	WRKY transcription factor (VvWRKY16)				
VIT_11S0118G00360	leucoanthocyanidin dioxygenase				
VIT_03S0063G01790	transducin protein				
VIT_08S0058G00470	Abscisic acid receptor PYL4 RCAR10				
VIT_06S0004G05660	glycosyl transferase family 1 protein				
VIT_00S0404G00100	Syntaxin of plants 124				
VIT_00S0211G00120	Glycine hydroxymethyltransferase				
VIT_02S0025G03520	haloacid dehalogenase hydrolase				
VIT_16S0022G01730	6-phosphogluconate dehydrogenase				
VIT_12S0028G03860	zinc finger (C3HC4-type RING finger) protein (RMA1)		*		
VIT_14S0066G01710	Leaf senescence protein		*		
VIT_19S0014G04790	Organic cation/carnitine transporter4	*	*		
VIT_14S0030G00400	WD40		*		
VIT_07S0031G02260	Unknown protein		*		
VIT_15S0046G00060	arginine/serine-rich splicing factor RSP31 (RSP31)				
VIT_13S0067G03350	Ribosomal protein L4/L1 (RPL4A) 60S				
VIT_07S0005G00280	Zinc finger (c3hc4-type ring finger) nitrogen limitation adaptation				
VIT_12S0028G03580	lectin-receptor like protein kinase 3				
VIT_08S0007G01150	Unc51-like kinase				
VIT_04S0008G04990	potassium channel (VvK1.2)		*		
VIT_17S0000G06340	MADS-box AGAMOUS-LIKE 30				
VIT_08S0007G04190	ATPP2-A15				
VIT_02S0025G03520	haloacid dehalogenase hydrolase				
VIT_08S0040G03270	E3 ubiquitin-protein ligase TRIP12				
VIT_13S0047G01130	Zfwd2 protein (ZFWD2)				
VIT_12S0059G02520	fringe				
VIT_01S0011G01940	Unknown protein				
VIT_18S0001G07300	MADS box interactor				
VIT_10S0071G00840	Unknown protein				
VIT_10S0071G00810	binding				
VIT_18S0001G01640	UDP-sugar pyrophospharylase				
VIT_19S0014G03290	NAC domain-containing protein (VvNAC17)				*
VIT_10S0071G00850	binding				
VIT_16S0050G00390	4-coumarate-CoA ligase				
VIT_02S0025G04660	senescence-inducible chloroplast stay-green protein 1		*		*
VIT_16S0100G00570	dehydration-responsive protein		*	*	
VIT_14S0060G01980	Unknown protein			*	
VIT_17S0000G03200	Unknown protein			*	
VIT_05S0077G00280	beta-amylase				
VIT_03S0038G01380	calcium-binding EF hand				
VIT_05S0020G01910	1,4-alpha-D-glucan maltohydrolase				
VIT_12S0034G02390	T-complex protein 11				
VIT_09S0002G06430	lactoylglutathione lyase				
VIT_17S0000G04050	Lipase family				
VIT_13S0019G02200	protein phosphatase 2CA AHG3 PP2CA				
VIT_18S0001G08490	early-responsive to dehydration				
VIT_00S0662G00040	Ethylene-responsive transcription factor RELATED TO APETALA2 4				
VIT_05S0020G03030	Leucine-rich repeat family protein				
VIT_01S0137G00620	auxin-independent growth promoter				
VIT_13S0064G00910	unknown				

Chapter 3

FUNCTIONAL CHARACTERIZATION OF *VvNAC03, VvNAC11, VvNAC13, VvNAC33* AND *VvNAC60* IN GRAPEVINE

1. INTRODUCTION

Grapevine has been cultivated worldwide for centuries as a source of fresh fruit, wine, juice and raisins. In a context of profound climate changes, one of the most challenge for viticulture will be maintaining a sustainable production of high quality grapes, given the importance of wine industry in the global economy. An ever-increase in seasonal temperature will dramatically shift the growing season by changing the normal pattern of grape development toward an earlier onset of flowering, véraison and harvest (Keller, 2010); therefore, global climate change prompted the use of many viticultural practices to modulate the environmental effects (van Leeuwen *et al.,* 2013) and the investigation of fundamental mechanisms that support agricultural traits, such as fruit ripening time. In this picture, a better understanding of the global transcriptomic reprogramming, marking the grapevine developmental shift from immature to mature growth, certainly represents a challenging task. The identification of the 'switch' genes (Palumbo *et al.,* 2014) has opened the possibility to start unravelling the regulatory network that controls this transition. This genes category includes transcription factors (TFs) such as plant-specific NAM/ATAF/CUC (NAC) TFs which have recently received considerable attention due to their significant roles in stress signaling and plant development (Jensen *et al.,* 2010). As mentioned in the chapter 2, five members of this genes family, *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60*, were selected as key factor candidates of the transcriptomic transition from the vegetative to mature organ phase during plant development. In order to shed light in the precise biological role of TFs, due to the current lack of grapevine mutant collections and the moderate recalcitrance of this species to stable transformation, transient expression assay represents an appropriate approach increasingly used in the past few years in defining unknown gene function. Although this assay can be performed in model plants, the results obtained can be sometimes controversial and might not reflect the real role of the

TFs in grapevine. This is probably because the regulative mechanisms could be different in a host species and the exogenous gene might mimic the functions of the orthologous endogenous regulator.

Indeed, grapevine is characterized by unique features whose study preferentially requires a homologous gene transfer system (Vidal *et al.,* 2010). Transient expression assay is based on temporary, high-level transcription of DNA sequences that do not necessarily integrate into the plant genome; leaf agro-infiltration using a needleless syringe or a vacuum pump represents an easy and rapid method significantly cheaper than most other ones for transient gene expression (Jelly *et al.,* 2014). Recently, some promising results have been obtained in *Vitis vinifera* (*V. vinifera*) cv. Sultana using this method for characterizing genes involved in phenylpropanoid pathway (Cavallini *et al.,* 2015) and in the regulation of vacuolar transport and flavonoid biosynthesis (Amato *et al.,* 2017). Based on these observations, we performed the transient ectopic expression of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* in *V. vinifera* cv. Sultana with the aim to focus on primary effects of each single transgene.

Another challenging approach for functional analysis is the grapevine stable transformation that allows the study of stable gene expression at whole plant level. Grapevine proved to be recalcitrant to biotechnological modifications, but significant progress has been made since the somatic embryogenesis of grapevine was described in the late 1970s (Hirabayashi *et al.,* 1976). Despite many protocols have been published, the method needs further improvements to be routinely used for gene functional studies. Induction of shoot germination from transformed embryos and acclimatization of the newly regenerated plantlets still remain the main bottlenecks. The percentage of plantlets regenerated from transgenic embryos usually ranges from 10 to 33% and successful transplanting and acclimatization can be achieved in nearly 90% of the cases following published guidelines (Bouquet *et al.,* 2006). Up to now this technique still remains restricted

to few research groups around the world since it keeps being considered an arduous and time consuming task. During this PhD project, the stable genetic transformation of *V. vinifera* cv. Shiraz was performed to elucidate *VvNAC33* and *VvNAC60* roles in grapevine, which are, among the five selected ones, the two putative 'switch' genes involved in the organ phase transition of the entire plant. Therefore, in this chapter we combined phenotypic and transcriptomic analysis of stable and transiently transformed grapevines to shed in light common and specific roles of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* during grapevine development and to pave the way for dissecting the complex regulatory network involved in this process.

2. MATERIALS AND METHODS

2.1 Plant material

For agroinfiltration of *Nicotiana benthamiana* (*N. 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 15 h/9h light/dark cycle.

For the transient overexpression of *NACs* in grapevine, plantlets of *V. vinifera* cv. Sultana were *in vitro* micropropagated and cultivated in HB medium in a growth chamber at 25°C with a 16-h photoperiod.

For the genetic transformation of *V. vinifera* cv. Shiraz, embryogenic calli were developed from anthers collected during the 2009 seasons in Coombe Vineyard (University of Adelaide, Urrbrae - South Australia).

2.2 Preliminary transient transformation of *Nicotiana Benthamiana*

For *N. benthamiana* transient transformation of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60*, the Gateway entry vectors pENTR/D-TOPO (Invitrogen) used were described in Chapter 2. The coding sequences were transferred into the binary overexpression vector pK7WG2.0 (Laboratory of Plant System Biology, Ghent University-https://gateway.psb.ugent.be/vector/show/pK7WG2/search/index/overexpression/any) by site-specific recombination. The constructs were inserted into *Agrobacterium tumefaciens* (*A. tumefaciens*) strain C58C1 by electroporation. For each construct, three full expanded leaves of three plants were infiltrated, using a syringe without needle by gentle infiltration in the intercellular spaces of leaf mesophyll. As control, the pK7WG2.0 vector containing a non coding sequence were used. Three days after the agroinfiltration we observed the phenotype and we took pictures.

2.3 Grapevine transformation

Transient overexpression of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60*

For grapevine transient transformation, we used the same vectors of *N. benthamiana* transient transformation, transferred to *A. tumefaciens* strain C58C1 by electroporation. Seven *in vitro* plants of grapevine cv. Sultana were immersed in each bacterial suspension and vacuum infiltrated (2 X 2 min at 90kPa). As control, plantlets transformed with pK7WG2.0 containing a non coding sequence were used. After agroinfiltration, plantlets were rinsed with sterile water and allowed to recover *in vitro* for seven days before collecting material for RNA extraction and transcriptomic analysis.

Preliminary GUS-assay

To find the most proper age of the plant and which leaves to sample showing the highest level of expression, a GUS-assay on grapevine of different ages transformed with pK7WG2.0 containing *GUS* coding sequence were performed. Basing on the results of this GUS-assay, in our experiments we transformed 5 weeks old plantlets and we sampled the younger well expanded leaves after 7 days.

Stable grapevine transformation of *VvNAC33* and *VvNAC60*

For the genetic transformation of *V. vinifera* cv 'Shiraz', embyogenic callus developed from anthers were kindly provided by Dr. Amanda Walker (CSIRO Plant Industry, Adelaide - Australia). Embryogenic calli were co-cultivated with *A. tumefaciens* harboring the binary overexpression vector pK7WG2 engineered to contain the Green Fluorescence Protein (GFP) sequence under the control of constitutive Arabidopsis Ubiquitin10 promoter; the presence of this reporter gene allows the selection of the transformed embryogenic material during the development of the embryos. Once initiated, the callus was

maintained on C1 medium in dark at 28°C and subcultured every 4 weeks. Well-developed material was selected for the transformation and maintained into GS1CA media for 14 days. For inoculation with *A. tumefaciens* strain EHA105, the embryogenic callus was collected in a Petri dish and submerged in 3 ml of bacterial suspension for 10 minutes. Bacterial suspension was withdrawn using a transfer pipette and any excess moisture was removed by blotting with sterilized Whatman 3MM filter paper. The agro-infiltrated callus was transferred to a new Petri dish containing 3 pieces of filter paper saturated with liquid modified GS1CA and incubated in the dark at 22°C. After 48 hours, the embryogenic callus was washed in liquid culture media with timentin (1000 µg /ml) and placed onto GS1CA medium for 9-10 days in the dark at 28°C. To select the transformed material, the callus was then moved into GS1CA supplemented with kanamycin 150 µg/ml; four weeks after the transformation they were subcultured into NN media with the selection antibiotic. Continuing to subculture the material every 4 weeks allowed the germination of GFP positive embryos which were selected at the microscope and collected on MS/2 with 5µM BAP. When primary shoots emerged, the embryos were transferred firstly in the same media without hormones to help the shoot elongation. Subsequently, plantlets were cut off and transferred into Magenta vessels containing rooting medium and cultured under the same conditions to allow further plant development. Vigorous transgenic plants with well-developed leaves and roots were then transplanted into 7-cm plastic pots containing grape soil mix and acclimated in the greenhouse under the shade for about 3 weeks before transfer to the light. For medium description and composition used in grapevine transformation refer to Bouquet *et al.* (2006).

2.4 Transcriptomic analyses

RNA extraction and reverse transcription

For gene expression analyses were performed on stable transgenic grapevines, total RNA was isolated from 100 mg of ground leaves using Spectrum™ Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. For gene expression analyses performed in grapevines transiently overexpressing *VvNACs*, total RNA was extracted with the same procedure from ~20-40 mg of ground leaves supposing to be mostly affected by the infection (GUS assay). RNA samples were quantified with the NanoDrop spectrophotometer (NanoDrop Thermo Scientific Technologies). 1 µg of RNA was treated with 1 unit (U) of DNase (Ambion) for 30 min at 37°C in 1X DNase Buffer. DNase-treated RNA was used for cDNA synthesis using the enzyme Super-Script™ III Reverse Transcriptase through a reaction protocol provided by the producing company (Invitrogen).

Quantitative PCR analysis of gene expression

Sultana plantlets and stable transgenic plants were screened for *NAC* transient overexpression by RT-PCR and qPCR. Primers used were reported in Table 1.

All qPCR was performed as described by Zenoni *et al.* (2010) using SYBR green qPCR master mix provided by Promega and a Mx3000P real-time PCR system (Stratagene). Each expression value, relative to *UBIQUITIN* (VIT_16s0098g01190) amplified with primers UBI FOR 5' - TCTGAGGCTTCGTGGTGGTA - 3' and UBI REV 5' - AGGCGTGCATAACATTTGCG - 3', was determined in triplicate. The PCR were set up and performed according to instrument and enzyme manufactures instruction with an appropriate annealing T based on Tm of each sets of primers. Amplification efficiency was calculated from raw data using the LingRegPCR_2015.3 software (Ramakers *et al.*, 2003). The relative

expression ratio value and SE values were calculated according to the Pfaffl equation (Pfaffl, 2001; Pfaffl, 2002).

Gene specificity	Primer Name	Primers sequence (5'-3')
VvNAC03	NAC03 For_Real time	TGCCCTGCTTCTCCGATATG
	NAC03 Rev_Real time	CTGGCATTCCTCCAAATATGG
VvNAC11	NAC11 For_Real time	CTGTCAATTCCCAGACTCCT
	NAC11 Rev_Real time	CCTGTGAGAACTCTGTTTTC
VvNAC13	NAC13 For_Real time	GGGAGACCTCCAAGTGATGA
	NAC13 Rev_Real time	CTGTGCAGGAAGCTTATTTGG
VvNAC33	NAC33 For_Real time	TGCCCTGCTTCTCCGATATG
	NAC33 Rev_Real time	CTGGCATTCCTCCAAATATGG
VvNAC60	NAC60 For_Real time	CTTCCACACTCGGTCATCTC
	NAC60 Rev_Real time	GGATGTCGTTGGATTGGCTG

Table 1: Primer sets for qPCR analysis of gene expression.

Microarray analysis

For microarray analysis RNA quality and quantity were determined using Nanodrop 2000 instrument (Thermo Scientific) and Bioanalyzer Chip RNA 7500 series II (Agilent).

Microarray analysis was performed on transient overexpressing grapevines cv. Sultana and on stable transgenic grapevines of cv. Shiraz. The cDNA synthesis, labeling, hybridization and washing reactions have been conducted according to the Agilent Microarray-Based Gene Expression Analysis Guide (V 6.5). A new Agilent custom microarray was designed on the 4pack 44K format (Agilent Sure Print HD 4X44K 60-mer G2514F-048771). This custom microarray was created by using an Agilent's web-based application able to create custom microarray designs and oligolibraries (https://earray.chem.agilent.com/earray/). In total, 34,651 specific *V. vinifera* probes, 60-mer in length, were produced by the software: 29,798 from the

Pinot noir predicted transcripts V1version, 4,392 from the new Pinot loci identified by Corvina transcriptome reconstruction and analysis performed by Venturini *et al.*, (2013) and 179 from Corvina private genes also identified by Venturini *et al.*, (2013). Scanning and Feature Extraction was performed by using an Agilent Scanner following the settings and parameters indicated in the instruction manual. After the extraction was completed successfully, the QC report for each extraction was analyzed to assess the quality of the overall hybridization procedure. 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 a correlation analysis was then performed to assess the consistency of the biological triplicates. Correlation matrixes were prepared using R software and Pearson's Correlation Coefficient (PCC) as the statistical metric. Data reported in the normalized data-matrix were used to determine the genes differently expressed in different samples by performing a T-test (TMeV).

2.5 Household genomic DNA extraction

Genomic DNA was extracted from *V. vinifera* cv. Corvina young leaves using a buffer constituted in Tris-HCl pH 8.0 200 mM, NaCl 250 mM, SDS 1% (w/v), EDTA 25 mM and β-mercaptoethanol 10 mM. 20-30 µg of leaf tissue were homogenized in 400 µl of extraction buffer. The sample was centrifuged at 13 000 rpm in a bench-top centrifuge for 10 minutes at room temperature (RT). 300 µl of supernatant were collected and the same volume of isopropanol was added. After 15 minute incubation at RT, the sample was centrifuged at 13 000 rpm for other 15 minutes; the supernatant was discarded and the pellet dried. 100 µl of sterile water were added to each sample and the pellet was left at 4°C o/n for the resuspension. The day after the sample was centrifuged at 13 000 rpm for 2 minutes and the supernatant was collected.

Genomic DNA was kept at 4°C or -20°C for long storage. This method can be used to obtain genomic DNA from both in vitro or greenhouse cultivated grapevines.

2.6 Vectors engineering for Dual-Luciferase Reporter Assay

Cloning of MYBA1 and MYBA2 regulative regions in a reporter vector

Genomic sequence of MYBA1 and MYBA2 regulative region was retrieved from the sequenced *V. vinifera* cv. Pinot Noir genome deposited at the Grape Genome Database - CRIBI website (http://genomes.cribi.unipd.it/grape/). Promoter prediction analyses were conducted exploiting specific tools available at the SoftBerry suite (http://www.softberry.com/), while the prediction of specific *cis*-acting elements for the regulative regions was achieved analyzing sequences at the PlantPAN database (http://plantpan2.itps.ncku.edu.tw/). The predicted regulative region of MYBA1 and MYBA2 were amplified from *V. vinifera* cv. Corvina genomic DNA using High Fidelity DNA Polymerase (Kapa HiFi DNA Polymerase – KAPA Biosystems) and the primers VvMYBA1.est.FOR 5'-CCCTTCATTAACATCAAAAGGTC -3' and VvMYBA1.promo.REV 5'-GAACCTCCTTTTTGAAGTGGTGACT -3' for MYBA1 (Kobayashi *et al.,* 2004) and VvMYBA2.est.FOR 5'- GCGATAAATTCACAAATACG -3' and VvMYBA2promo.REV 5'- TCATCGAGTCAACTCAACACA -3' for MYBA2. In order to advance the specificity of the reaction, a second amplification with primers VvMYBA1.promo.int.for 5'-CACCCGCCGATTAAATAGTTTAGGT-3' and VvMYBA1.promo.int.rev 5'- CGAGTCAACTCAACACAAGA - 3' for MYBA1 and VvMYBA2.promo.int.for 5'-ATGGAGGCGAAGAATGAGAT-3' and VvMYBA2.promo.int.rev 5'-GCCTTCTCCTTCTTTCTTGA - 3' for MYBA2 was performed. PCR analyses were set-up following the manual provided by the producing company. The generated PCR fragment were

purified with Wizard SV gel and PCR clean-up system (Promega) according to the producer instructions, directionally cloned into the Gateway entry vector pENTR/D-Topo (Invitrogen) thanks to the 5'-CACC-3' sequence in the second forward primer, and transferred by site-specific recombination into the binary reporter vector pPGWL7.0 (Laboratory of Plant System Biology, Ghent University- https://gateway.psb.ugent.be/vector/show/pPGWL7/search/index/transcriptional_reporters/any) to control the Firefly Luciferase gene (LUC) expression.

Effectors and reference constructs

The coding sequences of *VvNAC60* was previously amplified from a *V. vinifera* cv. Corvina berry cDNA and cloned into the binary overexpression vector pK7WG2.0 (Laboratory of Plant System Biology, Ghent University - https://gateway.psb.ugent.be/vector/show/pK7WG2/search/index/overexpression/any). For the realization of the reference vector, we used the binary overexpression vector pK7WG2.0 (Laboratory of Plant System Biology, Ghent University - https://gateway.psb.ugent.be/vector/show/pK7WG2/search/index/overexpression/any) cointaining the Renilla Luciferase gene (REN), previously engineered (Amato, 2016).

Plasmid DNA extraction

Bacterial cells are often used for biotechnological purpose and in this thesis commercial competent *Escherichia coli* cells (TOP10) provided by Invitrogen were used for both storing and replicating of all the plasmids engineered. Purification of plasmidic DNA from transformed bacteria was carried out using the "QIAprep® spin miniprep kit" (Qiagen) following the procedure described in the Handbook provided by the company.

Transient *Nicotiana benthamiana* transfection and Dual-Luciferase Reporter Assay

The transactivation of the regulative region of MYBA1 and MYBA2 were tested by Dual Luciferase Reporter Assay. For this analysis, all of the vectors of interest listed in Table 2 were inserted in *Agrobacterium tumefaciens* EHA105 strain (Hellens *et al.,* 2000) by electroporation (Bio-Rad electroporation instrument - 25 μF, 200 Ω and 2.5 kV). Bacterial cultures of transformed *Agrobacterium* were grown at 28°C for two days in rifampicin-containing LB medium (Bertani, 1951), pelleted by centrifugation at 4000 g and resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2, 100 μM acetosyringone, pH 5.6) to an OD600 of 0.2. Following incubation for 3h at RT, bacterial suspensions were syringe infiltrated into 5-6 weeks-old *N. benthamiana* plants; two leaves of two plants (4 biological replicates) were infiltrated with the same mix of *Agrobacterium* harboring specific constructs. Three days after the infiltration, 1cm diameter leaf discs were excised from each leaf and processed according to the manufacturer's instruction for the Dual Luciferase Reporter Assay (Promega). Firefly and Renilla luminescence were detected using a GENios Pro TECAN instrument (University of Verona, Biological Institute).

	Expression cassette	Backbone
Effectors vectors	35S:Vv*NAC60*	pK7WG2.0
Reference vector	35S:*REN*	
Reporter vector	*MYBA1prom*:LUC	pPGWL7.0
	MYBA2prom:LUC	

Table 2: Vectors used for MYBA1 and MYBA2 promoter analysis by Dual-Luciferase Reporter Assay.

2.7 Chlorophyll content

Leaves disk, taken from different plants, were frozen in liquid nitrogen and pigments were extracted with 80% aceton and kept in ice. The spectra were recorded at RT with an Aminco DW-2000 spectrophotometer. Chlorophyll and carotenoids content was determined by fitting the spectrum of the sample's acetone extract with the spectra of individual pigments, as described previously (Croce *et al.,* 2000). The program for the fitting of spectra was homemade. The program allows to shift the spectra of the single pigment in order to obtain the best fit.

3. RESULTS

3.1 Transient overexpression of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* in grapevine

In order to investigate the roles of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60*, transient transformation assays were set up.

We firstly used this approach in a heterologous system through the agroinfiltration of *N. benthamiana* leaves to have a preliminary result about the phenotypic effect of these TFs. The five selected genes were placed under the control of the constitutive CaMV 35S promoter in the pK7WG2 plasmid and, as a control, the same vector containing a non-coding sequence was used. Leaves of *N. benthamiana* were agroinfiltrated and, for some constructs, clear symptoms appeared after three days (Figure 1).

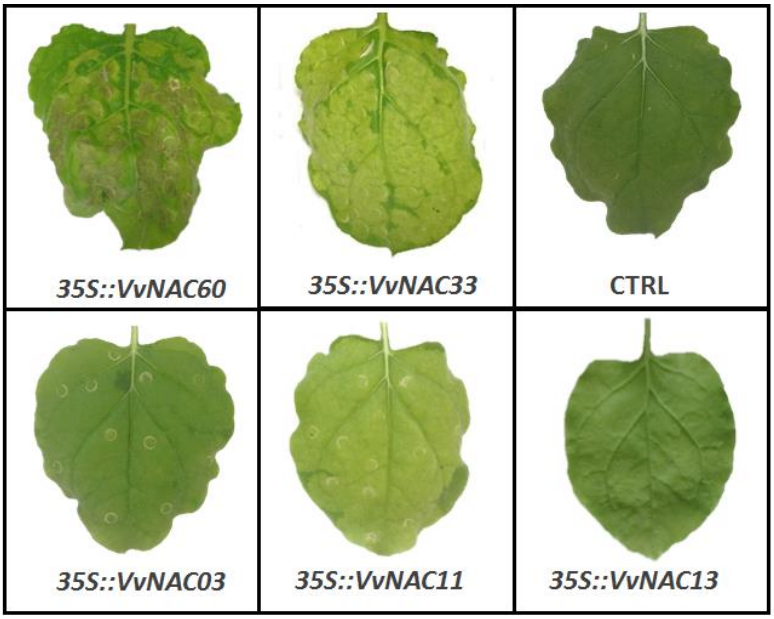


Figure 1: Phenotypes showed by *Nicotiana benthamiana* leaves agroinfiltrated with *35S:VvNAC60, 35S:VvNAC33, 35S:VvNAC13, 35S:VvNAC11, 35S:VvNAC03* and the control.

We noted that leaves agroinfiltrated with *VvNAC60* and *VvNAC33* showed the stronger phenotype; in particular, *VvNAC60* leaves presented browning necrotic-like regions similar to senescence behavior whereas *VvNAC33* leaves

exhibited a very strong yellowing effect. The other three *VvNACs* showed a phenotypic effect more similar to *VvNAC33*, with less and less intensity from *VvNAC03* to *VvNAC13*, in which it was almost not visible. These preliminary observations supported the hypothesis of an involvement of these *VvNACs* in the vegetative to mature phase transition in grapevine.

Then, in order to elucidate more in deep the functions of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60*, avoiding possible problems that may occur with gene expression in heterologous systems, we performed the assay directly in the native species. Hence, we transiently overexpressed the five selected *VvNACs* in *V. vinifera* cv. Sultana to obtain an overview on the putative primary effects of these TFs on grapevine leaf transcriptome. For each NAC factor, by vacuum *Agrobacterium*-mediated infection we transfected six young *in vitro* grown grapevine plantlets with the same constructs used for *N. Benthamiana* leaves. As control, six plantlets were transformed with the same vector containing a non-coding sequence. Seven days after infection the two younger and well expanded leaves were sampled and frozen. These leaves were selected on the basis of results obtained in a preliminary GUS-assay performed on grapevines of different ages transformed with the GUS coding sequence (Figure 2).

Figure 2: GUS-assay on grapevines of different ages transformed with pK7WG2.0 containing GUS coding sequence performed to find the most proper age of the plants and which leaves to sample. Basing on the results of this GUS-assay, in our experiments we transformed 5 weeks old plantlets and 7 days after infiltration we sampled the younger well expanded leaves.

Agroinfiltrated plants were screened for *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* overexpression by RT-PCR (data not shown) conducted on sampled leaves. For each TF, a qPCR was performed and we selected the three lines with the highest transcript amount of transgene in comparison to their respective *NAC* expression level in the three control lines (Figure 3). Plantlets did not show any obvious phenotypes.

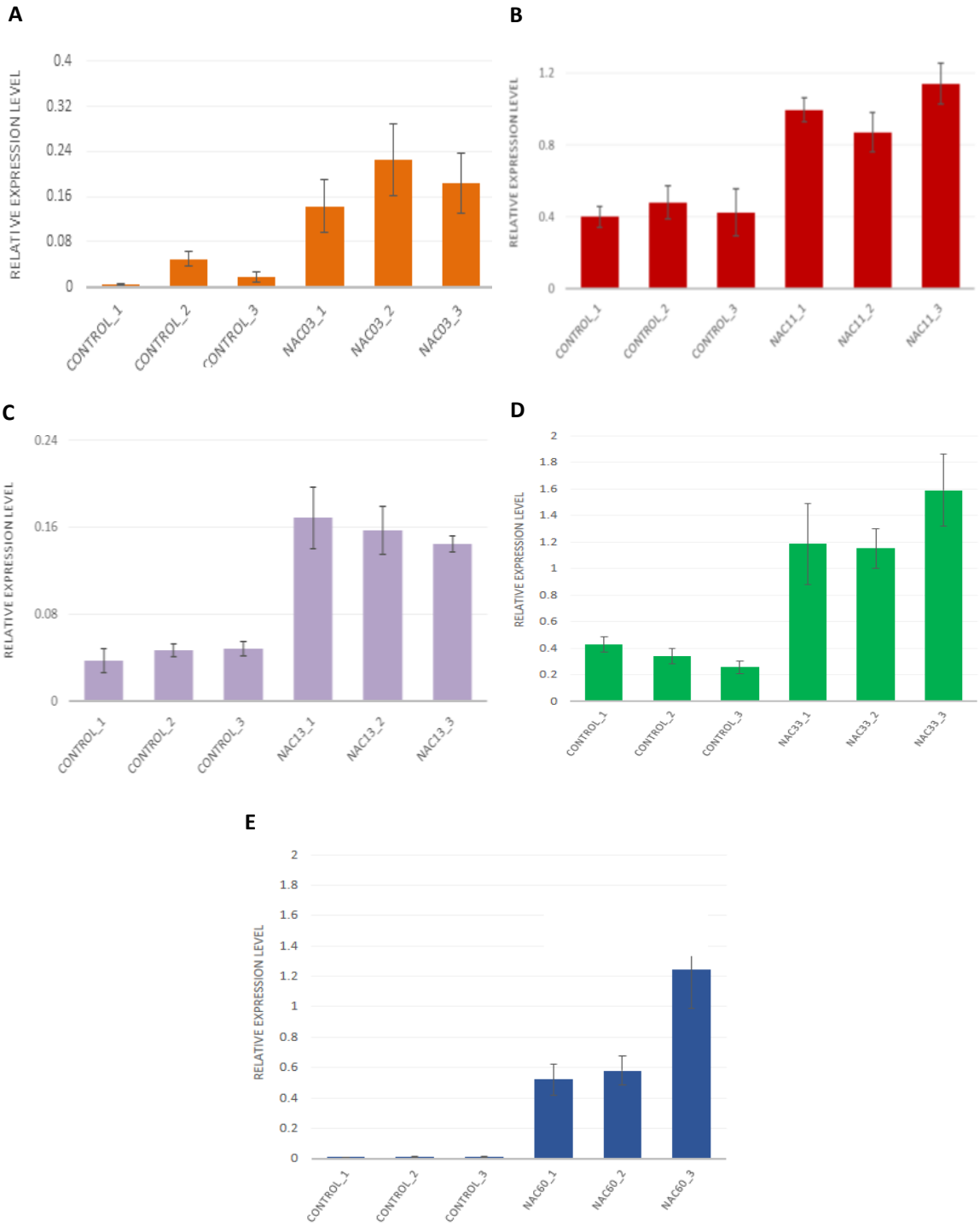


Figure 3: qPCR analysis of *NAC* expression level in leaves of overexpressing and control lines. Each expression value, relative to *UBIQUITIN* (VIT_16s0098g01190), was determined in triplicate ±S.E. The occurred overexpression of each NAC TF was verified by qPCR analysis because in the microarray platform the relative *NAC03* (A), *NAC11* (B), *NAC13* (C), *NAC33* (D) and *NAC60* (E) probes are located in the 3'-UTR region that we did not include in our overexpressing constructs.

To gain information about the impact of the ectopic expression of *NACs* on the leaf transcriptome and to have some hints about the possible target of these TFs a microarray analysis was performed on the leaf RNA of the selected lines. T-test analysis was carried out with a p correlation value of 0.05 (TMeV 4.3) comparing samples overexpressing *NACs* with the control lines. Considering a |fold change| > 2, in overexpressing plants we have identified up- and down-regulated genes, which were annotated using V1 version of the 12X draft annotation of the grapevine genome. We distributed them into 18 Gene Ontology functional categories and those with no similarity to known sequences or function (no hit/unknown protein) were removed from the subset. We focused our attention on up-regulated genes with a fold change > 2 as putative targets of these TFs; we particularly looked at those modulated genes having a pivotal role in metabolisms and pathways related to grapevine development and ripening.

In detail, regarding *VvNAC03* we identified 49 up-regulated genes and 47 down-regulated genes (Supplementary Table S1); among the up-regulated genes, the most represented functional categories were secondary metabolism, carbohydrate metabolic process and developmental process (Figure 4). We noted the genes affected by a higher modulation represented by the highest fold change were related to secondary metabolism, and in particular to flavonoid pathway; indeed we found five *UDP-GLUCOSE:FLAVONOID 7-O-GLUCOSYLTRANSFERASES*. Among the other highest modulated genes, we identified a *POLYGALACTURONASE* (VIT_01s0127g00400), an important hydrolytic enzyme involved in pectin degradation during fruit

softening, and a *STEROID 5ALPHA-REDUCTASE* (VIT_19s0014g00080), belonging to brassinosteroid biosynthesis (Table 3).

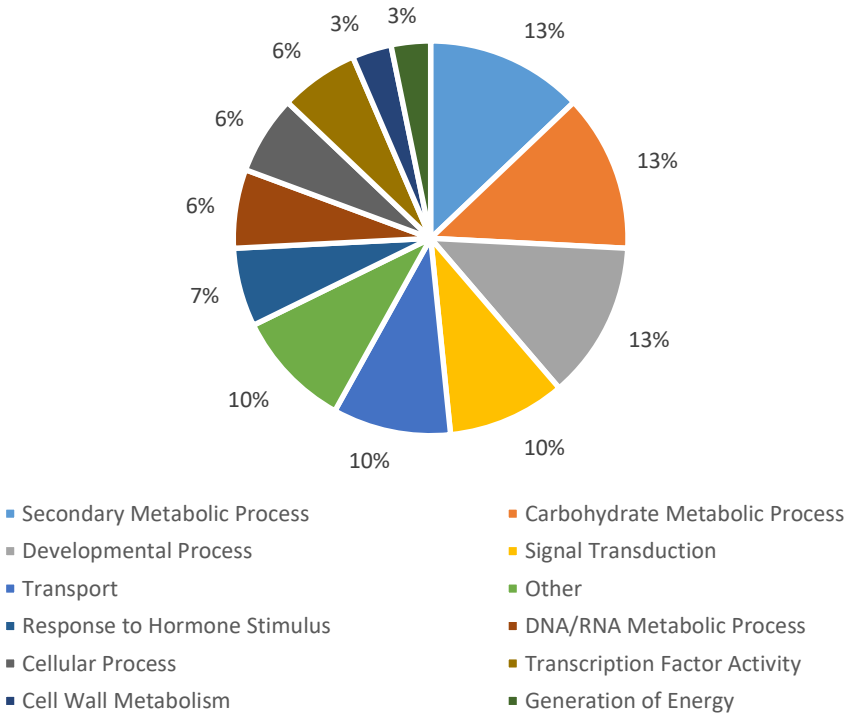


Figure 4: Distribution (%) of up-regulated genes in *VvNAC03* overexpressing plants into 18 Gene Ontology functional categories. Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset.

ID_code	Gene annotation	Functional category	FC
VIT_05s0062g00270	UDP-glucose:flavonoid 7-O-glucosyltransferase	Secondary Metabolic Process	8.40
VIT_05s0062g00340	UDP-glucose:flavonoid 7-O-glucosyltransferase		5.71
VIT_05s0062g00350	UDP-glucose:flavonoid 7-O-glucosyltransferase		5.61
VIT_05s0062g00710	UDP-glucose:flavonoid 7-O-glucosyltransferase		5.29
VIT_01s0127g00400	Polygalacturonase GH28	Cell Wall Metabolism	3.92
VIT_08s0007g05860	GASA like	Response to Hormone Stimulus	3.56
VIT_19s0014g00080	Steroid 5alpha-reductase		3.22
VIT_09s0018g01800	Acid phosphatase	Secondary Metabolic Process	3.22
VIT_05s0062g00660	UDP-glucose:flavonoid 7-O-glucosyltransferase		3.12
VIT_01s0011g03180	Lysine and histidine specific transporter	Transport	3.07

Table 3: The 10 most induced genes in *VvNAC03* overexpressing leaves compared to the control lines.

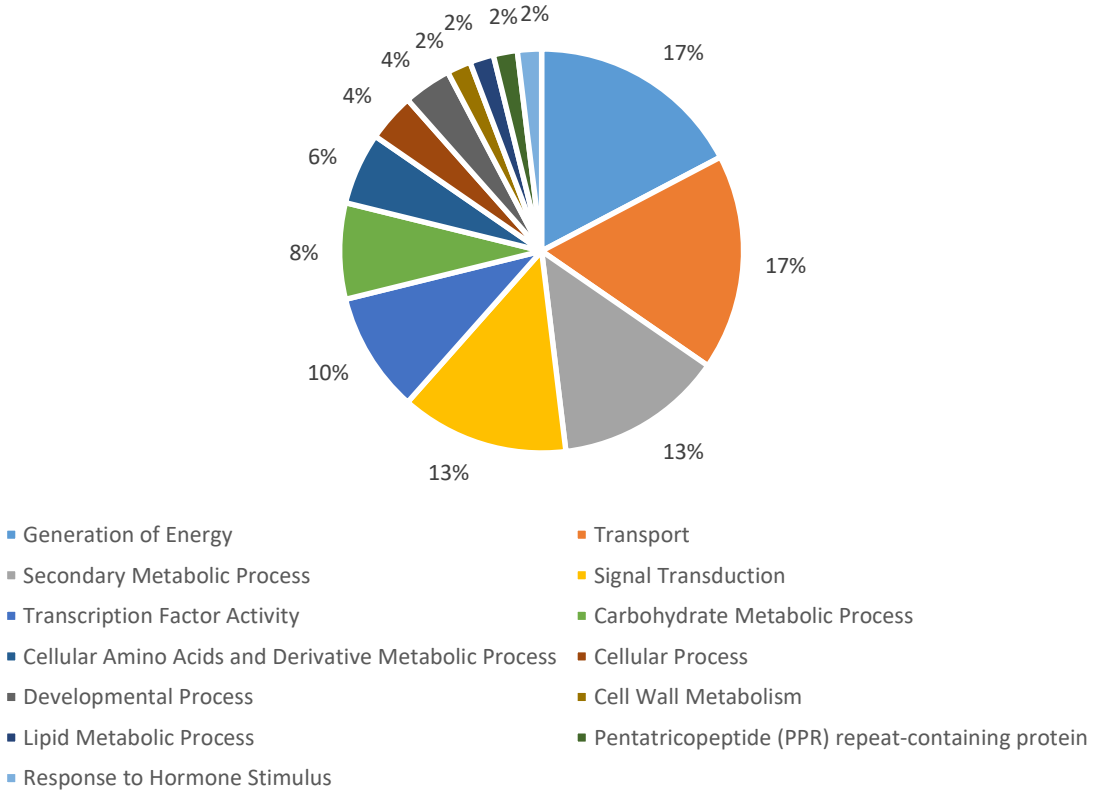


Figure 5: Distribution (%) of up-regulated genes in *VvNAC11* overexpressing plants into 18 Gene Ontology functional categories. Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset.

Regarding *VvNAC11*, concerning a |fold change| > 2 we identified 80 up-regulated genes and 33 down-regulated genes (Supplementary Table S2). Among the up-regulated genes belonging to the most represented functional categories (i.e. generation of energy, transport, secondary metabolic process, signal transduction and TF activity) (Figure 5), we observed the modulation of genes involved in secondary metabolism process belonging to different pathway, in particular flavonoid biosynthesis (*UDP-GLUCOSE FLAVONOID 3-O-GLUCOSYLTRANSFERASE 7*, VIT_11s0052g01600) and monoterpenoid biosynthesis (*GERANIOL 10-HYDROXYLASE*, VIT_15s0048g01600 and VIT_15s0048g01610). Moreover, noteworthy is a TF belonging to *BASIC HELIX-LOOP-HELIX FAMILY* (VIT_01s0026g02030) and a *ABCG/PDR-*

TYPE ABC TRANSPORTER (VIT_13s0074g00660), able to transport terpenoids (Çakir and Çakır B, 2013) (Table 4).

ID_code	Gene annotation	Functional category	FC
VIT_01s0150g00060	SOUL heme-binding	Generation of Energy	3.47
VIT_02s0087g00630	Alcohol oxidase		3.19
VIT_02s0012g02540	Chlororespiratory reduction 4 (CRR4)		2.60
VIT_08s0007g08540	Mg-chelatase subunit XANTHA-F		2.44
VIT_06s0004g06170	Thylakoid soluble phosphoprotein		2.28
VIT_05s0020g04040	Chlorophyllase (CLH2)		2.14
VIT_08s0040g00390	Magnesium-protoporphyrin IX ester cyclase		2.14
VIT_18s0001g15360	Thylakoid lumenal 29.8 kDa protein		2.09
VIT_12s0059g01810	Photosystem II psbZ		2.08
VIT_18s0001g10500	Abscisic acid 8` hydroxylase (CYP707A2)	Secondary Metabolic Process	4.84
VIT_09s0018g01800	Acid phosphatase		3.95
VIT_02s0087g00490	10-deacetylbaccatin III 10-O-acetyltransferase		3.38
VIT_15s0048g01600	Geraniol 10-hydroxylase		2.44
VIT_15s0048g01610	Geraniol 10-hydroxylase		2.43
VIT_11s0052g01600	UDP-glucose flavonoid 3-O-glucosyltransferase 7		2.21
VIT_00s0389g00030	CYP72A54		2.01
VIT_12s0035g02150	ferric reduction oxidase 7 FRO7	Transport	6.12
VIT_01s0026g02240	ANTR2 (anion transporter 2		3.04
VIT_15s0046g02390	ANTR2 (anion transporter 2)		2.85
VIT_13s0074g00660	ABC Transporter (VvPDR24 - VvABCG54)		2.62
VIT_03s0038g04210	Phototropin-2		2.43
VIT_00s0179g00370	ESCRT-I complex subunit TSG101		2.34
VIT_00s0291g00060	Inorganic phosphate transporter 2-1, chl prec		2.17
VIT_16s0100g00360	Per1		2.08
VIT_13s0019g05200	MATE efflux family protein		2.00

Table 4: Upregulated genes in *VvNAC11* overexpressing plant involved in generation of energy, secondary metabolism and transport.

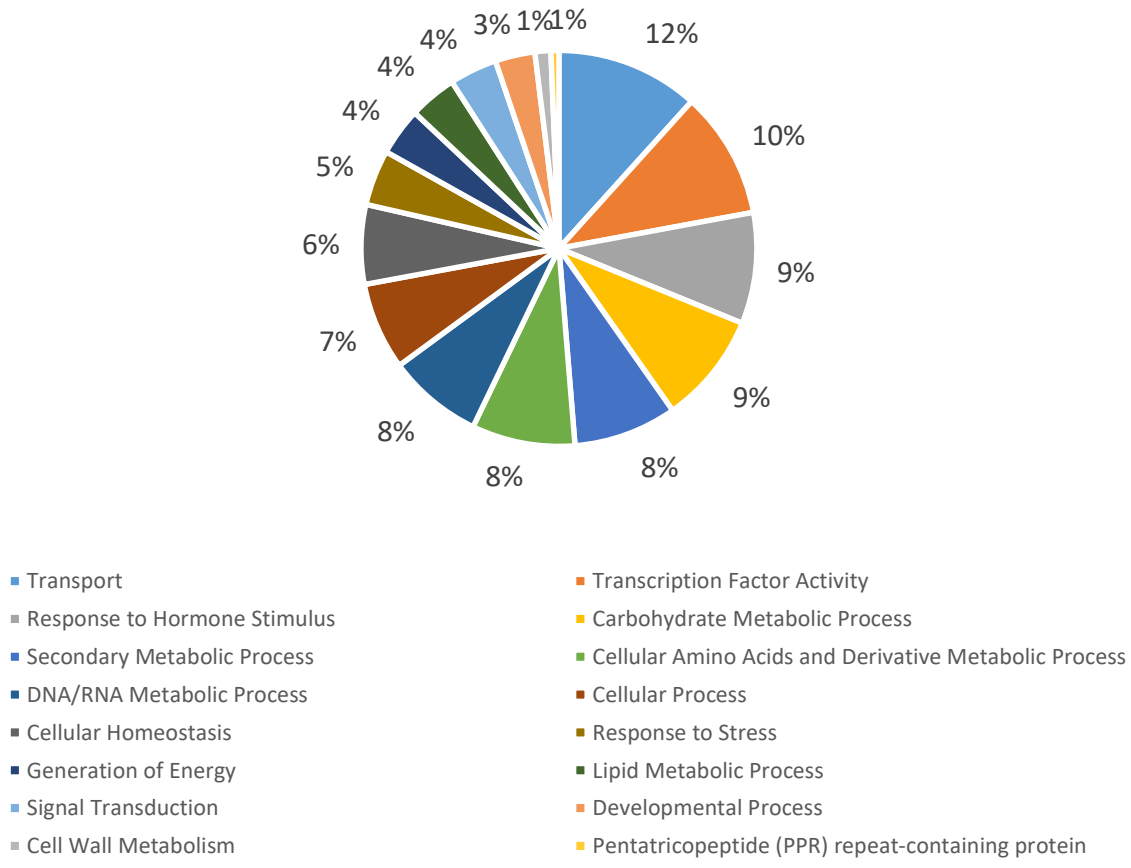


Figure 6: Distribution (%) of up-regulated genes in *VvNAC13* overexpressing plants into 18 Gene Ontology functional categories. Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset.

We observed that the transient overexpression of *VvNAC13* modulates a higher number of genes than *VvNAC03* and *VvNAC11*; in particular, concerning a |fold change| > 2, we identified 223 up-regulated genes and 101 down-regulated genes (Supplementary Table S3). Regarding the up-regulated genes, we noted that many categories are almost equally represented (Figure 6), indicating that many aspects of the cell metabolism could be influenced by the overexpression of *VvNAC13*.

Among the most induced gene we found *POLYGALACTURONASE QRT3* (VIT_01s0011g01310), involved cell wall metabolism and an *ABCB/MDR-TYPE ABC TRANSPORTER* (VIT_10s0003g02570), possibly responsible for the transport of auxins and alkaloids across membranes (Rea, 2007).

Moreover, the transcriptional analysis revealed up-regulation of many auxin responsive SAUR proteins that may have a role as regulators of the onset of ripening (Jones *et al.,* 2002; Audran-Delalande *et al.,* 2012). Of particular interest are those genes that may be involved in the secondary metabolism such as a *FLAVONOID 3'-HYDROXYLASE* (VIT_05s0094g01200), a *UDP-GLUCOSYL TRANSFERASE* (VIT_14s0068g00470) and a *CHALCONE ISOMESASE* (VIT_14s0066g00400). Moreover, it is noteworthy the induction of a *LATERAL ORGAN BOUNDARIES TRANSCRIPTION FACTOR* (VIT_17s0000g05490), whose family is a key regulator of plant organ development (such as pollen development, plant regeneration, photomorphogenesis, pathogen response, and specific developmental functions) (Xu *et al.,* 2016) and *AN AP2-LIKE ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR* (VIT_18s0001g10130) that has been described as putative positive markers of grape berry ripening (Fortes *et al.,* 2015) (Supplementary Table S3).

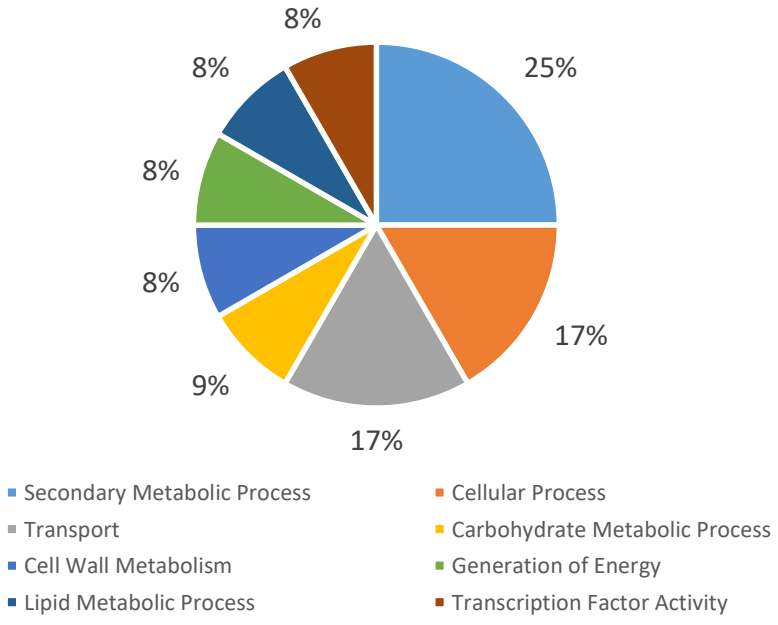


Figure 7: Distribution (%) of up-regulated genes in *VvNAC33* overexpressing plants into 18 Gene Ontology functional categories. Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset.

Regarding *VvNAC33*, concerning a |fold change| > 2 we identified only 12 up-regulated genes and 36 down-regulated (Supplementary Table S4). The most up-regulated gene was *FASCICLIN ARABINOGALACTAN-PROTEIN* (VIT_08s0040g02040), related to *CELL WALL ADHESION* (Johnson *et al.,* 2003). Among the most represented functional category (Figure 7), secondary metabolic process, we can underline the induction of two *UDP-GLUCOSE:FLAVONOID 7-O-GLUCOSYLTRANSFERASE* and a *SECOISOLARICIRESINOL DEHYDROGENASE*, a phenylpropanoid-related gene likely involved in the biosynthesis of lignans (Ferrer *et al.,* 2008) (Table 5). Interestingly, the highest down-regulated gene was *ACCUMULATION OF PHOTOSYSTEM ONE 2* (APO2, VIT_11s0037g00270), potentially involved in the assembly of photosystem apparatus. Indeed, it is a member of a novel gene family in which APO1 has been characterized as participant in [4Fe-4S] cofactor assembly or cotranslational incorporation into PsaA and/or PsaB proteins (Amann *et al.,* 2004) (Supplementary Table S4).

ID_code	Gene annotation	Functional category	FC
VIT_06s0061g01110	Lecithine cholesterol acyltransferase	Carbohydrate Metabolic Process	2.00
VIT_08s0040g02040	fasciclin arabinogalactan-protein (FLA11)	Cell Wall Metabolism	2.71
VIT_14s0060g01570	Kinesin motor protein	Cellular Process	2.25
VIT_01s0011g05150	Bet v I allergen		2.23
VIT_00s0873g00020	NADH dehydrogenase subunit 3	Generation of Energy	2.13
VIT_08s0056g01680	Lipoxygenase, LH2	Lipid Metabolic Process	2.01
VIT_05s0062g00590	UDP-glucose:flavonoid 7-O-glucosyltransferase	Secondary Metabolic Process	2.52
VIT_06s0080g00990	Secoisolariciresinol dehydrogenase		2.30
VIT_05s0062g00470	UDP-glucose:flavonoid 7-O-glucosyltransferase		2.00
VIT_02s0033g00050	Scarecrow transcription factor 3 (SCL3)	Transcription Factor Activity	2.43
VIT_02s0025g05110	MATE efflux family protein	Transport	2.35
VIT_00s0508g00050	Oligopeptide transporter 1		2.07

Table 5: Upregulated genes in *VvNAC33* overexpressing plants with fold change > 2.

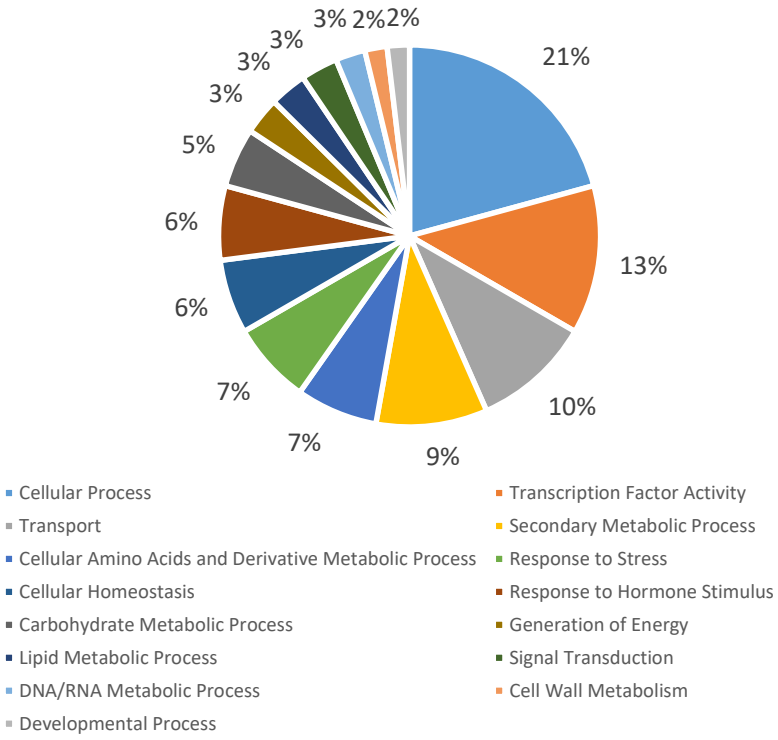


Figure 8: Distribution (%) of up-regulated genes in *VvNAC60* overexpressing plants into 18 Gene Ontology functional categories. Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset.

We noted that the transient overexpression of *VvNAC60* affected the expression of the highest number of genes and induced the highest modulation intensity. Concerning a |fold change| > 2, we identified 227 up -regulated genes and 221 down-regulated genes (Supplementary Table S5). Among up-regulated genes, firstly we identified many highly modulated *GERMIN PROTEINS*, involved in plant development, osmotic regulation, response to stress and programmed cell death (Dunwell *et al.,* 2008). Many genes were related to the TF activity (Figure 8), suggesting that *VvNAC60* could control downstream pathways through the modulation of the expression of intermediate regulators. Among them, we found two highly modulated *KNATs*, three *NAC DOMAIN-CONTAINING PROTEINS* and four MYB TFs, including *MYBPA1* (VIT_15S0046G00170), identified as a regulator of the proanthocyanidin biosynthesis (Bogs *et al.,* 2007).

ID_code	Gene annotation	Functional category	FC
VIT_07s0005g05890	FAD linked oxidase, N-terminal		139.07
VIT_14s0128g00540	Germin-like protein 3 [Vitis vinifera]		135.81
VIT_14s0128g00570	Germin		126.90
VIT_14s0128g00630	Germin-like protein 3 [Vitis vinifera]		85.00
VIT_14s0128g00610	Germin-like protein 3 [Vitis vinifera]		70.31
VIT_14s0128g00680	Germin		65.34
VIT_14s0128g00600	Germin-like protein 3 [Vitis vinifera]		57.11
VIT_14s0128g00980	Germin-like protein 3 [Vitis vinifera]		43.13
VIT_14s0128g00660	Germin-like protein 3 [Vitis vinifera]		40.83
VIT_14s0128g01040	Germin-like protein 8-11		27.73
VIT_14s0128g01010	Germin-like protein 3 [Vitis vinifera]		25.87
VIT_14s0128g01020	Germin-like protein 3 [Vitis vinifera]		24.68
VIT_14s0128g00640	Germin-like protein 3 [Vitis vinifera]		17.28
VIT_14s0060g02710	Germin		16.47
VIT_14s0128g00970	Germin-like protein 3 [Vitis vinifera]		12.27
VIT_04s0023g01510	DUF620	Cellular Process	6.95
VIT_13s0106g00190	Ankyrin repeat		6.53
VIT_14s0128g00990	Germin-like protein 3 [Vitis vinifera]		5.82
VIT_14s0060g02750	Germin-like protein 3 [Vitis vinifera]		4.24
VIT_14s0060g02730	Germin		3.09
VIT_07s0104g01350	Integral membrane family protein UPF0497		3.08
VIT_15s0048g02230	Calcineurin phosphoesterase		2.91
VIT_03s0063g00990	Blue (type 1) copper domain		2.52
VIT_02s0154g00490	Heat shock 22 kDa protein mitochondrial		2.52
VIT_18s0001g08300	Tubulin alpha-6 chain		2.50
VIT_02s0025g02840	Ankyrin 3, epithelial isoform a		2.40
VIT_03s0063g01010	Blue (type 1) copper domain		2.23
VIT_00s0415g00040	Glycine-rich protein		2.11
VIT_17s0000g00730	ATP binding / DNA binding		2.09
VIT_14s0128g01030	Germin-like protein 3 [Vitis vinifera]		2.09
VIT_04s0023g01230	T-complex protein 1 subunit delta		2.06
VIT_03s0063g00980	Blue (type 1) copper domain		2.06
VIT_01s0127g00210	KNAT2 (knotted1-like homeobox gene 6)		12.88
VIT_18s0001g08380	Homeobox protein knotted-1 like 1 (KNAT1)		11.92
VIT_14s0068g01290	Transcriptional factor B3		4.27
VIT_14s0083g00150	TCP family transcription factor 1		4.20
VIT_15s0048g02120	Myb domain protein 3R2	Transcription Factor Activity	4.15
VIT_17s0000g06200	Mini zinc finger 1 MIF1		3.96
VIT_13s0156g00370	myb family		3.93
VIT_00s0203g00120	Jumonji (jmjC)		3.67
VIT_06s0004g02580	BLH8 (BEL1-like homeodomain 8)		3.56
VIT_09s0018g01220	Zinc finger (C3HC4-type ring finger)		3.21
VIT_19s0014g03300	NAC domain-containing protein (VvNAC18)		3.18

VIT_15s0046g03190	myb domain protein 17	Transcription Factor Activity	2.76
VIT_17s0000g01230	putative MADS-box TM8a (VviTM8a)		2.72
VIT_15s0048g02290	NAC domain-containing protein (VvNAC54)		2.62
VIT_01s0011g00140	CRABS CLAW		2.55
VIT_15s0046g00170	VvMYBPA1		2.33
VIT_03s0167g00070	VviSVP5		2.33
VIT_14s0068g01580	basic helix-loop-helix (bHLH) family		2.22
VIT_18s0001g10300	basic helix-loop-helix (bHLH) family		2.22
VIT_19s0014g02200	NAC domain-containing protein (VvNAC16)		2.07
VIT_08s0040g00920	Glutathione S-transferase 25 GSTU7	Secondary Metabolic Process	4.66
VIT_15s0048g02480	Caffeate 3-O-methyltransferase 1		3.51
VIT_09s0054g01410	Beta-amyrin synthase		3.51
VIT_19s0093g00190	Glutathione S-transferase 25 GSTU25		3.30
VIT_12s0035g02100	Glutathione S-transferase Z1 GSTZ1		3.14
VIT_10s0003g03660	Beta-amyrin synthase		2.78
VIT_17s0000g07020	Cis-zeatin O-beta-D-glucosyltransferase		2.74
VIT_18s0001g00450	Vinorine synthase		2.71
VIT_09s0054g01730	Coniferyl alcohol acyltransferase		2.47
VIT_13s0047g01230	flavonoid 1-2 rhamnosyltransferase		2.35
VIT_15s0048g01440	Geraniol 10-hydroxylase		2.26
VIT_06s0080g00990	Secoisolariciresinol dehydrogenase		2.16
VIT_01s0011g06440	Chalcone reductase		2.12
VIT_09s0054g01420	Beta-amyrin synthase		2.07
VIT_05s0077g02190	Chalcone reductase		2.01
VIT_07s0005g05960	Cytokinin dehydrogenase 5 precursor	Response to Hormone Stimulus	176.17
VIT_00s2520g00010	Cytokinin oxidase		123.93
VIT_10s0003g03490	GA 2-oxidase		4.12
VIT_18s0001g14270	Gibberellin-regulated protein 1 (GASA1)		3.47
VIT_10s0116g00190	Homeobox protein shoot MERISTEMLESS (STM)		3.35
VIT_07s0005g03260	ERF/AP2 Gene Family (VvERF100)		3.17
VIT_07s0005g03230	ERF/AP2 Gene Family (VvERF099)		3.02
VIT_13s0067g01730	Steroid 5alpha-reductase		2.24
VIT_06s0004g05460	Protein phosphatase 2C DBP (VvPP2C-6)		2.23
VIT_07s0005g03210	ERF/AP2 Gene Family (VvERF097)		2.16

Table 6: Upregulated genes in *VvNAC60* overexpressing plant involved in cellular process, transcription factor activity, secondary metabolism and response to hormone stimulus.

ID_code	Gene annotation	FC				
		35S::VvNAC03	35S::VvNAC11	35S::VvNAC13	35S::VvNAC33	35S::VvNAC60
VIT_03s0038g00670	fructose-bisphosphate aldolase, chl pre	2.02	2.61			
VIT_15s0024g01860	MADS-box protein AGL24	2.06		4.81		
VIT_10s0092g00500	CYP71D10	2.06		2.01		
VIT_03s0017g00360	MADS-box protein SVP	2.10		5.06		
VIT_15s0046g02390	ANTR2 (anion transporter 2)	2.16	2.85			
VIT_09s0018g01800	Acid phosphatase	3.22	3.95			
VIT_19s0014g00080	Steroid 5alpha-reductase	3.22	5.25			
VIT_09s0054g01420	Beta-amyrin synthase			2.27		2.07
VIT_00s0346g00010	Lectin protein kinase			2.38		2.15
VIT_06s0080g00990	Secoisolariciresinol dehydrogenase				2.3	2.16
VIT_06s0061g01110	Lecithine cholesterol acyltransferase				2	2.18
VIT_02s0025g05110	MATE efflux family protein				2.35	2.50
VIT_14s0066g01950	Metalloendoproteinase 1 precursor			2.4		3.54
VIT_02s0012g00760	Haloacid dehalogenase hydrolase		2.05	2.14		
VIT_11s0118g00630	Unknown protein		2.36	2		

Table 7: Genes up-regulated (FC >2) by the overexpression of at least two NAC TFs of interest.

Therefore, in order to investigate the entire scenery of action of the regulatory network to which NACs belong, we compared the entire set of genes up-regulated in each independent transformation. We focused on those genes characterized by a FC > 2 in each experiment and we observed that transient overexpression of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* almost affected the same functional categories, but very few genes resulted modulated by more than one TFs (Table 7).

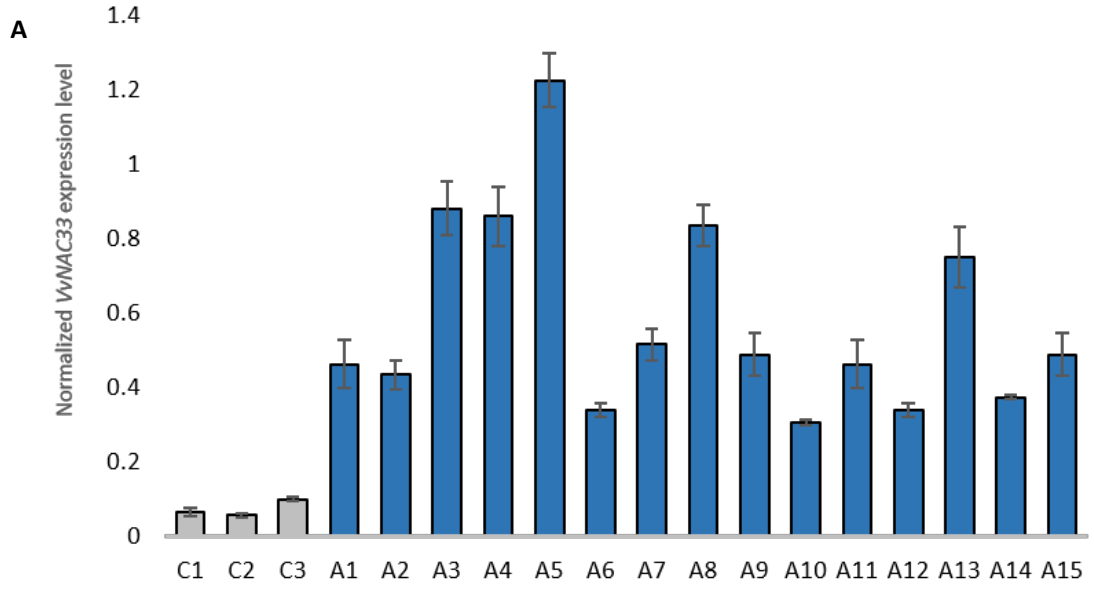
3.2 Characterization of stable transgenic grapevine overexpressing *VvNAC33* and *VvNAC60*

The functions of *VvNAC33* and *VvNAC60* were further investigated in the native species through the stable transformation of embryogenic calli of *V. vinifera* cv. Shiraz, with *A. tumefaciens*. We selected these two genes because they could represent general master regulators of the grapevine organ phase transition, according to the expression analysis and to the integrated network analysis performed on the grapevine atlas and berry development data sets (Palumbo *et al.,* 2014). The other three seems to be more specific of berry development and, given the long time the transformed plants take to make fruits, will be considered in future transformation experiments.

We overexpressed *VvNAC33* and *VvNAC60*, independently, in the pK7WG2 vector engineered for containing the GFP sequence. The presence of the reporter gene under the control of Arabidopsis Ubiquitin10 promoter allowed the additional selection of the transformed embryogenic material during the development of the embryos. The two vectors were transferred into the *A. tumefaciens* strain EHA105 by electroporation. As a negative control, we used the pk7WG2 vector overexpressing only the GFP sequence in order to avoid possible phenotypes due to the simple T-DNA insertion. A total of three independent transformation events for the overexpression and one for the control were performed and positively transformed embryogenic material was selected on the basis of both resistance to kanamycin and GFP reporter signal.

The transformation events yielded about 160 GFP positive embryos for VvNAC33, 55 of which produced emerging shoot, with a germination efficiency of about 35%. Despite the use of a double selection process for the identification of the fully transformed embryos (negative selection by kanamycin and positive selection by GFP signal), the confirmation of the transgene integration on the *in vitro* cultured material is useful to avoid further propagation of possible escapes.

After a screening by PCR, 40 positive plantlets were transferred to the pots and maintained under *in vitro*-like condition for about three weeks. Afterward, they were transferred to the greenhouse and acclimatized to low-hygrometric conditions (Bouquet *et al.,* 2006). After the acclimatization step, the plants were cultivated without other specific requirements in the greenhouse. Regarding VvNAC60, only 3 plantlets were germinated from 120 GFP positive embryos and only 2 of them were PCR positive. The majority of these embryos presented a regular shape but did not keep a consistency such as to allow the development of the shoots. Concerning the negative control, we obtained 70 transformed embryos and we selected 10 PCR positive plantlets. We followed the same procedure for acclimation in the greenhouse described above for *VvNAC33* for both *VvNAC60* overexpressing plants and control ones. As further step, we evaluated the actual level of transgene by qPCR. All the regenerated transformed grapevines overexpressing *VvNAC33* showed an enhanced level of expression, even with different intensity, in comparison with the control. In Figure 9A we summarized the most significant results obtained from this analysis. Regarding *VvNAC60*, unfortunately only one of two transformed plants showed significant expression level of the transgene (Figure 9B). As control, we chose three lines (C1, C2, C3) with low expression levels of *VvNAC33* and *VvNAC60*.



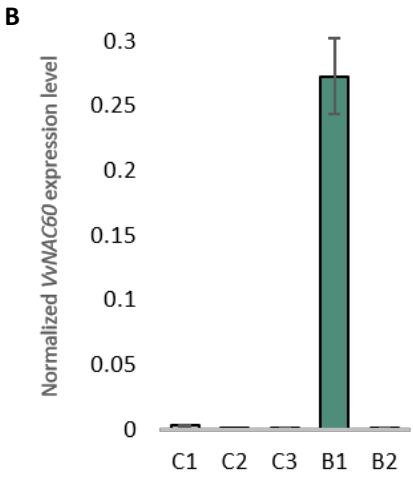


Figure 9: qPCR analysis of *VvNAC33* (A) and *VvNAC60* (B) expression level in leaves of transgenic (A1/A15 for *VvNAC33* – B1, B2 for *VvNAC60*) and control lines (C1, C2, C3). Each expression value, relative to *UBIQUITIN* (VIT_16s0098g01190), was determined in triplicate ±S.E.

Together with the molecular characterization, our work focused on the phenotyping of the stably transformed grapevines. We checked the presence of phenotypic changes in the transgenic plants in comparison to the control. Regarding *in vitro* cultivated plantlets, we did not observe any relevant difference in terms of morphology, growing habit and vigor, but we noticed a faint bleaching effect on some leaves overexpressing *VvNAC33* (Figure 10).

Figure 10: Phenotype of *in vitro* leaves in the control, in stable transformed overexpressing *VvNAC33* and *VvNAC60*.

However, sometimes potential phenotypic alterations may go undetected; we renewed *in vitro* cultivated plantlets every month by micro-cutting of the apical part and the growing conditions are quite different from the natural ones.

Therefore, we carefully observed the phenotypes on greenhouse cultivated plants. Considering the only one transformed plant overexpressing *VvNAC60,* we noticed a slightly stunted growth when compared to the control plant (Figure 11A), but no obvious phenotype was observed on leaves (Figure 11C). Interestingly, *VvNAC60* plants exhibited an early lignification of the stem in comparison with control plants of the same age, supporting an involvement of *VvNAC60* in the transition from herbaceous to mature/woody phase (Figure 11B).

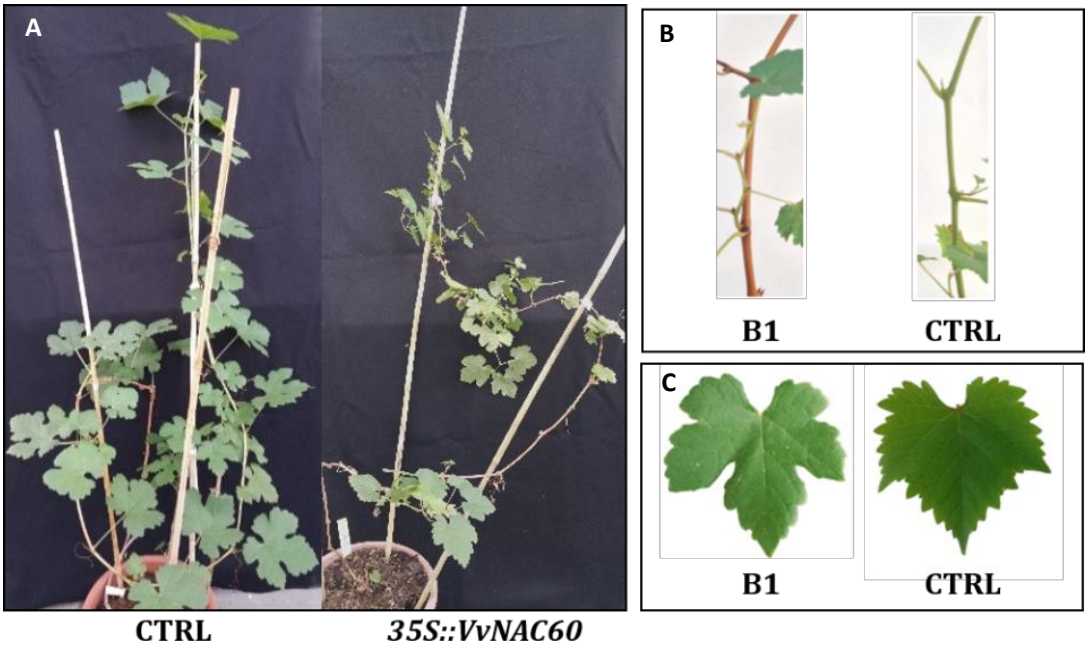


Figure 11: Phenotypes of grapevine overexpressed for *VvNAC60* (line B1) in comparison to the control. Transgenic plants were maintained in the same conditions in the greenhouse. A) *VvNAC60* overexpressed plant (*35S::VvNAC60)* presented a slightly stunted growth in comparison to the control (CTRL). B) Stem of *VvNAC60* overexpressing plant (B1) showed an earlier lignification than the control. C) Leaves of *VvNAC60* overexpressing plant (B1) with similar phenotype than the control.

VvNAC33 overexpressing plants showed growth rate, dimension and general habitus similar to the control (Figure 12A). However, we observed a clearly visible bleaching effect on adult fully expanded leaves, a phenotype that usually started appearing already in young leaves. These alterations presented various degrees of severity and, moreover, they were not present in all lines. In Figure 12B we reported the most apparent effect shown by three lines among those with

the highest *VvNAC33* expression levels (A4, A5, A8 lines; Figure 3). Since a similar phenotypic effect was observed in *N. benthamiana* leaves overexpressing the same transgene, these results support the attracting hypothesis of a key role played by *VvNAC33* in the organ phase transition during grapevine development.

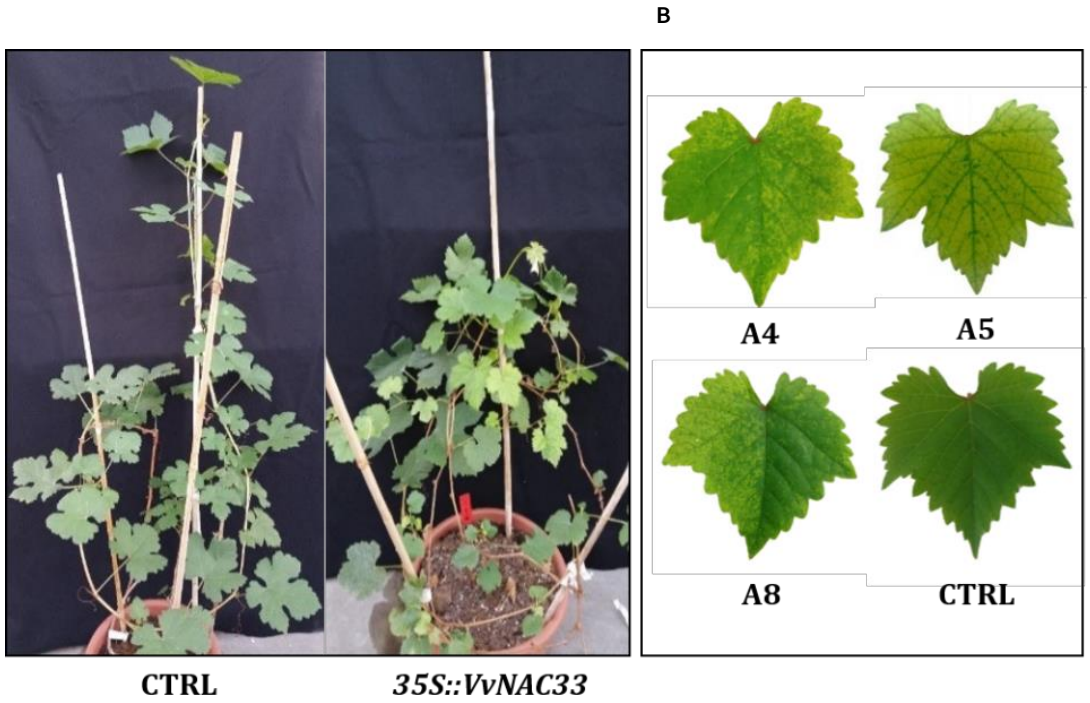
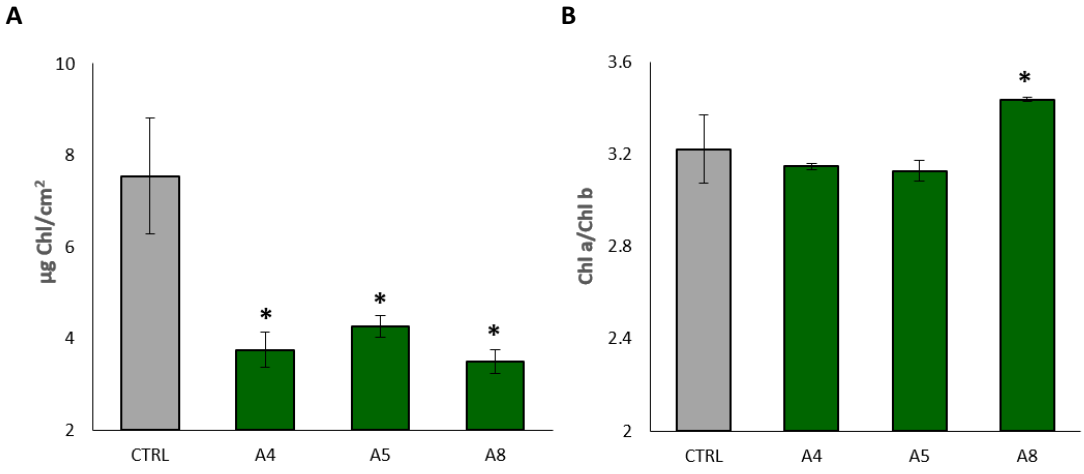


Figure 12: Phenotypes of grapevine overexpressed for *VvNAC33* in comparison to the control. Transgenic plants were maintained in the same conditions in the greenhouse. A) *VvNAC33* overexpressed plant (*35S::VvNAC33)* presented growth and dimension similar to the control (CTRL). B) Leaves of *VvNAC33* overexpressing plant (A4, A5, A8 lines) showed different level of yellowing effect in comparison to the control.

Based on the molecular and phenotypic features of the newly regenerated grapevines, for the further analysis we selected the three transgenic plants overexpressing *VvNAC33* which showed the stronger phenotype and with higher expression level of the transgene (A4, A5 and A8). Regarding *VvNAC60* we performed analysis on the only one line overexpressing this transgene (B1). As control, we used C1, C2 and C3 lines.

3.3 *VvNAC33* ectopic expression affects chlorophyll content in grapevine leaves

By focusing on the results obtained so far about *VvNAC33* function (i.e. the yellowing effect on *N. benthamiana* grapevine leaves overexpressing this gene and the strong *APO2* downregulation in transiently overexpressing grapevine plantlets), we hypothesized that this TF may affect chlorophyll accumulation. To verify that, in collaboration with the Laboratory of Photosynthesis at our University, we determined the chlorophyll content in transgenic leaves overexpressing *VvNAC33*. We compared the overexpressing lines A4, A5 and A8 with the control lines C1, C2 and C3. We observed that the total amount of chlorophyll was lower in the lines overexpressing *VvNAC33*, even if the ratio between chlorophyll a and chlorophyll b was not different (Figure 13A-B). However, by looking at the ratio between chlorophyll and carotenoids we noticed a decrease in the overexpressing lines in comparison with the control (Figure 13C). These results were consistent with the typical behavior of leaves undergoing senescence.



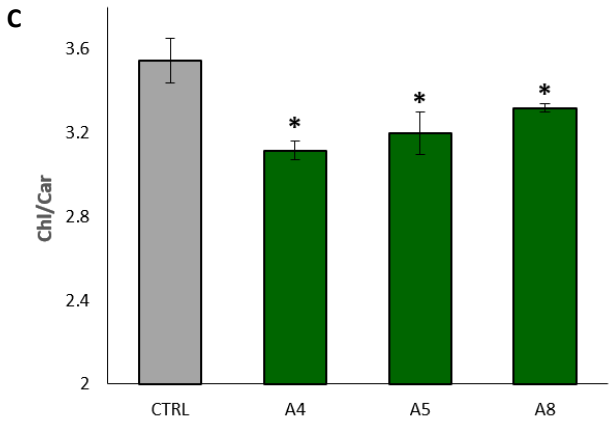
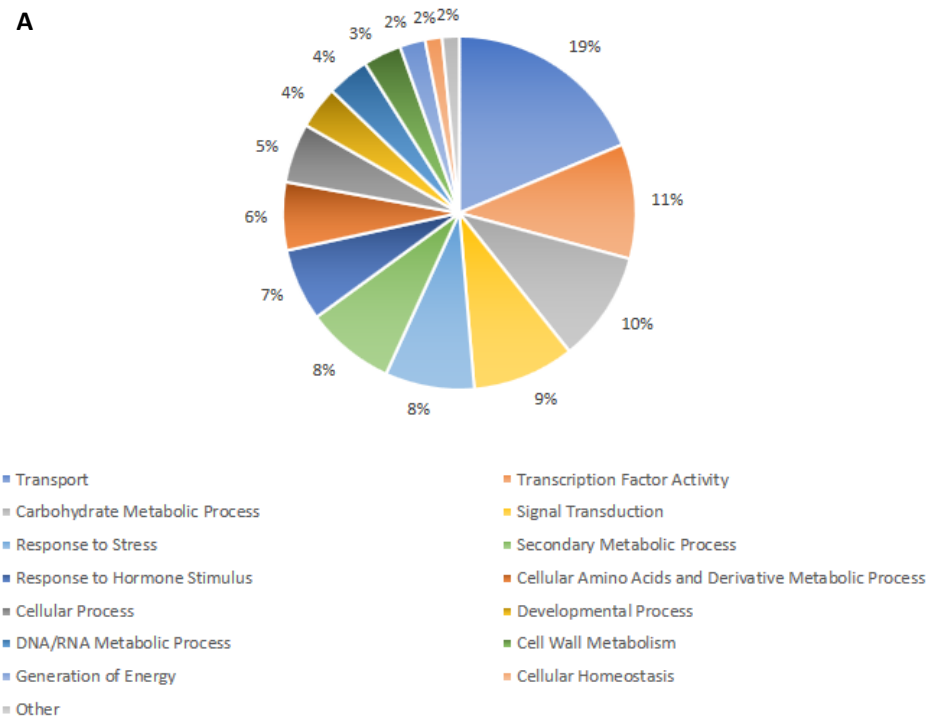


Figure 13: A) Chlorophyll content (µg Chl/cm^2) in control leaves (CTRL - the value represents the mean ±S.E. of C1, C2, C3 lines, four biological replicates for each one) and *VvNAC33* overexpressing leaves (A4, A5, A8 - each value represents the mean ±S.E. of four biological replicates); B) Ratio between chlorophyll a and chlorophyll b in the same leaves of A; C) Ratio between chlorophyll and carotenoid content in the same leaves of A. Asterisk indicates significantly different from the control line. *: $P < 0.05$.

3.4 Microarray analysis on *VvNAC33* and *VvNAC60* overexpressing plants

Microarray analyses were conducted on *VvNAC33* and *VvNAC60* overexpressing leaves to gain insight into any changes at transcriptomic level caused by the overexpression and therefore to highlight putative targets of these two TFs. We performed this experiment on the lines mentioned above (A4, A5, A8, B1, C1, C2, C3). Three clones of each selected line were grown into a growth chamber for two months, with the aim to minimize the transcriptomic changes due to fluctuations of the growing conditions. As biological replicates, we collected three young leaves at the same developmental stage choosing one clone for each line for *VvNAC33* (A4, A5, A8) and the control (C1, C2, C3). Moreover, *VvNAC33* overexpressing leaves showed phenotype. Regarding *VvNAC60*, since we obtained just one overexpressing line we collected three young leaves from each three generated clones. From each pool of leaves we extracted RNAs and all of them resulted suitable to proceed with retro transcription, labelling and hybridization according to the Agilent microarray analysis protocol. A multiclass

comparison analysis was carried out using Significance Analysis of Microarray (SAM) with a false discovery rate (FDR) of 0.1%. T-test analysis was carried out with a *p* correlation value of 0.05 (TMeV 4.3) comparing samples overexpressing NAC33 and NAC60 with the same control lines. A total of 1239 and 1537 differentially expressed genes were identified for NAC33 and NAC60, respectively, in the comparison with the control, with a fold change ≥ 2 or ≤ -2 (Supplementary Table 6; Supplementary Table 7). Differentially expressed transcripts were annotated on the basis of the V1 version of the 12X grapevine genome and grouped into 18 Gene Ontology (GO) functional categories (Figure 14 and 15). Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset. By removing the genes felt in unknown categories, regarding *35S::VvNAC33* plants we achieved 257 upregulated and 540 downregulated genes and for *VvNAC60* expression, 416 olinucleotides presented an increase and 585 a decrease in their hybridization signal.



B

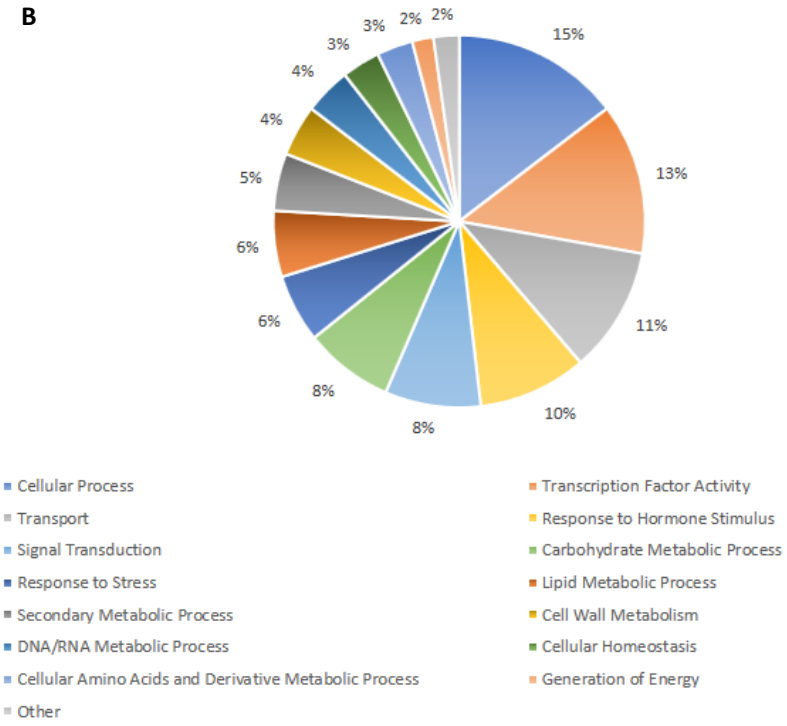
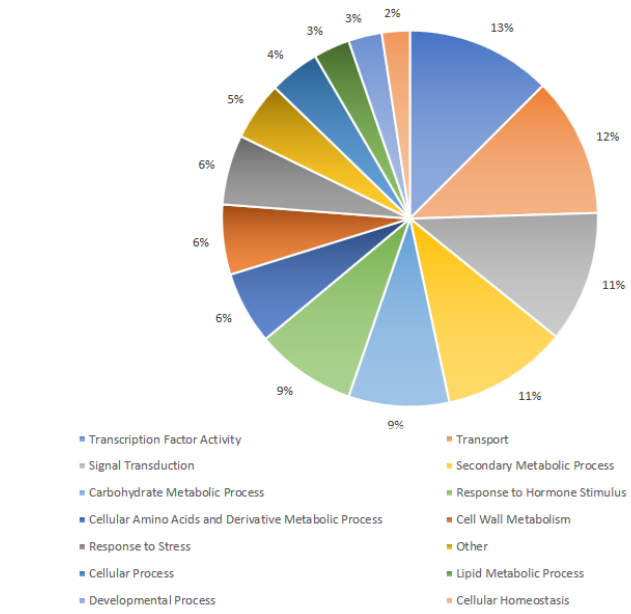


Figure 14: Distribution (%) of up-regulated (A) and down-regulated (B) genes in *VvNAC33* overexpressing plants into 18 Gene Ontology functional categories. Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset.

A



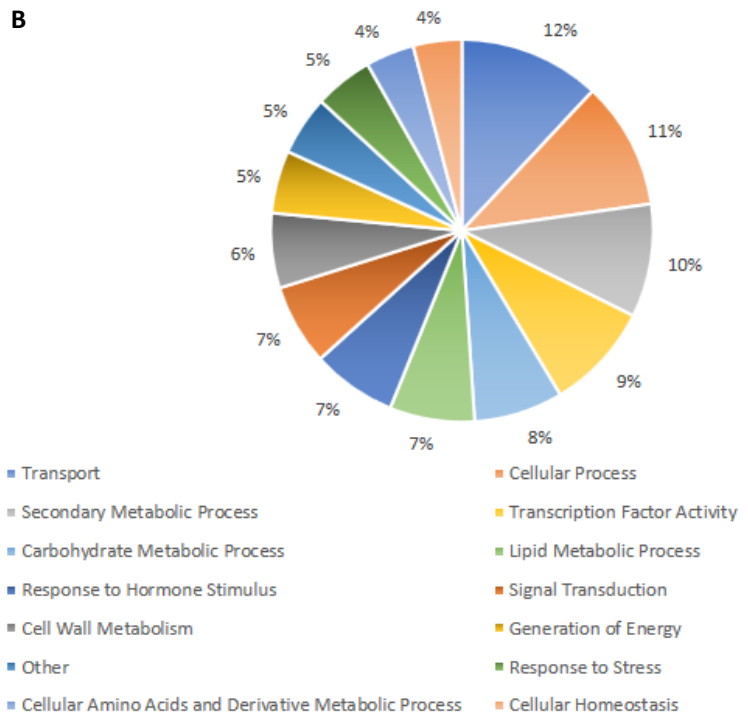


Figure 15: Distribution (%) of up-regulated (A) and down-regulated (B) genes in *VvNAC60* overexpressing plants into 18 Gene Ontology functional categories. Putative functional annotations were manually improved by BLAST analysis and those with no similarity to known sequences (no hit/protein unknown) were removed from the subset.

Interestingly, among the up-regulated genes, both modulated transcriptomes shared the same most represented functional categories, that is "Transport", "Transcription Factor Activity", "Carbohydrate Metabolic Process", "Signal Transduction" and "Secondary Metabolic Process".

Regarding the 60 most up-regulated genes in *VvNAC33* overexpressed leaves, transport was the most represented functional category (Table 8). Four *NITRATE TRANSPORTERS* (VIT_02s0087g00580; VIT_06s0004g03520; VIT_12s0059g01240; VIT_17s0000g09470) and two *ABC TRANSPORTERS* (VIT_09s0002g05540; VIT_09s0002g03570) were identified. Among carbohydrate metabolic process we observed the up-regulation of two *GALACTINOL SYNTHASE* (VIT_05s0020g00330; VIT_05s0077g00430) which are key enzyme in the synthesis of raffinose family oligosaccharides that function as osmoprotectants in plant cell (Nishizawa *et al.,* 2008); these two genes resulted

ID_code	Functional annotation	Gene Ontology	FC	
VIT_12s0034g01120	UDP-glycosyltransferase 71A13	Carbohydrate Metabolic Process	8.97	
VIT_12s0034g00040	UDP-glucose glucosyltransferase		8.62	
VIT_05s0077g00510	Beta-fructofuranosidase		5.50	
VIT_05s0077g00430	Galactinol synthase		4.41	*
VIT_05s0020g00330	Galactinol synthase		4.33	*
VIT_19s0015g01720	fructose-bisphosphate aldolase, cytoplasmic isozyme 1		4.14	
VIT_05s0020g03140	Sugar transporter 13		3.80	
VIT_00s0531g00060	Cellulose synthase CSLE1	Cell Wall Metabolism	5.21	
VIT_00s0469g00040	Cellulose synthase CSLE1		4.19	
VIT_12s0057g01430	Heavy-metal-associated domain-containing protein	Cellular Homeostasis	8.06	
VIT_13s0158g00080	Serine carboxypeptidase		3.96	
VIT_07s0129g00830	CYP81D2	Cellular Process	7.65	
VIT_18s0001g11580	CYP82A3		7.36	
VIT_08s0040g00130	Copper-binding family protein		7.15	
VIT_18s0001g09660	CYP81D2		5.11	
VIT_04s0008g06210	Nodulin		4.90	
VIT_14s0083g00640	Constans 2 (COL2)	Developmental	6.90	
VIT_02s0025g00960	V-type H+-transporting ATPase subunit E	Generation of Energy	6.75	
VIT_16s0013g01110	Ethylene-responsive transcription factor 5	Response to Hormone Stimulus	8.08	
VIT_09s0002g00700	Dormancy/auxin associated protein		5.73	
VIT_13s0019g03550	ERF/AP2 Gene Family (VvAP2-11)		4.24	
VIT_07s0104g00930	Gibberellin receptor GID1L2		4.07	
VIT_00s0253g00150	Methyl jasmonate esterase		3.76	
VIT_16s0013g01080	ERF/AP2 Gene Family (VvERF086)		3.73	
VIT_07s0031g01040	L-ascorbate oxidase	Response to Stress	3.86	
VIT_07s0031g01050	Ascorbate oxidase		3.72	
VIT_12s0034g00080	Flavonoid-glucosyltransferase	Secondary Metabolic Process	6.38	
VIT_08s0007g07730	CYP93A1 2-hydroxyisoflavanone synthase		6.32	
VIT_18s0001g09650	CYP81E1		3.93	
VIT_17s0000g07020	Cis-zeatin O-beta-D-glucosyltransferase		3.67	
VIT_19s0014g04430	S-locus protein kinase	Signal Transduction	5.40	
VIT_00s0366g00020	CRK10 (cysteine-rich RLK10)		5.36	
VIT_18s0122g00180	Calmodulin CML37		4.45	
VIT_17s0000g03330	Receptor serine/threonine kinase PR5K		4.02	
VIT_00s0286g00050	S-locus protein kinase		3.99	
VIT_00s0286g00110	S-locus protein kinase		3.96	
VIT_07s0129g00890	Protein kinase		3.69	
VIT_02s0025g04110	MAPKKK HA-tagged protein kinase		3.63	
VIT_07s0031g02610	NAC domain-containing protein (VvNAC39)	Transcription Factor Activity	7.62	
VIT_05s0049g01020	VvMyb15		7.33	
VIT_19s0014g03290	NAC domain-containing protein (VvNAC17)		6.85	*
VIT_17s0000g00270	GT2-like trihelix DNA-binding protein		3.80	
VIT_15s0046g00240	Lateral organ boundaries protein 1		3.77	
VIT_10s0003g00780	Glutamate receptor 3.4	Transport	10.76	
VIT_09s0002g05810	Boron transporter-like protein 4		8.90	
VIT_10s0003g00680	Glutamate receptor protein		8.79	
VIT_04s0008g04180	Arsenite transport protein (ArsB)		6.30	
VIT_03s0063g00250	Hydrogenobyrinic acid a,c-diamide synthase		5.89	
VIT_02s0087g00580	Nitrate transporter		5.45	
VIT_12s0059g01240	Nitrate transporter (NTP3)		4.99	
VIT_08s0058g00450	Substrate carrier, Mitochondrial		4.95	
VIT_09s0002g05540	ABC transporter g family pleiotropic drug resistance 12 PDR12		4.83	
VIT_05s0062g01150	Amino acid permease		4.40	
VIT_18s0001g12100	Auxilin		4.29	
VIT_11s0103g00010	Potassium-sodium symporter HKT2		4.27	
VIT_04s0069g00390	Glutamate receptor protein		3.89	
VIT_09s0002g03570	ABC transporter G member 15		3.76	
VIT_01s0010g03640	DnaJ homolog, subfamily A, member 3		3.73	
VIT_17s0000g09470	Nitrate transporter3.1		3.68	
VIT_06s0004g03520	Nitrate excretion transporter1		3.67	

Table 8: The 60 most up-regulated genes in *VvNAC33* overexpressing leaves. Genes identified by co-expression analysis performed in chapter 2 are indicated with an asterisk (*).

to be related to *VvNAC33* by co-expression analyses performed in chapter 2. In grape, raffinose is a minor carbohydrate, but its accumulation in leaves of *Vitis vinifera* could represent an early step of cold acclimation (Grant *et al.,* 2009). We also found two *CELLULOSE SYNTHASE* (VIT_00s0531g00060; VIT_00s0469g00040) among genes encoding proteins involved in cell wall metabolism and two members of *ERF/AP2 GENE FAMILY* (VIT_13s0019g03550; VIT_16s0013g01080), implicated in response to hormone stimulus, which have already been described (Licausi *et al.,* 2010). The up-regulation of a *FLAVONOID-GLUCOSYLTRANSFERASE* (VIT_12s0034g00080) was detected and it is involved in anthocyanin biosynthesis.

By looking at the 60 most up-regulated genes by *VvNAC60* (Table 9), we observed that many of them encoded proteins implicated in cell wall metabolism, that included a *PECTINESTERASE*, two *ENDO-1,4-BETA-GLUCANASE KORRIGAN (KOR)*, three *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE* and two *XYLOGLUCAN ENDO-TRANSGLYCOSYLASE*. Among genes involved in the response to hormone stimuli it is worth to note two *ETHYLENE RESPONSIVE FACTORS (ERFS), ERF018* (VIT_02s0025g04440) and *ERF113* (VIT_07s0031g01980), which is known to be upregulated during berry ripening (Licausi *et al.,* 2010). Regarding carbohydrate metabolic processes, we found the same two *GALACTINOL SYNTHASE* (VIT_05s0020g00330; VIT_05s0077g00430) up-regulated by *VvNAC33*. Concerning secondary metabolic process, two *CIS-ZEATIN O-BETA-D-GLUCOSYLTRANSFERASE* (VIT_18s0001g06090; VIT_18s0001g06120) were high up-regulated; zeatin is the most active and ubiquitous cytokinin and the glucosyltransferase peaked at véraison and down-regulated after véraison (Zhang *et al.,* 2008). Moreover, we found the up-regulation of *(9,10) (9`,10`) CLEAVAGE DIOXYGENASE (CCD4) (VvCCD4;* VIT_02s0087g00930), whose expression

increases dramatically during berry development, proportional to the loss of carotenoids (Young *et al*., 2012).

ID_code	Functional annotation	Gene Ontology	FC
VIT_08s0056g00220	Serine/threonine protein phosphatase PP1	Carbohydrate Metabolic Process	16.49
VIT_05s0077g00430	Galactinol synthase		8.87
VIT_05s0020g00330	Galactinol synthase		8.11
VIT_05s0062g01010	Aldo/keto reductase AKR		7.73
VIT_05s0020g02170	Sugar transporter ERD6-like 16		5.70
VIT_11s0016g00300	Pectinesterase family	Cell Wall Metabolism	11.47
VIT_11s0052g01180	Xyloglucan endotransglucosylase/hydrolase 23		8.10
VIT_11s0052g01190	Xyloglucan endotransglucosylase-hydrolase XTH3		7.43
VIT_11s0052g01300	Xyloglucan endotransglycosylase 6		7.35
VIT_00s2526g00010	Endo-1,4-beta-glucanase korrigan (KOR)		6.93
VIT_11s0052g01340	Xyloglucan endo-transglycosylase, C-terminal		6.69
VIT_11s0052g01260	Xyloglucan endotransglucosylase/hydrolase 23		6.49
VIT_00s2620g00010	Endo-1,4-beta-glucanase korrigan (KOR)		6.16
VIT_11s0052g01320	Xyloglucan endotransglycosylase 6		5.69
VIT_18s0001g08450	Branched-chain-amino-acid transaminase ATBCAT-2	Cellular Amino Acids and Derivative Metabolic Process	13.01
VIT_18s0001g08430	Branched-chain-amino-acid aminotransferase 2, chloroplast (Atbcat-2)		11.22
VIT_13s0019g02140	Tropinone reductase		10.11
VIT_05s0049g01980	3-isopropylmalate dehydratase large subunit 2		9.35
VIT_01s0011g00760	Beta-glucosidase		7.64
VIT_06s0004g00590	Lysine decarboxylase		6.08
VIT_00s0214g00120	F-box family protein	Cellular Homeostasis	7.13
VIT_01s0026g02700	CYP704G9	Cellular Process	11.66
VIT_17s0000g00830	Nodulin MtN3 family		6.10
VIT_07s0005g00660	Late embryogenis abundant protein 5	Developmental Process	6.77
VIT_14s0060g01910	Nodulin MtN3 family		5.68
VIT_12s0055g00800	Arachidonic acid-induced DEA1		5.06
VIT_18s0001g02960	Nucleotidyltransferase family protein, putative, expressed	DNA/RNA Metabolic Process	8.92
VIT_02s0154g00300	Small nuclear ribonucleoprotein Sm D3		5.28
VIT_08s0058g00930	Alanine--glyoxylate aminotransferase 2 3, mitochondrial	Generation of Energy	18.04
VIT_14s0066g01670	Alpha-dioxygenase	Lipid Metabolic Process	48.14
VIT_01s0011g03090	Allene oxide cyclase (jasmonates from fatty acids)		6.78
VIT_00s0194g00290	4-hydroxy-3-methylbut-2-enyl diphosphate reductase		6.19
VIT_07s0005g00870	Erg-1	Response to Hormone Stimulus	17.16
VIT_18s0001g14270	Gibberellin-regulated protein 1 (GASA1)		10.17
VIT_02s0025g04440	ERF/AP2 Gene Family (VvERF018),Dehydration Responsive Element-Binding TF		9.94
VIT_15s0046g01390	Ethylene-responsive transcription factor cytokinin response factor 4		9.73
VIT_19s0177g00030	Gibberellin 2-beta-dioxygenase 7		7.98
VIT_05s0049g01780	Caleosin		5.92
VIT_07s0031g01980	ERF/AP2 Gene Family (VvERF113)		5.73
VIT_07s0005g04800	SUPER1/YUCCA5 (suppressor of ER1)		5.73
VIT_18s0072g00260	Ethylene-responsive transcription factor related to APETALA2 6		5.66
VIT_18s0122g01210	Cuticular water permeability	Response to Stress	13.19
VIT_18s0075g00440	TIR-NBS-LRR disease resistance		8.41
VIT_18s0001g06090	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	27.66
VIT_18s0001g06120	Cis-zeatin O-beta-D-glucosyltransferase		24.48
VIT_10s0003g00470	Trans-resveratrol di-O-methyltransferase - VvROMT		9.23
VIT_08s0007g07730	CYP93A1 2-hydroxyisoflavanone synthase		6.88
VIT_02s0087g00930	(9,10) (9`,10`) cleavage dioxygenase (CCD4) (VvCCD4b)		6.46
VIT_01s0026g01380	Glutathione S-transferase 29 GSTU18		5.27
VIT_19s0014g03140	Lanthionine synthetase C	Signal Transduction	6.57
VIT_18s0089g01140	Wall-associated kinase		5.47
VIT_16s0098g00210	Receptor serine/threonine kinase		5.71
VIT_07s0197g00060	myb family	Transcription Factor Activity	7.00
VIT_03s0091g00670	Lateral organ boundaries protein 38		5.97
VIT_01s0026g02710	NAC domain-containing protein (VvNAC26)		5.93
VIT_12s0142g00360	putative MADS-box Agamous 1 (VviAG1)		5.07
VIT_06s0004g04590	Epsin N-terminal homology (ENTH) domain-containing	Transport	6.66
VIT_02s0025g04420	MATE efflux family protein		6.47
VIT_06s0004g03520	Nitrate excretion transporter1		5.79

Table 9: The 60 most up-regulated genes in *VvNAC60* overexpressing leaves. Genes identified by co-expression analysis performed in chapter 2 are indicated with an asterisk (*).

By focusing on the up-regulated genes with FC > 2 (Table 10), 19 'switch' genes were identified; in Table 10 are reported those ones found by consulting the global expression atlas (Fasoli *et al.,* 2012) and those ones by analyzing the berry transcriptional dataset (Massonnet, 2015; Palumbo *et al.,* 2014). Interestingly, *VvMYBA1* (VIT_02s0033g00410), *VvMYBA2* (VIT_02s0033g00390) and *VvMYBA3* (VIT_02s0033g00450) were found: they induced anthocyanin synthesis at the onset of the ripening by activating the UDP-glucose:flavonoid-3-O-glucosyltransferase gene (Walker *et al.,* 2007; Lijavetzky *et al.,* 2006; Kobayashi *et al*., 2004) and they have a pivotal role during the transition to berry ripening. *VvMYBA2* was identified also among genes up-regulated in plantlets transiently overexpressing *VvNAC60*. An enzyme involved in the phenylpropanoid biosynthesis, a *FLAVONOL SYNTHASE* (VIT_03s0017g00710), was found as 'switch' gene together with the mentioned above *VvCCD4b* and the two *ENDO-1,4-BETA-GLUCANASE KORRIGAN (KOR)*. Moreover, two genes belonging to transport were identified: *ALF5* (VIT_19s0014g02450) and *ORGANIC CATION/CARNITINE TRANSPORTER4* (VIT_19s0014g04790) and both of them resulted correlated to *VvNAC60* by co-expression analysis performed in chapter 2.

ID_code	Gene annotation	Functional annotation	FC		
VIT_05s0049g01980	3-isopropylmalate dehydratase large subunit 2	Cellular Amino Acids and Derivative Metabolic Process	9.35	A	
VIT_00s2526g00010	Endo-1,4-beta-glucanase korrigan (KOR)	Cell Wall Metabolism	6.93	R-W	
VIT_07s0005g00660	Late embryogenis abundant protein 5	Developmental Process	6.77	R-W	
VIT_02s0087g00930	(9,10) (9`,10`) cleavage dioxygenase (CCD4) (VvCCD4b)	Secondary Metabolic Process	6.46	A-R-W	
VIT_03s0091g00670	Lateral organ boundaries protein 38	Transcription Factor Activity	5.97	R-W	
VIT_00s0340g00050	Endo-1,4-beta-glucanase korrigan (KOR)	Cell Wall Metabolism	4.37	R-W	
VIT_04s0023g01110	PQ-loop repeat protein	Unknown Protein	3.80	W	
VIT_02s0025g04340	Osmotin	Response to Stress	3.60	A-R-W	
VIT_02s0033g00380	VvMybA2 (C-term)	Transcription Factor Activity	3.27	R-W	
VIT_03s0017g00710	flavonol synthase	Secondary Metabolic Process	3.07	A	
VIT_19s0014g04790	Organic cation/carnitine transporter4	Transport	2.94	A-R-W	*
VIT_02s0033g00410	VvMybA1	Transcription Factor Activity	2.85	R-W	
VIT_01s0127g00590	Protein disulfide isomerase	Cellular Process	2.71	A	
VIT_19s0014g02450	ALF5 (Aberrant lateral root formation 5)	Transport	2.62	A-W	*
VIT_14s0108g01070	NAC domain-containing protein (VvNAC11)	Transcription Factor Activity	2.48	R-W	
VIT_02s0033g00450	VvMybA3	Transcription Factor Activity	2.28	R-W	
VIT_02s0033g00390	VvMybA2	Transcription Factor Activity	2.23	R-W	
VIT_08s0007g05580	Embryo-abundant protein	Cellular Process	2.11	A	
VIT_15s0046g00150	DOF affecting germination 1	Transcription Factor Activity	2.10	R-W	

Table 10: Up-regulated genes (FC >2) by *VvNAC60* overexpression that resulted to be 'switch' genes. 'Switch' genes were identified by consulting the global genes expression atlas (A - Fasoli *et al.*,2012), the red (R) and white (W) berry transcriptome datasets (Massonnet, 2015; Palumbo *et al.,* 2014). Genes identified also by co-expression analyzed performed in chapter 2 are indicated by an asterisk (*).

By looking at the shared up-regulated genes between *VvNAC33* and *VvNAC60,* we noted that they were only 38 and they included a wide set of genes almost equally distributed in all 18 functional categories (Table 11).

ID_code	Functional annotation	Gene Ontology	FC		
			VvNAC33	*VvNAC60*	
VIT_05s0077g00430	Galactinol synthase	Carbohydrate Metabolic Process	4.41	8.87	*
VIT_05s0020g00330	Galactinol synthase		4.33	8.11	*
VIT_01s0011g00810	Heparanase 1		3.32	3.99	
VIT_05s0051g00010	Beta-amylase 1		3.06	2.06	
VIT_05s0049g00260	2-oxoglutarate-dependent dioxygenase		2.17	2.62	
VIT_00s0469g00040	Cellulose synthase CSLE1	Cell Wall Metabolism	4.19	2.60	
VIT_18s0075g00280	Branched-chain amino acid aminotransferase	Cellular Amino Acids and Derivative Metabolic Process	2.79	3.55	
VIT_10s0042g01050	Serine carboxypeptidase II		2.50	3.05	
VIT_01s0011g06260	Anthranilate synthase beta subunit		2.41	2.12	
VIT_13s0019g01980	Aspartic Protease (VvAP32)	Cellular Homeostasis	2.65	2.76	
VIT_06s0080g00790	MYB divaricata	Developmental Process	3.25	2.38	
VIT_10s0003g02420	SRG1 (senescence-related gene 1) oxidoreductase		2.61	2.79	
VIT_18s0001g02960	Nucleotidyltransferase family protein, putative, expressed	DNA/RNA Metabolic	2.32	8.92	
VIT_11s0016g03550	NADP adrenodoxin-like ferredoxin reductase	Generation of Energy	2.95	2.82	
VIT_09s0002g00700	Dormancy/auxin associated protein	Response to Hormone Stimulus	5.73	4.83	
VIT_13s0019g03550	ERF/AP2 Gene Family (VvAP2-11)		4.24	3.34	
VIT_07s0104g00930	Gibberellin receptor GID1L2		4.07	2.99	
VIT_12s0059g01850	Peroxisomal membrane protein	Response to Stress	3.52	2.00	
VIT_19s0027g01330	R protein PRF disease resistance protein		3.35	3.05	
VIT_13s0067g00260	Nematode-resistance protein		3.27	2.23	
VIT_06s0004g05730	Universal stress protein (USP) family protein		2.02	2.83	
VIT_08s0007g07730	CYP93A1 2-hydroxyisoflavanone synthase	Secondary Metabolic Process	6.32	6.88	
VIT_18s0001g09650	CYP81E1		3.93	2.87	
VIT_07s0129g00890	Protein kinase	Signal Transduction	3.69	4.71	
VIT_02s0025g04110	MAPKKK HA-tagged protein kinase		3.63	3.11	
VIT_00s0532g00070	CRK10 (cysteine-rich RLK10); kinase		2.29	3.32	
VIT_15s0046g00240	Lateral organ boundaries protein 1		3.77	2.82	
VIT_15s0048g02280	NAC domain-containing protein (VvNAC54)		3.08	3.18	
VIT_08s0058g00200	Transcription factor		2.40	2.20	
VIT_07s0129g00330	Lateral organ boundaries protein 39		2.34	4.69	
VIT_06s0004g07500	WRKY Transcription Factor (VvWRKY16)		2.11	2.32	**
VIT_02s0087g00580	Nitrate transporter	Transport	5.45	2.38	
VIT_12s0059g01240	Nitrate transporter (NTP3)		4.99	2.37	
VIT_06s0004g03520	Nitrate excretion transporter1		3.67	5.79	
VIT_09s0002g04500	Carnitine/acylcarnitine carrier, Mitochondrial		2.63	2.41	
VIT_19s0015g00040	ABC Transporter (VvMRP4 - VvABCC4)		2.28	2.24	

Table 11: Up-regulated genes shared between *VvNAC33* and *VvNAC60*. Genes identified also by co-expression analysis performed in chapter 2 are indicated with an asterisk (*) when they were related to *VvNAC33* and two asterisks (**) to *VvNAC60*.

The overexpression of both *VvNAC33* and *VvNAC60* led to the down-regulation of a set of genes larger than the set of up-regulated genes; in particular, we noticed that these two sets shared 253 down-regulated genes, much more than the 38

commonly up-regulated ones. Among them, many categories were almost equally represented, again indicating that many aspects of the cell metabolism could be influenced by the overexpression of *VvNAC33* and *VvNAC60*.

Among the 60 most down-regulated genes by both *NAC* TFs, we noted that they mainly included genes belonging to cellular process, cellular homeostasis and cell wall metabolism, as well as response to stress and response to hormone stimuli (Table 12, Table 13) By focusing on some relevant genes, interestingly, *VvNAC60* and *VvNAC33* shared the same most down-regulated gene, that was a *MYOSIN-RELATED* (VIT_06s0004g02360), a motor proteins implicated in the regulation of the cytoskeleton (Peremyslov *et al.,* 2011). In both modulated transcriptomes, several gibberellin-regulated proteins were found; gibberellin is present at high concentration in the flowers and during early berry development and its levels decrease throughout the subsequent berry development (Fortes *et al.,* 2015). Regarding *VvNAC33,* we observed the down-regulation of two genes involved in cell wall expansion and loosening, that were *VvEXPA11* (VIT_08s0007g00440) and *VvEXA13* (VIT_13s0019g01650); they were predominantly expressed during the green phase and probably involved in cell enlargement (Massonnet, 2015). Concerning *VvNAC60*, we noticed the down-regulation of the same *VvEXPA11* mentioned above and of three photosynthesis-related genes, that were two *LHCB3* (VIT_00s0181g00180; VIT_00s0181g00200) and a *LHCII type I CAB-1* (VIT_19s0014g00160). Moreover, we focused our attention on common genes between the down-regulated ones by *VvNAC33* and *VvNAC60* overexpression and the linked genes inversely correlated to these two *NACs* in the integrate co-expression network performed by Palumbo *et al.* (2014); we identified five and three of them, respectively, which are indicating with an asterisk in Tables 12 and Table 13.

ID_code	Gene annotation	Functional category	FC	
VIT_14s0083g00350	Beta-glucan-binding protein 5	Carbohydrate Metabolic Process	-11.34	
VIT_12s0059g01320	Glucan endo-1,3-beta-glucosidase 7 precursor		-6.76	
VIT_06s0004g06680	ACR4 (Arabidopsis CRINKLY4)		-5.34	
VIT_09s0002g02940	Myo-inositol oxygenase 1		-5.14	
VIT_15s0048g01750	fasciclin arabinogalactan-protein (FLA8)	Cell Wall Metabolism	-10.11	
VIT_02s0025g01380	Endo-1,4-beta-glucanase		-6.65	
VIT_10s0116g00520	Xyloglucan endotransglucosylase/hydrolase 8		-5.97	
VIT_08s0007g00440	Expansin (VvEXPA11)		-5.78	
VIT_11s0016g00590	Invertase/pectin methylesterase inhibitor		-7.21	
VIT_13s0019g01650	Expansin (VvEXPA13)		-6.04	
VIT_11s0037g00570	Anthranilate N-benzoyltransferase	Cellular Amino Acids and Derivative Metabolic Process	-8.02	*
VIT_01s0011g03210	Aspartic Protease (VvAP1)	Cellular Homeostasis	-7.46	
VIT_09s0018g01670	Aspartic Protease (VvAP26)		-5.58	
VIT_18s0001g07340	Aspartic Protease (VvAP43)		-5.55	
VIT_08s0007g00700	Aspartic Protease (VvAP21)		-5.39	*
VIT_03s0091g01290	Serine carboxypeptidase S10		-5.33	*
VIT_06s0004g02360	Myosin-related	Cellular Process	-61.16	
VIT_04s0008g03540	Transducin protein		-38.14	
VIT_08s0056g01140	Exostosin		-6.91	
VIT_07s0031g01680	CYP86A1		-5.60	
VIT_07s0104g00190	7S globulin precursor, basic		-6.12	
VIT_14s0068g01520	Ds RNA-binding domain-containing protein	DNA/RNA Metabolic Process	-14.65	
VIT_08s0032g00890	Alpha-L-arabinosidase		-6.88	*
VIT_13s0064g01260	DNA-damage-repair/toleration protein (DRT100)		-5.68	
VIT_18s0041g02150	Lipase GDSL	Lipid Metabolic Process	-9.64	
VIT_13s0047g00340	Ethylene-responsive transcription factor WRINKLED 1	Response to Hormone Stimulus	-11.44	
VIT_03s0038g00120	Gibberellin-regulated protein 4 (GASA4)		-8.18	
VIT_00s1317g00010	Gibberellin-regulated protein 4 (GASA4)		-8.00	
VIT_00s0189g00060	Gibberellin-regulated protein 4 (GASA4)		-7.76	
VIT_00s0189g00070	Gibberellin-regulated protein 4 (GASA4)		-7.64	
VIT_14s0108g00740	GASA4		-6.81	
VIT_18s0001g13980	Auxin responsive SAUR protein		-5.14	
VIT_10s0003g00650	Peroxidase	Response to Stress	-16.99	
VIT_11s0052g01620	Pathogenesis-related protein 1 precursor (PRP 1)		-8.36	
VIT_02s0025g01600	Harpin-induced 1		-6.32	
VIT_11s0052g00630	Metallothionein		-6.20	
VIT_12s0059g02420	Peroxidase ATP11A (gb|X98802).		-7.26	
VIT_03s0038g02170	Thaumatin		-6.04	
VIT_18s0001g03080	Chitin elicitor-binding CEBIP LysM domain-containing		-5.83	
VIT_04s0008g03950	RD22		-5.46	
VIT_16s0022g01970	Anthocyanidin 3-O-glucosyltransferase	Secondary Metabolic Process	-8.69	
VIT_08s0007g05160	Flavonoid 3',5'-hydroxylase		-6.84	
VIT_18s0041g00790	UDP-glycosyltransferase 88B1		-6.84	
VIT_04s0008g05830	Armadillo/beta-catenin repeat	Signal Transduction	-8.53	*
VIT_03s0063g00210	Receptor protein kinase		-7.23	
VIT_08s0007g07930	Clavata1 receptor kinase (CLV1)		-6.86	
VIT_12s0134g00010	Phototropic-responsive NPH3 protein		-5.32	
VIT_17s0000g09290	Protein kinase ATN1		-5.14	
VIT_08s0056g01130	Mini zinc finger 2 MIF2	Transcription Factor Activity	-11.65	
VIT_04s0008g01830	myb domain protein 32		-11.37	
VIT_07s0197g00040	Lateral organ boundaries domain gene 36		-7.31	
VIT_14s0108g00420	basic helix-loop-helix (bHLH) family		-6.87	
VIT_05s0020g02700	transcription factor MUTE		-6.56	
VIT_13s0067g02280	basic helix-loop-helix (bHLH) family		-5.80	
VIT_16s0022g02050	Lateral organ boundaries domain gene 36		-5.73	
VIT_17s0000g02660	myb domain protein 6 (VvMybC2-L2)		-6.02	
VIT_01s0011g04080	Zinc finger (C3HC4-type ring finger)		-5.62	
VIT_11s0037g01310	basic helix-loop-helix (bHLH) family		-5.23	
VIT_04s0008g04230	ABC Transporter (VvPDR28 - VvABCG58)	Transport	-7.57	
VIT_09s0002g00450	Subtilase		-7.33	

Table 12: The 60 most down-regulated genes in *VvNAC33* overexpressing leaves. Genes identified as VvNAC33 neighbor genes in the integrated co-expression network performed by Palumbo *et al.,* 2014 are indicated with an asterisk (*).

ID_code	Gene annotation	Functional category	FC	
VIT_04s0008g00870	Phosphoethanolamine/phosphocholine phosphatase	Carbohydrate Metabolic Process	-8.49	
VIT_12s0059g01220	Pyrophosphate-dependent phosphofructokinase		-7.69	
VIT_06s0004g06680	ACR4 (Arabidopsis CRINKLY4)		-7.39	
VIT_07s0005g01940	Pectinesterase family	Cell Wall Metabolism	-10.62	
VIT_08s0007g00440	Expansin (VvEXPA11)		-6.77	
VIT_10s0116g00520	Xyloglucan endotransglucosylase/hydrolase 8		-6.47	
VIT_18s0001g13380	Papain cysteine proteinase isoform I	Cellular Homeostasis	-20.05	
VIT_18s0001g13400	Papain cysteine proteinase isoform I		-13.67	
VIT_00s2015g00020	F-box family protein		-10.58	
VIT_13s0019g05130	Serine carboxypeptidase III		-10.03	
VIT_18s0089g01000	F-box family protein		-8.68	
VIT_06s0004g02360	Myosin-related	Cellular Process	-71.88	
VIT_05s0049g02320	HAD superfamily hydrolase		-25.06	
VIT_07s0005g01880	Patatin		-9.16	
VIT_01s0026g00570	Bet v I allergen		-8.99	
VIT_03s0038g01830	Proline-rich protein 4		-7.80	*
VIT_01s0011g01920	Phosphate-induced protein 1		-7.67	
VIT_06s0004g01050	Calcineurin phosphoesterase		-7.33	
VIT_00s0187g00160	Ripening-related protein		-7.09	
VIT_19s0015g01800	Nucleoside triphosphatase	DNA/RNA Metabolic Process	-8.38	
VIT_04s0008g07080	Aspartic Protease (VvAP5)		-7.43	
VIT_08s0032g00890	Alpha-L-arabinosidase		-6.82	*
VIT_12s0059g00210	Epoxide hydrolase		-6.67	
VIT_00s0181g00180	LHCB3 (light-harvesting chlorophyll binding protein 3)	Generation of Energy	-13.37	
VIT_00s0181g00200	LHCB3 (light-harvesting chlorophyll binding protein 3)		-12.85	
VIT_19s0014g00160	LHCII type I CAB-1		-7.72	
VIT_16s0098g00460	Lipase class 3	Lipid Metabolic Process	-30.22	
VIT_06s0004g06730	Microsomal omega-3 fatty acid desaturase		-17.50	
VIT_10s0003g02110	Lipase GDSL		-12.54	
VIT_12s0059g01590	Lipase GDSL		-10.34	
VIT_13s0047g00340	Ethylene-responsive transcription factor WRINKLED 1	Response to Hormone Stimulus	-12.10	
VIT_02s0012g00650	PBP1 (pinoid-binding protein 1)		-10.75	
VIT_14s0108g00740	GASA4		-8.55	*
VIT_07s0005g00090	Auxin-responsive GH3		-7.38	
VIT_03s0038g00120	Gibberellin-regulated protein 4 (GASA4)		-7.37	
VIT_00s1317g00010	Gibberellin-regulated protein 4 (GASA4)		-7.37	
VIT_10s0003g00650	Peroxidase	Response to Stress	-9.52	
VIT_11s0052g01620	Pathogenesis-related protein 1 precursor (PRP 1)		-8.71	
VIT_04s0008g03950	RD22		-7.08	
VIT_02s0025g04300	Thaumatin		-6.54	
VIT_00s0274g00080	Benzoquinone reductase	Secondary Metabolic Process	-8.56	
VIT_06s0004g05050	Abscisic acid 8` hydroxylase (CYP707A2) (VvA8H-CYP707A2.1)		-7.34	
VIT_00s0218g00130	Anthocyanidine rhamnosyl-transferase		-6.94	
VIT_08s0056g01130	Mini zinc finger 2 MIF2	Transcription Factor Activity	-16.88	
VIT_02s0033g00300	myb family		-11.45	
VIT_14s0108g00420	basic helix-loop-helix (bHLH) family		-6.99	
VIT_07s0197g00040	Lateral organ boundaries domain gene 36		-6.74	
VIT_17s0119g00080	Organic cation transport protein OCT1	Transport	-45.98	
VIT_04s0008g04230	ABC Transporter (VvPDR28 - VvABCG58)		-13.41	
VIT_08s0058g01030	Saposin B domain-containing protein		-11.61	
VIT_09s0002g01030	Subtilisin serine proteinase		-10.64	
VIT_11s0103g00050	High-affinity K+ transporter 1 (HKT1)		-10.06	
VIT_18s0001g10350	Subtilase family protein		-9.73	
VIT_19s0090g01280	Lipid-binding serum glycoprotein family protein		-8.72	
VIT_00s0131g00180	Annexin ANN4		-8.41	
VIT_15s0048g01170	Subtilisin serine protease		-8.12	
VIT_18s0001g02140	Metal transporter Nramp1		-8.02	
VIT_13s0064g00940	ferric reductase defective 3		-7.24	
VIT_11s0016g04160	Sulfate transporter 3.5		-7.08	
VIT_13s0084g00090	Nodulin MtN21 family		-7.05	

Table 13: The 60 most down-regulated genes in *VvNAC60* overexpressing leaves. Genes identified as NAC33 neighbor genes in the integrated co-expression network performed by Palumbo *et al.,* 2014 are indicated with an asterisk (*).

3.5 *VvMYBA1* acts down-stream of *VvNAC60*

Microarray analyses performed on transgenic Shiraz grapevines overexpressing *VvNAC60* revealed the upregulation of some MYB family members, among which *VvMYBA1* (VIT_02s0033g00410) and *VvMYBA2* (VIT_02s0033g00390), already well characterized for their direct and crucial role during the transition to berry ripening and key regulators of the biosynthesis of anthocyanins (Walker *et al.,* 2007; Kobayashi *et al.,* 2004). An up-regulation of *VvMYBA2*, albeit not very high (FC=1.42), was also found in Sultana plantlets transiently overexpressing *VvNAC60*. It was expected not to find a modulation of *VvMYBA1* in this white variety because in white cultivars the allelic variation caused by the insertion of the Gret1 Gypsy-type retrotransposon into the promoter of *VvMYBA1* prevents *VvMYBA1* transcription (Kobayashi *et al.,* 2004) which controls anthocyanin biosynthesis in ripening berries. On the contrary, microarray analysis could detect the mutated allele of *VvMYBA2* which encodes a protein with a non-conservative amino acid change, unable to trigger the transcription of anthocyanin structural genes (Walker *et al.,* 2007).

Based on these observations, we wondered whether *VvMYBA1* and *VvMYBA2* could act down-stream *VvNAC60*, raising it in a putative role of controlling the biosynthesis of anthocyanins. Validating this assumption is another important step to define NACs regulatory network involved in the transition to the ripening phase in grapevine berries. Hence, to investigate whether these two MYBs are targeted by *VvNAC60*, we analyzed the ability of this protein to activate the two regulative regions. The regulative regions of *VvMYBA1* and *VvMYBA2* were isolated from *Vitis vinifera* cv. Corvina and cloned into a reporter vector to control the Firefly Luciferase gene (LUC) expression. In the identification of each promoter, we considered the sequence about 1/1.5 Kb up-stream the transcription start site (TSS). The TSS for *VvMYBA1* and *VvMYBA2* transcript predicted in the V1 genome assembly was further confirmed exploiting data from an RNA-seq

experiment previously performed in our research group (data not shown). After sequencing, we aligned the two sequences and the result is reported in Figure 16. Before proceeding in trans-activation experiments, we performed a preliminary bioinformatically analysis to verify the presence of NAC DNA-binding sites (Table 14).

```
MYBA2p          CGCCGATTAAATAGTTTAGGTTTAGAAAATGATATATTTGGAATAACAACTTTTTTTAAT
MYBA1p          ------------------------------------------------------------

MYBA2p          TAAGAAAAAAAGAAGATAAAAATGATATTATAAAAATTTCATTAAAGCCTATAATGTTCC
MYBA1p          ------------------------------------------------------------

MYBA2p          CACATAAGTCTAAAAACGTCTTCTTCAATAAGATATCTTATTGCTGCCTCTATATTAAGC
MYBA1p          ------------------------------------------------------------

MYBA2p          TCTTTTTTTTTCTTTTTCTTTTTTCCTTCTTTAATGTCAAAAGGTCTTTGGAGAAAATGA
MYBA1p          -----------------------------------------------TGGAGAAAATGA
                                                               ************

MYBA2p          TGCA------TGCAGAATTAAGGAATTACTGGTGTGCAAAATCATAACTTTCTTCTATTT
MYBA1p          TGCAACGACCTGCAGAATTAAGAAGTTAATGATGTGCAAAATCATAACTTTCTTCTATTT
                ****      ************ * *** ** ****************************

MYBA2p          TTATTAACAATAACAAATTTTTTTTCTTAACACTACTAATCAAAATATTCACTTGAAAAC
MYBA1p          TTATTAACAATAA---ATTTTTTTTCTTAACACTACTAATCCAAATATTCACTACAAAAC
                *************   ************************* ***********  *****

MYBA2p          TCATATTTGTTATTTTATTAATTTTTTTAAAATATAATAATTTAATATCTACACATTACT
MYBA1p          TAATATTTGTTATTTTATTAATTTTTA-AAAATATAATAATTTAATATCTACACATTACT
                * ************************  *******************************

MYBA2p          TATTTTATCAAAAAATTTCAAAGGTTCAGGTGAAATTTTTATACTTTTATCAATTTCCAT
MYBA1p          TATTTTATCAAAAT-TTTCAAAGGTTCATGTGAAAATTTTATACTTTTATCAATTTCCAT
                *************  ************* ****** ************************
                                              A
MYBA2p          CATTTTTCAAATTTTAAAACAATTTTAATTTATATGCAAATTTTTAT-------------
MYBA1p          CATTTTTCAAATTTTAAAACAATTTTAATTTATATGCAAATTTCTACTGATATTTCCATA
                ****************************************** **
MYBA2p          --ACTTGTATATATTTGAGGG----AAAAT------------------------------
MYBA1p          AAACTCGAAAATCAGTGAGGGTAACAAAGTCAATTTTTTTAACATTGGGTATATGTATAT
                  *** * * **   ******    *** *

MYBA2p          ---------AATTGTCAGTGAGGAGAGTACATTGTAGGAAATGACACACGTCCCAAGCAA
MYBA1p          ATTTGAGGGAATTGTCAGTGAGAAGAATACATGGTAGGAAATGACACACGTCCCAAGCAA
                         ************* *** ***** ****************************

MYBA2p          CAGATGGATGGTTGAGAATGAACCCGGTCATTGAATTGACAATAGAAAAAGTTCTAAAAT
MYBA1p          CAGATGGATGGTTGAGAATGAACCCGGTCATTGAATTGACATTAGAAAATGTTCTAAAAT
                **************************************** ******* **********
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MYBA2p          GTATTTTTATCGACGTTACTGGTCAGAAACAAAGTCCTCCGCGAGCCAGAGGCATATCCT
MYBA1p          ATATTTTTATCGACGTTACTGATCAGAAACAAAGTCCTCCGCCAGCCAGAGGCATATCCT
                 ******************** ******************** ****************

MYBA2p          AATGATTGTACCTTTCTTCGCTGACAATTCCCATGAATTAGCTGCTGCCACTGCATAGCG
MYBA1p          AATGATTGTACCTTTCCTCACTGACAATTCCCATTAATTAGCTGCTGCCACTGCATAGCG
                **************** ** ************** ************************

MYBA2p          GCCATAATATAATGGTAGAGGACCCATGGAGCTTCCCTTTTCAGTGAACATGGGTTAGTC
MYBA1p          GCTATAATATAATGGTAGAGGGCCCATGGAGCTTTCCTTTTCAGTGAACATGGGTTAGTC
                ** ****************** ************ ************************

MYBA2p          GACAAAAGAAAATGTTAAAGTTGAAAGAGGAGCGGTGGCCCTCAAAGGTTCCCATCACTT
MYBA1p          GACAAAAGAAAATGTTCAAGTTGAAAGANGAGCGGTGGCCCTCAAAGGTTCCCGTCACTT
                **************** *********** ********************** ******

MYBA2p          GGTTGCTTTTTGTC-AAGGAAACAGTGGTATCAGAATCCAAATCTTCTACGTAATGTCCC
MYBA1p          GGTTGCTTTTTGTCTAAGGAAACAGTGGTATCAGAATCCAAATCTTCTACGTAATGTCCC
                ************** ******************************************

MYBA2p          ATTCATCCTACCAATGTCCAAATGAATTCCTCTGGACATTAAAATATGGTAGCACGTGGT
MYBA1p          ATTCATCCTACCAATGTCCATATGAATTCCTCTGGACGTTAAAAAATGGTTGCACGTGGT
                ******************** *************** ****** ***** *********

MYBA2p          TGTCTTCGGGATCACACCAGTTTATACATTTGCACCACAAAATAGAGATTGTTCATAAAG
MYBA1p          TGTCTTCAGGATCACACCAGTTTATACATTTGGACCACAAAATAGAGATTGTTCATCAAG
                ******* ************************ ********************** ***

MYBA2p          GATACTAGTCAGCAATTAATTCCTAAATTTCGCTGTACATTTATAGTAAGTTGATACATA
MYBA1p          GATACTAGTCAGCAATTAATTCCTAAAT-------------------------------
                ****************************

MYBA2p          ATGGGTAAATATCTCTTATGACACACACCCTTTGTCCATGATGTCCATCGCATTCGGAAG
MYBA1p          ----------ATCTCTTATGACACACACCCTTTGTCCATGAACTCCAGCGCATTTGGAAG
                          ******************************  **** ****** *****
                                                        B
MYBA2p          CCAGGTAATGCACCATAAGAAACGTGTCGAATCAACCAATTAGGGGTCTGGTGTCCGAGT
MYBA1p          CCAG-TAATGCACCATAAGAAACGTGTCGAATAAACCAATTAGGGGTCTGGTGTCCGAGT
                **** ************************** **************************

MYBA2p          CATGAGATAGAACAGGTTCGAGGTTGTTATATATCAATCAATAATTAGAGAAGGAGCCGG
MYBA1p          CATGAGATAGAACAGGTTCGAGGTTGTTATATATCAATCAATAATTAGAGAAGGAGCCGG
                ************************************************************

MYBA2p          TCTCTTGTGTTGAGTTGACTCG
MYBA1p          TCTCTTGTGTTGAGTTGACTCG
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Figure 16: Nucleotidic alignment between VvMYBA2 and VvMYBA2 regulative regions. The putative TATA box is marked in red. The two NAC binding sites found only in MYBA2 promoter are marked in the box A (NAC binding site predicted by PlantPAN - sequences 6, Table 14) and in the box B (NAC binding sites predicted by Puranik *et al.,* 2011 - sequence 7, Table 14).

	Hit Sequence	Strand	MYBA1	MYBA2
NACBDs	1. tcTACGTaat	+	1	1
	2. tACGTAat	-	1	1
	3. agTTGACtc	-	1	1
	4. tcTACGTaat	-	1	1
	5. aTTACTtat	+	1	1
	6. CATGTg	+	1	/
	7. GAACTCCAGC	+	1	/

Table 14: Specific binding sites corresponding to NAC binding sites (NACBDs) predicted by PlantPAN (sequences 1-5) and by Puranik *et al.* (2011 - sequence 7).

This analysis revealed the presence of specific NAC recognition elements reported in literature as CGT[AG] motif (Bu *et al.,* 2008; Olsen *et al.,* 2005), shared by both promoters, and CATGTG motif (Figure 16-box A, Tran *et al.,*2004) and [C/G][A/T] [T/A][G/C]TC[C/G][A/T][C/G][G/C] motif (Figure 16-box B, Puranik *et al.*, 2011), found only in MYBA1 promoter.

Therefore, we analyzed the ability of *VvNAC60* to activate VvMYBA1 and VvMYBA2 regulative regions performing Dual-Luciferase reporter assay in transfected *N. benthamiana* leaves (Figure 17).

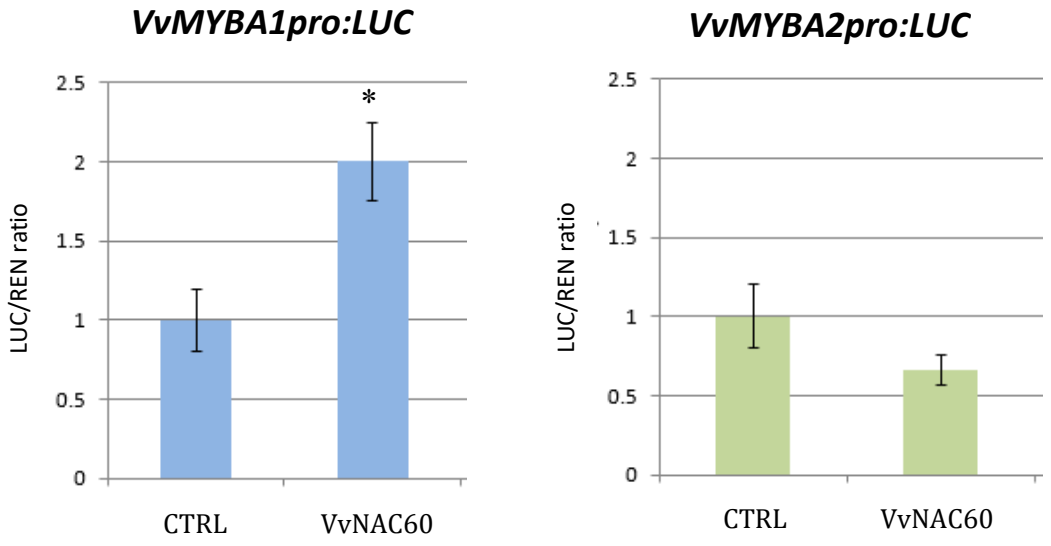

Figure 17: Candidate target genes promoter activation tested by Dual-luciferase reporter assay in *Agrobacterium* sp.-infiltrated *Nicotiana benthamiana* leaves. *LUC* values are reported relative

to the *REN* control and normalized on specific negative control. Each value represents the mean ± S.E. of four biological replicates. Asterisk indicate significant differences in promoter activation compared with the negative control ($P < 0.05$).

We noticed that VvNAC60 protein was not able to directly activate VvMYBA2 regulative region, but a significant LUC induction was observed for VvMYBA1 promoter. This result strongly suggested that *VvMYBA1* was transcriptionally related to NAC60 *in vivo* and we could hypothesis that *VvNAC60* played a central role in the control of the biosynthesis of anthocyanins.

4. DISCUSSION

The NAC (NAM/ATAF/CUC) family is one of the largest classes of TFs in plant kingdom with important functions as components of the regulation of various biological processes. Recently, some grapevine NAC members have been indicated as putative master regulators of the transcriptome shift driving the plant into a maturation program. The challenge of this chapter is an attempt to understand the roles of the five NAC TFs reported in chapter 2 in the regulatory network controlling the transcriptomic reprogramming which takes place along plant and berry development. In summary, *VvNAC33* and *VvNAC60* were selected as putative master regulators able to promote the immature-to mature transition in the entire plant (including berries), *VvNAC11* and *VvNAC13* as putative master regulators of the ripening inception in the berry, whereas *VvNAC03* as close homologue gene of tomato *NOR*.

A useful and rapid way to gain information about gene function is the transient expression assay. We transiently overexpressed these genes in *N. benthamiana* leaves to obtain a preliminary idea about their functions. The browning regions and the yellowing effects shown by agroinfiltrated leaves were consistent with our working hypothesis proposing an involvement in organ phase transition during grapevine growth.

Due to the peculiar characteristics of this plant species, a homologous system is more appropriate to avoid the misinterpretation of results because of a foreign genetic background in heterologous systems. Based on these observations, we performed a transient overexpression of *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* directly in grapevine. The agro-infiltrated plantlets did not show any obvious phenotype; a global analysis of the transcriptomic data by microarray revealed a small set of genes specifically up- and down-regulated by the transient overexpression of each *NAC* factor. We obtained a not always similar range in fold change and a different number of differentially expressed genes

among the five modulated transcriptomes which could represent transgene-related features as well as features related to the leaf sampling. The RNA used to perform microarray analyses was extracted from a pool of two entire leaves we supposed to be the most affected by the transgene basing on a preliminary GUS-assay. We checked the occurred overexpression of the transgene by qPCR, as mentioned above, but a significant improvement we are trying to carry out is performing transient transformation using a reporter genes in the overexpressing construct; in this way, we could sample only those plant regions actually highly affected by the infection. Despite of this technique should certainly be optimized to reduce technical problems, this approach allowed us to obtain a general view on the early effects of each transgene on grape transcriptome. We noted that overexpression affected a wide range of processes and we focused our attention on those genes linked with functions in pathways and metabolic processes with central role during grapevine maturation. We noted that secondary metabolic process was up-regulated by all the five *NACs*; in particular, we identified many genes implicated in phenylpropanoid pathway, anthocyanin and terpenoid accumulation. It is interesting to underline that *VvNAC60* up-regulated *VvNAC03* and *VvNAC03* down-regulated *VvNAC60*; we hypothesized that these two genes could be involved in the fine tuning of their own expression. Regarding *VvNAC60,* we noticed the up-regulation of many germin proteins, involved in development, osmotic regulation, photoperiodic oscillation, defence and apoptosis; this observation could give a first explanation about the phenotype showed by *N. benthamiana* leaves, even though we should consider that the agro-infiltrated plantlets might have suffered some effect due to the stress after the agro-infiltration. Concerning *VvNAC33*, it is worth to note that the most down-regulated gene was the photosynthesis related *APO2* that could be related to early disrupting of the photosynthetic apparatus.

We further investigated the functions of *VvNAC33* and *VvNAC60* that are the two NACs that, according to the evidences collected so far, seem to be key

participants in regulating transition from immature to mature phase not only in the berry but also in the whole plant. Despite this approach is still time-consuming and represents an arduous task, we successfully regenerated three groups of transformed plants: those one overexpressing *VvNAC33*, those one overexpressing *VvNAC60* and those one transformed with GFP only (negative control). We used the embryogenic calli of *Vitis vinifera* cv. Shiraz, kindly provided by Dr. Amanda Walker (CSIRO Plant Industry, Adelaide – Australia). For the first time, we used the pK7WG2 binary vector that slightly increased the transformation efficiency compared to the vector previously used in our laboratory. *VvNAC60* overexpression would need a particular clarification: indeed, since we obtained only two transformed plants, only one of which overexpressing the transgene, we hypothesized that overexpression of this NAC caused a severe phenotype hampering the development of shoots from positive embryos. Other cycles of transformation will be necessary to validate this hypothesis.

Plants overexpressing the two TFs showed different kind of alterations at phenotypic level. The overexpression of *VvNAC33* did not affect plant architecture, but caused a severe modification in leaves color. This tissue indeed showed a different bleaching degree, well-visible at adult fully-expanded stage and sometimes already evident at young stage. Moreover, we noted a good correlation between level of transgene expression and phenotype appearance: the lines showing the most obvious alterations in leaves color (A4, A5, A8) corresponded to those one with the highest *VvNAC33* expression level. A similar phenotype appeared also on *N. benthamiana* transiently *VvNAC33* overexpression leaves; therefore, we could consider this modification as an evidence that supports our working hypothesis of an involvement of this gene in playing a crucial role in grapevine transition to the mature phase. Loss of green color and the associated yellowing of the leaves are typical symptoms related to leaf senescence and is caused by chlorophyll degradation (Noodén *et al.,* 1997).

By analyzing three overexpressing lines (A4, A5, A8) we noted, as expected, an increase of this pigment in comparison with the control, even though the chlorophyll a:b ratio remains high and did not show significant differences with the control; usually this ratio declines with leaf age because the structural loss of the chloroplast stroma lamellae, containing photosystem I and most of the chlorophyll a, occurred earlier than of the grana lamellae, containing photosystem II (Bricker and Newman, 1982). Further analysis will be necessary to validate and better understand this result. We measured also the ratio between chlorophyll and carotenoids and we noticed a decrease in the overexpressing lines; this result is consistent with the typical behavior of leaves undergoing senescence in which all photosynthetic pigments decline (Biswal 1994; Britton 1989); in particular, studies demonstrated a greater loss of chlorophyll than carotenoids, which results in yellow coloration of senescing leaves, revealing a relative stability of carotenoids (Biswal, 1995). Moreover, in some senescence-like processes, such as the ripening of tomatoes, a synthesis of new carotenoids has been measured; these potent antioxidants were retained as part of the cellular equipment defending against photodamage during leaf senescence. Deeper investigation will be required to better understand chlorophyll catabolism and to check the function of photosystems I and II, also considering that we found a gene putatively involved in the early disrupting of the photosystem apparatus as the most down-regulated one in *VvNAC33* transiently overexpressing plantlets.

VvNAC60 overexpressing plants showed different phenotypic alterations on the appearance of the plant; the habitus of the only transformed and overexpressing plant was slightly affected by a reduced dimension in comparison to the control plant. In all the three clones generated from this line we noticed an obvious phenotype not on leaves, as for *VvNAC33,* but on the stem: it was characterized by an early lignification in comparison to the control, representing a clear feature of a mature/woody plant growth phase. Further analysis will be doubtless required

to validate this observation and other cycles of transformation would recommend to obtained a higher of transformed plants to characterize.

One important information to better characterize the roles of these two NACs as transcriptional regulators is the identification of downstream targets; we proceeded with a microarray analysis aimed at investigating the whole set of genes modulated by *VvNAC33* and *VvNAC60* overexpression. Firstly, as putative targets, we focused on up-regulated genes referable to a specific functional category, excluding those one not yet annotated, and we observed above all that *VvNAC33* and *VvNAC60* overexpression affected the same five most represented categories (transport, TF activity, signal transduction, carbohydrate metabolic process and secondary metabolic process). We looked at those pathways involved in the maturation process, and particularly during fruit ripening, such as increased respiration, chlorophyll degradation, sugar accumulation, biosynthesis of anthocyanins, essential oils, and flavor and aroma components, increased activity of cell wall-degrading enzymes, and a transient increase in ethylene production (Brady, 1987). Indeed, the global transcriptome reprogramming involved a wide spectrum of biochemical changes, especially when the plant 'switch' from primary to secondary metabolism. In our analysis, according to the tissue softening and cell expansion that characterized the onset of the ripening, an up-regulation of genes coding proteins implicated in cell wall metabolism were noted. Furthermore, pathways controlling berry color and aroma were found; in particular, regarding anthocyanins biosynthesis, we observed that *VvNAC33* up-regulated a flavonoid-glucosyltransferase and *VvNAC60* modulated the expression of three key regulators of this pathway, *VvMYBA1*, *VvMYBA2* and *VvMYBA3* (Kobayashi *et al*., 2002). Moreover, *VvCCD1* and *VvCCD4b* were up-regulated by *VvNAC33* and *VvNAC60* respectively and they encoded carotenoids cleavage dioxygenases whose products represent potent flavor and aroma compounds (Young *et al.,* 2012). We found two important TFs in the transcriptional regulation of stilbene biosynthesis, that were *VvMYB14*

(VIT_07s0005g03340, FC=2.82), modulated by *VvNAC33* and *VvMYB15* (VIT_05s0049g01020, FC=7.33 NAC60), modulated by *VvNAC60* (Holl *et al.,* 2013). Together with the other TFs identified in this analysis, several *NACs* were observed; they could take place in the regulatory networks controlling plant developmental processes consistently with examples previously reported (Nakano *et al.,* 2015; Tripathi *et al.,* 2014) supporting the idea of a complex network in grapevine developmental regulation. *VvNAC33* and *VvNAC60* also up-regulated some *ETHYLENE RESPONSE FACTORS* (ERF), indicating that this hormone may play a crucial role in grape, which is a non-climacteric fruit, particularly in the late stages of ripening. The last interesting gene belonging to the transport functional category was *ORGANIC CATION/CARNITINE TRANSPORTER4* that we found up-regulated by *VvNAC60* in stable overexpressing plant, by *VvNAC611*in transiently overexpressing plants and strongly related to *VvNAC60* in co-expression analysis; it is also a 'switch' gene identified by analyzing global expression atlas and berry transcriptome datasets (Palumbo *et al.,* 2014; Fasoli *et al.,* 2012). Further studies could be interesting to perform on this transporter to better understanding its role in the regulation network controlling organ phase transition in grapevine development.

Although only 38 genes were shared between the up-regulated by *VvNAC33* and *VvNAC60*, the overexpression of the two NAC TFs induced a higher number of down-regulated genes and the two set of genes shared 253 of them. This result was consistent with the evidences reported by Palumbo *et al.* (2014) which indicated that the transition to mature growth mainly involved the suppression of vegetative pathways rather that maturation-specific ones. Indeed, the plant moving through mature phase has to switch off all the plethora of processes involved in vegetative growth. Firstly, we noted that *VvNAC60* down-regulated some genes involved in photosynthesis machinery, and particularly some proteins regarding the antenna systems. We also noted a strong down-regulation of genes linked with function in cellular process, cellular homeostasis and cell wall

metabolism. In particular, we observed that these two NACs shared the same most down-regulated gene, which was a myosin-related implicated in the cytoskeleton regulation. Other genes involved in the cell wall expansion and loosening were found, such as some expansin proteins that we know they are mainly expressed in green phase (Massonnet, 2015).

A crucial step in the study of a regulatory network is the selection and the validation of some putative target genes identified with microarray analysis. We focused our attention on the MYB family which plays a key role in the transcriptional regulation of anthocyanins (Martin and Paz-Ares, 1997) and, particularly, on *VvMYBA1* and *VvMYBA2*. The two regulators of the expression of the UFGT gene (Kobayashi *et al*., 2002) were up-regulated in transgenic plants overexpressing *VvNAC60* and *VvMYBA2* was positively modulated even in transiently overexpressing plants. By performing a Dual-Luciferase reporter assay in transfected *N. benthamiana* leaves we analyzed the ability of *VvNAC60* to activate VvMYBA1 and VvMYBA2 regulative regions and we observed an induction of *VvMYBA1* and not of the other MYB TF by *VvNAC60*. We tried to make some hypothesis by studying the preliminary bioinformatics analysis conducted on the promoter regions of MYB TFs. The upstream portion which is found only in MYBA2 promoter or those portions of different length - even reduced at one single nucleotide - between the two regulative regions could be involved in the diverse activation. Another possibility could be found in the two NAC binding sites (Box A and B, Figure 16) identified only in MYBA1 promoter. Further investigation in this analysis is clearly required. A validation of the physical interaction between the resulting restricted promoter and the TF of interest might be also represented an important improvement in our study.

Taken together, all the findings reported in this chapter provided real evidence that *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* participate in the regulation of the transcriptome reprogramming during grapevine development.

Although we did not clearly identify a common set of putative unequivocally target genes, we defined a draft of the range of processes in which these TFs are involved to promote grapevine development, taking a step towards knowing their mode of regulation.

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SUPPLEMENTARY DATA

Supplementary Table S1: Differentially expressed genes (> |2| fold) in *VvNAC03* transiently overexpressing plants compared to the control line.

ID_code	Gene annotation	FC
VIT_05s0062g00270	UDP-glucose:flavonoid 7-O-glucosyltransferase	8.40
VIT_05s0062g00340	UDP-glucose:flavonoid 7-O-glucosyltransferase	5.71
VIT_05s0062g00350	UDP-glucose:flavonoid 7-O-glucosyltransferase	5.61
VIT_05s0062g00710	UDP-glucose:flavonoid 7-O-glucosyltransferase	5.29
VIT_01s0127g00400	Polygalacturonase GH28	3.92
VIT_08s0007g05860	GASA like	3.56
VIT_19s0014g00080	Steroid 5alpha-reductase	3.22
VIT_09s0018g01800	Acid phosphatase	3.22
VIT_05s0062g00660	UDP-glucose:flavonoid 7-O-glucosyltransferase	3.12
VIT_01s0011g03180	Lysine and histidine specific transporter	3.07
VIT_06s0080g00780	NAC domain-containing protein (VvNAC74)	2.97
VIT_16s0050g02370	Inorganic phosphate transporter 1-3	2.94
VIT_03s0063g02680	Radialis-like protein 5	2.83
VIT_17s0000g05110	CYP78A4	2.56
VIT_16s0050g02510	WRKY DNA-binding protein 53	2.53
VIT_14s0006g02530	Non-specific lipid-transfer protein 2 (LTP 2)	2.49
VIT_09s0002g06970	Palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, chloroplast	2.47
VIT_12s0028g03510	S-locus lectin protein kinase	2.43
VIT_00s0399g00020	Protease inhibitor/seed storage/lipid transfer protein (LTP)	2.36
VIT_04s0008g07220	Chloroplast nucleoid DNA binding protein	2.17
VIT_12s0034g00140	UDP-glucose glucosyltransferase	2.17
VIT_15s0046g02390	ANTR2 (anion transporter 2)	2.16
VIT_10s0116g01650	5'-adenylylsulfate reductase (APR1)	2.11
VIT_01s0011g06310	Inositol polyphosphate related phosphatase	2.11
VIT_03s0017g00360	MADS-box protein SVP (short vegetative phase)	2.10
VIT_03s0017g00450	MADS-box protein SVP (short vegetative phase)	2.08
VIT_15s0048g00630	Protease inhibitor/seed storage/lipid transfer protein (LTP)	2.06
VIT_10s0092g00500	CYP71D10	2.06
VIT_15s0024g01860	MADS-box protein AGL24	2.06
VIT_12s0055g00020	UDP-glucose glucosyltransferase	2.04
VIT_18s0041g00370	Double strand break repair protein (XRCC4)	2.02
VIT_03s0038g00670	fructose-bisphosphate aldolase, chloroplast precursor	2.02
VIT_13s0156g00610	S-receptor kinase	2.02
VIT_00s0748g00020	Receptor kinase RK20-1	2.01
VIT_14s0083g01100	Alpha-1,4-glucan-protein synthase 1	-2.02
VIT_13s0019g01420	No hit	-2.02
VIT_05s0049g00580	No hit	-2.02
VIT_07s0005g01890	Patatin	-2.02
VIT_00s0240g00060	No hit	-2.03
VIT_03s0038g00060	No hit	-2.03
VIT_14s0030g00200	Sugar transporter ERD6-like 5	-2.04
VIT_12s0059g00560	Fimbrin 2	-2.07
VIT_00s0125g00180	Zinc finger (C3HC4-type ring finger)	-2.09
VIT_00s1428g00010	Ankyrin repeat	-2.09
VIT_04s0043g00220	Amine oxidase	-2.11
VIT_17s0000g08600	Signal recognition particle receptor beta subunit	-2.12
VIT_05s0077g00010	Protein transporter	-2.14
VIT_14s0030g00320	Sugar transporter ERD6-like 5	-2.14
VIT_02s0033g00300	myb family	-2.19
VIT_19s0014g00600	BED finger-nbs-lrr resistance protein [Populus trichocarpa]	-2.21
VIT_00s0239g00070	CTV.22	-2.22
VIT_15s0024g01230	No hit	-2.26
VIT_13s0158g00100	MADS-box agamous-like 15	-2.29
VIT_07s0005g01840	Patatin	-2.32
VIT_04s0008g03230	Unknown protein	-2.34
VIT_19s0027g00220	Unknown protein	-2.35
VIT_07s0151g00100	Ankyrin	-2.42
VIT_03s0063g01720	S-N-methylcoclaurine 3'-hydroxylase	-2.45
VIT_17s0053g00910	Cyclic nucleotide-binding transporter 1	-2.46
VIT_01s0011g05990	No hit	-2.47
VIT_06s0004g07810	GTP binding protein EngB	-2.47
VIT_06s0009g02370	EMB2261 (embryo defective 2261)	-2.49
VIT_11s0118g00010	No hit	-2.50
VIT_16s0022g01210	myb domain protein 85	-2.53
VIT_00s0322g00020	HHP4 (heptahelical protein 4)	-2.57
VIT_08s0007g06850	Colon cancer-associated protein Mic1 containing protein	-2.58

VIT_05s0020g03050	Unknown	-2.59
VIT_04s0008g02070	Avr9/Cf-9 induced kinase 1	-2.65
VIT_08s0058g01220	No hit	-2.68
VIT_18s0001g06320	Cupin, RmlC-type	-2.71
VIT_19s0014g03810	No hit	-2.84
VIT_03s0038g02020	Amidase	-2.88
VIT_16s0100g00230	Glucose-methanol-choline (GMC) oxidoreductase family protein	-3.03
VIT_17s0000g08430	No hit	-3.54
VIT_03s0038g03030	Mechanosensitive ion channel	-3.56
VIT_16s0022g01050	Acetolactate synthase 1, chloroplast precursor	-3.87
VIT_08s0040g02650	Unknown protein	-4.05
VIT_13s0019g02180	Tropinone reductase	-4.17
VIT_02s0154g00470	No hit	-4.57
VIT_03s0063g00580	Rad54	-5.73
VIT_12s0028g03640	Ripening induced protein	-6.77

Supplementary Table S2: Differentially expressed genes (> |2| fold) in *VvNAC11* transiently overexpressing plants compared to the control line.

ID_code	Gene annotation	FC
VIT_12s0035g02150	ferric reduction oxidase 7 FRO7	6.12
VIT_19s0014g00080	Steroid 5alpha-reductase	5.25
VIT_18s0001g10500	Abscisic acid 8` hydroxylase (CYP707A2) (VvA8H-CYP707A2.3)	4.84
VIT_03s0038g02010	Amidase	4.38
VIT_03s0038g02000	Amidase	4.13
VIT_05s0020g02920	Unknown protein	4.02
VIT_09s0018g01800	Acid phosphatase	3.95
VIT_01s0150g00060	SOUL heme-binding	3.47
VIT_18s0001g05710	Hydrolase, alpha/beta fold	3.39
VIT_08s0007g04580	UGT73C2 (UDP-glucosyl transferase 73C2)	3.38
VIT_02s0087g00490	10-deacetylbaccatin III 10-O-acetyltransferase	3.38
VIT_18s0001g12490	O-methyltransferase	3.31
VIT_00s0179g00320	Unknown protein	3.28
VIT_16s0022g01640	Receptor serine/threonine kinase	3.24
VIT_02s0087g00630	Alcohol oxidase	3.19
VIT_16s0100g00330	Unknown protein	3.08
VIT_01s0026g02240	ANTR2 (anion transporter 2	3.04
VIT_15s0046g02390	ANTR2 (anion transporter 2)	2.85
VIT_00s0211g00160	Serine hydroxymethyltransferase 1	2.82
VIT_01s0011g05450	Unknown protein	2.76
VIT_14s0006g03180	CXE carboxylesterase CXE17	2.76
VIT_17s0000g06930	Unfertilized embryo sac 10 UNE10	2.68
VIT_09s0054g01760	Unknown protein	2.66
VIT_06s0004g02290	Unknown	2.63
VIT_13s0074g00660	ABC Transporter (VvPDR24 - VvABCG54)	2.62
VIT_03s0038g00670	fructose-bisphosphate aldolase, chloroplast precursor	2.61
VIT_02s0012g02540	Chlororespiratory reduction 4 (CRR4)	2.60
VIT_07s0129g00470	Unknown protein	2.55
VIT_10s0003g03310	EMB2756 (embryo defective 2756)	2.53
VIT_06s0080g00570	Unknown protein	2.51
VIT_18s0041g01150	Lectin protein kinase	2.50
VIT_11s0103g00760	CIA2 (chloroplast import apparatus 2)	2.48
VIT_01s0010g01140	Unknown	2.47
VIT_00s0338g00030	Cellular retinaldehyde-binding/triple function, C-terminal	2.45
VIT_18s0001g09160	Cyclin, N-terminal	2.45
VIT_08s0007g08540	Mg-chelatase subunit XANTHA-F	2.44
VIT_15s0048g01600	Geraniol 10-hydroxylase	2.44
VIT_03s0038g04210	Phototropin-2	2.43
VIT_07s0129g00990	Protein kinase	2.43
VIT_15s0048g01610	Geraniol 10-hydroxylase	2.43
VIT_08s0105g00430	Omega-3 fatty acid desaturase, chloroplast precursor	2.40
VIT_08s0058g00280	Lectin protein kinase	2.37
VIT_11s0118g00630	Unknown protein	2.36
VIT_17s0000g08580	ApaG domain protein	2.35
VIT_00s0179g00370	ESCRT-I complex subunit TSG101	2.34
VIT_02s0025g02640	Unknown protein	2.31
VIT_14s0083g00260	No hit	2.31
VIT_16s0022g01650	Receptor serine/threonine kinase PR5K	2.30
VIT_08s0058g00950	Curculin-like (mannose-binding) lectin family	2.30
VIT_03s0063g02460	Transcription termination factor mitochondrial mTERF	2.30
VIT_06s0004g06170	Thylakoid soluble phosphoprotein	2.28
VIT_03s0091g00230	Unknown protein	2.26
XLOC_005307	PREDICTED: hypothetical protein [Vitis vinifera]	2.25
VIT_13s0067g03730	Unknown	2.25
VIT_16s0022g00510	Heat shock 22 kDa protein	2.25
VIT_11s0052g01600	UDP-glucose flavonoid 3-O-glucosyltransferase 7	2.21
VIT_14s0060g01050	Pentatricopeptide (PPR) repeat-containing protein	2.19
VIT_11s0016g03250	Lachrymatory factor synthase	2.19
VIT_14s0108g01180	Unknown protein	2.18
VIT_00s0291g00060	Inorganic phosphate transporter 2-1, chloroplast precursor	2.17
VIT_14s0060g02550	Unknown	2.16
VIT_05s0020g04040	Chlorophyllase (CLH2)	2.14
VIT_08s0040g00390	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase	2.14
VIT_12s0134g00340	S-locus lectin protein kinase	2.14
VIT_18s0001g10510	Thioredoxin family	2.11
VIT_18s0001g15360	Thylakoid lumenal 29.8 kDa protein	2.09

VIT_16s0100g00360	Per1	2.08
VIT_12s0059g01810	Photosystem II psbZ	2.08
VIT_08s0007g06930	Dirigent pDIR3	2.06
VIT_09s0002g00420	Senescence-associated protein	2.06
VIT_01s0011g00460	Unknown protein	2.05
VIT_02s0012g00760	Haloacid dehalogenase hydrolase	2.05
VIT_06s0004g00260	Shoot1 protein	2.04
VIT_01s0026g02030	basic helix-loop-helix (bHLH) family	2.03
VIT_14s0060g02630	Unknown protein	2.02
VIT_15s0046g02100	Late embryogenesis abundant group 14	2.02
VIT_00s0389g00030	CYP72A54	2.01
VIT_07s0031g02160	Protein phosphatase 2C DBP	2.01
VIT_13s0019g05200	MATE efflux family protein	2.00
VIT_17s0000g10260	AarF domain containing kinase	2.00
VIT_00s1458g00010	CRK10 (cysteine-rich RLK10)	-2.01
VIT_19s0015g00710	Cellulose synthase CSLE1	-2.02
VIT_10s0003g04830	Protein kinase Xa21	-2.06
VIT_12s0034g02220	RKF3 (receptor-like kinase IN in flowers 3)	-2.09
VIT_13s0067g01650	Glutaredoxin	-2.10
VIT_15s0021g01260	Unknown	-2.12
VIT_06s0004g07810	GTP binding protein EngB	-2.19
VIT_01s0011g04780	Unknown protein	-2.19
VIT_10s0003g00980	Unknown protein	-2.22
VIT_04s0008g05300	Phosphate translocator	-2.26
VIT_00s0226g00100	Regulator of chromosome condensation (RCC1)	-2.26
VIT_01s0011g02950	Zinc finger (C3HC4-type ring finger)	-2.32
VIT_14s0068g00650	Unknown	-2.33
VIT_12s0028g02710	Isoflavone methyltransferase/orcinol O-methyltransferase oomtA	-2.34
VIT_11s0052g01220	Xyloglucan endotransglycosylase 6	-2.35
VIT_10s0116g00980	Unknown protein	-2.36
VIT_05s0020g02700	transcription factor MUTE	-2.37
VIT_05s0049g00580	No hit	-2.39
VIT_11s0016g00880	Strictosidine synthase; Soluble quinoprotein glucose dehydrogenase	-2.45
VIT_12s0028g02760	Isoflavone methyltransferase/orcinol O-methyltransferase oomtB	-2.58
VIT_14s0030g00520	Disease resistance protein (NBS-LRR class)	-2.64
VIT_14s0068g01920	Peroxidase	-2.64
VIT_19s0014g00600	BED finger-nbs-lrr resistance protein [Populus trichocarpa]	-2.82
VIT_05s0020g03050	Unknown	-2.82
VIT_08s0040g02650	Unknown protein	-2.91
VIT_18s0041g00800	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase	-2.93
VIT_14s0068g01900	Peroxidase 50	-2.97
VIT_00s0240g00060	No hit	-2.98
VIT_00s0271g00110	flavodoxin-like quinone reductase 1	-3.05
VIT_04s0008g02070	Avr9/Cf-9 induced kinase 1	-3.24
VIT_14s0068g01880	No hit	-3.62
VIT_02s0154g00470	No hit	-4.31
VIT_03s0038g03130	Flavin containing monooxygenase 3	-6.42

Supplementary Table S3: Differentially expressed genes (> |2| fold) in *VvNAC13* transiently overexpressing plants compared to the control line.

ID_code	Gene annotation	FC
VIT_18s0001g01740	Histone-lysine N-methyltransferase ASHH2 EFS (Early flowering	5.91
VIT_09s0002g00360	Remorin	5.38
VIT_02s0033g01230	No hit	5.38
VIT_16s0050g02540	Nodulin MtN3 family	5.33
VIT_03s0167g00100	MADS-box protein SVP (short vegetative phase)	5.12
VIT_03s0017g00360	putative MADS-box Short Vegetal Phase 4 (VviSVP4)	5.06
VIT_03s0063g01830	AOS (allene oxide synthase)	5.05
VIT_15s0024g01860	putative MADS-box JOIN 4 (VviSVPS4)	4.81
VIT_01s0011g01310	Polygalacturonase QRT3	4.56
VIT_03s0038g03480	Auxin-induced SAUR	4.35
VIT_10s0042g00960	DNAJ heat shock N-terminal domain-containing protein	4.27
VIT_07s0104g00610	Glutamine synthetase	4.17
VIT_11s0118g00080	Disease resistance protein	4.08
VIT_05s0094g01200	flavonoid 3'-hydroxylase cytochrome P450	3.99
VIT_00s0201g00090	Annexin ANN4	3.94
VIT_07s0005g03410	Globulin 11S	3.86
VIT_08s0007g07630	Tryptophan/tyrosine permease family	3.81
VIT_10s0003g02570	ABC Transporter (VvMDR12 - VvABCB12)	3.80
VIT_18s0001g13380	Papain cysteine proteinase isoform I	3.76
VIT_09s0002g05890	No hit	3.59
VIT_02s0012g03210	No hit	3.54
VIT_17s0000g00710	DNA-binding protein	3.52
VIT_14s0068g00470	UDP-glucosyl transferase	3.47
VIT_12s0035g00880	Lactoylglutathione lyase	3.40
VIT_09s0002g02120	Beta-galactosidase	3.39
VIT_12s0034g01900	Globulin-like protein	3.38
VIT_04s0008g05160	Unknown protein	3.38
VIT_11s0016g05280	Peroxidase	3.33
VIT_17s0000g06680	Unknown protein	3.32
VIT_13s0067g03690	Pinene synthase	3.31
VIT_14s0128g00230	DNA binding	3.29

VIT_18s0001g09520	CYP81B2v2	3.29
VIT_03s0038g03470	Auxin-induced SAUR	3.29
VIT_09s0096g00950	No hit	3.27
VIT_15s0107g00560	No hit	3.25
VIT_05s0102g00170	Calcium Dependent Protein Kinase (VvCPK4)	3.21
VIT_04s0008g00220	IAA6	3.20
VIT_04s0008g01290	CGA1 (cytokinin-responsive GATA factor 1)	3.17
VIT_07s0005g00550	Ribosomal protein S12 (RPS12C) 40S	3.14
VIT_02s0154g00030	No hit	3.14
VIT_18s0072g01120	GASA2 (GAST1 protein homolog 2)	3.13
VIT_02s0012g02200	No hit	3.13
VIT_04s0008g00100	AMP binding protein	3.11
VIT_01s0011g04260	Zinc finger (CCCH-type) family protein	3.10
VIT_17s0000g01170	Thylakoid lumenal 17.9 kDa protein, chloroplast	3.04
VIT_11s0118g00030	Exostosin family protein	3.00
VIT_07s0031g03080	No hit	2.99
VIT_03s0038g01090	Auxin responsive SAUR protein	2.97
VIT_16s0050g01870	CAAX amino terminal protease	2.95
VIT_09s0018g00090	ATATH6 (ABC2 homolog 6)	2.94
VIT_17s0000g04460	Calmodulin	2.93
VIT_18s0001g13400	Papain cysteine proteinase isoform I	2.88
VIT_17s0000g00270	GT2-like trihelix DNA-binding protein	2.87
VIT_05s0020g00500	Alpha-L-arabinofuranosidase	2.87
VIT_11s0118g00060	Exostosin-like	2.86
VIT_19s0015g01720	fructose-bisphosphate aldolase, cytoplasmic isozyme 1	2.86
VIT_04s0023g00530	Auxin responsive SAUR protein	2.85
VIT_04s0008g00230	Amino acid permease 8	2.84
VIT_11s0118g00070	Exostosin family protein	2.83
VIT_06s0004g05740	IMP dehydrogenase/GMP reductase	2.82
VIT_14s0006g03140	HEAT repeat-containing protein	2.81
VIT_02s0025g03750	Unknown protein	2.77
VIT_04s0008g05340	Bundle-sheath defective protein 2	2.77
VIT_06s0004g06890	Potassium transporter (KUP1)	2.74
VIT_05s0049g00080	PhSUP1 SUPERMAN	2.74
VIT_16s0013g02110	Aldo/keto reductase	2.73
VIT_14s0128g00240	Cation exchanger (CAX11)	2.72
VIT_03s0038g01230	Auxin responsive SAUR protein	2.72
VIT_02s0025g00450	SGT1a	2.72
VIT_18s0001g14030	Lysine decarboxylase	2.70
VIT_03s0038g01210	Auxin responsive SAUR protein	2.69
VIT_18s0001g05870	WPP domain-associated protein	2.69
VIT_02s0033g00920	No hit	2.69
VIT_12s0134g00240	Avr9/Cf-9 rapidly elicited protein 20	2.68
VIT_00s0746g00010	Retroelement pol polyprotein	2.68
VIT_16s0050g01330	Retrovirus Pol polyprotein from transposon TNT 1-94	2.67
VIT_14s0030g01390	Ribosome biogenesis protein Bms1	2.67
VIT_13s0047g00500	3-hydroxyisobutyryl-CoA hydrolase	2.66
VIT_03s0038g01150	Auxin-responsive	2.65
VIT_10s0042g00100	Superoxide dismutase, Fe-Mn family	2.63
VIT_05s0062g00450	No hit	2.61
VIT_13s0047g00410	3-hydroxyisobutyryl-coenzyme A hydrolase	2.60
VIT_17s0000g05490	Lateral organ boundaries domain family protein (LBD27)	2.59
VIT_05s0020g01620	Transcription termination factor mitochondrial mTERF	2.58
VIT_07s0104g01400	Glutaredoxin	2.58
VIT_09s0002g01030	Subtilisin serine proteinase	2.56
VIT_18s0089g01200	Berberine bridge enzyme	2.56
VIT_13s0064g00190	RNA polymerase sigma subunit SigD	2.55
VIT_05s0020g04810	Lipase GDSL 7	2.55
VIT_09s0002g00290	NBS-LRR type disease resistance protein	2.55
VIT_04s0008g03560	Lactoylglutathione lyase	2.53
VIT_05s0077g00110	Unknown protein	2.53
VIT_00s0445g00040	Unknown	2.50
VIT_01s0011g04010	Catalytic	2.49
VIT_15s0046g01360	No hit	2.49
VIT_14s0128g00620	Germin-like protein 3 [Vitis vinifera]	2.48
VIT_12s0059g02500	Constans-like 11	2.48
VIT_10s0116g01710	Galactosyltransferase family protein	2.48
VIT_05s0102g00280	No hit	2.47
VIT_17s0000g04270	Peptidyl-prolyl cis-trans isomerase B	2.47
VIT_02s0012g02150	Reduced sugar response 4 RSR4	2.47
VIT_05s0020g01030	No hit	2.46
VIT_19s0014g01240	Morphogenesis of root hair 1 MRH1	2.46
VIT_05s0020g01350	Nuclear transcription factor Y subunit B-5	2.44
VIT_18s0001g03440	No hit	2.44
VIT_14s0066g00400	Chalcone isomerase	2.43
VIT_17s0119g00370	Unknown	2.42
VIT_00s0266g00090	ARK3 (Arabidopsis Receptor Kinase 3)	2.42
VIT_06s0061g00080	Saccharopine dehydrogenase	2.42
VIT_03s0038g03460	Auxin-induced SAUR	2.42
VIT_09s0002g01450	Unknown protein	2.42
VIT_08s0040g00790	FK506-binding protein genes family (VvFKBPc)	2.42

VIT_13s0106g00420	Metalloendopeptidase	2.41
VIT_14s0066g01950	Metalloendoproteinase 1 precursor	2.40
VIT_18s0001g11340	No hit	2.40
VIT_19s0015g00330	RNA recognition motif (RRM)-containing protein	2.39
VIT_14s0068g01720	Unknown protein	2.39
VIT_08s0007g01010	Aldo/keto reductase	2.38
VIT_00s0346g00010	Lectin protein kinase	2.38
VIT_07s0005g05040	ARR24 Type A	2.36
VIT_19s0090g00760	No hit	2.36
VIT_15s0046g02380	CYP86A8	2.36
VIT_09s0070g00340	Unknown protein	2.35
VIT_01s0011g03300	Plastid-specific 30S ribosomal protein 3	2.35
VIT_09s0002g07930	Arogenate dehydrogenase 1, chloroplastic	2.34
VIT_01s0011g00040	No hit	2.34
VIT_00s0869g00010	Unknown	2.34
VIT_07s0130g00240	Zinc Finger Homeodomain Transcription Factor (VvZHD4)	2.33
VIT_14s0083g00850	Lipase GDSL 7	2.33
VIT_14s0128g00420	Glycosyl transferase family 8 protein	2.33
VIT_08s0007g05360	Strictosidine synthase	2.33
VIT_05s0020g03580	Unknown protein	2.33
VIT_07s0031g01150	FK506-binding protein genes family (VvFKBP16-2)	2.32
VIT_05s0029g00380	No hit	2.32
VIT_01s0011g06520	Galacturonic acid reductase [Vitis vinifera]	2.32
VIT_13s0067g03050	Leucine-rich repeat transmembrane protein kinase	2.32
VIT_14s0068g00280	Unknown protein	2.31
VIT_14s0006g00030	KH domain-containing protein	2.31
VIT_01s0026g00670	Unknown protein	2.31
VIT_10s0003g04220	Lipase class 3	2.31
VIT_13s0101g00270	Notchless-like protein	2.31
VIT_15s0021g00380	Unknown	2.30
VIT_00s0228g00060	Unknown	2.30
VIT_16s0022g01580	3-ketoacyl-CoA synthase	2.30
VIT_14s0006g00510	No hit	2.29
VIT_01s0011g02250	Metal-nicotianamine transporter YSL7	2.29
VIT_10s0116g00630	Unknown protein	2.29
VIT_00s0304g00040	Succinate dehydrogenase iron-sulfur protein, Mitochondrial	2.29
VIT_15s0024g00660	Esterase	2.29
VIT_07s0031g01550	No hit	2.28
VIT_02s0012g02970	No hit	2.28
VIT_09s0002g06230	RPS5 (resistant to p. syringae 5)	2.28
VIT_01s0010g01570	Pentatricopeptide (PPR) repeat-containing	2.28
VIT_19s0014g01090	No hit	2.27
VIT_09s0054g01420	Beta-amyrin synthase	2.27
VIT_06s0004g05330	Tropinone reductase	2.27
VIT_00s0878g00020	Stachyose synthase precursor	2.27
VIT_03s0167g00110	Receptor protein kinase RK20-1	2.26
VIT_00s0560g00010	Unknown	2.25
VIT_04s0043g01010	violaxanthin de-epoxidase (VDE1) (VvVDE1)	2.25
VIT_00s0286g00120	S-locus protein kinase	2.24
VIT_01s0011g06670	No hit	2.23
VIT_13s0019g01300	No hit	2.23
VIT_19s0015g02030	UDP-galactose transporter 6	2.23
VIT_05s0094g00100	FK506-binding protein genes family (VvFKBP16-3)	2.23
VIT_04s0008g05490	L-ascorbate peroxidase.	2.23
VIT_13s0064g01610	Glycosyl hydrolase family 1 protein	2.22
VIT_01s0146g00160	Exocyst subunit EXO70 family protein C1	2.21
VIT_19s0015g00720	No hit	2.21
VIT_13s0019g04880	No hit	2.21
VIT_18s0001g06030	Erg-1	2.20
VIT_13s0019g03300	Glycosyl hydrolase family 17	2.20
VIT_01s0244g00110	Unknown protein	2.20
VIT_01s0127g00730	ATMYB66/WER/WER1 (WEREWOLF 1)	2.18
VIT_05s0094g01350	Thiol methyltransferase 1	2.18
VIT_04s0043g00370	Ammonium transporter 1;2	2.18
VIT_08s0040g00580	No hit	2.18
VIT_01s0011g00550	Hydrolase, alpha/beta fold family	2.18
VIT_04s0008g06140	No hit	2.17
VIT_00s0188g00110	Glucose-inhibited division family A	2.17
VIT_14s0219g00220	CCT motif constans-like	2.17
VIT_15s0046g01920	ferric reduction oxidase 2	2.16
VIT_06s0004g04080	GTP cyclohydrolase II	2.16
VIT_08s0007g04340	FK506-binding protein genes family (VvFKBP16-4)	2.16
VIT_05s0049g01130	Aldo/keto reductase	2.16
VIT_08s0007g02970	Unknown protein	2.15
VIT_14s0006g02480	Phosphatidic acid phosphatase alpha	2.15
VIT_18s0001g10130	ERF/AP2 Gene Family (VvERF006)	2.15
VIT_16s0039g01050	NADP dependent malic enzyme	2.15
VIT_05s0102g01090	Unknown	2.14
VIT_03s0038g04180	Pigment defective 191 (PDE191)	2.14
VIT_02s0012g00760	Haloacid dehalogenase hydrolase	2.14
VIT_04s0008g06150	No hit	2.13
VIT_05s0077g00960	Ferredoxin 4Fe-4S, iron-sulfur binding	2.13

VIT_06s0004g05440	Tropinone reductase	2.13
VIT_04s0008g05730	Sucrose-phosphate synthase	2.12
VIT_11s0016g04510	No hit	2.11
VIT_04s0023g00080	carotene hydroxylase (CYP97A3; LUT5) (VvLUT5)	2.10
VIT_11s0016g01620	Unknown	2.09
VIT_15s0046g02910	Ribosomal protein L21, chloroplast / CL21 (RPL21) 50S	2.09
VIT_17s0000g02660	myb domain protein 6 (VvMybC2-L2)	2.09
VIT_08s0007g00920	Tropinone reductase	2.08
VIT_01s0182g00050	Rubisco	2.08
VIT_14s0066g01780	Heavy-metal-associated domain-containing protein	2.07
VIT_01s0244g00130	Inducer of CBF expression 1 ICE1	2.06
VIT_13s0106g00290	Histone deacetylase HDA14	2.05
VIT_12s0034g01240	GNS1/SUR4 membrane	2.05
VIT_04s0069g00540	Glutamate receptor protein	2.05
VIT_07s0031g02440	Amino acid permease 3	2.05
VIT_10s0116g00600	RIC10 (ROP-Interactive crib motif-containing)	2.04
VIT_00s0183g00200	No hit	2.04
VIT_13s0019g00260	Photosystem II reaction center PsbP	2.04
VIT_14s0006g03060	Ribosomal protein S3, Chloroplast 30S	2.04
VIT_00s0211g00080	Serine hydroxymethyltransferase 2	2.04
VIT_18s0122g00680	Oxygen-evolving complex PsbP	2.04
VIT_17s0000g01420	Snurportin-1 (RNA U transporter 1)	2.04
VIT_04s0044g00650	S-receptor kinase	2.04
VIT_01s0011g05820	Unknown protein	2.03
VIT_05s0020g03440	Photosystem II 11 kDa protein PSB27	2.03
VIT_13s0074g00090	Glycosyl transferase family 47 protein	2.03
VIT_06s0009g02340	Tryptophan/tyrosine permease family	2.03
VIT_04s0008g01840	TT2 (transparent testa 2)	2.02
VIT_11s0016g01880	lycopene epsilon-cyclase (LECY) (VvLECY1)	2.01
VIT_19s0014g01940	Unknown	2.01
VIT_10s0092g00500	CYP71D10	2.01
VIT_03s0017g02300	Unknown protein	2.01
VIT_13s0074g00780	Late embryogenic abundant protein	2.00
VIT_08s0007g05260	Glutamate synthase (GLU1), ferredoxin-dependent	2.00
VIT_11s0118g00630	Unknown protein	2.00
VIT_18s0001g08340	Unknown	2.00
VIT_16s0098g01180	Aldo/keto reductase	2.00
VIT_18s0089g01400	Protein kinase	2.00
VIT_10s0003g01860	RKF1 (receptor-like kinase in flowers 1)	-2.00
VIT_06s0004g01620	No hit	-2.02
VIT_06s0004g02400	Unknown protein	-2.02
VIT_06s0004g07830	SOS2 (salt overly sensitive 2)	-2.03
VIT_18s0089g00210	Endo-1,4-beta-glucanase	-2.05
VIT_00s0772g00010	Protein kinase	-2.05
VIT_00s0254g00040	Heat shock cognate 70 kDa protein 3 (HSC70-3) (HSP70-3)	-2.05
VIT_01s0011g06440	Chalcone reductase	-2.06
VIT_01s0026g01550	Homeodomain leucine zipper protein HB-1	-2.07
VIT_17s0000g07210	Flavonoid 3'-hydroxylase b (F3'Hb) [Vitis vinifera]	-2.07
VIT_00s0199g00190	14-3-3 protein GF14 kappa (GRF8)	-2.07
VIT_14s0128g00700	EMB2261 (embryo defective 2261)	-2.08
VIT_13s0074g00540	Lysine histidine transporter 1	-2.08
VIT_00s2547g00010	WRKY DNA-binding protein 21	-2.09
VIT_06s0009g01720	Nucleoside triphosphatase	-2.10
VIT_18s0001g01470	No hit	-2.10
VIT_15s0024g00440	R protein PRF disease resistance protein	-2.11
VIT_00s0323g00060	Invertase/pectin methylesterase inhibitor	-2.11
VIT_12s0057g01190	No hit	-2.12
VIT_19s0014g01880	Homeobox protein	-2.12
VIT_00s0179g00150	Heat shock transcription factor A6B	-2.13
VIT_04s0023g01760	Unknown	-2.14
VIT_08s0058g00590	Macrophage migration inhibitory factor	-2.17
VIT_04s0069g00210	Glutamate receptor 2.8	-2.17
VIT_01s0010g02750	Lipoxygenase	-2.18
VIT_08s0058g00020	Proteasome 26S regulatory subunit (RPN5)	-2.18
VIT_14s0081g00190	No hit	-2.19
VIT_07s0031g00980	Aspartate aminotransferase	-2.19
VIT_18s0001g13020	No hit	-2.19
VIT_06s0004g01000	Dirigent protein	-2.21
VIT_18s0075g00520	Retrotransposon protein, Unclassified	-2.21
VIT_03s0038g04050	Unknown	-2.21
VIT_01s0010g02270	DELLA protein RGL1 (RGA-like protein 1)	-2.22
VIT_04s0008g00620	myb domain protein 68	-2.22
VIT_15s0046g02060	ATRAD3 (A.thaliana Ras Associated with Diabetes protein 3	-2.22
VIT_00s1553g00010	Subtilisin-like proteinase AIR3	-2.23
VIT_01s0010g02040	Hydroxyproline-rich glycoprotein	-2.23
VIT_05s0020g01700	No hit	-2.24
VIT_10s0003g02190	Nodulin MtN3 family	-2.24
VIT_01s0011g06500	NADPH-dependent codeinone reductase	-2.27
VIT_14s0006g02650	Receptor-like protein kinase	-2.29
VIT_18s0089g00170	1,4-beta-mannan endohydrolase	-2.29
VIT_08s0007g03260	Unknown protein	-2.31
VIT_06s0004g06480	CYP71E	-2.32
VIT_15s0046g00150	DOF affecting germination 1	-2.36
VIT_07s0104g00920	No hit	-2.36
VIT_02s0033g00050	Scarecrow transcription factor 3 (SCL3)	-2.36
VIT_12s0028g02670	NAC domain-containing protein (VvNAC35)	-2.40

VIT_01s0010g03020	Calcium-binding protein CML	-2.42
VIT_19s0014g01180	Pathogenesis-related	-2.42
VIT_04s0210g00080	Strictosidine synthase	-2.43
VIT_05s0094g00770	Short-chain dehydrogenase/reductase (SDR)	-2.43
VIT_02s0025g00870	No hit	-2.45
VIT_07s0031g00220	ERF/AP2 Gene Family (VvAP2-13)	-2.45
VIT_08s0007g00820	Proteinase inhibitor	-2.45
VIT_18s0001g02570	IAA-amino acid hydrolase 6	-2.48
VIT_07s0031g01310	No hit	-2.50
VIT_09s0002g02940	Myo-inositol oxygenase 1	-2.51
VIT_04s0210g00070	Strictosidine synthase	-2.53
VIT_03s0063g01760	No hit	-2.54
VIT_18s0001g09680	No hit	-2.55
VIT_10s0003g03270	Inward rectifying potassium channel	-2.61
VIT_14s0083g00460	Tryptophan synthase beta chain 2	-2.62
VIT_12s0134g00400	Zinc finger (B-box type)	-2.62
VIT_18s0001g01510	No hit	-2.70
VIT_04s0210g00060	Mucin-like protein	-2.77
VIT_02s0025g04330	Thaumatin VVTL1 [Vitis vinifera]	-2.78
VIT_16s0050g00180	HcrVf1 protein	-2.79
VIT_15s0048g00100	No hit	-2.82
VIT_08s0007g00430	C2 domain containing protein	-2.87
VIT_04s0023g01680	No hit	-2.88
VIT_01s0011g06600	Glutamate decarboxylase	-2.94
VIT_08s0007g06760	Cation efflux family protein MTPc3	-2.96
VIT_04s0008g01140	Beta-fructosidase (BFRUCT1)	-3.00
VIT_16s0050g00200	HcrVf3 protein	-3.03
VIT_17s0000g00690	Pyruvate dehydrogenase E1 beta subunit isoform 2	-3.04
VIT_09s0096g00390	Disease resistance protein (NBS-LRR class)	-3.09
VIT_00s0480g00020	Hydroxyproline-rich glycoprotein family protein	-3.18
VIT_08s0007g04550	NCS1 nucleoside transporter family protein	-3.19
VIT_07s0104g01230	Auxin response factor 2	-3.42
VIT_01s0026g00850	Zinc finger protein 5	-3.45
VIT_09s0002g00510	Lipase GDSL 1	-3.67
VIT_16s0050g00020	Cullin-4	-3.72
VIT_13s0019g00540	Ethylene-responsive protein	-3.72
VIT_04s0044g01230	Unknown	-3.85
VIT_14s0108g00120	WRKY Transcription Factor (VvWRKY44)	-4.02
VIT_00s2527g00010	Beta-fructosidase (BFRUCT3)	-4.04
VIT_05s0077g01860	ERF/AP2 Gene Family (VvERF058)	-4.04
VIT_17s0000g00650	No hit	-4.18
VIT_13s0067g02560	Unknown protein	-4.20
VIT_04s0008g02070	Avr9/Cf-9 induced kinase 1	-4.35
VIT_00s0211g00030	Symbiosis receptor kinase	-4.48
VIT_02s0025g04250	Osmotin	-4.58
VIT_14s0068g01770	WRKY Transcription Factor (VvWRKY43)	-4.62
VIT_02s0025g04260	Osmotin [Vitis vinifera]	-4.79
VIT_13s0067g03450	ARR9 typeA	-5.54
VIT_01s0026g02400	Glutathione S-transferase 10 GSTU10	-5.78
VIT_14s0030g02150	Calmodulin	-6.10
VIT_02s0025g04360	S-N-methylcoclaurine 3'-hydroxylase	-6.45
VIT_00s0270g00120	Alpha-amylase/subtilisin inhibitor	-8.58
VIT_05s0077g01600	s8_Pathogenesis protein 10 [Vitis vinifera]	-11.66

Supplementary Table S4: Differentially expressed genes (> |2| fold) in *VvNAC33* transiently overexpressing plants compared to the control line.

ID_code	Gene annotation	FC
VIT_08s0040g02040	fasciclin arabinogalactan-protein (FLA11)	2.71
VIT_05s0062g00590	UDP-glucose:flavonoid 7-O-glucosyltransferase	2.52
VIT_02s0033g00050	Scarecrow transcription factor 3 (SCL3)	2.43
VIT_02s0025g05110	MATE efflux family protein	2.35
VIT_06s0080g00990	Secoisolariciresinol dehydrogenase	2.30
VIT_14s0060g01570	Kinesin motor protein	2.25
VIT_01s0011g05150	Bet v I allergen	2.23
VIT_00s0873g00020	NADH dehydrogenase subunit 3	2.13
VIT_00s0508g00050	Oligopeptide transporter 1	2.07
VIT_08s0056g01680	Lipoxygenase, LH2	2.01
VIT_06s0061g01110	Lecithine cholesterol acyltransferase	2.00
VIT_05s0062g00470	UDP-glucose:flavonoid 7-O-glucosyltransferase	2.00
VIT_04s0023g03570	Oligopeptide transporter 7 ATOPT7	-2.00
VIT_17s0000g07320	Unknown protein	-2.00
VIT_18s0001g09020	Exo70 exocyst complex subunit G1	-2.01
VIT_17s0000g09760	ABC Transporter (VvMDR2 - VvABCB2)	-2.01
VIT_17s0000g07450	Glycerophosphoryl diester phosphodiesterase	-2.02
VIT_16s0050g02110	Timeless ATIM	-2.05
VIT_12s0134g00380	S-locus lectin protein kinase family	-2.10
VIT_12s0028g02870	Isoflavone methyltransferase/ Orcinol O-methyltransferase 1 oomt1	-2.10
VIT_07s0005g01790	Acyl-CoA synthetase long-chain member 2	-2.11
XLOC_008617	#N/A	-2.12
VIT_01s0011g05020	Wuschel related homeobox 1	-2.12
VIT_17s0000g02360	Receptor protein kinase	-2.15

VIT_01s0011g02240	Metal-nicotianamine transporter YSL7	-2.19
VIT_14s0006g01690	Zinc finger (C2H2 type) family	-2.24
VIT_04s0008g05200	No hit	-2.28
VIT_01s0010g00960	Peroxidase 48 (Atperox P48)	-2.28
VIT_04s0023g01660	Scarecrow-like transcription factor 7 (SCL7)	-2.30
VIT_16s0022g02500	No hit	-2.32
VIT_07s0141g00760	Unknown protein	-2.33
VIT_16s0050g00210	HcrVf3 protein	-2.35
VIT_05s0094g00770	Short-chain dehydrogenase/reductase (SDR)	-2.37
XLOC_003444	#N/A	-2.37
VIT_14s0068g02240	HcrVf1 protein	-2.38
VIT_11s0065g00520	Small G protein / RhoGAP	-2.44
XLOC_008259	#N/A	-2.46
XLOC_002382	#N/A	-2.58
VIT_12s0034g01300	TIR-NBS-LRR disease resistance	-2.78
VIT_00s0269g00100	Ankyrin	-2.87
VIT_02s0109g00410	No hit	-2.90
VIT_19s0090g01550	Mo25	-2.96
VIT_14s0066g02660	Hcr2-p1.2	-3.00
VIT_16s0050g00340	Disease resistance family protein	-3.04
XLOC_021936	#N/A	-3.18
VIT_13s0019g03930	No hit	-3.21
VIT_14s0066g02670	Leucine Rich Repeat receptor-like kinase	-3.32
VIT_11s0037g00270	Accumulation of photosystem one 2 (APO2)	-4.37

Supplementary Table S5: Differentially expressed genes (> |2| fold) in *VvNAC60* transiently overexpressing plants compared to the control line.

ID_code	Gene annotation	FC
VIT_07s0005g06010	FAD linked oxidase, N-terminal	247.20
VIT_07s0005g05960	Cytokinin dehydrogenase 5 precursor	176.17
VIT_19s0090g01350	Aspartyl protease	164.47
VIT_07s0005g05890	FAD linked oxidase, N-terminal	139.07
VIT_14s0128g00540	Germin-like protein 3 [Vitis vinifera]	135.81
VIT_14s0128g00570	Germin	126.90
VIT_00s2520g00010	Cytokinin oxidase	123.93
VIT_14s0128g00630	Germin-like protein 3 [Vitis vinifera]	85.00
VIT_14s0128g00610	Germin-like protein 3 [Vitis vinifera]	70.31
VIT_15s0048g02640	No hit	69.32
VIT_14s0128g00680	Germin	65.34
VIT_14s0128g00600	Germin-like protein 3 [Vitis vinifera]	57.11
VIT_14s0128g00980	Germin-like protein 3 [Vitis vinifera]	43.13
VIT_14s0128g00660	Germin-like protein 3 [Vitis vinifera]	40.83
VIT_01s0011g02420	Unknown	35.64
VIT_14s0128g00940	No hit	33.38
VIT_14s0128g01040	Germin-like protein 8-11	27.73
VIT_14s0128g01010	Germin-like protein 3 [Vitis vinifera]	25.87
VIT_14s0128g01020	Germin-like protein 3 [Vitis vinifera]	24.68
VIT_10s0116g01620	Lyase	17.28
VIT_14s0128g00640	Germin-like protein 3 [Vitis vinifera]	17.28
VIT_14s0060g02710	Germin	16.47
VIT_06s0004g04210	No hit	16.10
VIT_05s0049g00810	No hit	13.43
VIT_01s0127g00210	KNAT2 (knotted1-like homeobox gene 6)	12.88
VIT_14s0128g00970	Germin-like protein 3 [Vitis vinifera]	12.27
VIT_18s0001g08380	Homeobox protein knotted-1 like 1 (KNAT1)	11.92
VIT_17s0000g01750	Auxin-independent growth promoter	11.80
VIT_17s0119g00080	Organic cation transport protein OCT1	11.59
VIT_05s0077g00780	No hit	10.74
VIT_12s0035g01290	No hit	9.45
VIT_08s0007g03450	TFL1B protein [Vitis vinifera]	8.78
VIT_04s0023g01510	DUF620	6.95
VIT_13s0106g00190	Ankyrin repeat	6.53
VIT_05s0165g00220	Protein binding protein	6.28
VIT_06s0080g00120	Male sterility 2 (MS2) Acyl-CoA reductase	6.04
VIT_00s0187g00060	No hit	6.04
VIT_14s0128g00990	Germin-like protein 3 [Vitis vinifera]	5.82
VIT_02s0025g04330	Thaumatin VVTL1 [Vitis vinifera]	5.79
VIT_05s0077g00430	Galactinol synthase	5.26
VIT_04s0023g01500	Polyol transporter 6 (PLT6)	5.13
VIT_14s0060g00660	No hit	5.04
VIT_16s0013g00050	Unknown	5.04
VIT_01s0010g02730	Chaperone BCS1 mitochondrial	4.94
VIT_16s0039g02850	Unknown	4.90
VIT_05s0020g00330	Galactinol synthase	4.81
VIT_15s0048g01710	Alcohol dehydrogenase	4.77

VIT_01s0011g02410	Unknown	4.77
VIT_08s0040g00920	Glutathione S-transferase 25 GSTU7	4.66
VIT_16s0022g01100	Acetohydroxyacid synthase 1	4.65
VIT_11s0016g04930	Binding / zinc ion binding	4.30
VIT_01s0011g05120	Major latex	4.28
VIT_14s0068g01290	Transcriptional factor B3	4.27
VIT_14s0060g02750	Germin-like protein 3 [Vitis vinifera]	4.24
VIT_14s0083g00150	TCP family transcription factor 1	4.20
VIT_01s0011g05440	Unknown protein	4.17
VIT_15s0048g02120	Myb domain protein 3R2	4.15
VIT_10s0003g03490	GA 2-oxidase	4.12
VIT_14s0108g00690	Amino acid permease	4.09
VIT_15s0046g01160	NADPH HC toxin reductase	4.02
VIT_17s0000g06200	Mini zinc finger 1 MIF1	3.96
VIT_13s0156g00370	myb family	3.93
VIT_04s0008g05700	ACT domain-containing protein	3.84
VIT_12s0059g00490	Unknown	3.79
VIT_16s0098g00160	Receptor serine/threonine kinase	3.77
VIT_03s0063g01730	Viral-response family protein-like	3.74
VIT_00s0203g00120	Jumonji (jmjC)	3.67
VIT_09s0002g05290	2-oxoglutarate-dependent dioxygenase	3.63
VIT_01s0026g02500	Amino acid transport protein	3.57
VIT_14s0108g00630	Amino acid permease	3.56
VIT_06s0004g02580	BLH8 (BEL1-like homeodomain 8)	3.56
VIT_14s0066g01950	Metalloendoproteinase 1 precursor	3.54
VIT_15s0048g02480	Caffeate 3-O-methyltransferase 1	3.51
VIT_09s0054g01410	Beta-amyrin synthase	3.51
VIT_05s0020g03970	Sulfate transporter 3.1 (AST12) (AtST1)	3.48
VIT_18s0001g14270	Gibberellin-regulated protein 1 (GASA1)	3.47
VIT_09s0002g02450	Phosphatase	3.42
VIT_10s0116g00190	Homeobox protein shoot MERISTEMLESS (STM)	3.35
VIT_12s0055g01080	Cationic peroxidase 2	3.31
VIT_19s0093g00190	Glutathione S-transferase 25 GSTU25	3.30
VIT_09s0018g01220	Zinc finger (C3HC4-type ring finger)	3.21
VIT_07s0005g03290	Inorganic phosphate transporter 1-4	3.18
VIT_19s0014g03300	NAC domain-containing protein (VvNAC18)	3.18
VIT_07s0005g03260	ERF/AP2 Gene Family (VvERF100)	3.17
VIT_06s0061g00570	Amino acid permease	3.16
VIT_03s0063g02320	BURP domain containing protein	3.16
VIT_05s0062g00930	Nicotianamine aminotransferase B	3.15
VIT_12s0035g02100	Glutathione S-transferase Z1 GSTZ1	3.14
VIT_15s0048g00180	No hit	3.10
VIT_14s0060g02730	Germin	3.09
VIT_07s0104g01350	Integral membrane family protein UPF0497	3.08
VIT_18s0001g12930	Heavy-metal-associated domain-containing protein	3.06
VIT_10s0003g03530	Lupeol synthase	3.05
VIT_07s0005g03230	ERF/AP2 Gene Family (VvERF099)	3.02
VIT_10s0003g05080	Wall-associated receptor kinase-like 10	3.01
VIT_12s0057g01580	No hit	2.98
VIT_12s0028g02310	No hit	2.98
VIT_14s0060g01190	Nicotianamine synthase	2.97
VIT_19s0014g01350	Ribulose bisphosphate carboxylase, large chain	2.97
VIT_00s1916g00010	DNA binding	2.97
VIT_00s0262g00150	Glycine-rich protein	2.97
VIT_15s0048g02230	Calcineurin phosphoesterase	2.91
VIT_00s0294g00040	Receptor serine/threonine kinase	2.91
VIT_16s0013g00180	Pectinesterase PPME1	2.89
VIT_18s0001g14260	No hit	2.88
VIT_05s0094g01030	UDP-glucose:salicylic acid glucosyltransferase	2.88
VIT_09s0054g01360	Cycloartenol synthase	2.86
VIT_00s0323g00070	Pectin methylesterase inhibitor	2.84
VIT_15s0046g01040	Lipase class 3	2.81
VIT_01s0011g06180	Blight-associated protein p12 precursor	2.80
VIT_10s0003g03660	Beta-amyrin synthase	2.78
VIT_15s0046g03190	myb domain protein 17	2.76
VIT_14s0036g00580	Betaine aldehyde dehydrogenase	2.75
VIT_18s0001g08430	Branched-chain-amino-acid aminotransferase 2	2.75
VIT_12s0057g00250	NDB3 (alternative NAD(P)H dehydrogenase 32)	2.74
VIT_17s0000g07020	Cis-zeatin O-beta-D-glucosyltransferase	2.74
VIT_17s0000g01230	MIKC-Type MADS Box Genes Family (VvTM8)	2.72
VIT_18s0001g01380	Oxidoreductase N-terminal domain-containing	2.72
VIT_15s0046g00970	Lipid transfer protein	2.71
VIT_18s0001g00450	Vinorine synthase	2.71
VIT_03s0038g02020	Amidase	2.68
VIT_11s0016g04470	Ornithine decarboxylase	2.67
VIT_19s0140g00160	BCL-2-associated athanogene 7 ATBAG7	2.66

VIT_18s0001g05140	Ribosomal protein L17-2 60S	2.65
VIT_10s0116g01080	No hit	2.64
VIT_15s0048g02290	NAC domain-containing protein (VvNAC54)	2.62
VIT_12s0055g00980	Peroxidase precursor	2.60
VIT_03s0017g01500	N-6 Adenine-specific DNA methylase	2.59
VIT_13s0320g00020	Unknown	2.58
VIT_00s2507g00010	F-box family protein	2.56
VIT_15s0021g00330	Proton-dependent oligopeptide transport family protein	2.55
VIT_01s0011g00140	CRABS CLAW	2.55
VIT_09s0002g08520	F-box domain containing protein	2.53
VIT_03s0063g00990	Blue (type 1) copper domain	2.52
VIT_11s0037g00590	Anthranilate N-hydroxycinnamoyl/benzoyltransferase	2.52
VIT_05s0077g00040	No hit	2.52
VIT_02s0154g00490	Heat shock 22 kDa protein mitochondrial	2.52
VIT_02s0025g05110	MATE efflux family protein	2.50
VIT_18s0001g08300	Tubulin alpha-6 chain	2.50
VIT_12s0035g01280	R protein disease resistance protein	2.50
VIT_12s0034g00060	UDP-glucose glucosyltransferase	2.48
VIT_01s0011g02350	Unknown	2.48
VIT_09s0054g01730	Coniferyl alcohol acyltransferase	2.47
VIT_10s0116g00800	CYP77A3	2.47
VIT_02s0033g00780	14-3-3 protein GF14 omega (GRF2)	2.45
VIT_18s0001g09910	L-asparaginase	2.44
VIT_09s0002g07080	No hit	2.43
VIT_04s0043g01030	Dual-specific kinase DSK1	2.42
VIT_19s0014g04110	Elongation factor 1-alpha (EF-1-alpha)	2.41
VIT_00s2248g00010	Rust resistance kinase Lr10	2.41
VIT_02s0025g02840	Ankyrin 3, epithelial isoform a	2.40
VIT_18s0001g01140	Peroxidase 64	2.40
VIT_18s0075g00440	TIR-NBS-LRR disease resistance	2.39
VIT_04s0023g01110	PQ-loop repeat protein	2.38
VIT_18s0072g01220	ABC Transporter (VvWBC28 - VvABCG28)	2.35
VIT_13s0047g01230	flavonoid 1-2 rhamnosyltransferase	2.35
VIT_15s0046g00170	VvMybPA1	2.33
VIT_03s0167g00070	MADS-box protein SVP (short vegetative phase)	2.33
VIT_11s0037g01090	No hit	2.32
VIT_15s0048g02360	ARV1	2.32
VIT_18s0041g00940	Non-symbiotic hemoglobin class 1	2.32
VIT_14s0036g00640	No hit	2.31
VIT_15s0048g01180	Subtilisin serine protease	2.31
VIT_01s0127g00590	Protein disulfide isomerase	2.31
VIT_04s0069g00660	Unknown	2.30
VIT_01s0026g01460	Thioredoxin H-type 2 (Trx-H-2)	2.27
VIT_07s0129g00830	CYP81D2	2.27
VIT_15s0048g01440	Geraniol 10-hydroxylase	2.26
VIT_13s0067g01730	Steroid 5alpha-reductase	2.24
VIT_13s0019g03050	Heat shock protein 17.6 kDa class I	2.24
VIT_06s0004g05460	Protein phosphatase 2C DBP	2.23
VIT_03s0063g01010	Blue (type 1) copper domain	2.23
VIT_14s0068g01580	basic helix-loop-helix (bHLH) family	2.22
VIT_19s0090g00800	F-box protein	2.22
VIT_13s0074g00310	Translation initiation factor eIF-2 gamma subunit	2.22
VIT_18s0001g10300	basic helix-loop-helix (bHLH) family	2.22
VIT_16s0148g00420	Rust resistance kinase Lr10	2.19
VIT_06s0061g01110	Lecithine cholesterol acyltransferase	2.18
VIT_08s0007g03000	Chaperone protein dnaJ-related	2.17
VIT_07s0005g03210	ERF/AP2 Gene Family (VvERF097)	2.16
VIT_06s0004g07720	S-adenosyl-L-methionine-dependent methyltransferase	2.16
VIT_06s0080g00990	Secoisolariciresinol dehydrogenase	2.16
VIT_00s0346g00010	Lectin protein kinase	2.15
VIT_15s0048g01200	Subtilisin serine endopeptidase (XSP1)	2.15
VIT_04s0008g06560	No hit	2.14
VIT_05s0165g00230	SAR1 (secretion-associated ras)	2.14
VIT_01s0011g02740	Phosphoenolpyruvate carboxylase	2.13
VIT_01s0011g06440	Chalcone reductase	2.12
VIT_00s0415g00040	Glycine-rich protein	2.11
VIT_01s0127g00600	DC1 domain-containing protein	2.11
VIT_16s0098g01250	Metal-nicotianamine transporter YSL3	2.11
VIT_01s0010g02030	Gamma-thionin precursor	2.10
VIT_05s0020g04110	ELIP1 (early light-inducible protein)	2.10
VIT_15s0048g00780	AT-hook DNA-binding protein	2.10
VIT_17s0000g00730	ATP binding / DNA binding	2.09
VIT_09s0002g02460	Phosphatase	2.09
VIT_04s0008g00060	Proteasome 26S regulatory subunit S5A (RPN10)	2.09
VIT_14s0128g01030	Germin-like protein 3 [Vitis vinifera]	2.09
VIT_09s0002g06730	Longin	2.09
VIT_03s0063g01160	Nodulin 1A, Senescence-associated	2.09
VIT_05s0020g00180	No hit	2.08

VIT_09s0054g01420	Beta-amyrin synthase	2.07
VIT_00s0559g00010	Membrane bound O-acyl transferase family protein	2.07
VIT_19s0014g02200	NAC domain-containing protein (VvNAC16)	2.07
VIT_07s0031g00150	Disease resistance	2.06
VIT_15s0021g00160	No hit	2.06
VIT_04s0023g01230	T-complex protein 1 subunit delta	2.06
VIT_03s0063g00980	Blue (type 1) copper domain	2.06
VIT_00s0337g00040	Unknown protein	2.05
VIT_04s0044g01820	RNA polymerase I specific transcription initiation factor	2.03
VIT_12s0055g00990	Peroxidase	2.02
VIT_05s0077g02190	Chalcone reductase	2.01
VIT_15s0107g00380	basic helix-loop-helix (bHLH) family	-2.00
VIT_04s0023g01390	Cupin family protein	-2.01
VIT_08s0007g07940	No hit	-2.01
VIT_03s0038g04630	Isoflavone reductase related protein	-2.01
VIT_03s0038g02950	Pentatricopeptide (PPR) repeat-containing protein	-2.02
VIT_17s0053g00630	Rhodanese domain containing protein	-2.02
VIT_00s0203g00050	Receptor-interacting protein	-2.03
VIT_19s0090g01830	No hit	-2.04
VIT_16s0039g01710	myb domain protein 9	-2.04
VIT_13s0019g03730	No hit	-2.05
VIT_19s0015g01610	No hit	-2.06
VIT_18s0122g00110	Unknown protein	-2.06
VIT_07s0130g00020	NADPH2:quinone reductase	-2.06
VIT_00s2630g00010	Acetyl-CoA carboxylase	-2.07
VIT_00s0125g00350	Proteasome 26S regulatory subunit (RPN2)	-2.08
VIT_15s0024g00500	No hit	-2.08
VIT_05s0051g00900	No hit	-2.08
VIT_12s0059g02530	fringe-related protein	-2.08
VIT_15s0024g00470	R protein PRF disease resistance protein	-2.09
VIT_02s0025g04830	Copper chaperone for superoxide dismutase	-2.09
VIT_15s0045g00940	No hit	-2.10
VIT_16s0022g02250	basic helix-loop-helix (bHLH) family	-2.10
VIT_02s0025g04080	CYP87A2	-2.10
VIT_17s0000g06140	Glutathione S-transferase 9 GSTU9	-2.10
VIT_18s0166g00160	No hit	-2.11
VIT_04s0008g07210	D8-sphingolipid desaturase	-2.12
VIT_16s0098g01210	No hit	-2.13
VIT_12s0028g02360	ABC Transporter (VvWBC16 - VvABCG16)	-2.13
VIT_15s0045g01360	No hit	-2.14
VIT_06s0009g01680	No hit	-2.14
VIT_18s0001g03240	ERF/AP2 Gene Family (VvERF008)	-2.15
VIT_16s0039g00830	No hit	-2.15
VIT_03s0088g00550	MADS-box agamous-like 62	-2.16
VIT_01s0137g00520	CYP71B35	-2.16
VIT_00s0426g00070	No hit	-2.16
VIT_13s0047g00370	Enoyl-CoA hydratase	-2.17
VIT_03s0167g00210	No hit	-2.18
VIT_10s0042g01100	Zinc transporter 10 PRECURSOR	-2.18
VIT_10s0003g01210	Unknown protein	-2.18
VIT_05s0124g00230	F-box domain containing protein	-2.19
VIT_02s0025g04440	ERF/AP2 Gene Family (VvERF018)	-2.20
VIT_15s0021g00310	Ent-kaurenoic acid oxidase	-2.20
VIT_18s0001g03440	No hit	-2.20
VIT_14s0030g02140	Cellulose synthase CSLD1	-2.21
VIT_00s0131g00320	Annexin ANN3	-2.22
VIT_04s0023g00660	No hit	-2.23
VIT_03s0063g01860	AOS (allene oxide synthase)	-2.24
VIT_18s0001g09020	Exo70 exocyst complex subunit G1	-2.25
VIT_18s0041g01800	Taxadiene 5-alpha-hydroxylase	-2.26
VIT_12s0034g01300	TIR-NBS-LRR disease resistance	-2.26
VIT_11s0016g03090	No hit	-2.27
VIT_13s0019g02860	UDP-glucoronosyl and UDP-glucosyl transferase	-2.28
VIT_07s0031g02730	Reticulon-like protein B1 RTNLB1	-2.28
VIT_00s0286g00030	S-locus protein kinase	-2.28
VIT_07s0151g00760	Lipid transfer protein	-2.29
VIT_02s0025g00680	Tiny root hair 1	-2.30
VIT_14s0060g00220	Nitrate transporter 1:2	-2.31
VIT_17s0000g00560	UPF0497 family	-2.31
VIT_08s0007g01600	Unknown protein	-2.31
VIT_03s0038g01220	Auxin-induced protein 15A	-2.33
VIT_09s0002g03220	Acid phosphatase	-2.35
VIT_07s0141g00090	Fatty acid elongase 1	-2.35
VIT_02s0025g02710	NAC domain-containing protein (VvNAC24)	-2.37
VIT_03s0038g01260	Auxin responsive SAUR protein	-2.37
VIT_10s0042g00010	Strictosidine synthase	-2.39
VIT_18s0122g01220	DNA-binding bromodomain-containing protein	-2.40
VIT_00s0173g00030	No hit	-2.40
VIT_16s0098g00910	No hit	-2.40

VIT_16s0098g00910	No hit	-2.40
VIT_13s0139g00120	No hit	-2.41
VIT_01s0011g02250	Metal-nicotianamine transporter YSL7	-2.42
VIT_01s0010g02250	No hit	-2.42
VIT_19s0140g00220	No hit	-2.43
VIT_12s0059g00970	Cellulose synthase CSLB04	-2.44
VIT_05s0049g01340	RIC5 (ROP-interactive crib motif-containing protein 5)	-2.44
VIT_18s0001g15740	No hit	-2.45
VIT_05s0062g00800	No hit	-2.45
VIT_09s0002g06720	SPX1 (SYG1/Pho81/XPR1) domain-containing protein	-2.45
VIT_19s0027g00850	No hit	-2.45
VIT_06s0004g07510	No hit	-2.45
VIT_08s0056g01650	Lateral organ boundaries domain protein 20 (LBD20)	-2.48
VIT_19s0014g03940	Sporocyteless	-2.49
VIT_09s0096g00520	Coniferyl alcohol acyltransferase	-2.50
VIT_10s0092g00410	No hit	-2.50
VIT_06s0061g00750	Superoxide dismutase [Cu-Zn], chloroplast precursor	-2.51
VIT_00s0286g00100	S-locus protein kinase	-2.52
VIT_07s0255g00110	WD40	-2.52
VIT_14s0108g00860	NAC domain-containing protein (VvNAC10)	-2.53
VIT_17s0000g02630	TFL1 (Terminal flower 1)	-2.54
VIT_15s0046g00790	Unknown protein	-2.54
VIT_07s0005g00190	Unknown	-2.54
VIT_12s0035g00090	No hit	-2.56
VIT_15s0021g00150	Disease resistance protein RGA4	-2.58
VIT_19s0015g02530	No hit	-2.59
VIT_00s0426g00010	No hit	-2.60
VIT_01s0010g00120	No hit	-2.64
VIT_09s0018g01800	Acid phosphatase	-2.65
VIT_15s0046g03510	No hit	-2.67
VIT_10s0003g03320	Unknown	-2.68
VIT_01s0026g02620	Expansin (VvEXPA1)	-2.70
VIT_00s0410g00020	No hit	-2.73
VIT_17s0053g00280	No hit	-2.75
VIT_04s0023g00490	Auxin responsive SAUR protein	-2.78
VIT_13s0064g01530	Beta-glucosidase	-2.78
VIT_12s0057g01010	Nuclear transcription factor Y subunit B-3	-2.79
VIT_08s0007g07720	CYP93A1 2-hydroxyisoflavanone synthase	-2.79
VIT_14s0066g02480	Unknown protein	-2.80
VIT_16s0050g01800	Major Facilitator Superfamily MFS	-2.83
VIT_03s0038g00770	No hit	-2.87
VIT_14s0060g00700	Unknown	-2.90
VIT_00s0556g00010	Pollen Ole e 1 allergen and extensin	-2.93
VIT_00s0160g00270	No hit	-2.94
VIT_12s0028g02870	Isoflavone methyltransferase/ Orcinol O-methyltransferase 1	-3.08
VIT_03s0038g01420	Phytochelatin synthetase	-3.13
VIT_09s0002g01220	Unknown protein	-3.16
VIT_06s0004g07740	Cationic peroxidase 1 precursor	-3.19
VIT_04s0069g00010	Glutamate receptor protein	-3.19
VIT_17s0000g10200	Steroid sulfotransferase	-3.20
VIT_00s0480g00070	Polyphenol oxidase II, chloroplast precursor	-3.21
VIT_16s0100g00110	Hydroxymethylglutaryl coenzyme A synthase	-3.24
VIT_16s0050g01330	Retrovirus Pol polyprotein from transposon TNT 1-94	-3.26
VIT_05s0165g00050	No hit	-3.36
VIT_01s0011g00960	RPM1 (resistance to p. syringae pv maculicola 1)	-3.49
VIT_02s0109g00410	No hit	-3.57
VIT_02s0033g01220	No hit	-3.61
VIT_08s0058g00990	Peroxidase	-3.77
VIT_17s0000g02150	Ribosomal protein L19	-3.80
VIT_04s0023g00510	Auxin responsive SAUR protein	-3.82
VIT_15s0046g00820	Unknown protein	-3.82
VIT_19s0014g03820	Myb domain protein 102	-4.11
VIT_06s0004g03350	Lateral organ boundaries protein 1	-4.23
VIT_17s0000g09800	No hit	-4.26
VIT_18s0001g14430	Unknown	-4.27
VIT_18s0072g00420	No hit	-4.54
VIT_15s0021g01820	No hit	-4.91
VIT_01s0010g03970	No hit	-5.02
VIT_08s0007g04120	CYPLXXVIA2	-5.32
VIT_13s0067g02810	NADPH:quinone oxidoreductase	-12.99

Supplementary Table S6: Differentially expressed genes (> |4| fold) in *VvNAC33* stably overexpressing plants compared to the control line.

ID_code	Gene annotation	FC
VIT_10s0003g00780	Glutamate receptor 3.4	10.76
VIT_12s0034g01120	UDP-glycosyltransferase 71A13	8.97
VIT_09s0002g05810	Boron transporter-like protein 4	8.90
VIT_10s0003g00680	Glutamate receptor protein	8.79
VIT_12s0034g00040	UDP-glucose glucosyltransferase	8.62
VIT_16s0013g01110	Ethylene-responsive transcription factor 5	8.08
VIT_12s0057g01430	Heavy-metal-associated domain-containing protein	8.06
VIT_07s0129g00830	CYP81D2	7.65
VIT_07s0031g02610	NAC domain-containing protein (VvNAC39)	7.62
VIT_18s0001g11580	CYP82A3	7.36
VIT_05s0049g01020	VvMyb15	7.33
VIT_08s0040g00130	Copper-binding family protein	7.15
VIT_14s0083g00640	Constans 2 (COL2)	6.90
VIT_19s0014g03290	NAC domain-containing protein (VvNAC17)	6.85
VIT_02s0025g00960	V-type H+-transporting ATPase subunit E	6.75
VIT_12s0034g00080	Flavonoid-glucosyltransferase	6.38
VIT_08s0007g07730	CYP93A1 2-hydroxyisoflavanone synthase	6.32
VIT_04s0008g04180	Arsenite transport protein (ArsB)	6.30
VIT_03s0063g00250	Hydrogenobyrinic acid a,c-diamide synthase	5.89
VIT_09s0002g00700	Dormancy/auxin associated protein	5.73
VIT_05s0077g00510	Beta-fructofuranosidase	5.50
VIT_02s0087g00580	Nitrate transporter	5.45
VIT_19s0014g04430	S-locus protein kinase	5.40
VIT_00s0366g00020	CRK10 (cysteine-rich RLK10)	5.36
VIT_00s0531g00060	Cellulose synthase CSLE1	5.21
VIT_18s0001g09660	CYP81D2	5.11
VIT_12s0059g01240	Nitrate transporter (NTP3)	4.99
XLOC_000856	no hit	4.97
VIT_08s0058g00450	Substrate carrier, Mitochondrial	4.95
VIT_04s0008g06210	Nodulin	4.90
VIT_09s0002g05540	ABC transporter g family pleiotropic drug resistance 12 PDR12	4.83
VIT_08s0040g02180	Mlo3	4.63
VIT_18s0122g00180	Calmodulin CML37	4.45
XLOC_010722	PREDICTED: hypothetical protein [Vitis vinifera];tmv resistance protein	4.42
VIT_05s0077g00430	Galactinol synthase	4.41
VIT_05s0062g01150	Amino acid permease	4.40
VIT_05s0020g00330	Galactinol synthase	4.33
VIT_18s0001g12100	Auxilin	4.29
VIT_11s0103g00010	Potassium-sodium symporter HKT2	4.27
VIT_13s0019g03550	ERF/AP2 Gene Family (VvAP2-11)	4.24
VIT_00s0469g00040	Cellulose synthase CSLE1	4.19
VIT_19s0015g01720	fructose-bisphosphate aldolase, cytoplasmic isozyme 1	4.14
VIT_07s0104g00930	Gibberellin receptor GID1L2	4.07
VIT_17s0000g03330	Receptor serine/threonine kinase PR5K	4.02
VIT_06s0004g02360	Myosin-related	-61.16
VIT_04s0008g03540	Transducin protein	-38.14
VIT_10s0003g00650	Peroxidase	-16.99
VIT_01s0011g01920	Phosphate-induced protein 1	-16.80
VIT_14s0068g01520	Double-stranded RNA-binding (DsRBD) domain-containing protein	-14.65
VIT_05s0124g00100	Unknown	-13.05
VIT_05s0049g00740	No hit	-12.58
VIT_08s0056g01130	Mini zinc finger 2 MIF2	-11.65
VIT_13s0047g00340	Ethylene-responsive transcription factor WRINKLED 1	-11.44
VIT_04s0008g01830	myb domain protein 32	-11.37
VIT_14s0083g00350	Beta-glucan-binding protein 5	-11.34
VIT_05s0049g00730	No hit	-11.07
VIT_19s0090g00730	No hit	-10.20
VIT_15s0048g01750	fasciclin arabinogalactan-protein (FLA8)	-10.11
VIT_18s0041g02150	Lipase GDSL	-9.64
VIT_16s0022g01970	Anthocyanidin 3-O-glucosyltransferase	-8.69
VIT_04s0008g05830	Armadillo/beta-catenin repeat	-8.53
VIT_11s0052g01620	Pathogenesis-related protein 1 precursor (PRP 1)	-8.36
VIT_13s0064g00460	Unknown protein	-8.24
VIT_03s0038g00120	Gibberellin-regulated protein 4 (GASA4)	-8.18
VIT_11s0037g00570	Anthranilate N-benzoyltransferase	-8.02
VIT_00s1317g00010	Gibberellin-regulated protein 4 (GASA4)	-8.00
VIT_00s0261g00040	Unknown protein	-7.87
VIT_00s0189g00060	Gibberellin-regulated protein 4 (GASA4)	-7.76
VIT_00s0189g00070	Gibberellin-regulated protein 4 (GASA4)	-7.64
VIT_04s0008g04230	ABC Transporter (VvPDR28 - VvABCG58)	-7.57
VIT_12s0134g00560	Unknown protein	-7.48
VIT_01s0011g03210	Aspartic Protease (VvAP1)	-7.46
VIT_05s0049g00570	Unknown	-7.43
VIT_09s0002g00450	Subtilase	-7.33
VIT_07s0197g00040	Lateral organ boundaries domain gene 36	-7.31
VIT_05s0049g00610	No hit	-7.29
VIT_12s0059g02420	Peroxidase ATP11A (gb|X98802).	-7.26
VIT_03s0063g00210	Receptor protein kinase	-7.23
VIT_11s0016g00590	Invertase/pectin methylesterase inhibitor	-7.21
VIT_08s0056g01140	Exostosin	-6.91
VIT_08s0032g00890	Alpha-L-arabinosidase	-6.88
VIT_14s0108g00420	basic helix-loop-helix (bHLH) family	-6.87
VIT_08s0007g07930	Clavata1 receptor kinase (CLV1)	-6.86
VIT_08s0007g05160	Flavonoid 3',5'-hydroxylase	-6.84
VIT_18s0041g00790	UDP-glycosyltransferase 88B1	-6.84
VIT_14s0108g00740	GASA4	-6.81
VIT_12s0059g01320	Glucan endo-1,3-beta-glucosidase 7 precursor	-6.76
VIT_10s0071g00860	Disease resistance protein	-6.71
VIT_02s0025g01380	Endo-1,4-beta-glucanase	-6.65
VIT_05s0020g02700	transcription factor MUTE	-6.56

VIT_02s0025g01600	Harpin-induced 1	-6.32
VIT_11s0052g00630	Metallothionein	-6.20
VIT_07s0104g00190	7S globulin precursor, basic	-6.12
VIT_13s0019g01650	Expansin (VvEXPA13)	-6.04
VIT_03s0038g02170	Thaumatin	-6.04
VIT_17s0000g02660	myb domain protein 6 (VvMybC2-L2)	-6.02
VIT_10s0116g00520	Xyloglucan endotransglucosylase/hydrolase 8	-5.97
VIT_18s0001g03080	Chitin elicitor-binding CEBIP LysM domain-containing	-5.83
VIT_13s0067g02280	basic helix-loop-helix (bHLH) family	-5.80
VIT_08s0007g00440	Expansin (VvEXPA11)	-5.78
VIT_16s0022g02050	Lateral organ boundaries domain gene 36	-5.73
VIT_13s0064g01260	DNA-damage-repair/toleration protein (DRT100)	-5.68
VIT_01s0011g04080	Zinc finger (C3HC4-type ring finger)	-5.62
VIT_07s0031g01680	CYP86A1	-5.60
VIT_09s0018g01670	Aspartic Protease (VvAP26)	-5.58
VIT_18s0001g07340	Aspartic Protease (VvAP43)	-5.55
VIT_04s0008g03950	RD22	-5.46
XLOC_007318	no hit	-5.40
VIT_08s0007g00700	Aspartic Protease (VvAP21)	-5.39
VIT_14s0128g00280	Unknown	-5.35
VIT_06s0004g06680	ACR4 (Arabidopsis CRINKLY4)	-5.34
VIT_03s0091g01290	Serine carboxypeptidase S10	-5.33
VIT_12s0134g00010	Phototropic-responsive NPH3 protein	-5.32
VIT_11s0037g01310	basic helix-loop-helix (bHLH) family	-5.23
XLOC_002742	PREDICTED: hypothetical protein [Vitis vinifera]	-5.18
VIT_17s0000g09800	No hit	-5.18
VIT_18s0001g13980	Auxin responsive SAUR protein	-5.14
VIT_17s0000g09290	Protein kinase ATN1	-5.14
VIT_09s0002g02940	Myo-inositol oxygenase 1	-5.14
VIT_06s0004g01600	No hit	-5.13
VIT_10s0003g01550	IMK2 (inflorescence meristem receptor-like kinase 2)	-5.12
VIT_15s0045g01600	Eceriferum 1 (CER1 protein) Sterol desaturase	-5.08
XLOC_013069	protein	-5.06
VIT_19s0015g01310	Amino acid permease 7	-5.04
VIT_01s0026g00570	Bet v I allergen	-4.98
VIT_18s0001g14910	Mannitol dehydrogenase	-4.96
VIT_12s0028g02240	Thioredoxin TTL3 (Tetratricopetide-repeat thioredoxin-like 3)	-4.94
VIT_03s0038g01280	Auxin responsive SAUR protein	-4.91
VIT_00s0665g00030	Unknown protein	-4.90
VIT_08s0007g04040	flavonoid 3-monooxygenase	-4.87
VIT_14s0068g02010	IMP dehydrogenase/GMP reductase	-4.86
VIT_06s0004g01270	Callose synthase catalytic subunit	-4.81
VIT_06s0004g08210	Receptor protein kinase	-4.80
VIT_00s0218g00130	Anthocyanidine rhamnosyl-transferase	-4.78
VIT_16s0022g00230	Structural maintenance of chromosomes SMC2	-4.78
VIT_04s0008g05440	Ethylene-responsive transcription factor SHINE 3	-4.76
VIT_04s0023g00550	Auxin-induced SAUR	-4.76
VIT_11s0037g01230	basic helix-loop-helix (bHLH) family	-4.75
VIT_16s0013g00500	Unknown protein	-4.74
VIT_08s0007g04200	Late meristem identity1 HB51/LMI1 (VvATHB-5)	-4.71
VIT_03s0038g03600	Serine/threonine kinase	-4.71
VIT_13s0067g03050	Leucine-rich repeat transmembrane protein kinase	-4.69
VIT_13s0074g00390	CYP77A2	-4.68
VIT_14s0030g01880	Calmodulin-binding region IQD26	-4.67
VIT_00s1312g00010	basic helix-loop-helix (bHLH) family	-4.67
VIT_12s0055g01190	Alpha-L-arabinosidase	-4.63
VIT_02s0025g02540	Unknown protein	-4.63
VIT_06s0061g01230	Cellulose synthase CSLA02	-4.53
VIT_05s0077g00780	No hit	-4.53
VIT_07s0197g00020	Lateral organ boundaries domain gene 36	-4.52
VIT_03s0088g00260	Serine carboxypeptidase S10	-4.51
VIT_19s0090g00630	Tetraacyldisaccharide 4'-kinase	-4.50
VIT_08s0007g08540	Mg-chelatase subunit XANTHA-F	-4.49
VIT_13s0067g02390	Unknown	-4.49
VIT_14s0068g01230	fructose-2,6-bisphosphatase	-4.49
XLOC_019231	no hit	-4.44
VIT_06s0009g03390	No hit	-4.43
VIT_15s0045g01460	Eceriferum 1 (CER1 protein) Sterol desaturase	-4.40
VIT_04s0008g03940	BURP domain-containing protein	-4.39
VIT_18s0001g06430	Homeobox-leucine zipper protein ATHB-6	-4.36
VIT_03s0038g01290	Auxin responsive SAUR protein	-4.35
VIT_08s0058g00960	basic helix-loop-helix (bHLH) family	-4.34
VIT_12s0059g00650	No hit	-4.34
VIT_11s0118g00600	PLATZ transcription factor	-4.29
VIT_02s0025g02700	Glutaredoxin family protein	-4.29
VIT_04s0008g00870	Phosphoethanolamine/phosphocholine phosphatase	-4.29
VIT_07s0031g00270	Anthranilate N-hydroxycinnamoyl/benzoyltransferase	-4.28
VIT_14s0006g00520	Glucan endo-1,3-beta-glucosidase 3 precursor	-4.28
VIT_15s0045g01450	No hit	-4.26
VIT_14s0083g01110	Brassinosteroid-6-oxidase	-4.26
XLOC_016122	no hit	-4.26
VIT_04s0023g00540	Auxin-induced protein 6B	-4.24
VIT_19s0014g02070	flavonol 3-O-glucosyltransferase	-4.23
VIT_05s0062g00960	Exostosin (Xyloglucan galactosyltransferase KATAMARI 1)	-4.23
VIT_07s0031g00540	Rapid ALkalinization Factor RALFL34	-4.22
VIT_08s0007g03430	Germin	-4.20
VIT_17s0000g08290	Dof zinc finger protein DOF5.6	-4.20
VIT_00s0184g00110	Auxin-induced protein (AIR12)	-4.18
VIT_08s0007g08790	putative MADS-box Agamous-like 15b (VviAGL15b)	-4.18
VIT_14s0083g00490	Phosphoglycerate mutase	-4.17
VIT_00s1365g00010	fimbrin 1	-4.12
VIT_03s0038g01100	Auxin responsive SAUR protein	-4.10

VIT_15s0045g01440	Eceriferum 1 (CER1 protein) Sterol desaturase	-4.09
XLOC_010035	no hit	-4.07
VIT_05s0102g00880	Strictosidine synthase	-4.07
VIT_16s0022g02200	Subtilase	-4.07
VIT_15s0021g00050	Eceriferum 1 (CER1 protein) Sterol desaturase	-4.05
VIT_01s0127g00240	Retrotransposon protein, Unclassified	-4.05
VIT_10s0003g05030	Leucine-rich repeat family protein	-4.04
VIT_09s0002g06990	Phosphatidic acid phosphatase / PAP2	-4.01
VIT_18s0164g00050	No hit	-4.00
VIT_05s0062g01430	Glycosyl hydrolase family 17 protein	-4.00
VIT_08s0007g05200	Nudix hydrolase 16	-4.00
XLOC_016674	no hit	-4.00
VIT_09s0002g05700	Phototropic-responsive NPH3	-4.00
VIT_19s0090g01670	Receptor-like kinase 902	-4.00

Supplementary Table S6: Differentially expressed genes (> |4| fold) in *VvNAC60* stably overexpressing plants compared to the control line.

ID_code	Gene annotation	FC
VIT_14s0066g01670	Alpha-dioxygenase	48.14
VIT_16s0050g01820	No hit	41.92
VIT_00s0214g00130	F-box family protein	40.54
VIT_18s0001g06090	Cis-zeatin O-beta-D-glucosyltransferase	27.66
VIT_18s0001g06120	Cis-zeatin O-beta-D-glucosyltransferase	24.48
VIT_16s0022g00390	No hit	18.43
VIT_08s0058g00930	Alanine--glyoxylate aminotransferase 2 3, mitochondrial	18.04
VIT_07s0005g00870	Erg-1	17.16
VIT_08s0007g06430	No hit	16.73
VIT_08s0056g00220	Serine/threonine protein phosphatase PP1	16.49
VIT_13s0019g03530	No hit+B113	16.18
VIT_06s0004g08400	No hit	15.73
VIT_18s0122g01210	Cuticular water permeability	13.19
VIT_18s0001g08450	Branched-chain-amino-acid transaminase ATBCAT-2	13.01
VIT_01s0026g02700	CYP704G9	11.66
VIT_11s0016g00300	Pectinesterase family	11.47
VIT_18s0001g08430	Branched-chain-amino-acid aminotransferase 2, chloroplast (Atbcat-2)	11.22
VIT_18s0001g14270	Gibberellin-regulated protein 1 (GASA1)	10.17
VIT_13s0019g02140	Tropinone reductase	10.11
VIT_02s0012g01380	No hit	10.03
VIT_02s0025g04440	ERF/AP2 Gene Family (VvERF018),Dehydration Responsive Element-Binding TF	9.94
XLOC_000856	no hit	9.92
VIT_15s0046g01390	Ethylene-responsive transcription factor cytokinin response factor 4	9.73
VIT_05s0049g01980	3-isopropylmalate dehydratase large subunit 2	9.35
VIT_10s0003g00470	Trans-resveratrol di-O-methyltransferase - VvROMT	9.23
VIT_18s0001g02960	Nucleotidyltransferase family protein, putative, expressed	8.92
VIT_05s0077g00430	Galactinol synthase	8.87
VIT_04s0008g04040	RD22 [Vitis vinifera]	8.59
VIT_18s0075g00440	TIR-NBS-LRR disease resistance	8.41
VIT_13s0067g02050	Unknown protein	8.41
VIT_05s0020g00330	Galactinol synthase	8.11
VIT_11s0052g01180	Xyloglucan endotransglucosylase/hydrolase 23	8.10
VIT_18s0001g14260	No hit	8.01
VIT_19s0177g00030	Gibberellin 2-beta-dioxygenase 7	7.98
VIT_12s0028g00680	No hit	7.80
VIT_05s0062g01010	Aldo/keto reductase AKR	7.73
VIT_01s0011g00760	Beta-glucosidase	7.64
VIT_00s0250g00090	Oxidoreductase, 2OG-Fe(II) oxygenase	7.63
VIT_10s0003g03990	No hit	7.62
VIT_14s0006g02340	No hit	7.55
VIT_11s0052g01190	Xyloglucan endotransglucosylase-hydrolase XTH3	7.43
VIT_11s0052g01300	Xyloglucan endotransglycosylase 6	7.35
XLOC_002948	no hit	7.32
VIT_12s0059g01640	Unknown protein	7.20
VIT_00s0214g00120	F-box family protein	7.13
VIT_07s0197g00060	myb family	7.00
XLOC_001777	vq motif-containing protein	6.95
VIT_00s2526g00010	*Endo-1,4-beta-glucanase korrigan (KOR)*	6.93
VIT_08s0007g07730	CYP93A1 2-hydroxyisoflavanone synthase	6.88
VIT_09s0018g00910	No hit	6.85
VIT_01s0011g03090	Allene oxide cyclase (jasmonates from fatty acids)	6.78

VIT_07s0005g00660	*Late embryogenis abundant protein 5*	6.77
VIT_11s0052g01340	Xyloglucan endo-transglycosylase, C-terminal	6.69
VIT_06s0004g04590	Epsin N-terminal homology (ENTH) domain-containing	6.66
VIT_19s0014g03140	Lanthionine synthetase C	6.57
VIT_11s0052g01260	Xyloglucan endotransglucosylase/hydrolase 23	6.49
VIT_02s0025g04420	MATE efflux family protein	6.47
VIT_02s0087g00930	*(9,10) (9`,10`) cleavage dioxygenase (CCD4) (VvCCD4b)*	6.46
VIT_04s0008g04060	RD22	6.33
VIT_01s0011g02890	Unknown protein	6.25
VIT_00s0194g00290	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	6.19
VIT_00s2620g00010	Endo-1,4-beta-glucanase korrigan (KOR)	6.16
VIT_07s0104g00280	No hit	6.10
VIT_17s0000g00830	Nodulin MtN3 family	6.10
VIT_06s0004g00590	Lysine decarboxylase	6.08
VIT_03s0091g00670	*Lateral organ boundaries protein 38*	5.97
VIT_01s0026g02710	NAC domain-containing protein (VvNAC26)	5.93
VIT_18s0001g01440	No hit	5.92
VIT_05s0049g01780	Caleosin	5.92
XLOC_009902	no hit	5.88
VIT_06s0004g03520	Nitrate excretion transporter1	5.79
VIT_07s0031g01980	ERF/AP2 Gene Family (VvERF113)	5.73
VIT_07s0005g04800	SUPER1/YUCCA5 (suppressor of ER1)	5.73
VIT_16s0098g00210	Receptor serine/threonine kinase	5.71
VIT_05s0020g02170	Sugar transporter ERD6-like 16	5.70
VIT_11s0052g01320	Xyloglucan endotransglycosylase 6	5.69
VIT_14s0060g01910	Nodulin MtN3 family	5.68
VIT_18s0072g00260	Ethylene-responsive transcription factor related to APETALA2 6	5.66
VIT_03s0017g00660	Nematode resistance-like protein	5.63
VIT_12s0059g00470	Unknown protein	5.63
VIT_09s0002g04160	Thioesterase family	5.61
VIT_18s0089g01140	Wall-associated kinase	5.47
VIT_08s0007g00910	No hit	5.36
VIT_02s0154g00300	Small nuclear ribonucleoprotein Sm D3	5.28
VIT_01s0026g01380	Glutathione S-transferase 29 GSTU18	5.27
XLOC_018004	PREDICTED: hypothetical protein [Vitis vinifera]	5.13
VIT_12s0142g00360	putative MADS-box Agamous 1 (VviAG1)	5.07
VIT_12s0055g00800	Arachidonic acid-induced DEA1	5.06
VIT_18s0001g00850	Laccase	5.02
VIT_13s0106g00610	Tetracycline transporter	5.01
VIT_02s0025g01090	Unknown protein	5.00
VIT_05s0094g00650	Stearoyl-acyl-carrier protein desaturase	4.97
VIT_10s0003g02910	FLS2 (flagellin-sensitive 2)	4.96
VIT_05s0094g00770	Short-chain dehydrogenase/reductase (SDR)	4.86
VIT_16s0098g01170	Homeobox-leucine zipper protein HB-12 (VvATHB-10)	4.85
VIT_09s0002g00700	Dormancy/auxin associated protein	4.83
VIT_19s0140g00120	Gibberellin 2-beta-dioxygenase 1	4.82
XLOC_007391	no hit	4.79
VIT_12s0057g00950	No hit	4.78
VIT_07s0129g00890	Protein kinase	4.71
VIT_09s0002g00570	Lipase GDSL 1	4.70
XLOC_010633	no hit	4.70
VIT_07s0129g00330	Lateral organ boundaries protein 39	4.69
VIT_01s0011g05930	S-adenosyl-L-methionine:carboxyl methyltransferase	4.66
XLOC_002244	no hit	4.64
VIT_08s0007g01070	DC1 domain-containing protein	4.63
VIT_18s0001g03180	Nodulin MtN21 family	4.61
VIT_11s0052g01200	Xyloglucan endotransglucosylase/hydrolase 23	4.48
VIT_14s0068g02330	Chloride channel protein B	4.47
XLOC_007301	no hit	4.41
VIT_14s0066g00890	Cytochrome c oxidase subunit Vc	4.40
VIT_05s0020g02720	Aspartic Protease (VvAP11)	4.40
VIT_13s0064g00910	Unknown	4.39
VIT_09s0002g00710	ACP2 (Acyl carrier protein 2)	4.38
VIT_00s0340g00050	*Endo-1,4-beta-glucanase korrigan (KOR)*	4.37
VIT_10s0116g01150	Unknown	4.34
VIT_00s0762g00030	S-locus lectin protein kinase	4.29
VIT_08s0007g01360	Unknown protein	4.28
XLOC_021550	no hit	4.23
VIT_16s0050g01720	Receptor serine/threonine kinase PR5K	4.19
VIT_16s0050g02120	HcrVf3 protein	4.17
VIT_12s0055g00620	Glutamate synthase (NADH), chloroplast	4.16
VIT_11s0016g03950	Dehydration-responsive protein (RD22)	4.15

VIT_07s0005g04880	Glutathione S-transferase 25 GSTU7	4.14
VIT_18s0001g13740	Basic Leucine Zipper Transcription Factor (VvbZIP48)	4.13
VIT_00s0316g00020	Chloride channel protein CLC-A	4.12
VIT_11s0016g00900	Aldehyde Dehydrogenase (VvALDH7B5)	4.09
VIT_00s0872g00010	Unknown	4.08
VIT_18s0089g01030	ERF/AP2 Gene Family (VvERF017),Dehydration Responsive Element-Binding TF (VvDREB30)	4.08
VIT_05s0094g00200	Chitinase class IV	4.06
VIT_18s0001g00370	Ceramidase	4.05
VIT_01s0011g06590	Protease inhibitor/seed storage/lipid transfer protein (LTP)	-4.01
VIT_03s0063g01880	Acyl-CoA synthetase long-chain member 2	-4.02
VIT_18s0001g03080	Chitin elicitor-binding CEBIP LysM domain-containing	-4.03
VIT_02s0012g00640	PBP1 (pinoid-binding protein 1)	-4.05
VIT_00s0261g00040	Unknown protein	-4.06
XLOC_013069	protein	-4.09
VIT_03s0088g00410	Pyruvate kinase isozyme A, chloroplast precursor	-4.09
VIT_12s0055g01190	Alpha-L-arabinosidase	-4.15
VIT_09s0002g06750	ERF/AP2 Gene Family (VvERF042)	-4.15
XLOC_021560	no hit	-4.15
VIT_10s0003g04350	Photosystem I subunit X (PSAK)	-4.16
VIT_13s0019g04460	Phenylalanine ammonia-lyase 2 (PAL2)	-4.18
VIT_12s0034g01230	Thaumatin ATLP-1	-4.20
VIT_04s0008g06530	formin-2	-4.23
XLOC_021532	PREDICTED: hypothetical protein [Vitis vinifera]	-4.24
VIT_01s0011g04080	Zinc finger (C3HC4-type ring finger)	-4.25
VIT_10s0071g01120	Alpha-galactosidase	-4.27
VIT_08s0007g02550	Ovate family protein 6 OFP6	-4.29
XLOC_016046	protein	-4.30
VIT_09s0002g03750	GATA transcription factor 11	-4.32
VIT_07s0031g02160	Protein phosphatase 2C DBP	-4.32
VIT_02s0025g00820	Cation/hydrogen exchanger (CHX18)	-4.32
VIT_01s0011g03540	Lateral organ boundaries protein 41	-4.33
VIT_00s0346g00040	Receptor protein kinase RK20-1	-4.36
VIT_18s0001g15510	Unknown	-4.37
VIT_03s0038g04000	Cysteine endopeptidase, papain-type (XCP1)	-4.37
VIT_05s0029g00250	No hit	-4.38
VIT_02s0025g04720	Leucoanthocyanidin dioxygenase (VvLDOX) [Vitis vinifera]	-4.39
VIT_11s0016g04090	DNA repair protein MutS	-4.39
XLOC_000601	protein	-4.39
VIT_14s0108g01020	Expansin (VvEXPA16)	-4.42
VIT_08s0007g02670	Secoisolariciresinol dehydrogenase	-4.43
VIT_10s0003g03690	Beta-1,3-glucanase precursor	-4.45
VIT_01s0026g00490	Nodulin	-4.50
VIT_03s0038g03540	Dehydrin (VvDHN3)	-4.50
VIT_08s0056g01480	Cation exchanger, CAX7	-4.51
VIT_18s0001g00800	Tetracycline transporter protein	-4.52
VIT_02s0025g00260	Polygalacturonase GH28	-4.54
VIT_16s0022g01970	Anthocyanidin 3-O-glucosyltransferase	-4.54
VIT_02s0087g00330	Glycosyl transferase family 1 protein	-4.55
VIT_06s0004g01250	Omega-6 fatty acid desaturase, endoplasmic reticulum (FAD2)	-4.58
VIT_00s0322g00020	HHP4 (heptahelical protein 4)	-4.59
VIT_05s0124g00100	Unknown	-4.59
VIT_05s0020g01980	Acyl carrier protein, mitochondrial 3	-4.62
VIT_08s0056g01140	Exostosin	-4.69
VIT_08s0007g04040	flavonoid 3-monooxygenase	-4.69
VIT_00s0372g00070	Linalool synthase (VvTPS58), E,E)-Geranyl linalool syn	-4.69
VIT_03s0063g00210	Receptor protein kinase	-4.70
VIT_15s0045g01460	Eceriferum 1 (CER1 protein) Sterol desaturase	-4.70
VIT_14s0060g00330	Aspartic Protease (VvAP34)	-4.70
VIT_03s0038g02170	Thaumatin	-4.74
VIT_17s0000g02660	myb domain protein 6 (VvMybC2-L2)	-4.76
VIT_16s0039g00820	CYP89A5	-4.77
VIT_04s0008g03550	Aquaporin TIP4;1	-4.79
VIT_01s0127g00400	Polygalacturonase GH28	-4.82
VIT_16s0098g00930	Papain cysteine proteinase isoform I	-4.83
VIT_08s0007g08030	No hit	-4.85
VIT_09s0054g00640	N-acetyltransferase ESCO1	-4.91
XLOC_016089	PREDICTED: hypothetical protein [Vitis vinifera]	-4.93
VIT_15s0045g01440	Eceriferum 1 (CER1 protein) Sterol desaturase	-4.93
VIT_09s0002g02390	Phospholipase D epsilon	-4.96
VIT_08s0007g00700	Aspartic Protease (VvAP21)	-4.97
VIT_14s0060g00680	Lipase GDSL	-4.97
VIT_06s0004g06960	BCL2 binding anthogene	-5.02
VIT_15s0021g00050	Eceriferum 1 (CER1 protein) Sterol desaturase	-5.04
VIT_08s0007g07740	CYP93A1 2-hydroxyisoflavanone synthase	-5.06
VIT_10s0071g00860	Disease resistance protein	-5.10
VIT_08s0007g06340	Unknown protein	-5.10
VIT_15s0045g01450	No hit	-5.11

VIT_01s0011g03210	Aspartic Protease (VvAP1)	-5.14
VIT_18s0041g00580	Proton-dependent oligopeptide transport (POT) family protein	-5.16
VIT_04s0023g01600	Membrane protein	-5.18
VIT_13s0067g02390	Unknown	-5.19
VIT_14s0030g02090	Calcium-transporting ATPase 12 ACA12	-5.21
VIT_15s0024g01860	putative MADS-box JOIN 4 (VviSVPS4)	-5.22
VIT_10s0116g01670	Prephenate dehydratase with ACT region	-5.23
VIT_03s0017g00360	putative MADS-box Short Vegetal Phase 4 (VviSVP4)	-5.23
XLOC_006658	ubiquitin carboxyl-terminal hydrolase-related protein	-5.26
VIT_03s0167g00100	MADS-box protein SVP (short vegetative phase)	-5.28
VIT_09s0002g05700	Phototropic-responsive NPH3	-5.28
VIT_03s0017g00450	putative MADS-box JOIN 2 (VviSVPS2)	-5.30
VIT_12s0055g01110	LHCB6 (light harvesting complex PSII)	-5.31
XLOC_000354	at1g78880-like protein	-5.31
VIT_11s0016g05360	Phospholipase D alpha 1 precursor (PLD 1) (Choline phosphatase 1)	-5.33
VIT_17s0000g09800	No hit	-5.34
VIT_11s0016g00590	Invertase/pectin methylesterase inhibitor	-5.35
VIT_16s0022g02050	Lateral organ boundaries domain gene 36	-5.36
VIT_08s0007g08540	Mg-chelatase subunit XANTHA-F	-5.37
VIT_03s0017g01950	Pectinesterase family	-5.37
VIT_12s0059g00590	Allergenic protein Pt2L4	-5.38
VIT_04s0023g03040	Lactoylglutathione lyase	-5.42
VIT_14s0066g02020	Proton-dependent oligopeptide transport (POT) family protein	-5.46
VIT_19s0090g01400	Wax synthase isoform 1	-5.56
VIT_00s0131g00030	Annexin ANN4	-5.57
VIT_01s0026g02500	Amino acid transport protein	-5.59
VIT_04s0069g00860	Sarcosine oxidase	-5.61
XLOC_007697	hypothetical protein VITISV_039794 [Vitis vinifera]	-5.63
VIT_12s0059g02040	Unknown	-5.69
VIT_09s0002g06990	Phosphatidic acid phosphatase / PAP2	-5.83
VIT_14s0128g00280	Unknown	-5.83
VIT_19s0090g01670	Receptor-like kinase 902	-5.84
VIT_04s0008g05830	Armadillo/beta-catenin repeat	-5.86
XLOC_013000	PREDICTED: hypothetical protein [Vitis vinifera]	-5.88
VIT_17s0000g08960	Raffinose synthase	-5.90
VIT_02s0025g02650	Cellulase CEL2	-5.93
VIT_14s0030g00340	Sugar transporter ERD6-like 8	-6.06
VIT_04s0008g01860	CYP72A58	-6.11
XLOC_006417	rapid alkalinization factor 1	-6.12
VIT_13s0139g00420	Annexin ANN4	-6.15
VIT_00s0189g00060	Gibberellin-regulated protein 4 (GASA4)	-6.17
VIT_18s0001g08550	Squalene monooxygenase	-6.19
XLOC_019150	unnamed protein product [Vitis vinifera]	-6.23
XLOC_015922	no hit	-6.24
VIT_18s0041g01780	Aspartic protease	-6.29
VIT_00s0847g00020	Linalool synthase (VvTPS63), (E)- Nerolidol syn	-6.37
VIT_15s0048g00630	Protease inhibitor/seed storage/lipid transfer protein (LTP)	-6.44
VIT_00s0189g00070	Gibberellin-regulated protein 4 (GASA4)	-6.44
VIT_01s0146g00420	No hit	-6.46
VIT_10s0116g00520	Xyloglucan endotransglucosylase/hydrolase 8	-6.47
VIT_02s0025g04300	Thaumatin	-6.54
VIT_02s0025g02540	Unknown protein	-6.62
XLOC_009499	no hit	-6.63
VIT_12s0059g00210	Epoxide hydrolase	-6.67
VIT_07s0197g00040	Lateral organ boundaries domain gene 36	-6.74
VIT_08s0007g00440	Expansin (VvEXPA11)	-6.77
VIT_12s0028g03120	No hit	-6.81
VIT_08s0032g00890	Alpha-L-arabinosidase	-6.82
VIT_00s0218g00130	Anthocyanidine rhamnosyl-transferase	-6.94
VIT_14s0108g00420	basic helix-loop-helix (bHLH) family	-6.99
VIT_13s0084g00090	Nodulin MtN21 family	-7.05
VIT_04s0008g03950	RD22	-7.08
VIT_11s0016g04160	Sulfate transporter 3.5	-7.08
VIT_00s0187g00160	Ripening-related protein	-7.09
VIT_18s0001g00160	Unknown protein	-7.18
VIT_13s0064g00940	ferric reductase defective 3	-7.24
VIT_06s0004g01050	Calcineurin phosphoesterase	-7.33
VIT_06s0004g05050	Abscisic acid 8` hydroxylase (CYP707A2) (VvA8H-CYP707A2.1)	-7.34
VIT_00s1317g00010	Gibberellin-regulated protein 4 (GASA4)	-7.37
VIT_03s0038g00120	Gibberellin-regulated protein 4 (GASA4)	-7.37
VIT_07s0005g00090	Auxin-responsive GH3	-7.38
VIT_06s0004g06680	ACR4 (Arabidopsis CRINKLY4)	-7.39

VIT_04s0008g07080	Aspartic Protease (VvAP5)	-7.43
VIT_03s0091g00960	No hit	-7.44
VIT_16s0022g00190	No hit	-7.51
VIT_01s0011g01920	Phosphate-induced protein 1	-7.67
VIT_12s0059g01220	Pyrophosphate-dependent phosphofructokinase beta subunit	-7.69
VIT_04s0008g03940	BURP domain-containing protein	-7.71
VIT_19s0014g00160	LHCII type I CAB-1	-7.72
XLOC_013457	no hit	-7.78
VIT_02s0025g02100	No hit	-7.79
VIT_03s0038g01830	Proline-rich protein 4	-7.80
XLOC_006678	hypothetical protein VITISV_041092 [Vitis vinifera]	-7.92
VIT_18s0001g02140	Metal transporter Nramp1	-8.02
VIT_15s0048g01170	Subtilisin serine protease	-8.12
VIT_19s0015g01800	Nucleoside triphosphatase	-8.38
VIT_14s0060g01780	Unknown protein	-8.41
VIT_00s0131g00180	Annexin ANN4	-8.41
VIT_04s0008g00870	Phosphoethanolamine/phosphocholine phosphatase	-8.49
VIT_14s0108g00740	GASA4	-8.55
VIT_00s0274g00080	*Benzoquinone reductase*	-8.56
VIT_16s0022g02090	Embryo-specific 3	-8.57
VIT_18s0089g01000	F-box family protein	-8.68
VIT_02s0236g00030	Unknown	-8.71
VIT_11s0052g01620	Pathogenesis-related protein 1 precursor (PRP 1)	-8.71
VIT_19s0090g01280	Lipid-binding serum glycoprotein family protein	-8.72
VIT_12s0035g00920	Unknown protein	-8.97
VIT_01s0026g00570	Bet v I allergen	-8.99
VIT_07s0005g01880	Patatin	-9.16
VIT_10s0003g00650	Peroxidase	-9.52
VIT_18s0001g10350	Subtilase family protein	-9.73
VIT_09s0002g06680	Embryo-specific 3	-9.74
VIT_13s0019g05130	Serine carboxypeptidase III	-10.03
VIT_11s0103g00050	High-affinity K+ transporter 1 (HKT1)	-10.06
VIT_12s0059g01590	Lipase GDSL	-10.34
VIT_19s0014g05010	Unknown protein	-10.40
VIT_00s2015g00020	F-box family protein	-10.58
VIT_07s0005g01940	Pectinesterase family	-10.62
VIT_09s0002g01030	Subtilisin serine proteinase	-10.64
VIT_02s0012g00650	PBP1 (pinoid-binding protein 1)	-10.75
VIT_14s0060g01790	Unknown protein	-11.01
VIT_19s0090g00730	No hit	-11.01
VIT_02s0033g00300	myb family	-11.45
VIT_08s0058g01030	Saposin B domain-containing protein	-11.61
VIT_13s0047g00340	Ethylene-responsive transcription factor WRINKLED 1	-12.10
VIT_10s0003g02110	Lipase GDSL	-12.54
VIT_04s0008g02760	Unknown protein	-12.69
VIT_00s0181g00200	LHCB3 (light-harvesting chlorophyll binding protein 3)	-12.85
VIT_00s0181g00180	LHCB3 (light-harvesting chlorophyll binding protein 3)	-13.37
VIT_04s0008g04230	ABC Transporter (VvPDR28 - VvABCG58)	-13.41
VIT_18s0001g13400	Papain cysteine proteinase isoform I	-13.67
VIT_05s0051g00700	No hit	-13.95
VIT_05s0051g00680	Unknown protein	-15.80
VIT_14s0060g01870	Unknown protein	-16.23
VIT_14s0060g01040	No hit	-16.74
VIT_08s0056g01130	Mini zinc finger 2 MIF2	-16.88
VIT_06s0004g06730	Microsomal omega-3 fatty acid desaturase	-17.50
VIT_05s0077g00780	No hit	-17.77
XLOC_009162	PREDICTED: hypothetical protein [Vitis vinifera]	-17.77
VIT_14s0060g01840	Unknown protein	-18.46
VIT_18s0001g13380	Papain cysteine proteinase isoform I	-20.05
VIT_05s0049g02320	HAD superfamily hydrolase	-25.06
VIT_14s0060g01850	Unknown	-25.29
XLOC_007579	hypothetical protein VITISV_023265 [Vitis vinifera]	-26.17
VIT_16s0098g00460	Lipase class 3	-30.22
VIT_17s0119g00080	Organic cation transport protein OCT1	-45.98
XLOC_019261	no hit	-70.45
VIT_06s0004g02360	Myosin-related	-71.88

Chapter 4

FUNCTIONAL COMPLEMENTATION OF *non-ripening* (*nor*) TOMATO MUTANT

1. INTRODUCTION

Fruit development and ripening are complex and exclusive processes to plant species. This field has received considerable research attention not only for practical agricultural purposes but also for better understanding the coordinated regulation of different pathways during plant developmental programs.

Ripening involves a profound transformation of the seed-bearing structure of fleshy fruit species into an edible and desirable organ to seed dispersing animals and valuable as an agriculture product (Seymour, 1993). These biochemical and physiological changes, although variable among fruit from different species, generally include some key general characteristic events, such as drastic alteration in color, cell wall structure, sugar content and susceptibility to post-harvest pathogens (Seymour *et al.,* 2013; Klee and Giovannoni, 2011). Many different coordinated molecular pathways lead to these modifications; thanks to the availability of sequencing of numerous fleshy fruit genome, an active frontier in fruit ripening research is the elucidation of the molecular basis of specific and shared conserved regulatory mechanisms (Giovannoni, 2007; Seymour, 1993). Fleshy fruits have traditionally been classified into two groups based on their ripening physiologies. Climacteric fruit such as tomato, banana, apple and pear are characterized by a burst of respiration often coinciding with a dramatic increase in ethylene synthesis at the onset of ripening, whereas non-climacteric fruits such as strawberry, citrus and grape, do not (Giovannoni, 2004). Many studies involving targeted repression of ethylene synthesis genes (Watkins *et al.,* 2000; Klee *et al.,* 1991; Tucker and Brady, 1987) and the tomato *Never-ripe* (*Nr)* ethylene receptor mutant (Lanahan *et al.,* 1994) have demonstrated that this phytohormone is necessary for ripening of climacteric fruits. A lot more needs to be explored to establish about the regulation of the ripening in non-climacteric fruit which seems to be mainly independent of

ethylene (Giovannoni, 2004; Lelievre, 1997). However, recently studies on strawberry (Merchante *et al.,* 2013), bell pepper (Aizat *et al.,* 2013) and grape (Chervin *et al.,* 2004) demonstrated the production of a small amount of ethylene at certain stages, such as just before the start of véraison in grapevine. Other studies in strawberry showed that auxin plays a crucial role in the maturation of this fruit (White, 2002).

Among the fleshy fruits, tomato has proven to be a useful model to dissect the molecular mechanisms of fruit development and ripening. Tomato has diploid genetics, a short generation time, a large expressed sequence tag (EST) collection and a well-annotated genome sequence (http://solgenomics.net). It is easily transformed allowing rapid generation of transgenic plants for functional analyses (Mueller *et al.,* 2005; Fei *et al.,* 2004). Like grape berries, tomato fruits become palatable after complex physiological and biochemical changes, characterized by a shift from a vegetative to a mature growth phase (Palumbo *et al.,* 2014). By looking at tomato fruit development, we can distinguish four phases: the first is the fruit set phase, corresponding to the development of the ovary. In the second one the fruit tissue undergoes a wide cell division which lasts between seven and ten days after fertilization. Then, an extensive cell expansion occurs, driven by the accumulation of water in the vacuole (Cheniclet *et al.,* 2005). The fruit, which has reached its final size and contained mature seeds, undergoes ripening, the last phase of fruit development.

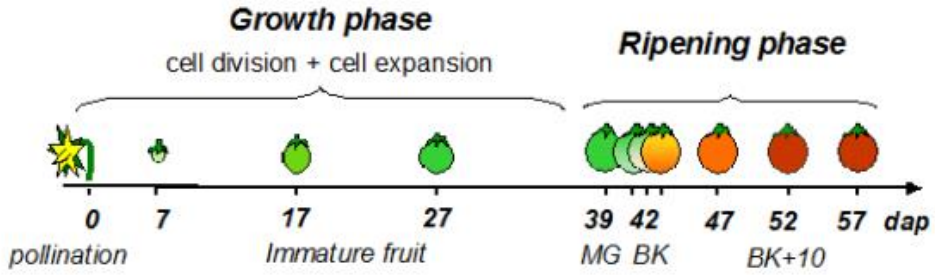

Figure 1: Phases of fruit development. Scheme illustrating the different developmental stages of tomato fruit (cv Ailsa Craig) from the time of pollination of the flower to ripe fruit. MG: mature green; BK: breaker, dap: days after anthesis. Figure from Martel (2010).

Tomato fruit development stages were subdivided into key stages by counted days post anthesis (DPA) and the visual color changes during development and ripening set by USDA (Figure 1).

Briefly, mature green (MG) stage corresponds to the fully expanded fruit with a green surface, containing mature seeds. The breaker stage (BK) represents the first visible sign of ripening, characterized by a carotenoid accumulation and a production of high levels of ethylene. During the red ripe fruit stage (RR) the ripening program has been completed. Depending on the cultivar, a tomato fruit takes three to ten day to reach RR from BK is typically three to ten days.

Several tomato-ripening mutants have been characterized to shed light on the transcriptional control of fruit ripening (Moore *et al.,* 2002; Giovannoni 2001). Among them, three non-allelic single-gene mutations, ripening inhibitor (*rin*), colorless non-ripening (*Cnr*) and non-ripening (*nor*), have been shown acting early in the transcriptional activation cascade that regulates ripening-related processes. In particular, studies demonstrated that *nor* might even act upstream of *RIN* in tomato regulatory network (Osorio *et al.,* 2011). The *rin, nor* and *Cnr* mutants were able to reach the MG stage with mature seeds and final-size fruit containing mature seeds, but they did not ripe neither naturally nor in response to exogenous phytohormone. The genes underlying all three mutations have been isolated via positional cloning and found to encode transcription factors (TFs). The *rin* locus encodes a MADS-box TF (*LeMADS-RIN*) (Vrebalov *et al.,* 2002), the *Cnr* a SBP TF (Manning *et al.*, 2006) and *nor* mutation (Tigchelaar *et al.,* 1973) was identified as a NAC domain family TF (Martel *et al.,* 2011). *NOR* was identified after a naturally occurring mutation at the *nor* locus of tomato by analyzed many genes involved in the ripening process. The non-ripening phenotype results from a 2 base pairs deletion in the *NOR* gene, which causes a frame shift that affects

NOR protein synthesis (U.S. Pat. No. 6,762,347). It is a recessive mutation located on tomato chromosome 10.

In the previous chapters, we showed how some members of VvNAC TFs could represent master regulators of the organ phase transition to immature to mature growth in grapevine plant, including berries. Therefore, in order to investigate the ability of these genes to regulate fruit ripening initiation, we tested if grapevine *NAC03, NAC11, NAC33* and *NAC60* could complement the *nor* mutation in tomato.

2. MATERIALS AND METHODS

2.1 Tomato Transformation

Transformation of *nor* (*nor*/*nor*) mutant tomato (*Solanum lycopersicum* cv. Ailsa Craig) cotyledon explants was performed by Boyce Thompson Institute transformation facility (Cornell University, Ithaca, NY - USA) as described below.

Plant Material

1) Sterilize seed: a) Immerse 0.9 - 1.0g of seed in 25 ml 20% bleach with 2 drops Tween (100 seeds, ~ 350mg). Shake on a rotary shaker at 250 rpm for 20 min. b) Rinse 3 times with sterile Milli-Q water.
2) Sow seed in Magenta boxes containing 1/2 MSO (approximately 25 - 30 seeds/box).
3) One day prior to inoculation with *Agrobacterium tumefaciens* (*A. tumefaciens*) cut cotyledons from 6-8-day-old seedlings. It is important that the first true leaves have not enlarged or opened. Place explants on plates of 2Z medium, adaxial side down and include 10 explants on one plate for controls. Culture 24°C + 2°C, 16 hr photoperiod.

Agrobacterium

The transformations were performed with the vectors overexpressing *VvNAC03, VvNAC11, VvNAC33* and *VvNAC60* used in chapter 3 for *Nicotiana benthamiana* and *Vitis vinifera* cv. Sultana transient transformation. They were transferred to *A. tumefaciens* strain LBA4404 by electroporation.

Transformation

1) Incubate explants in *Agrobacterium* culture/MS-O,2%: a) Pipette 25 ml of *Agrobacterium* culture into a sterile Magenta box. b) Transfer explants from 2 to 3 plates into inoculum in Magenta box. c) Incubate for 5 min with occasional

shaking. d) Remove explants to a sterile paper towel. e) Return explants to plates containing 2Z - medium, adaxial side down. f) Seal plates with Parafilm.
2) Cocultivate explants in the dark at 19°C for 48 hrs.
3) Transfer 25 explants to each plate of selection media (2Z), adaxial side up. Seal plates with Micropore tape. Culture at 24°C + 2°C, 16hr photoperiod, for one week.
4) Transfer to fresh 2Z selection medium for 2 weeks, then transfer to 1Z selection medium (15 explants per plate).
5) Transfer explants to new 1Z selection medium plates every 2 weeks. When shoots begin to appear and touch the lid of the plate, transfer explants to 1Z selection medium in Magenta boxes.

Regeneration and Rooting

1) Initial shoots should appear within 4-6 weeks.
2) Excise shoots from explants when shoots are at least 2 cm and include at least 1 node. Place in Magenta boxes containing Tomato Rooting Media with selective agent and Timentin.
3) Roots should begin to appear in 5-9 days.

The presence of the transgenes was verified in the T1 generations by PCR using the primers reported in Table 1, chapter 3.

2.2 Quantitative RT-PCR (qRT-PCR)

Total RNA from tomato vegetative tissue, flowers and fruit was isolated using procedures and reagents described by Chang *et al.* (1993). RNA was then digested with RQ1 DNase (Promega) and quantitative RT-PC was performed using SYBR Green on the AB7900 using 18S as the internal control. We used the same gene specific primers reported in Table 1, chapter 3.

2.3 Determination of ethylene

Fruits were collected and placed in open 250-ml jars for 3 hr to minimize the effect of wound ethylene caused by picking. Jars were then sealed and incubated at room temperature for a “t” time. 1 ml of headspace gas was injected into Agilent 6850 series gas chromatograph equipped with a flame ionization detector. Samples were compared with reagent grade ethylene standards of known concentration and normalized for fruit weight and incubation time (t).

3. RESULTS AND DISCUSSION

To check the ability of *VvNAC03, VvNAC11, VvNAC33* and *VvNAC60* to fulfil the function of *NOR* in tomato, transgenic lines with constructs driven by the constitutive CaMV35S promoter were produced by Boyce Thompson Institute transformation facility at Cornell University (Ithaca, New York-USA). The analyzes reported in this chapter were performed in Dr. Giovannoni Laboratory at the same University.

T_0 plants were grown to maturity and two independent lines for each transgene was selected by phenotype. The selected lines, showed in Figure 2, were characterized by a slight pericarp pigmentation, except for the line #1 of *VvNAC60* that showed a reddish fruit surface, in comparison to the *nor* mutant, that appeared completely green (Figure 4).

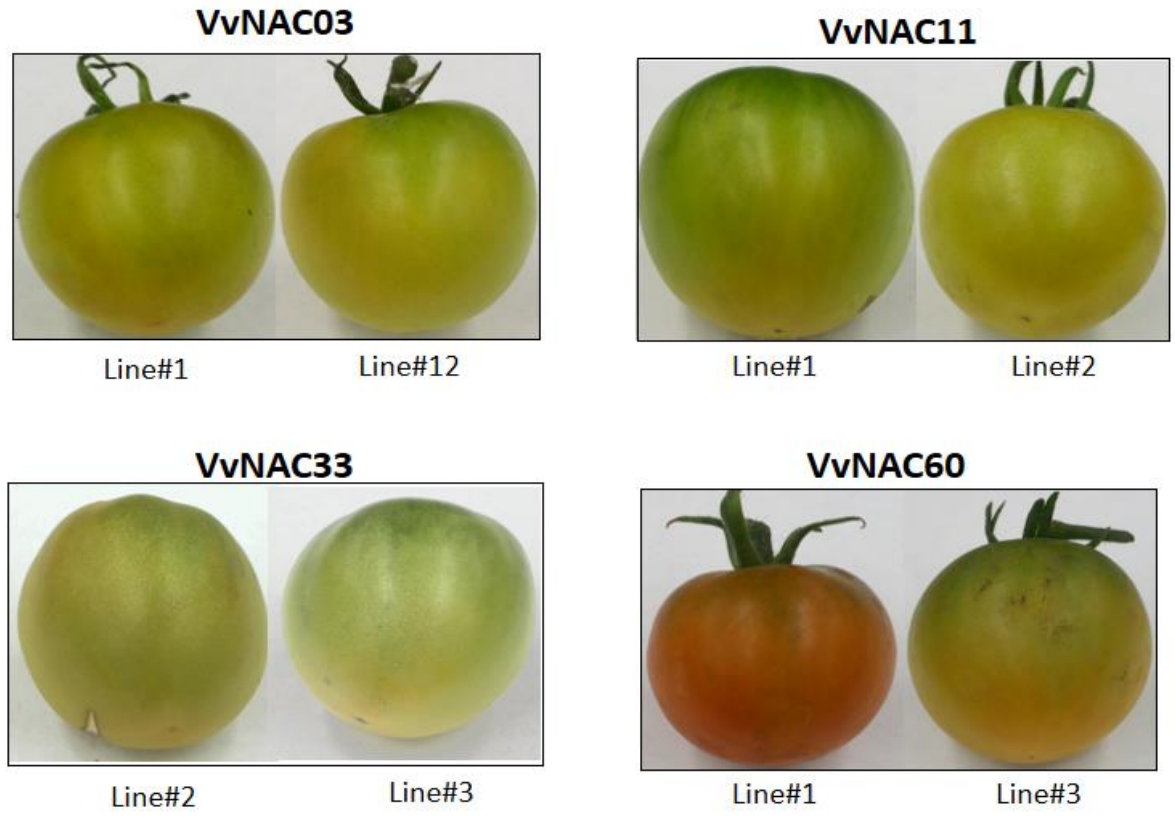

Figure 2: Phenotype of 35S::*VvNAC03,* 35S::*VvNAC11,* 35S::*VvNAC33* and 35S::*VvNAC60* T_0 tomato fruit (*Solanum lycopersicum* cv. Ailsa Craig).

Seeds obtained from the two selected T_0 lines were planted and T_1 plants were grown in a greenhouse; a total of 97 putative transgenic T_1 plants have been obtained from T_0 generation, as reported in Table 1.

Construct	Line	n° plants
35S::*VvNAC03*	#1	12
	#12	9
35S::*VvNAC11*	#1	9
	#2	12
35S::*VvNAC33*	#2	15
	#3	16
35S::*VvNAC60*	#1	10
	#3	14

Table 1: number of T_1 plants obtained from T_0 generation.

All 97 plants were screened by PCR to confirm the T-DNA integration in the genome of these plants and not more than 6 plants for each T_1 line were

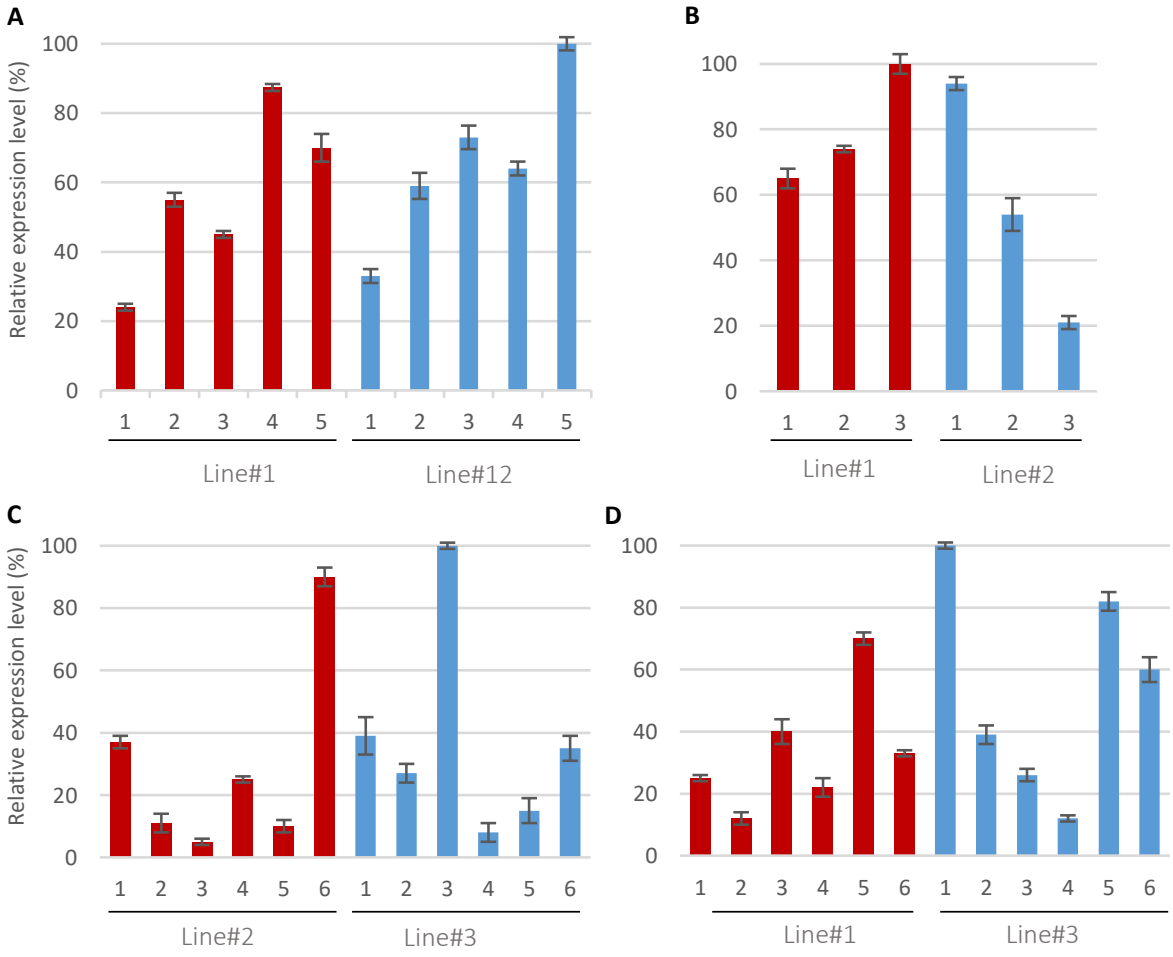

Figure 3: Quantitative RT-PCR analysis of *VvNAC03* (A), *VvNAC11* (B), *VvNA33* (C) and *VvNAC60* (D) transcript levels in *nor* mutant background. qRT-PCR values are reported relative to 18S and normalized on the expression level of the maximum one of both lines for each transgene.

selected and grown to maturity for the further analysis. Since for *VvNAC03* and *VvNAC11* we encountered some technical problems, we could have analyzed only 5 and 3 plants for each line, respectively. Expression of each transgene in leaves was measured by qRT-PCR. In Figure 3 we reported the relative expression level (%) for each plant of each line; we detected expression of the transgenes in all plants, even if at different level. They were grown to maturity in the greenhouse and the fruits were analyzed for complementation of the *nor* mutation.

Regarding plants architecture, we did not observe some evident alterations in comparison to *nor* mutant plants, except for a slightly smaller dimension of 35S::*VvNAC60* plants that will have to be confirmed.

By focusing on the fruits, we monitored their growth by counting days after the initial visual observation of lycopene accumulation, corresponding to breaker stage. We noted that *VvNAC60* fruits reached this stage about one week before the others and that they showed smaller dimension than the other same-age fruits, as reported in Figure 4. We collected the fruits around four and ten days after the breaker and the phenotypes shown are reported in Figure 3. Same-age wild type and *nor/nor* fruits were used as comparison in these phenotypic analyses.

VvNAC11 and *VvNAC33* transgenic fruits were indistinguishable from *nor* mutants in both the stages analyzed (Figure 4). On the other hand, we observed that *VvNAC03* and *VvNAC60* pericarps showed different degrees of yellowness around four days after the breaker. However, five days later, even if *VvNAC60* fruits were redder that *VvNAC03* ones, they were not able to complete the ripening phenotype: indeed, their pulps remained greenish and the pericarp did not reach the same red color as wild type fruits.

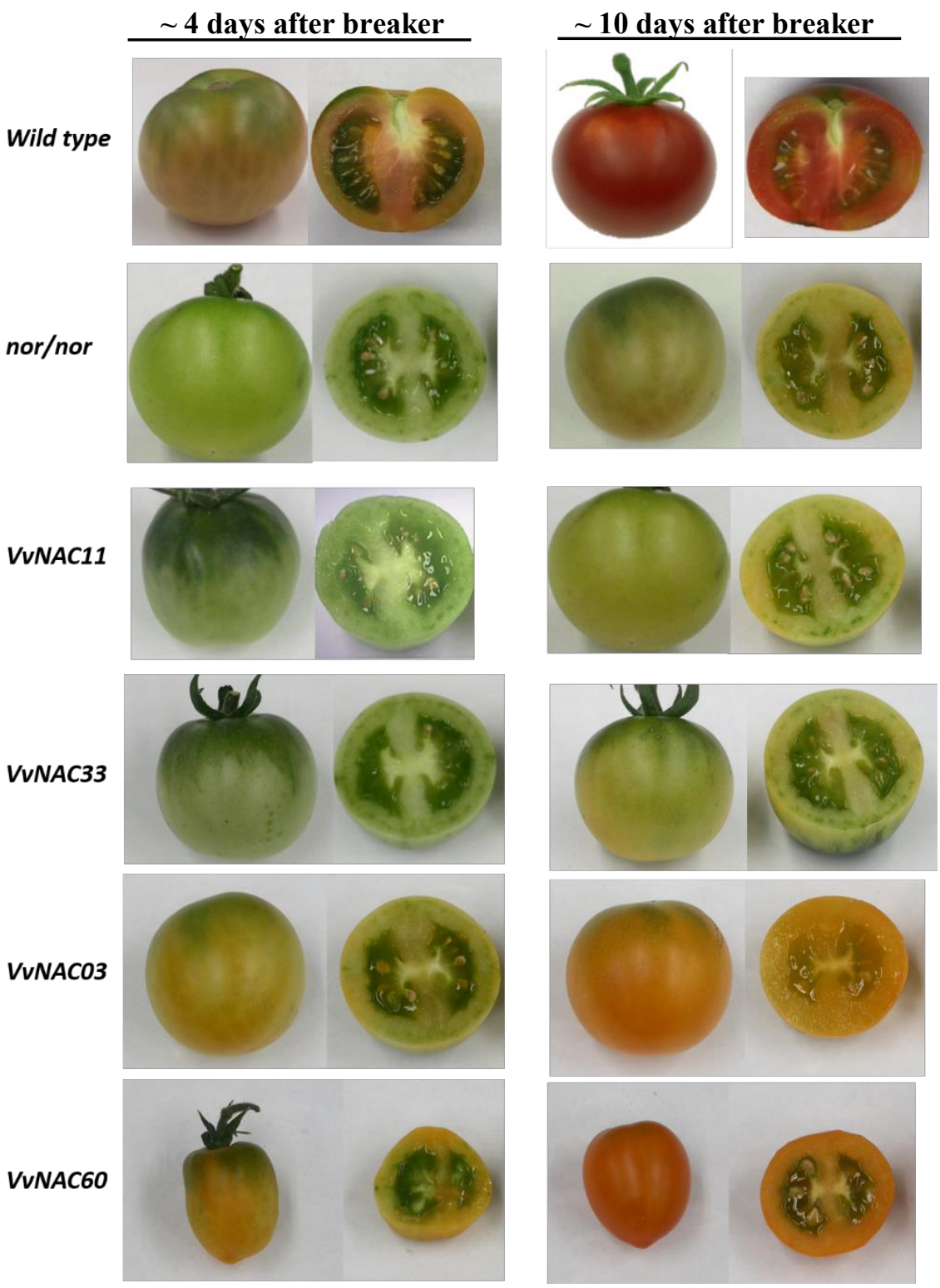

Figure 4: Phenotype of tomato fruits corresponding to wild type (*Solanum lycopersicum* cv. Ailsa Craig), *nor/nor* and T_1 fruit transformed with 35S::*VvNAC03,* 35S::*VvNAC11,* 35S::*VvNAC33* and 35S::*VvNAC60* in *nor* tomato mutant background. They were collected at the different stages: around four and ten days after breaker.

Since *nor* mutant fails to undergo an increase in ripening-related ethylene production, we measured the production of this gas in *VvNAC03*, *VvNAC11*, *VvNAC33* and *VvNAC60* fruits after breaker, corresponding to the stage in which the production starts in a wild type fruit. We noticed that *VvNAC03* and *VvNAC60* transgenic fruits showed a higher level of ethylene production in comparison to *VvNAC11* and *VvNAC33*. This result supported the phenotypes above-showed, suggesting that these two *NACs* were able to weakly start ripening.

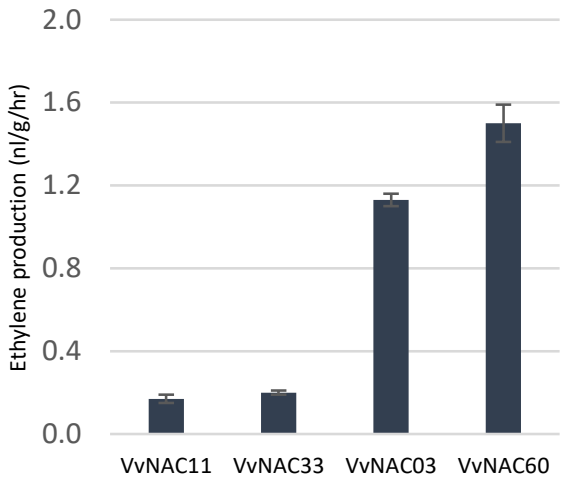

Figure 5: Ethylene production (in nl per g per hr) compared among *VvNAC03*, *VvNAC11*, *VvNA33* and *VvNAC60* transgenic fruits in *nor* background in tomato. Each value represents the mean ±S.E. of five biological replicates, except of three for *VvNAC11*. Ethylene production of a wild type fruit is around 6 nl/g/hr (Vrebalov *et al.,* 2009).

Based on these results, we can affirm that *VvNAC11* and *VvNAC33* could not complement the *nor* mutant phenotype since they failed to ripe, like *nor* mutant, indicating that these two grapevine *NACs* and the tomato one are not functionally conserved. Regarding *VvNAC11*, we noted that the overexpressing plants have had some problems to get fruits, maybe due to some difficulties during pollination. Further analysis will be required to confirm and shed light on this observation. By analyzing the phylogenetic tree developed in chapter 2, we noticed that these two *NACs* did not belong to the same *NOR* clade; therefore, we could hypothesize that they are too evolutionary distant to act in the context of the tomato system.

Concerning *VvNAC03* and *VvNAC60*, they partially complemented *nor* mutation in tomato since the fruits showed a carotenoid accumulation in their pericarp, which represents a clear sign of fruit ripening. Moreover, they produced a slight amount of ethylene, which is another ripening-related aspect. By analyzing the above-mentioned phylogenetic tree, *VvNAC03* was one of the two closest homologues to *NOR* and *VvNAC60* belonged to the same *NOR* clade. These finding could in part explain the different results obtained by the functional complementation of *nor* mutant. However, since the two grapevine *NACs* were not able to reach the fully ripe stage in tomato, the homology between *VvNAC03* and *VvNAC60* and *NOR* seemed not enough to maintain full functionality, maybe given the evolutionary distance between tomato and grapevine. *VvNAC03* and *VvNAC60* could have sufficiently diverged to be unable to completely act in the context of the tomato network of interaction proteins. Alternatively, these grapevine *NACs* could act downstream in comparison to *NOR* in the regulatory network and so they were not able to activate themselves the transcriptional activation cascade that regulates ripening-related processes. In this regard, we should also consider that we were characterized grapevine genes in a tomato background.

Further analysis will be required to better understand how the selected *VvNACs* are involved in the regulatory mechanisms of fruit development and ripening. In order to evaluate if NAC factors are able to rescue the metabolic processes affected by *nor* mutation, a RNAseq analysis has been performed on transformed fruits. Moreover, we will analyze the expression level of some well-characterized ripening-related regulator genes. T_2 plants are growing and they will be characterized.

It would be interesting to perform the same experiment with some other *VvNACs*, given the complexity of NACs regulatory network that controls maturation, as described in the previous chapter.

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Chapter 5

GENERAL DISCUSSION

In the current contest of extensive climate changes which widely affect agriculture, grapevine cultivation is assuming a notable attention due to its high sensibility to global warming effects. In order to maintain a sustainable viticulture with a stable production of high quality grape, an increase of the knowledge on the overall regulation of the developmental program occurring in grapevine organs and, in particular, of the transition to the mature phase is required. This transition is featured also by grape berries during the seasonal development and marks the onset of ripening.

This work was focused on the characterization of five transcription factors (TFs) belonging to the NAC (NAM/ATAF/CUC) gene family, that could play the role of master regulators controlling this organ phase transition in grapevine (Nuruzzaman *et al.,* 2013). The selection criteria were based on the analysis of their expression profiles, on the results of an integrated network analysis on multiple transcriptome datasets (Massonnett, 2015; Palumbo *et al.,* 2014; Fasoli *et al.,* 2012) and on a phylogenetic study comparing grapevine *NACs* and tomato *NOR*, known as a member of the same gene family and playing a key role in the regulation of fruit ripening (Giovannoni, 2004; Giovannoni *et al.,* 1995). In detail, *VvNAC33* and *VvNAC60* were selected as 'switch' genes of the entire plant development, *VvNAC11* and *VvNAC13* of berry development and *VvNAC03* as a close homologue of tomato *NOR*.

Different approaches have been carried out to investigate the function of these *NAC* genes in order to pave the way for unravelling the complex *NAC* regulatory mechanisms. The five genes were firstly characterized in term of features of the corresponding encoded protein sequences and expression profiles in different grapevine organs and developmental stages. As expected, all five NAC proteins possessed the conserved NAC domain at the N-terminus; moreover, the genes were expressed at low levels in

vegetative/green tissues and their expression increased in the mature/woody phase, independently from the color skin.

Then, a transient overexpression assay combined with microarray analysis was performed by obtaining an overview on the primary effects of these TFs on leaves transcriptome. A wide range of biological processes resulted affected by the ectopic expression of the transgenes; in particular, an up-regulation of many ripening-related genes involved in flavonoid and anthocyanin biosynthesis, cell wall metabolism, hormone biosynthesis or degradation has been revealed, encouraging our working hypothesis.

Embryogenic calli of *Vitis vinifera* were transformed with *Agrobacterium tumefaciens* harboring a binary vector in which the *VvNAC33* and *VvNAC60* were overexpressed. Although this process is still considered an arduous task and time-consuming, transgenic grapevines were successfully generated. To get a comprehensive description of them, the obtained plants have been molecularly and phenotypically characterized. *VvNAC33* overexpressing leaves showed a yellowing effect due to a chlorophyll breakdown, supporting the phenotype observed on *Nicotiana benthamiana* (*N. benthamiana*) leaves overexpressing the same transgene. *VvNAC60* overexpressing plants showed a slightly plant growth and an earlier stem lignification in comparison to the same-age control plant, supporting the senescence-related phenotype observed on *N. benthamiana* leaves overexpressing the same transgene. These results encouraged the idea of a crucial role of NACs in the transition from vegetative to mature development in grapevine since they reflected typical behaviors of plants undergoing ripening and/or senescence. Moreover, a microarray experiment was carried out comparing the different transcriptomes with a control plant. This analysis revealed common and specific patterns of modulated transcripts belonging to different functional categories; in particular, genes encoded proteins involved in tissue softening and cell expansion, as well as in color and aroma were found. All these processes are

implicated in the transition of plant to mature growth and, therefore, these evidences were in according to our working hypothesis. Interestingly, we noticed that 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 studied modulated transcriptomes. These findings indicated that several genes related to processes that characterize the vegetative phase, such as photosynthesis, cell cycle and development, have been down-regulated when plant switches to mature growth, in according to Palumbo *et al.* (2014). It was found that the transition to mature growth mainly involved the suppression of vegetative pathways rather than maturation ones. As further analysis, it would be useful to use specific approaches aimed to the repression of NAC TF activity. Silencing or repression constructs could be prepared using the native promoter with the aim of driving the transgene in the same tissue and developmental stage during which the endogenous gene is expressed, in order to have a specific action.

Since the economic value of grapevine mostly depends on quality of its berries and, moreover, we found several modulated genes belonging to mechanisms and metabolic pathways notably important for grape berry quality parameters (phenylpropanoid, flavonoid and stilbenoid pathways), additional useful insights could be obtained by phenotypic and molecular characterization of the fruits of the transgenic plants to get more specific information about the roles played by *VvNAC33* and *VvNAC60* directly in grape berries. In this regard, since grapevine plant takes around three-four years to produce the first fruits, we used the heterologous system tomato to have some results in a shorter time. Indeed, the tomato system has proved to be one of the best fleshy fruit model to study the regulation of fruit ripening. Hence, a functional complementation analysis on *nor* mutant tomato plants overexpressing *VvNAC03, VvNAC11, VvNAC13, VvNAC33* and *VvNAC60* were carried out.

From preliminary results, *VvNAC03* and *VvNAC60* showed a partial ripening phenotype, revealing a partial complementation of the *nor* mutation.

By investigating the result of microarray analysis performed on transiently and stably overexpressing plants, it is worth noting that transport and TF activity were present among the most represented functional categories. This finding has been confirmed also by the co-expression analysis. Regarding the strong relation between *NACs* and transport-related genes, we hypothesized that *NACs* could control primary processes, involved in plant maturation, able to activate vacuolar transport. An interesting gene seem to be the *ORGANIC CATION/CARNITINE TRANSPORTER4* (VIT_19s0014g04790), correlated to *VvNAC03, VvNAC11* and *VvNAC60* and up-regulated by *VvNAC60* in stable overexpressing plant and by *NAC11* in transiently overexpressing plants. Concerning the high number of TFs identified, the co-expression analysis revealed a correlation between *NAC03* and *NAC11* and between *NAC33* and *NAC60* and the microarray analysis showed a *NAC03* up-regulation by *NAC60* and a *NAC60* down-regulation by *NAC03*, hypothesizing that these two genes could be involved in the fine tuning of their own expression. Moreover, several 'switch' genes identified by Palumbo *et al.* (2014) were emerged in the co-expression and microarray analysis, supporting again the existence of a complex regulatory network during plant development.

Overall, among the analyzed five *NACs*, we observed strong evidences regarding *VvNAC03, VvNAC33* and *VvNAC60* as master regulator of immature-to-mature transition phase in grapevine. In particularr, the first one seems to have a role in the regulation of fruit ripening, on the basis of the results obtained from the transiently overexpression assay and *nor* mutant complementation. *VvNAC33* could be considered a negative regulator of photosynthesis, due to the phenotypic effects on *Nicotiana benthamiana* (*N. benthamiana*) and stably transformed grapevine leaves. *VvNAC60* seems to

play a role in ripening/senescence process, by considering the phenotypic effects on *N. benthamiana* leaves overexpressing this transgene, the developmental growth of stably transformed grapevine plants and the partial complementation of tomato *nor* mutant. Moreover, it seems to be involved in the positive regulation of anthocyanin synthesis, in according to the results obtained by microarray analysis on transiently and stably overexpressing plants and confirmed by the Dual Luciferase Assay. By using this method, *VvMYBA1*, a known grapevine regulator of the anthocyanin biosynthetic pathway, resulted to act downstream to Vv*NAC60*.

Taken together, all these approaches aimed to unravel the regulatory network controlling the transcriptomic shift that occurs in grapevine when plant moves into the developmental mature phase. By comparing transcriptomic analysis obtained from transient and stable grapevine transformation we were not able to identify target genes commonly and consistently modulated by the two approaches; we should consider that the effects of these approaches could be slightly different, since the first one is more focused on the primary (early) effect on transcriptome and the second one may catch secondary transcriptomic changes, derived from altered metabolisms or developmental processes. More efforts to better understand the specific and the overlapping *NAC* functions are certainly required. Therefore, other players of this complex regulation system could be selected and validated by performing the Dual Luciferase Assay or by ChIP-seq which combined microarray with chromatin immunoprecipitation.

In conclusion, all the results obtained in this work helped us to get a comprehensive picture about the molecular mechanisms underlying the developmental transcriptomic shift in grapevine. The findings could pave the way to get more insight to support vineyard management in the context of the climate changes that are occurring in recent decades, which cause severe effects on viticulture (Ollat *et al.,* 2014; Ollat *et al.,* 2011; Webb *et al.,* 2007).

However, the elucidation of the complexity of TFs network are still in their infancy and many other studies should be performed on this research area.

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