IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF MASTER REGULATORS OF THE ONSET OF BERRY RIPENING IN GRAPEVINE (*Vitis vinifera L.*)

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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF MASTER REGULATORS OF THE ONSET OF BERRY RIPENING IN GRAPEVINE (*Vitis vinifera L.*)

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ABSTRACT

Grapevine is one of the most important and cultivated fruit crops in the world. Its economic importance is especially related to winemaking and the production of high-quality grape is one of the major concerns of the viticulturists. In the last years, continuous temperatures increasing are altering the maturation process; in particular, higher temperatures have caused an anticipation of the onset of berry ripening, called *veraison*, with reducing grape color and increasing volatilization of aroma compounds. This change could modify the physiological characteristics of grape, its final quality and consequently wine quality. In this contest of climate changes, the development of strategies to prevent these negative effects is indispensable. Many agronomic practices have been tested, but they are very complex and expensive and their application on large scale could be economically unsustainable. Accordingly, the identification of alternative approaches seems to be essential. The interpretation of the molecular mechanisms controlling the onset of berry ripening could provide allow the development of more specific and targeted intervention strategies. To this aim, many molecular studies have been performed. One of the most important is represented by the generation of the grapevine gene expression atlas (Fasoli et al., 2012); this study showed a transcriptomic reprogramming during the vegetative-to-mature transition, suggesting the existence of key regulator genes. Further studies (Palumbo et al., 2012; Massonnet et al., 2017) showed that this phase transition seem to be regulated by specific genes, defined switch genes: they are mainly transcription factors and they could be master regulators of the ripening process in grapevine. Furthermore, some of these transcription factors are characterized by a strong induction during the first phase of *veraison*, confirming their specific role in the regulation of the onset of berry ripening (Fasoli et al., 2018). The identification of the functions of these transcription factors could provide important details about the molecular mechanism controlling the maturation process in grapevine. Among these transcription factors, five of them, *VviNAC33*, *VviNAC60*, *VviAGL15*, *VviWRKY19* and *VvibHLH75*, have been selected for functional characterization. They are five switch genes and, excluding *VviAGL15*, they are markers of the first transition during *veraison*. Furthermore, they belong to 4 of the most important transcription factors families in plants. Their functional analysis in grapevine has been performed using stable genetic transformation and transient gene expression approaches. The application, improvement and development of these approaches has supported the functional characterization of the five selected genes.

Regarding the stable transformation, to identify a standard method, 3 different protocols in 3 different cultivars (Shiraz, Garganega and Sangiovese), using *GFP* as reporter gene, have been tested. Different parameters, including the type of embryogenic tissue, different *Agrobacterium* OD_{600} and media, have been analyzed. The results showed that the regeneration of transgenic somatic embryos and plants occurred only in Shiraz and Garganega cultivars using embryogenic calli as transformation material, indicating that this complex process is cultivar-dependent. Furthermore, there weren't remarkable differences in terms of regenerated plants between the protocol tested. Stable genetic transformation was used for the functional analysis of both *VviNAC33* and *VviNAC60*. In a previous work (D'Incà, 2017), both NAC genes have been overexpressed in grapevine plants; the overexpression of *VviNAC33* has altered the chlorophyll metabolism, while the overexpression of *VviNAC60* has caused stunted growth and anthocyanins leaf accumulation, indicating that both genes are involved in the regulation of vegetative-to-mature transition. Furthermore, their overexpression showed an upregulation of many genes involved in the maturation process. In this PhD project, both NAC gene have been fused with EAR motif, the strongest transcriptional repression domain in plants, and stably expressed in Garganega and Shiraz plants. The results showed that some putative target genes of both NAC transcription factors are less expressed than WT plants, indicating that EAR motif represents a good approach to study the function of a transcription factor and to identify their target genes. Regarding transient gene expression, this method was used for the functional analysis of *VviAGL15*, *VviWRKY19* and *VvibHLH75*. Leaf agroinfiltration, the historic and the most used assay of this method, was optimized using *YFP* as reporter gene and tested in different cultivars by a vacuum system. The analysis of YFP transient expression showed that the fluorescence signal is especially localized in the first and second leaf from apex and the day post infiltration of maximum YFP expression is cultivar dependent. Moreover, agroinfiltration was tested using grapevine berry, a more complex tissue than leaf, obtained from fruiting cuttings. This approach was performed using again *YFP* as reporter gene and two different agroinfiltration methods: syringe with needle and vacuum system. The YFP transient expression analysis showed that the efficiency of this approach is not very high, but the visualization of fluorescence signal only in the inner part of vacuum agroinfiltrated berries, indicates that this approach can be further improved and subsequently used for gene functional analysis directly in berry. However, *VviAGL15*, *VviWRKY19* and *VvibHLH75* have been functionally characterized

using the improved leaf agroinfiltration protocol and plants of Thompson seedless cultivar. Each transcription factor was co-expressed with *YFP* gene: the visualization of its expression has allowed to select only agroinfiltrated leaves. Next microarray analysis of overexpressing leaves showed that many upregulated genes are involved in processes associate with ripening, and an exhaustive molecular interpretation of these preliminary results seem to indicate that *VviAGL15*, *VviWRKY19* and *VvibHLH75* are master regulators of the onset of berry ripening, controlling many aspects of the maturation programs. Finally, the last topic of this thesis is the regeneration of grapevine plants from embryogenic calli-derived protoplasts. This approach was tested in two different grapevine cultivars, Garganega and Sangiovese. The results showed that plant regeneration occurred in both cultivars, but the efficiency was higher in Garganega. Furthermore, protoplast transfection with a vector harboring a cassette for YFP overexpression showed a high and uniform YFP expression until 72 hours post transfection. The successful of these results indicate that protoplast technology can be used for functional studies, including genome editing, an innovative and emergent system to genetic modifications and improvement of plants.

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Chapter 1

INTRODUCTION

Grapevine development and berry ripening

Grapevine (*Vitis vinifera* L.) is a woody perennial plant from the family *Vitaceae*. It is one of the most important fruit crops in the world, and viticulture and enology play an important role in the economy of many developed and emerging countries (Martínez-Esteso et al, 2013).

Grapevine has a biennal reproductive cycle: buds formed in the first year give rise to shoots bearing fruit in the second year. Its annual growth cycle is represented by a period of active growth from spring to fall, followed by a rest period in the winter. During the dormant season, the organs undergo an acclimation process to survive freezing temperatures. In the spring, following the increase of day length and temperatures, the dormancy is released. After that, the budburst takes place and the first shoots start to grow. Early shoot growth is relatively slow, but soon it enters a phase of rapid growth which typically continues until just after fruit set to a halt by about the time the fruit begins to ripen. As the shoot grows, the flower cluster development takes place, rapidly also forming individual flower. Following the flowering, during which processes of pollination and fertilization take place, the next phases are the fruit set and the berry development. This latter phase and the ripening are the most important processes of the annual growth cycle of grapevine. Grape berry development and ripening are represented by double sigmoid growth pattern (Conde et al., 2007; Kennedy, 2002; Figure 1). The first growth phase is characterized by not only rapid cell division, which increases the number of cells, but also by an expansion of existing cells; during this phase, the berry is formed, the seed embryos are produced, and several solutes are accumulated. The most prevalent compounds are tartaric and malic acids, followed by hydroxycinnamic acids, tannins, amino acids, micronutrients and aroma compounds. The first phase is followed by a lag phase with little or no growth. The second growth phase coincides with the onset of ripening, called *veraison*, a French word used to describe the change in berry skin colour; it is characterized by important biochemical and physiological changes such as softening, coloring and engustment of berry. As grape berries develop, they change in size and composition: in fact, during this phase, berry

approximately doubles in size between *veraison* and harvest and most of compounds accumulated in berries during the first growth phase (malic acid, tannins and aroma compounds) are significantly reduced while others, especially fructose and glucose sugars and anthocyanins, are considerably increased.

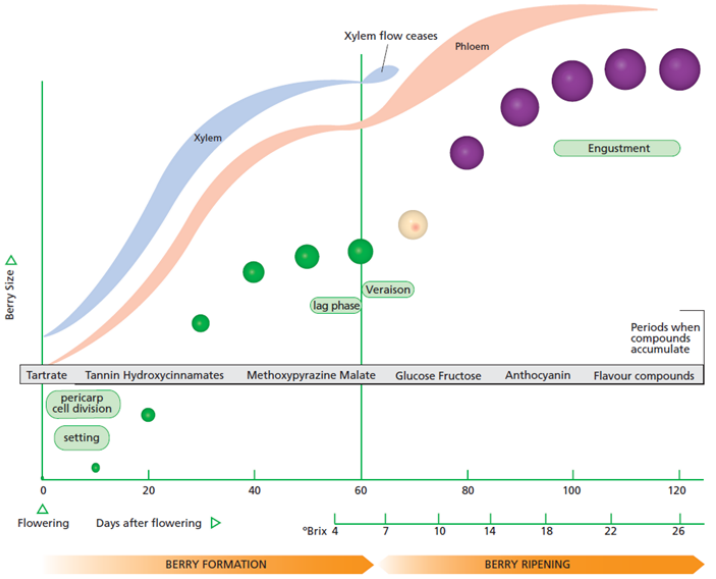

Figure 1: diagram showing the two phases of grape berry development and ripening (Kennedy, 2002).

Berry ripening is a complex developmental process affected by many endogenous and exogenous factors. Hormonal signaling but also many environmental influences, such as sunlight, temperature, inorganic nutrients and water, are the main factors involved in the regulation of berry ripening (Jackson, 2014; Kuhn et al., 2014). Grape is a non-climacteric fruit and the respiratory burst and rise in ethylene production are absent at the onset of ripening. However, ethylene and other two hormones, abscisic acid (ABA) and brassinosteroids, have been suggested to promote ripening through complex interactions (Fortes et al., 2015; Conde et al., 2007), while auxins delay ripening associated processes, such us berry size, sugar accumulation and anthocyanin content (Kuhn et al., 2014). The levels of ABA increase after *veraison* in berry tissues where it plays a role in seed maturation, acquisition of seed dormancy and resistance to water stress deficit. ABA is also specifically involved in maturation control, regulating positively sugar and phenolics accumulation in grape (Conde et al., 2007). Despite

ethylene levels are always very low in ripening grape berries, itmay influence berry acidity and the development of grape flavor and aroma (Conde et al., 2007; Fortes et al., 2015). Finally, brassinosteroids are hormones involved in plant growth and development, but they dramatically increase at the onset of berry ripening suggesting they may play a primary role in the regulation of this process (Kuhn et al., 2014).

Berry ripening is also strongly affected by environmental factors: in particular, light exposure regulates the flavonoid pathway and promotes flavonol and anthocyanin synthesis that results in a a deeper berry skin coloration in red cultivars (Jackson, 2014; Kuhn et al., 2014). Temperatures is another important parameter: low temperatures are necessary to increase total soluble solid and anthocyanin content and to decrease total acidity, while high temperatures have negative effects on berry ripening, causing a reduction in berry weight, total soluble solid, anthocyanins and flavonol contents (Kuhn et al., 2014). Water and inorganic nutrients supply also affect berry ripening: a moderate water deficit after *veraison* can be beneficial to grape quality, because it enhances anthocyanin synthesis and limits berry enlargement, but it also increases stilbenoids and sugar contents (Jackson, 2014). Regarding inorganic nutrients, low soil nitrogen and phosphorus deficiencies are known to increase anthocyanin content while high potassium levels can increase berry juice pH and, thereby, lower wine color (Jackson, 2014).

Finally, the very detailed transcriptomic maps produced in the last years evidenced that berry development and ripening are characterized by a fine genetic regulation (Fasoli et al., 2012; Massonnet et al., 2017; Fasoli et al., 2018); however, excluding some processes, such as for example the regulation of anthocyanin synthesis(Walker et al., 2007; Kobayashi et al., 2004), and the identification of the gene responsible of the *flb* (*fleshless berry*) mutant phenotype (Fernandez et al., 2006), the precise function and contribution of the huge amount of genes modulated during berry development and ripening remain unknown.

Climate changes, grape quality and intervention strategies

Control of the ripening timing, berry size and coloration, acidity and the relative assortment of volatile and non-volatile aroma and flavor compounds in wine grape cultivars are major concerns to viticulturists (Conde et al., 2007). Continued and specific study of the key control points in grape ripening are crucial to improve grape and wine quality. One of the most

important factors affecting these parameters is represented by weather and climate change projections for the 21st century is expected to have important impacts on viticulture. In fact, grapevine physiology and fruit metabolism/composition are highly influence by the mean temperature along the growing season and extreme heat or heat weaves may also permanently affect vine physiology and yield attributes (Fraga et al., 2012). Winemaking regions under extremely hot temperatures may lead to a significant increase in the risk of organoleptic degradation and wine spoilage. In particular, higher temperatures may inhibit the formation of anthocyanin thus reducing grape color and increasing volatilization of aroma compounds. Under a future warmer climate, springtime warming may lead to earlier budburst and continue increases in temperature cause a trend towards earlier flowering, *veraison* and harvest. The timing of *veraison* may be of importance, because earlier *veraison* implies that the critical ripening period shifts towards the hotter part of the season (Keller, 2010). This change of the timing of grape ripening and harvest date may affect grape quality and yield and consequently wine quality. Altogether, the profound climate changes, the modification of grape quality and therefore the production of high-quality wine, could have substantial consequences for the global economy of wine industry.

To reduce the negative effects of climate changes, many adaptation strategies, represented especially by agricultural practices, have been performed. Among them, there are the late winter pruning, late irrigation, late defoliation, the use of sunscreens for leaf protection, the use of specific products affecting the maturation phases (especially auxin and cytokinin) and the increase of buds to produce more grapes and to slow down ripening (Palliotti et al., 2012). All these short-term management practices are finalized to regulate/delay the maturation, avoiding the alteration of grape quality caused by temperatures increase. The positive results obtained by their application indicate that these agricultural practices are efficient; however most of them are based on principles related to traditional viticulture. The interpretation of the molecular mechanisms involved in the regulation of berry ripening could provide important information about these processes and allow the development of more effective intervention strategies.

Molecular studies of berry development and ripening

The recent grapevine genome sequencing (Jaillon et., 2007) has allowed to perform many molecular studies related to grapevine development and berry ripening. One of the most important is represented by the generation of grapevine gene expression atlas (Fasoli et al., 2012, see Chapter 2 for a more detailed description). This complex work has showed a deep transcriptomic shift during the immature-to-mature shift in all grapevine organs, suggesting the existence of key regulators genes involved in the regulation of this complex phase transition. These specific genes, named switch genes (Palumbo et al., 2014), are expressed at low level in immature organs but their expression increase considerably in mature organs, indicating that they are a specific role in the regulation of transcriptomic changes during ripening process in grapevine. To obtain more information about the regulation of berry ripening process, the specific switch genes involved in the immature-to-mature transition in both red and white berry have been identified (Palumbo et al., 2014; Massonnet et al., 2017, see Chapter 2 for a more detailed description). The comparison of switch genes of grapevine expression atlas and switch genes berry specific shows that many genes are common while others are specific of only one transcriptomic dataset. Switch genes of both expression atlas and berry transcriptome include genes belong to many functional categories, from carbohydrate metabolic process and cell wall metabolism to response to hormone stimulus and secondary metabolic process, but the functional category overrepresented in both datasets is transcription factor activity. These results suggest a fundamental role of transcription factors in the regulation of immature-to-mature in grapevine organs, including berry.

These works have provided important information about the transcriptional changes between green and mature berries, but the specific molecular mechanism controlling the onset of berry ripening remain unknown. To further improve these molecular knowledges related to berry ripening and to better identify the key genes involved in the regulation of this process, transcriptomic analysis of berry at different developmental stages (from fruit set to full maturity) has been performed (Fasoli et al., 2018, see Chapter 2 for a more detailed description), confirming again the transcriptional shift during immature-to-mature transition and suggesting the existence of specific key regulators genes. Furthermore, the transcriptomic analysis of berry around veraison (early veraison, mid-veraison and late veraison), showed that the onset of berry ripening could be represented by two molecular transitions starting from 14

days before veraison (Figure 2). Positive biomarkers of transition 1 were characterized by a stronger induction 14 days before veraison than positive biomarkers of transition 2, indicating the first set of genes trigger the expression of the second set, which in turn mediates the different processes that characterize ripening. As previously described for switch genes, the positive biomarkers of both transitions are represented by genes involved in many processes, such as response to hormone stimulus, cell wall metabolism and secondary metabolic processes, but one of the most represented functional categories are transcription factors. Furthermore, many of them were already identified as switch gene of expression atlas and of both red and white berry transcriptome. This data indicates that transcription factors are key genes during the immature-to-mature transition and they have a very important role in the regulation of berry development and ripening.

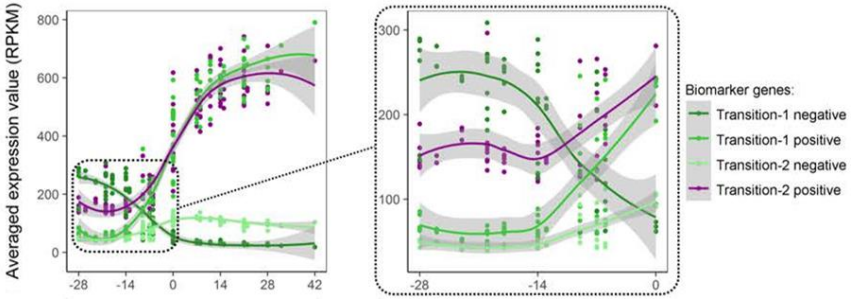

Figure 2: the averaged expression profile of transition-specific putative biomarker genes shown over the whole of development (left plot) and during pre-*veraison* phase (right plot).

Gene transfer technologies in grapevine

After the identification of a specific gene of interest, the next phase is represented by its functional characterization. In fact, the genetic improvement of grapevine can be performed only after the complete characterization of the gene of interest. Gene transfer technologies are very useful for this purpose. In grapevine, the most important are stable genetic transformation and the transient gene expression (Jelly et al., 2014). Stable transformation allows the study of stable gene expression at the whole plant level, in different tissues and at different developmental stages; it is based on *Agrobacterium tumefaciens*-mediated transformation of embryogenic culture derived by explants of stamens and pistils or leaves. It is a complex, long and random process, with a very low efficiency; it is characterized by numerous limitations, include poor embryogenic potential of genotypes, wide variations among varieties in their

response to genetic transformation, *Agrobacterium*-induced post-cocultivation necrosis of embryogenic cultures, and poor plant recovery from transformed somatic embryos. However, the grapevine stable transformation and the regeneration of transformed plants has been reported in some works (Iocco et al., 2001; Li et al., 2006, 2008, 2015; Dhekney et al., 2009; Kandel et al., 2016). On the other hand, transient expression provides the most efficient way to study many genes in a very short time (Jelly et al., 2014). It is based on temporary, high-level transcription of DNA sequences that do not necessarily integrate into the plant genome. Methods for transient gene expression in plants mainly involve *Agrobacterium* tumefaciens-mediated transformation: during a short period immediately following the cultivation with *Agrobacterium tumefaciens*, many copies of the transgene are actively transcribed in the plant cells, allowing a high expression of gene of interest. Leaf agro-infiltration represents a major historic breakthrough in transient expression assays. It is easy and rapid, and it is based on the forced infiltration of *Agrobacterium tumefaciens* into the intercellular spaces of the leaf parenchyma, using a needleless syringe or a vacuum pump. Many reports (Zottini et al., 2008; Bertazzon et al., 2012; Santos-Rosa et al., 2008; Ben-Amar et al., 2013) have stated the success of grapevine leaf agroinfiltration using both methods above mentioned.

Both stable genetic transformation and transient gene expression approaches cane be used for the functional analysis of gene of interest. However, despite its simplicity and rapidity, transient expression represents a good strategy for a rapid and preliminary study of gene function but a complete characterization of gene of interest can be performed by the stable transformation. Furthermore, the generation of genetically modified grapevine plants harboring important traits is fundamental for the genetic improvement of grapevine (Vidal et al., 2010).

Genetic improvement of grapevine

In the last years, the economic importance of grapevine has considerably increased the studies related with its genetic modification. The historic approach used for grapevine genetic improvement is represented by conventional breeding. This method was largely used during 19th century to generate grapevine plants resistant to fungal diseases, especially against phylloxera (Riaz et al., 2007). Breeding requires a cross between parent plants characterized by genomes can interact between them, with the formation of progeny that combine both the positive and negative traits from each parent. Based on searched trait, the progeny

performance is evaluated during the growth, keeping the plants with desired trait and discarding the others. The presence of undesirable traits, the long time before the fruit is produced and the difficulty to obtain a specific set of genes to confer improved properties, make the use of this system in grapevine very complex and time-consuming. An alternative, more prominent approach of genetic improvement is represented by genetic engineering; this method has been further implemented after the complete sequencing of grapevine genome (Jaillon et al, 2007). As previously described, stable transformation, the most representative technology of genetic engineering, can be used for the functional analysis of gene of interest, but also for the generation of genetically modified plants, obtained by transferring of single gene coding for specific traits, with the minimum alteration of the original genome. Transgenic plants could show many advantages than non-transformed wild type plants, such us biotic or abiotic stress resistance or higher capacity of production, but the introduction of exogenous DNA sequences, the use of a transformation agent, *Agrobacterium tumefaciens*, the incomplete understanding of the genetic mechanisms underlying the trait of interest and the substantial scepticism among the general public, hinders or limits the complete use of these type of plants (Holme et al., 2013). An alternative to transgenic technology is represented by cisgenesis: the definition of this approach is: "Full CDS including introns of a gene originating from the sexually compatible gene pool of the recipient plant" (Schouten et al., 2006). This method avoids the use of exogenous DNA, but the use of *Agrobacterium* as transformation agent to obtain cisgenic plants and the scepticism related to transformed plants could still limit the application of this technology. A recent and innovative approach of genetic modification of plants, including grapevine, is represented by genome editing. This method enables targeted genome modification using sequence-specific nucleases, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) system (Yin et al., 2017). These sequence-specific nucleases generate double-stranded breaks (DSBs) at targeted genome sites, which are generally repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR), which lead to gene knockout or gene replacement, respectively. In particular, the CRISPR/Cas system can be apply in transformable plants for functional characterization of gene of interest and to improve delivered traits; to this aim, it is possible to use both *Agrobacterium*-based traditional transformations systems, to deliver in

plant plasmids containing specific CRISPS-Cas expression cassette, and DNA and *Agrobacterium*-free systems, based on use of ribonucleoparticles (RNPs), a mixture of Cas protein and RNA guide. The last approach is very prominent for the genetic improvement of grapevine, because the absence of *Agrobacterium* and of sequences of exogenous DNA and the minimal modification of the genome, would allow the generation of plants indistinguishable from those obtained by conventional breeding, exempting them from the current genetic modified organisms (GMOs) regulations.

Outline of the thesis

The great goal of this PhD project was the identification and functional characterization of putative master regulators of the onset of berry ripening. This main topic was flanked by the application, development and improvement of technologies for functional studies and for genetic improvement of grapevine. The genes selected are the transcription factors *VviNAC33*, *VviNAC60*, *VviAGL15a*, *VviWRKY19*, *VvibHLH75*: they are five switch genes of red and white berry transcriptomes (Palumbo et al., 2014; Massonnet et al., 2017) and, excluding *VviAGL15a*, they were identified as markers of first transition of *veraison* (Fasoli et al., 2018). The functional characterization of genes selected was performed using stable genetic transformation and transient gene expression approaches. The phenotypic and molecular analysis of transgenic plants allowed to obtain important information about their roles in the regulation of the onset of berry ripening.

Chapter 2 describes the analysis of different transcriptomic dataset of grapevine and berry development and ripening, the selection of the best candidates for functional characterization in grapevine and the analysis of their expression profile in different grapevine organs and developmental stages and correlated genes.

Chapter 3 reports the application of gene transfer technology in grapevine. To identify a standard and defined protocol, stable genetic transformation was tested using different methods in different cultivars and *gfp* as reporter gene. Regarding the transient gene expression, the use of *yfp* as reporter gene allowed to improve the leaf agroinfiltration, identifying the best expressing leaves at the day post infiltration of maximum expression, and to develop the berry agroinfiltration.

In the **Chapter 4**, the functional analysis of *VviNAC33* and *VviNAC60* by stable genetic transformation was performed. In a previous work (D'Incà, 2017), their overexpression showed an upregulation of many genes involved in the maturation process, suggesting their involvement in the regulation of grapevine development. Here, their conversion into transcriptional repressors confirmed previous results, showing a downregulation of some target of both NAC genes, and allowed to complete the characterization of these two transcription factors.

In the **Chapter 5,** *VviAGL15a*, *VviWRKY19* and *VvibHLH75* were functionally characterized by transient overexpression, using the improved leaf agroinfiltration approach based on YFP

expression. The exhaustive molecular analysis of overexpressing leaves allowed to identify their target genes and to better define their specific roles in the transcriptional regulatory network controlling the onset of berry ripening.

Finally, the **Chapter 6** describes the application of a protocol to isolate grapevine protoplast from embryogenic calli and to regenerate plants by somatic embryogenesis. Plant regeneration and the positive results obtained after protoplast transfection using a plasmid carrying the *yfp* reporter gene, indicate that this technology can be used for many studies, including the application of genome editing by CRISP-Cas system.

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Chapter 2

IDENTIFICATION OF MASTER REGULATORS OF THE ONSET OF BERRY RIPENING AND CANDIDATES' SELECTION FOR FUNCTIONAL CHARACTERIZATION

ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the most economically important fruit crops in the world. Qualitative characteristics of grape are acquired during berry development and ripening phases and they are widely affected by both agronomic practicals and environmental factors. In particular, the negative effects related to ripening anticipation, caused by high temperature season, are well known. The onset of ripening (*veraison*) is a complex developmental process, influenced by many exogenous and endogenous factors, whose molecular bases are only partially known. In the last years, several molecular studies showed that *veraison* is characterized by a profound transcriptomic reprogramming and some genes, which are promptly induced during this process, could be key master regulators of berry ripening. The identification of their functions could allow to control the timing of ripening initiation. Among these genes, the functional category overrepresented are transcription factors and some of them have been selected to functionally characterized. They are *VviNAC33*, *VviNAC60*, *VviAGL15a*, *VviWRKY19* and *VvibHLH75*, belonging to four of the most important transcription factors families in plants. The analysis of their expression profile in different grapevine organs at different developmental stages and the coexpression analysis showed that they are characterized by a high expression level only at *veraison* and in the mature berry and that their highly correlated putative target genes are involved in processes associated with ripening. These results suggest their specific role in the regulation of this process.

1. INTRODUCTION

Grapevine (*Vitis vinifera L.*) is one of the major fruit crops in the world. The economic importance of grapevine is closely correlated to fruit quality; grape is highly valued for its multiple uses as a fresh fruit and processed food product such as jelly, juice, raisins, and especially wine. The grape quality characteristics are acquired during berry formation, growth and ripening phases and they are widely affected by agronomic and environmental factors. In a context of profound climate changes, these specific characteristics could be highly altered, with dramatic consequences for wineries basing the identification of their products on grape varieties cultivated in determinate geographical areas. In particular, the high temperatures cause a ripening anticipation, with deep changes in the biochemical and physiological characteristics of grape and consequently the final qualities of grape. In some cases, these changes can be modulated by adopting specific agronomical practices that may not be economically sustainable. The interpretation of the molecular mechanisms controlling the maturation process in grapevine could provide better strategies to control and manipulate grape ripening preserving/enhancing specific quality characteristics.

Veraison is a key event during the berry ripening and it coincides with the onset of ripening. During *veraison* the main events involved in the shift from immature to mature berry take place. This complex transition is marked by the colouring and softening of berries, related to important biochemical and physiological changemost of which are well known. On the other hand, the molecular mechanisms involved in the regulation of this process are only partially known and in the last years, many studies have been performed to discover the transcriptional programs associated to *veraison* and the ripening process.

One of them is represented by the generation of grapevine global genes expression atlas (Fasoli et al., 2012). This analysis was carried out in 54 different samples of *Vitis vinifera* cv Corvina representing green and mature organ and tissue at different development stages using a comprehensive grapevine genome microarray. This research has revealed a clear distinction between the green/vegetative and woody/mature sample transcriptomes, suggesting a fundamental shift in global gene expression as the plant switches from the immature to the mature developmental program. These results indicate the existence of specific regulatory genes that promote the vegetative-to-mature transcriptomic transition. The identification of the key genes involved in deep transcriptome shift that occurs in grapevine was carry out using

a gene network analysis (Palumbo et al, 2014). Using the differential expressed genes among woody/mature organs and vegetative/green organs, a co-expression network has been generated. The specific topological properties of the co-expression network were analyzed and a subset of 113 genes was classified as switch genes. These switch genes of the global gene expression atlas were expressed at a low level in vegetative/green tissues but at significantly higher levels in mature/woody organs, suggesting they participate in the regulation of the transition from immature to mature development. Among these, the functional categories overrepresented are secondary metabolic process, carbohydrate metabolic process and transcription factors activity. Regarding the transcription factors, two *LATERAL ORGAN BOUNDARIES DOMAIN*, two *NAC DOMAIN-CONTAINING PROTEINS* and many *ZINC FINGER* have been identified.

To elucidate the immature-to-mature transition in berry, the most important organ in grapevine, and to identify the key genes of this process, the same approach was used (Palumbo et al, 2014; Massonnet et al., 2017). Berries from 5 red and 5 white varieties were sampled at different phenological stages and the transcriptomic profiles were obtained by RNA-Seq. After the identification of differential expressed genes, the gene co-expression network has revealed the existence of 190 switch genes for red varieties and 212 switch genes. They are likely to be involved in the regulation of the grape berry development transition and they are expressed at low level during the immature phase and were significantly induced at *veraison*. Among the switch genes of both red and white varieties, the functional categories overrepresented are carbohydrate metabolic process, cell wall metabolism, secondary metabolic process and transcription factor activity. Among the transcription factors of both sets of switch genes, 1 *MADS-box* gene, 3 *LATERAL ORGAN BOUNDARIES DOMAIN*, 4 *NAC DOMAIN-CONTAINING PROTEINS*, 3 *MYBA* genes, 3 *WRKY* genes, 3 *bHLH* genes and many *ZINC FINGER* have been identified. Among the switch genes, the identification of a high number of transcription factors could indicate the existence of a specific transcriptional regulatory controlling the onset of berry ripening.

To further improve the molecular knowledge related to berry ripening and to better identify the key genes involved in the regulation of this process, transcriptomic analysis by RNA-Seq of berry of two different cultivars, Cabernet Sauvignon and Pinot Noir, at different developmental stages (from fruit set to full maturity) has been performed (Fasoli et al., 2018). The results

showed that during the progress of berry development and maturation, transcripts are divided into four classes: genes expressed during pre-*veraison* (Class 1), during *veraison*/mid-ripening (Classes 2 and 3) and during later ripening (class 4). The expression of class 1 transcripts rapidly decreases during berry development, transcripts of classes 2 and 3 show a peak at *veraison* and subsequently declining while genes of class 4 are expressed during late-ripening stages with increasing expression throughout development. These data confirming the transcriptional shift during immature-to-mature transition and suggesting again the existence of specific key regulators genes. To obtain more information about the molecular mechanisms that regulate the transition from lag phase to ripening, the berry transcriptome around *veraison* (early *veraison*, mid-*veraison* and late *veraison*) have been analyzed. The results showed that *veraison* could be resolved into two back-to-back molecular transitions starting from 14 days before veraison. Each transition contains positive and negative molecular biomarkers; negative biomarkers of transition 1 showed a very low expression level while negative biomarkers of transition 2 showed a more complex trend with a small initial increase of expression until *veraison* followed by a very slight decline thereafter. Indeed, the positive biomarkers of transitions 1 and 2 showed similar upward expression, but they differed during the pre-*veraison* phase. Positive biomarkers of transition 1 started at very low expression levels, but they were characterized by a strong induction 14 days before *veraison* and their average expression value doubled in less than 1 week. In contrast, positive biomarkers of transition 2 started with a higher level of expression but their upregulation 14 days before *veraison* occurs at a slower rate than the transition-1 positive biomarkers. This result could indicate that the first set of genes trigger the expression of the second set, which in turn mediates the different processes that characterize ripening. Furthermore, as previously described for switch genes, among the positive biomarkers of both transitions, there are genes involved in many processes, such as response to hormone stimulus, cell wall metabolism and secondary metabolic processes, but one of the most represented functional categories are transcription factors. Furthemore, many of these transcription factors have been identified as switch genes of grapevine expression atlas and of both red and white berry transcriptomes. This data indicates that these genes have a very important roles in the regulation of berry development and ripening.

Altogether, these results have provided important and detailed information about the specific set of genes, represented especially by transcription factors, putatively controlling the

development and ripening of grape berry. At this point, an in-depth functional characterization of specific transcription factors is necessary for a complete interpretation of the molecular mechanism involved in the regulation of the onset of berry ripening.

In this chapter, the close inspection of the expression and co-expression behavior of switch genes has allowed to select specific transcription factors representing candidates to be functionally characterized to define their putative role of master regulators of grapevine berry ripening.

2. MATERIALS AND METHODS

2.1 Gene selection criteria

The selection of candidate genes for functional analysis was performed using four criteria: 1) their belonging to the functional category of transcription factors, 2) their role as switch genes in both grapevine expression atlas (Fasoli et al., 2012) and in the transcriptomic dataset of red and white berries (Massonnet et al., 2017), 3) their identification as marker of first and/or second transition during the onset of berry ripening (Fasoli et al., 2018) and 4) the specific biological role of the gene family they belong to.

2.2 Expression analysis of selected genes in grapevine

The expression profiles of *VviNAC33*, *VviNAC60*, *VviAGL15a*, *VviWRKY19* and *VvibHLH75* were analyzed in the *Vitis vinifera* cultivar Corvina (clone 48) gene expression atlas of different organs at various developmental stages (Fasoli et al., 2012). 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 selected genes were also analyzed in a berry specific expression map. Transcriptomic data were obtained by RNA-Seq performed on whole berry samples collected from 10 different grapevine varieties (Sangiovese, Barbera, Negroamaro, Refosco, Primitivo, Vermentino, Garganega, Glera, Moscato, Passerina) at four different developmental stages (Massonnet et al., 2017).

Finally, the expression analysis of *VviNAC33*, *VviNAC60*, *VviAGL15a*, *VviWRKY19* and *VvibHLH75* was performed using a berry specific expression map of both Cabernet Sauvignon and Pinot

Noir cultivars (Fasoli et al., 2018). Transcriptomic data were obtained by RNA-Seq using berry samples collected every 7 to 10 days, from fruit set to full maturity.

2.3 Co-expression analysis

The gene co-expression analyses of *VviNAC33*, *VviNAC60*, *VviAGL15a*, *VviWRKY19* and *VvibHLH75* was performed using the global gene expression dataset of *V. vinifera* cv. Corvina obtained by microarray approach (Fasoli et al., 2012) by means of the CorTo software (http://www.usadellab.org/cms/index.php?page=corto), setting Pearson's coefficient as correlation metric.

3. RESULTS

3.1 Candidate selection

The candidate genes selected for functional characterization belong to the functional category of transcription factors, one of the most represented among the list of switch genes (Palumbo et al., 2014; Massonnet et al., 2017). The selection has been performed by analyzing and combining the list of transcription factors identified as switch genes in the grapevine expression atlas and in the transcriptomic dataset from red and white berries (Table 1). Another level of selection comes from the information obtained by Fasoli et al. (2018) that indicates some of these transcription factors as marker of first and/or second transition during the onset of berry ripening. Finally, the transcription factors to be functionally analyzed have been selected based on specific role of the gene family they belong to.

Table 1: Transcription factors identified as switch genes of grapevine expression atlas (A), of red (R) and white (W) berry transcriptome and marker of the first and/or second transition of the onset of ripening.

VIT	FUNCTIONAL ANNOTATION	A	R	W	Marker of the 1° transition	Marker of the 2° transition
VIT_17S0000G00430	basic helix-loop-helix (bHLH) family (VvibHLH75)		*	*	*	
VIT_11S0037G01230	basic helix-loop-helix (bHLH) family			*		
VIT_05S0077G00750	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_12S0028G00980	myb family	*				
VIT_07S0031G01930	myb TKI1 (TSL-KINASE INTERACTING PROTEIN 1)		*	*		
VIT_02S0033G00380	Myb VvMYBA1		*	*		
VIT_14S0108G01070	NAC domain-containing protein (VviNAC11)		*	*		
VIT_02S0012G01040	NAC domain-containing protein (VviNAC13)		*	*		
VIT_19S0027G00230	NAC domain-containing protein (VviNAC33)	*	*	*	*	
VIT_08S0007G07670	NAC domain-containing protein (VviNAC60)	*	*	*	*	
VIT_13S0158G00100	putative MADS-box Agamous-like 15a (VviAGL15a)		*	*		
VIT_02S0033G00410	VvMybA1		*	*		
VIT_02S0033G00390	VvMybA2		*	*		*
VIT_02S0033G00450	VvMybA3		*	*		
VIT_17S0000G01280	WRKY Transcription Factor (VviWRKY75)		*			
VIT_07S0005G01710	WRKY Transcription Factor (VviWRKY19)		*	*	*	*
VIT_12S0059G00880	WRKY Transcription Factor (VviWRKY37)			*		
VIT_13S0064G01210	Zf A20 and AN1 domain-containing stress-associated protein 2		*			
VIT_13S0047G01130	Zfwd2 protein (ZFWD2)	*				
VIT_10S0071G00580	Zfwd2 protein (ZFWD2)	*				
VIT_12S0059G02510	Zinc finger (B-box type)		*			
VIT_00S0347G00030	Zinc finger (B-box type)	*		*		
VIT_00S0203G00210	Zinc finger (B-box type)	*		*		
VIT_06S0061G00760	Zinc finger (C2H2 type) family		*			
VIT_06S0004G04180	Zinc finger (C2H2 type) protein (ZAT11)	*				
VIT_14S0219G00040	Zinc finger (C3HC4-type ring finger)	*	*		*	
VIT_05S0020G04730	Zinc finger (C3HC4-type ring finger)		*	*		*
VIT_08S0040G01950	Zinc finger (C3HC4-type ring finger)	*	*	*	*	
VIT_18S0001G01060	Zinc finger (C3HC4-type ring finger)	*	*	*		

VIT_12S0028G03860	Zinc finger (C3HC4-type ring finger) protein (RMA1)		*			*
VIT_03S0091G00260	Zinc finger protein 4		*	*	*	

Based on the criteria previously described, five transcription factors have been selected for functional characterization. The first two genes are represented by *VviNAC33* (VIT_19S0027G00230) and *VviNAC60* (VIT_08S0007G07670). They are two switch genes of grapevine expression atlas and of both red and white berry transcriptome; furthermore, they are two markers of the first transition of the onset of ripening. NAC transcription factors family is one of the most important transcription factors families in plants; their involvement in plant growth regulation and response to abiotic and biotic stress emerged after many studies in *Arabidopsis thaliana* and rice. Regarding grapevine, a comprehensive analysis of NAC transcription factors has been performed (Wang et al., 2013), but the specific biological function of each of them remains unknown. The functional analysis of these two transcription factors has been initiated in a previous project (D'Incà, 2017) that highlighted an effective role of these two genes in the regulation of vegetative-to-mature transition. A more detailed description of these preliminary results and other information about NAC transcription factors are reported in the Chapter 4.

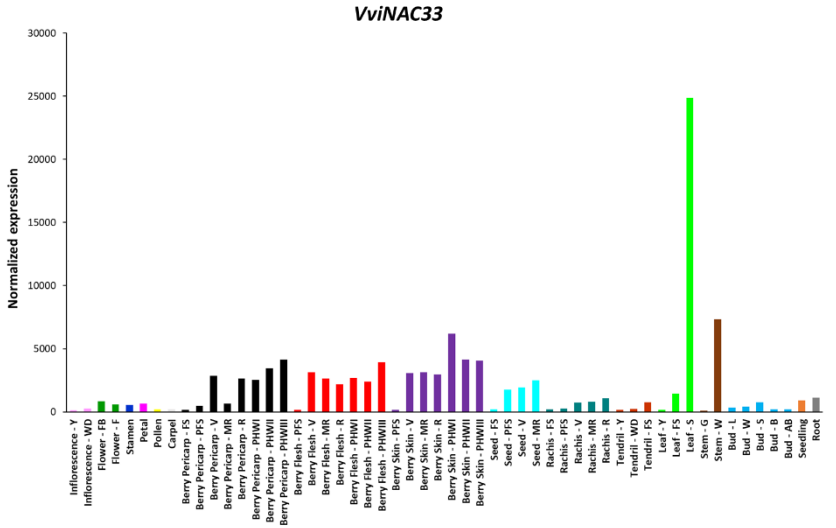
The other three transcription factors selected are *VviAGL15a* (VIT_07S0005G01710), *VviWRKY19* (VIT_13S0158G00100) and *VvibHLH75* (VIT_17S0000G00430). They are three switch genes of both red and white berry transcriptomes; furthermore, *VviWRKY19* and *VvibHLH75* are two markers of the first transition and *VviWRKY19* is also a marker of the second transition of the onset of ripening. These three transcription factors belong to three large families of transcription factors in plants: *VviAGL15a* belongs to the MADS-box transcription factors family, involved especially in process related to reproductive development (Gramzow and Theissen, 2010), *VviWRKY19* belongs to the WRKY transcription factor family, whose main roles are biotic and abiotic stress responses (Schluttenhofer and Yuan, 2015) and *VvibHLH75* belongs bHLH transcription factors family, involved in many processes, from hormone signaling and regulation of secondary metabolism to flower and fruit development (Carretero-Paulet et al., 2010). Each of these transcription factors families has been described in grapevine (Grimplet et al., 2016; Wang et., 2014; Wang et al., 2018). A more detailed description of these transcription factors and their gene families is reported in Chapter 5.

3.2 Expression profiles of the selected transcription factors

The expression analysis of *VviNAC33*, *VviNAC60, VviAGL15a*, *VviWRKY19*, *VvibHLH75*, was determined by inspecting the grapevine gene expression atlas (Fasoli et al., 2012), the transcriptome dataset of both red and white varieties (Massonnet et al, 2017) and the transcriptomic data related to berry ripening of Cabernet Sauvignon and Pinot Noir (Fasoli et al., 2018)

The expression profiles of each transcription factor in the gene expression atlas are shown in Figure 1. Regarding *VviNAC33* (Figure 1A), it shows a very high expression during senescence phase in leaves, but it also expressed in berry pericarp, flesh and skin during post-*veraison* phases and in woody stem. The expression of *VviNAC60* (Figure 1B) occurs especially in berry pericarp, flesh and skin during post-*veraison* phases but this gene is also expressed in woody stem, senescent leaf, rachis and seed during post-*veraison* phases. Regarding *VviAGL15a* (Figure 1C), its expression occurs mainly in berry pericarp, flesh and skin, starting from *veraison* phase and continuing until post-harvest phases; furthermore, VviAGL15 show a very high expression in stamen and pollen and while its expression in seed decrease from fruit set phase to *veraison* phase. The expression of *VviWRKY19* (Figure 1D) is very high in berry pericarp, flesh and skin during all post-*veraison* phases, but it is very high also in rachis at post-*veraison* phases. Moreover, *VviWRKY19* is expressed in seed at *veraison* and mid-ripening phases, in woody stem and bud and in roots. Finally, regarding *VvibHLH75* (Figure 1E), this gene is preferentially expressed in berry pericarp, flesh and skin during all post-*veraison* phases and in rachis after *veraison*. The high expression of *VviNAC33* and *VviNAC60* in each mature/woody organ/tissue confirms their putative role of master regulators of the vegetative-to-mature transition of various organs while the preferential expression of *VviAGL15a*, *VviWRKY19* and *VvibHLH75* in berry during post-*veraison* phases confirms their role of putative master regulators of the onset of berry ripening.

A


VviNAC33
Normalized expression
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25000
20000
15000
10000
5000
0
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
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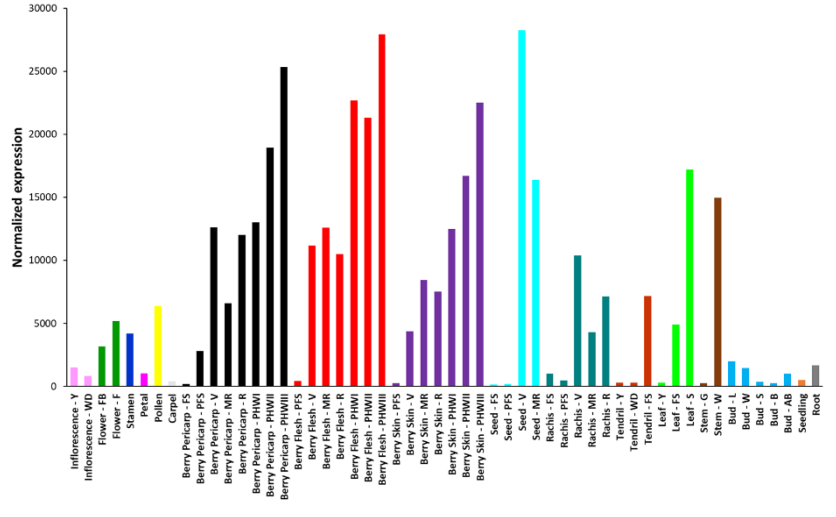
Stem - W
Bud - L
Bud - W
Bud - S
Bud - B
Bud - AB
Seedling
Root

B


VviNAC60
Normalized expression
30000
25000
20000
15000
10000
5000
0
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
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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
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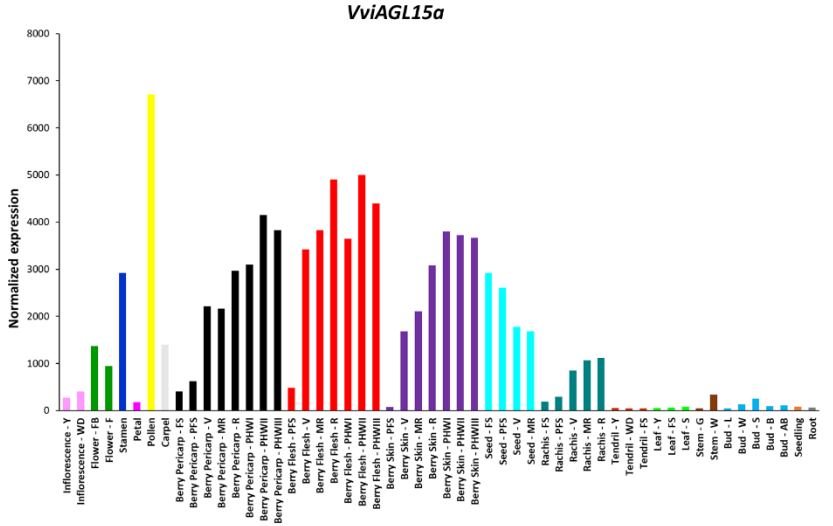
Stem - G
Stem - W
Bud - L
Bud - W
Bud - S
Bud - B
Bud - AB
Seedling
Root

C


VviAGL15a
Normalized expression
8000
7000
6000
5000
4000
3000
2000
1000
0
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
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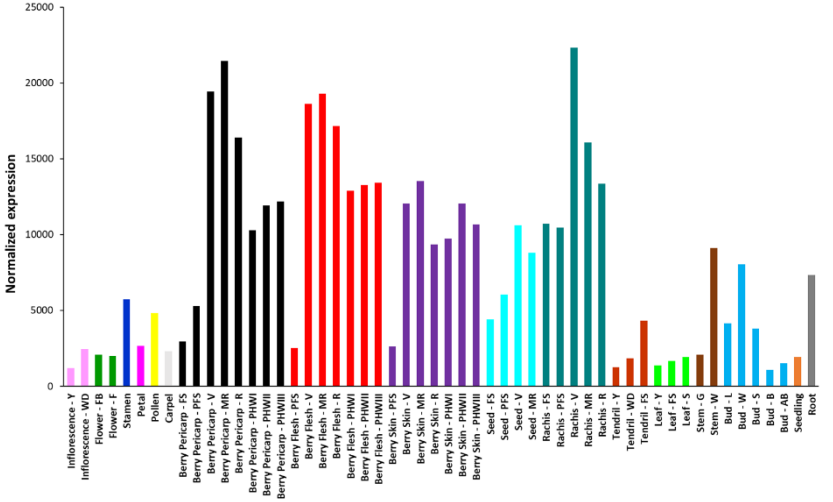
Berry Skin - PHWII
Berry Skin - PHWIII
Seed - FS
Seed - PFS
Seed - V
Seed - MR
Rachis - FS
Rachis - PFS
Rachis - V
Rachis - MR
Rachis - R
Tendril - Y
Tendril - WD
Tendril - FS
Leaf - Y
Leaf - FS
Leaf - S
Stem - G
Stem - W
Bud - L
Bud - W
Bud - S
Bud - B
Bud - AB
Seedling
Root

D


VviWRKY19
Normalized expression
25000
20000
15000
10000
5000
0
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

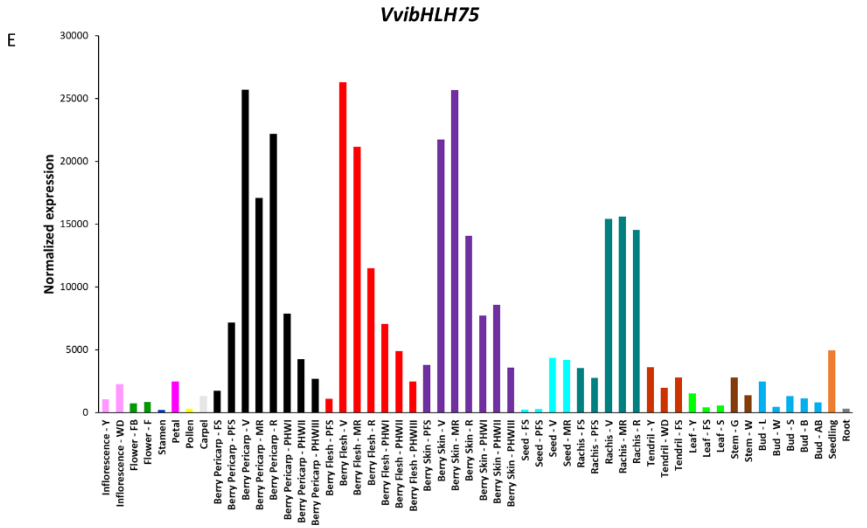
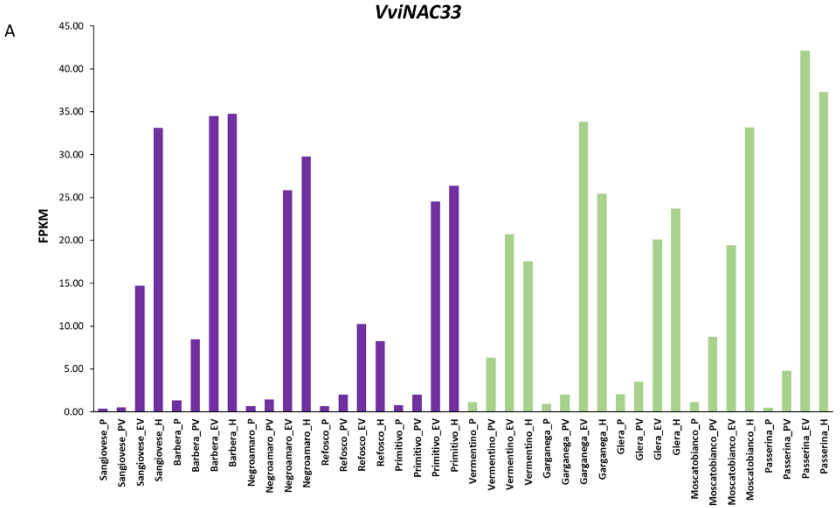


Figure 1: *VviNAC33* (A), *VviNAC60* (B) *VviAGL15a* (C), *VviWRKY19* (D) and *VvibHLH75* (E) expression profiles in 54 grape organs at different developmental stages; transcriptomic data were obtained by a global expression map of Vitis vinifera cv. Corvina by microarray (Fasoli et al., 2012).

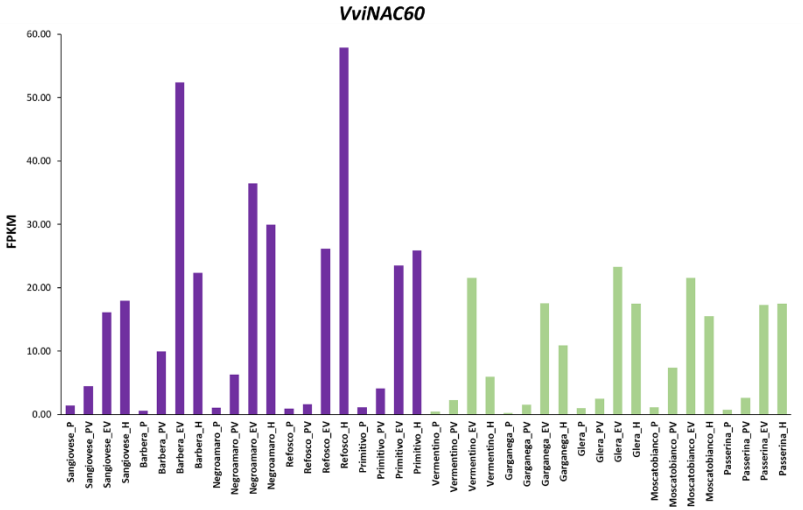
Description of ATLAS abbreviations:
Bud – L = latent bud; – W = winter bud; – S = bud swell; – B = bud burst; – AB = bud after-burst; **Inflorescence** – Y = young inflorescence; – WD = well developed inflorescence; **Flower** – FB = flowering begins; – F = flowering; **Stamen** = pool of stamen from 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 = postharvest 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 expression profile of *VviNAC33, VviNAC60, VviAGL15a*, *VviWRKY19* and *VvibHLH75* retrieved from the berry transcriptomic survey of Massonnet et al., 2017 show that each transcription factors is mainly expressed in both red and white berries at end of *veraison* and harvest phases (Figure 2). The expression of *VviNAC33* (Figure 2A) and *VviNAC60* (Figure 2B) is slightly different among the varieties and it shows maximum level of expression at end of

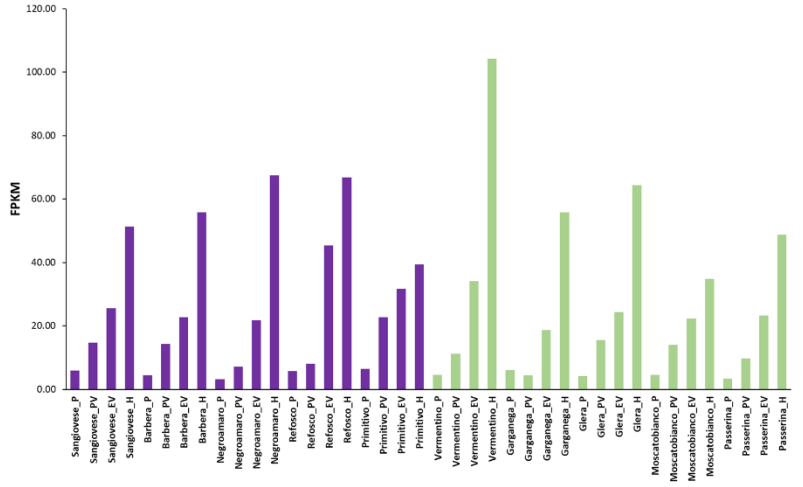
veraison or harvest phase depending on the cultivars. In particular, *VviNAC33* show a higher expression during harvest phase in all varieties, excluding Refosco, Vermentino, Garganega and Passerina, while the expression of *VviNAC60* is higher at end of *veraison* phase in all varieties, excluding Sangiovese, Refosco, Primitivo and Passerina. The expression of *VviNAC33* and *VviNAC60* follow the same trend showed in Figures 1A and 1B, with high expression in berry tissues during post-*veraison* phases. Regarding *VviAGL15a* (Figure 2C), its maximum expression value in both red and white varieties occurs during harvest phase, confirming the results previously described using the grapevine expression atlas (Figure 1C). *VviWRKY19* (Figure 2D) and VvibHLH75 (Figure 2E) are preferentially expressed during end of *veraison* phase in each variety, confirming again the results previously described (Figure 1D, E). These results confirm again the high expression of the five transcription factors only in ripening berry and support their role of master regulators of the vegetative-to-mature transition in berry.



B


VviNAC60
FPKM
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Sangiovese_P
Sangiovese_PV
Sangiovese_EV
Sangiovese_H
Barbera_P
Barbera_PV
Barbera_EV
Barbera_H
Negroamaro_P
Negroamaro_PV
Negroamaro_EV
Negroamaro_H
Refosco_P
Refosco_PV
Refosco_EV
Refosco_H
Primitivo_P
Primitivo_PV
Primitivo_EV
Primitivo_H
Vermentino_P
Vermentino_PV
Vermentino_EV
Vermentino_H
Garganega_P
Garganega_PV
Garganega_EV
Garganega_H
Glera_P
Glera_PV
Glera_EV
Glera_H
Moscatobianco_P
Moscatobianco_PV
Moscatobianco_EV
Moscatobianco_H
Passerina_P
Passerina_PV
Passerina_EV
Passerina_H

C


VviAGL15a
FPKM
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Sangiovese_P
Sangiovese_PV
Sangiovese_EV
Sangiovese_H
Barbera_P
Barbera_PV
Barbera_EV
Barbera_H
Negroamaro_P
Negroamaro_PV
Negroamaro_EV
Negroamaro_H
Refosco_P
Refosco_PV
Refosco_EV
Refosco_H
Primitivo_P
Primitivo_PV
Primitivo_EV
Primitivo_H
Vermentino_P
Vermentino_PV
Vermentino_EV
Vermentino_H
Garganega_P
Garganega_PV
Garganega_EV
Garganega_H
Glera_P
Glera_PV
Glera_EV
Glera_H
Moscatobianco_P
Moscatobianco_PV
Moscatobianco_EV
Moscatobianco_H
Passerina_P
Passerina_PV
Passerina_EV
Passerina_H

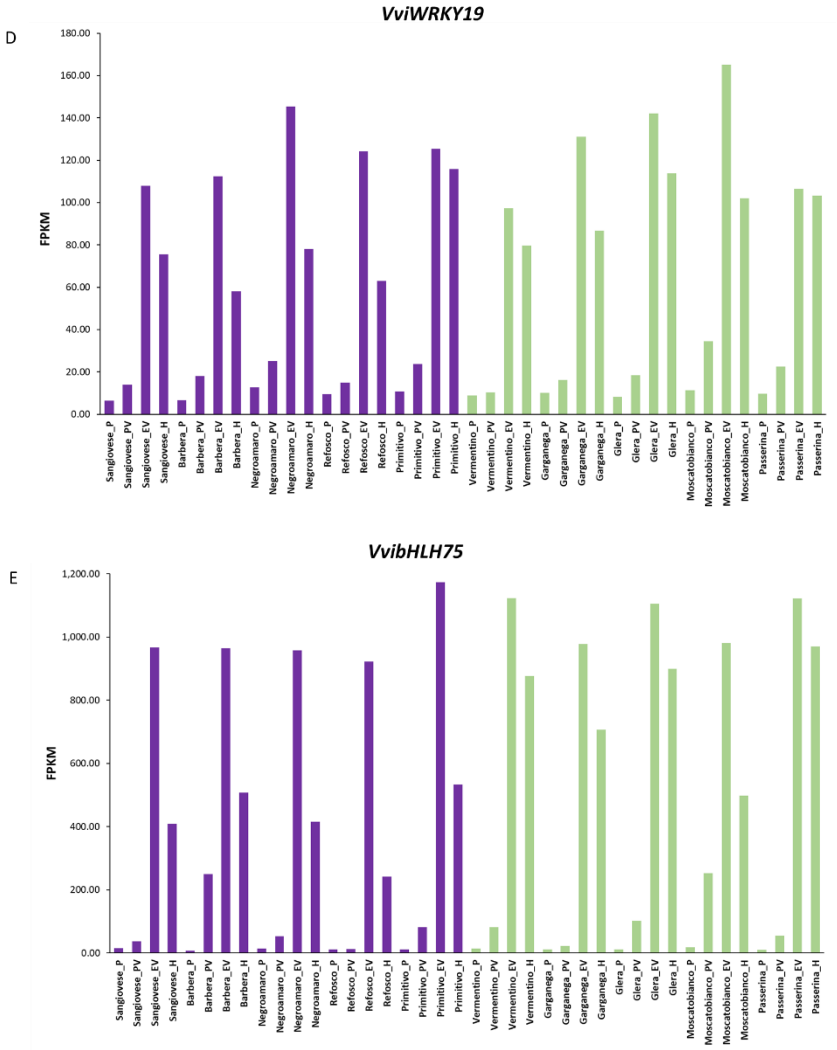
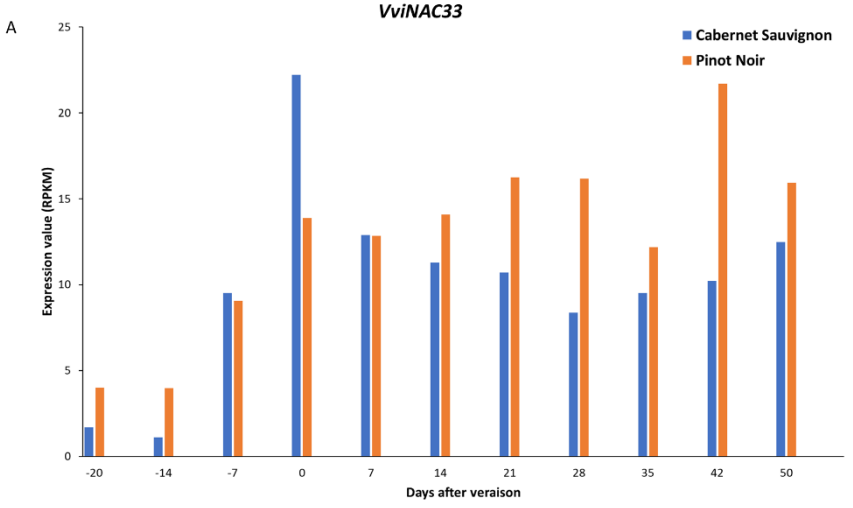


Figure 2: *VviNAC33* (A), *VviNAC60* (B) *VviAGL15a* (C), *VviWRKY19* (D) and *VvibHLH75* (E), expression profiles in 10 different grapevine varieties at four developmental stages. Transcriptomic data were retrieved by a berry specific expression map obtained by RNAseq (Massonnet et al., 2017). Abbreviations: **P**, pea-sized berry; **PV**, pre-veraison; **EV**, end of veraison; **H**, harvest.

Finally, the expression analysis of *VviNAC33*, *VviNAC60*, *VviAGL15a*, *VviWRKY19* and *VvibHLH75* using the transcriptomic data related to berry ripening process of Cabernet Sauvignon and Pinot Noir (Fasoli et al., 2018) shows that each transcription factor, excluding *VviAGL15a*, is characterized by a sudden increase of expression just before *veraison* (Figure 3). These data are consistent with their classification as positive bio-markers of first transition of *veraison* and suggest that they act as master regulators during the onset of ripening. After *veraison*, the expression of *VviNAC33* (Figure 3A), *VviNAC60* (Figure 3B), *VviWRKY19* (Figure 3D) and *VvibHLH75* (Figure 3E) can further increase or decrease, but their level of expression remain higher than the pre-*veraison* stages, indicating that they may have a role during the whole ripening process. The steep decrease of expression of *VvibHLH75* (Figure 3E), suggests that it plays a major role during the initial phases of ripening. Finally, concerning *VviAGL15a*, it is the only transcription factor characterized by the absence of a sudden expression before *veraison*, but its expression increases during the ripening process, suggesting its involvement in the regulation of specific processes associated with late ripening stages (Figure 3C).



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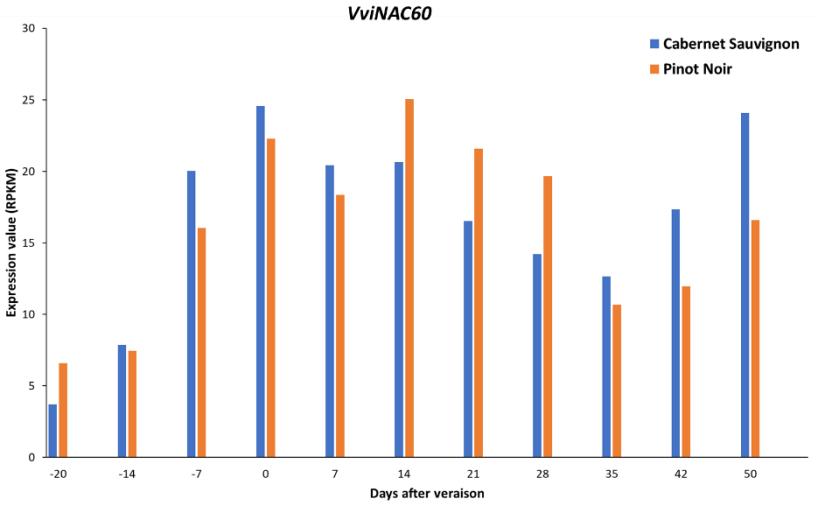
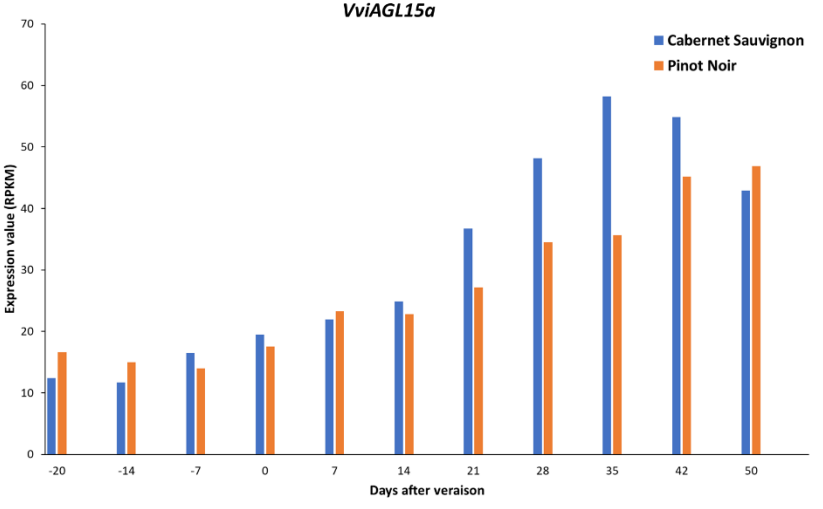

VviNAC60
Cabernet Sauvignon
Pinot Noir
Expression value (RPKM)
0
5
10
15
20
25
30
-20
-14
-7
0
7
14
21
28
35
42
50
Days after veraison

C


VviAGL15a
Cabernet Sauvignon
Pinot Noir
Expression value (RPKM)
0
10
20
30
40
50
60
70
-20
-14
-7
0
7
14
21
28
35
42
50
Days after veraison

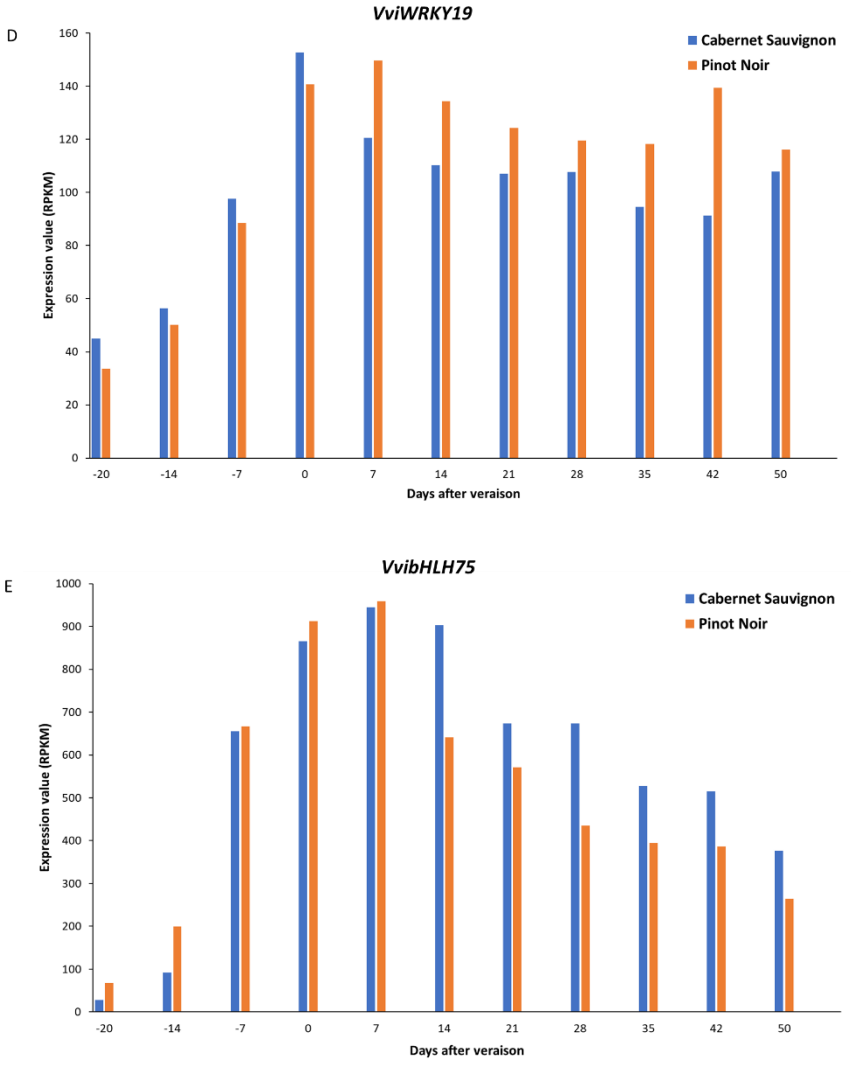


Figure 3: *VviNAC33* (A), *VviNAC60* (B), *VviAGL15a* (C), *VviWRKY19* (D) and *VvibHLH75* (E) expression profiles related to berry ripening, from fruit set to full maturity, of Cabernet Sauvignon and Pinot Noir. Transcriptomic data were retrieved by a berry specific expression map obtained by RNAseq (Fasoli et al., 2018).

3.3 Co-expression analysis

To obtain preliminary information about genes transcriptionally related to the selected transcription factors, that may include their targets, regulators or partners, a co-expression analysis by the CorTo software has been performed on the transcriptomic dataset of the grapevine gene expression atlas (Fasoli et al., 2012). For each transcription factors, only the first thirty co-expressed genes, excluding those with no similarity to known sequences or function (no hit/unknown protein), are reported.

Regarding *VviNAC33*, the first thirty co-expressed genes are indicated in table 2. Among them, there two *RECEPTOR KINASE RK20-1* (VIT_00S2634G00010, VIT_00S0398G00030) and two *RECEPTOR SERINE/THREONINE KINASE* (VIT_00S0409G00050, VIT_05S0049G01190), involved in signal transduction, two proteins related to senescence, *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 1* (VIT_02S0025G04660) and senescence-related gene 1 (*SRG1*, VIT_13S0019G02010), two *GALACTINOL SYNTHASE* (VIT_05S0077G00430, VIT_05S0020G00330), related to sugar signaling, and one NAC transcription factor, *VviNAC36* (VIT_12S0028G00860).

Table 2: the first thirty genes co-expressed with *VviNAC33*.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_15S0048G01010	2-hydroxy isoflavone/dihydroflavonol reductase	Secondary Metabolic Process	0.881
VIT_18S0001G08300	tubulin alpha-6 chain	Cellular Process	0.83
VIT_00S2634G00010	receptor kinase RK20-1	Signal Transduction	0.821
VIT_14S0060G01530	DNA polymerase III subunit epsilon	DNA/RNA Metabolic Process	0.812
VIT_00S0398G00030	receptor kinase RK20-1	Signal Transduction	0.807
VIT_14S0006G01610	PMI2 (plastid movement impaired 2)	#N/D	0.802
VIT_18S0001G13780	Cytochrome P450, family 83, subfamily B, polypeptide 1	Secondary Metabolic Process	0.801
VIT_04S0008G05400	serine hydrolase [Vitis vinifera]	Cellular Amino Acids and Derivative Metabolic Process	0.793
VIT_02S0025G04660	senescence-inducible chloroplast stay-green protein 1	Developmental Process	0.78
VIT_00S0409G00050	Receptor-like serine-threonine protein kinase	Signal Transduction	0.778

VIT_01S0011G06460	Deoxymugineic acid synthase	Cellular Amino Acids and Derivative Metabolic Process	0.773
VIT_15S0046G00080	yippee	#N/D	0.769
VIT_19S0090G01170	UPF0041	#N/D	0.761
VIT_01S0137G00670	QUINOLINATE SYNTHASE	Secondary Metabolic Process	0.758
VIT_05S0077G00430	galactinol synthase	Carbohydrate Metabolic Process	0.758
VIT_05S0020G00330	galactinol synthase	Carbohydrate Metabolic Process	0.754
VIT_05S0049G01190	Receptor serine/threonine kinase	Signal Transduction	0.753
VIT_01S0010G03640	DnaJ homolog, subfamily A, member 3	Transport	0.738
VIT_05S0077G01140	bZIP transcription factor BZIP53	Transcription Factor Activity	0.737
VIT_18S0122G01170	12-oxophytodienoate reductase 2	Lipid Metabolic Process	0.732
VIT_14S0030G01490	cysteine synthase	Cellular Amino Acids and Derivative Metabolic Process	0.732
VIT_00S0324G00060	UDP-glycosyltransferase 85A8	Carbohydrate Metabolic Process	0.73
VIT_13S0019G02010	SRG1 (senescence-related gene 1)	Developmental Process	0.729
VIT_13S0019G05070	Nodulin family protein	#N/D	0.724
VIT_19S0090G01180	light induced protein like	#N/D	0.716
VIT_06S0061G01470	ABC transporter G member 22	Transport	0.713
VIT_12S0028G00860	NAC domain-containing protein (VvNAC36)	Transcription Factor Activity	0.712
VIT_15S0048G01960	CYP87A3	#N/D	0.711
VIT_16S0100G00350	ABC transporter B member 8	Transport	0.71
VIT_01S0026G02500	amino acid transport protein	Transport	0.71

The genes highly correlated with *VviNAC60* (Table 3), include the *LATERAL ORGAN BOUNDARIES DOMAIN 15* (VIT_06S0004G07790), a switch gene emerged from the analysis of the of expression atlas and berry transcriptomic datasets and a marker of the first transition, one NAC transcription factor, *VviNAC61* (VIT_08S0007G07640), and two *GEM-LIKE PROTEIN 5* (VIT_14S0068G01360, VIT_01S0146G00410).

Table 3: the first thirty genes co-expressed with *VviNAC60*.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_16S0050G00390	4-coumarate-CoA ligase	Secondary Metabolic Process	0.892
VIT_14S0068G01360	GEM-like protein 5	Signal Transduction	0.89
VIT_01S0127G00680	SRO2 (SIMILAR TO RCD ONE 2)	Secondary Metabolic Process	0.88
VIT_13S0019G04620	OTU cysteine protease	Cellular Homeostasis	0.877
VIT_18S0072G01010	Peptide chain release factor eRF subunit 1	DNA/RNA Metabolic Process	0.864
VIT_19S0014G04790	Organic cation/carnitine transporter4	Transport	0.863
VIT_06S0004G07790	lateral organ boundaries DOMAIN 15	Transcription Factor Activity	0.861
VIT_14S0108G00490	Cullin 3a	Cellular Homeostasis	0.86
VIT_08S0007G01150	Unc51-like kinase	Signal Transduction	0.858
VIT_08S0007G05250	cig3	DNA/RNA Metabolic Process	0.85
VIT_01S0146G00410	GEM-like protein 5	Cellular Process	0.849
VIT_19S0014G02190	tyrosine aminotransferase	Cellular Amino Acids and Derivative Metabolic Process	0.846
VIT_08S0007G07640	NAC domain-containing protein (VvNAC61)	Transcription Factor Activity	0.844
VIT_12S0028G03580	lectin-receptor like protein kinase 3	#N/D	0.843
VIT_13S0019G01810	scarecrow transcription factor 14 (SCL14)	Transcription Factor Activity	0.842
VIT_16S0022G01690	Band 7 family	Cellular Process	0.841
VIT_11S0016G03000	Phosphoinositide 3-kinase regulatory subunit 4	Transport	0.84
VIT_02S0025G03530	Gamma-glutamylcysteine synthetase	Cellular Amino Acids and Derivative Metabolic Process	0.839
VIT_12S0059G01100	PRLI-interacting factor K	Transcription Factor Activity	0.834
VIT_00S1278G00010	Brassinosteroid insensitive 1-associated receptor kinase 1	Response to Hormone Stimulus	0.833
VIT_01S0011G03660	IMP dehydrogenase/GMP reductase	DNA/RNA Metabolic Process	0.833
VIT_11S0016G00400	zinc finger (FYVE type)	Transcription Factor Activity	0.831
VIT_14S0030G00400	WD40	Cellular Process	0.83
VIT_04S0008G03770	Aspartate aminotransferase P1	Generation of Energy	0.829

VIT_12S0028G00980	myb family	Transcription Factor Activity	0.827
VIT_14S0060G00420	pyruvate dehydrogenase kinase	Carbohydrate Metabolic Process	0.827
VIT_14S0060G00040	Retrotransposon	DNA/RNA Metabolic Process	0.826
VIT_09S0002G00750	P-GLYCOPROTEIN 19	Transport	0.826
VIT_12S0035G01350	Cysteinyl-tRNA synthetase	Cellular Amino Acids and Derivative Metabolic Process	0.825
VIT_16S0100G00570	dehydration-responsive protein	#N/D	0.824

Regarding *VviAGL15a*, the thirty co-expressed genes (Table 4) include a *MADS BOX INTERACTOR* (VIT_18S0001G07300), probably involved in specific activities together with other MADS-box proteins, two *INVERTASE/PECTIN METHYLESTERASE INHIBITOR* (VIT_02S0012G00500, VIT_15S0021G00540), involved in the cell wall metabolism, one NAC transcription factors, *VviNAC18* (VIT_19S0014G03300), and one *SUGAR TRANSPORTER 1* (VIT_02S0025G04430).

Table 4: the first thirty genes co-expressed with *VviAGL15a*.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_18S0001G07300	MADS box interactor	#N/D	0.873
VIT_09S0018G01370	STE20/SPS1 proline-alanine-rich protein kinase	#N/D	0.866
VIT_08S0007G00540	haloacid dehalogenase hydrolase	Carbohydrate Metabolic Process	0.846
VIT_14S0128G00900	MORC family CW-type zinc finger 4	#N/D	0.842
VIT_06S0009G03650	permease nonimprinted in Prader-Willi/Angelman	Transport	0.835
VIT_15S0021G01500	RWD domain-containing protein	#N/D	0.833
VIT_06S0004G01640	UDP-glucoronosyl and UDP-glucosyl transferase	#N/D	0.83
VIT_02S0012G00500	invertase/pectin methylesterase inhibitor	Cell Wall Metabolism	0.828
VIT_17S0000G07540	paired amphipathic helix protein Sin3a	Transcription Factor Activity	0.827
VIT_19S0014G03300	NAC domain-containing protein (VvNAC18)	Transcription Factor Activity	0.826
VIT_16S0039G00720	Folate-biopterin transporter	Transport	0.822
VIT_02S0025G04430	Sugar transporter 1	Carbohydrate Metabolic Process	0.82
VIT_00S0577G00020	Phosphoglycerate mutase	Carbohydrate Metabolic Process	0.819

VIT_18S0001G09480	PUMILIO 8 (APUM8)	DNA/RNA Metabolic Process	0.816
VIT_04S0023G03120	Histone H3	DNA/RNA Metabolic Process	0.816
VIT_05S0051G00640	purple acid phosphatase 23-ATPAP23/PAP23	Secondary Metabolic Process	0.816
VIT_18S0001G09490	metal transporter Nramp2	Transport	0.815
VIT_08S0040G03260	Nuclear transcription factor Y subunit B-5	Transcription Factor Activity	0.814
VIT_14S0068G01540	PBS1 (avrPphB susceptible 1)	Response to Stress	0.811
VIT_05S0020G04120	DnaJ homolog, subfamily A, member 1	Transport	0.811
VIT_13S0067G01780	histidine acid phosphatase	Secondary Metabolic Process	0.811
VIT_08S0040G00770	cysteine protease inhibitor	Cellular Homeostasis	0.81
VIT_14S0083G00880	Phosphatidylinositol 4-kinase type-II	Carbohydrate Metabolic Process	0.81
VIT_08S0007G07440	PUMILIO 12 (APUM12)	Transcription Factor Activity	0.809
VIT_00S0188G00150	auxilin	Transport	0.809
VIT_15S0021G00540	invertase/pectin methylesterase inhibitor	Cell Wall Metabolism	0.807
VIT_18S0001G10560	Myosin heavy chain	Cellular Process	0.807
VIT_01S0011G04370	Phosphatidylserine synthase 2	Carbohydrate Metabolic Process	0.807
VIT_06S0004G03990	Nudix hydrolase 9	Response to Stress	0.804
VIT_08S0007G03010	hydrolase, alpha/beta fold	#N/D	0.803

The genes highly correlated with *VviWRKY19* (Table 5), include two NAC transcription factors, *VviNAC05*(VIT_17S0000G06400) and *VviNAC01* (VIT_01S0146G00280), two WRKY transcription factors, *VviWRKY14* (VIT_05S0077G00730) and *VviWRKY52* (VIT_17S0000G01280), the *POLYGALACTURONASE PG1* (VIT_08S0007G08330), the *INVERTASE/PECTIN METHYLESTERASE INHIBITOR* (VIT_16S0022G00960), one *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 32* (VIT_06S0061G00550) and the *CELLULOSE SYNTHASE CSLG2* (VIT_05S0049G00050), four proteins involved in cell wall metabolism, and one *SUCROSE-PHOSPHATE SYNTHASE* (VIT_11S0118G00200).

Table 5: the first thirty genes co-expressed with *VviWRKY19*.

VIT	FUNCTIONAL ANALYSIS	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_10S0003G02450	flavonol synthase	Secondary Metabolic Process	0.857

VIT_17S0000G06400	NAC domain-containing protein (VvNAC05)	Transcription Factor Activity	0.854
VIT_06S0061G00550	xyloglucan endotransglucosylase/hydrolase 32	Cell Wall Metabolism	0.847
VIT_18S0001G06060	UDP-glycosyltransferase 85A1	#N/D	0.83
VIT_01S0127G00590	Protein disulfide isomerase	#N/D	0.826
VIT_05S0077G00730	WRKY transcription factor (VvWRKY14)	Transcription Factor Activity	0.825
VIT_01S0011G05110	major latex protein 22	Response to Stress	0.819
VIT_08S0040G01950	zinc finger (C3HC4-type RING finger)	Transcription Factor Activity	0.817
VIT_08S0007G08330	polygalacturonase PG1	Cell Wall Metabolism	0.814
VIT_05S0049G00050	cellulose synthase CSLG2	Cell Wall Metabolism	0.814
VIT_18S0001G00560	alpha-amylase / 1,4-alpha-D-glucan glucanohydrolase	Carbohydrate Metabolic Process	0.81
VIT_08S0007G08840	Glycosyl transferaseHGA1	#N/D	0.8
VIT_17S0000G01280	WRKY transcription factor (VvWRKY52)	Transcription Factor Activity	0.794
VIT_08S0007G00200	ankyrin repeat	Cellular Process	0.791
VIT_13S0067G00140	proline-rich family protein	Transcription Factor Activity	0.79
VIT_00S0324G00050	UDP-glucose glucosyltransferase	Carbohydrate Metabolic Process	0.788
VIT_18S0001G12240	Peptide chain release factor eRF subunit 1	DNA/RNA Metabolic Process	0.788
VIT_01S0146G00280	NAC domain-containing protein (VvNAC01)	Transcription Factor Activity	0.785
VIT_18S0001G05690	protein phosphatase 2C	Signal Transduction	0.785
VIT_08S0007G02570	CCR4-NOT transcription complex subunit 7	DNA/RNA Metabolic Process	0.782
VIT_11S0118G00200	sucrose-phosphate synthase	Carbohydrate Metabolic Process	0.777
VIT_16S0022G00960	invertase/pectin methylesterase inhibitor	Cell Wall Metabolism	0.776
VIT_00S0216G00040	ER lumen protein retaining receptor	#N/D	0.774
VIT_13S0064G01210	Zf A20 and AN1 domain-containing stress-associated protein 2	Transcription Factor Activity	0.773
VIT_01S0011G06260	anthranilate synthase beta subunit	Cellular Amino Acids and Derivative Metabolic Process	0.773
VIT_03S0038G03570	monocopper oxidase SKS5 (SKU5 Similar 5)	Transport	0.77
VIT_08S0040G01170	hydroxymethylglutaryl-CoA lyase	Lipid Metabolic Process	0.77
VIT_18S0001G02680	BTB/POZ domain-containing protein	Transcription Factor Activity	0.767
VIT_00S2364G00010	U-box domain-containing protein 8	Cellular Homeostasis	0.767

VIT_17S0000G01080	HVA22E (HVA22-LIKE PROTEIN E)	Response to Hormone Stimulus	0.766

Finally, regarding *VvibHLH75*, the first thirty co-expressed genes are reported in Table 6; among them, there are two members of the ERF/AP2 gene family, *VviERF045* (VIT_04S0008G06000) and *VviERF008* (VIT_18S0001G03240), one WRKY transcription factor, *VviWRKY14* (VIT_05S0077G00730), two *BASIC HELIX-LOOP-HELIX* (VIT_01S0244G00010, VIT_11S0037G01230), the *CELLULOSE SYNTHASE CSLG2* (VIT_05S0049G00050), the *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 32* (VIT_06S0061G00550), one expansin, *VviEXPA19* (VIT_18S0001G01130), the *HEXOSE TRANSPORTER HT2* (VIT_18S0001G05570), and the *AUXIN-RESPONSIVE SAUR9* (VIT_04S0023G03230).

Table 5: the first thirty genes co-expressed with *VvibHLH75*.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_04S0008G06000	ERF/AP2 Gene Family (VvERF045)	Response to Hormone Stimulus	0.901
VIT_05S0020G02290	Endonuclease	DNA/RNA Metabolic Process	0.895
VIT_18S0001G06060	UDP-glycosyltransferase 85A1	#N/D	0.892
VIT_18S0001G03240	ERF/AP2 Gene Family (VvERF008)	Response to Hormone Stimulus	0.888
VIT_05S0049G00050	cellulose synthase CSLG2	Cell Wall Metabolism	0.876
VIT_11S0052G00630	Metallothionein	Response to Stress	0.876
VIT_02S0109G00230	early-responsive to dehydration protein / ERD protein	#N/D	0.874
VIT_07S0031G00420	SHN1 (SHINE 1)	Response to Hormone Stimulus	0.872
VIT_12S0057G00260	external rotenone-insensitive NADPH dehydrogenase	Generation of Energy	0.867
VIT_08S0007G08280	remorin	Other Processes	0.859
VIT_08S0040G01170	hydroxymethylglutaryl-CoA lyase	Lipid Metabolic Process	0.857
VIT_05S0077G00730	WRKY transcription factor (VvWRKY14)	Transcription Factor Activity	0.857
VIT_18S0001G05690	protein phosphatase 2C	Signal Transduction	0.853
VIT_17S0000G05580	Isopiperitenol dehydrogenase	Secondary Metabolic Process	0.851
VIT_01S0244G00010	basic helix-loop-helix (bHLH) family (VvCEB1)	Transcription Factor Activity	0.845
VIT_09S0018G00780	HcrVf1 protein	Response to Stress	0.835
VIT_00S0324G00050	UDP-glucose glucosyltransferase	Carbohydrate Metabolic Process	0.834
VIT_06S0061G00550	xyloglucan endotransglucosylase/hydrolase 32	Cell Wall Metabolism	0.829

VIT_18S0001G01130	Expansin (VvEXPA19)	Cell Wall Metabolism	0.828
VIT_11S0118G00200	sucrose-phosphate synthase	Carbohydrate Metabolic Process	0.826
VIT_11S0037G01230	basic helix-loop-helix (bHLH) family	Transcription Factor Activity	0.817
VIT_04S0023G03230	Auxin-responsive SAUR9	Response to Hormone Stimulus	0.817
VIT_18S0001G05990	UDP-glycosyltransferase 85A1	Carbohydrate Metabolic Process	0.817
VIT_17S0000G08290	Dof zinc finger protein DOF5.6	Transcription Factor Activity	0.814
VIT_13S0156G00610	S-receptor kinase	Signal Transduction	0.814
VIT_07S0031G01160	Hyperosmotically inducible periplasmic protein	Transport	0.813
VIT_17S0000G09350	DEHYDRATION-INDUCED 19	Signal Transduction	0.813
VIT_17S0000G06200	MINI ZINC FINGER 1 MIF1	Transcription Factor Activity	0.81
VIT_18S0001G05570	Hexose transporter HT2	Carbohydrate Metabolic Process	0.809
VIT_17S0000G01930	Potassium transporter 2	Transport	0.807

4. DISCUSSION

Grapevine Berry development and ripening are long and complex processes, affected by many exogenous and endogenous factors. The onset of ripening, called *veraison*, is a crucial event during the maturation process; its biochemical and physiological characteristics are well known, but the molecular mechanisms controlling this process are still poor explored. Recent studies have shown a depth transcriptional shift during the vegetative-to-mature transition in most of grapevine organs and tissues (Fasoli et al., 2012) and the existence of a specific set of genes, named switch genes, highly expressed only in mature organs, that may have a role in the regulation of this phase transition (Palumbo et al., 2014). More information about the ripening process initiation in berry has been obtained by the analysis of red and white berry transcriptomes (Palumbo et al., 2014; Massonnet et al., 2017) showing the existence of switch genes in common with the switch genes of expression atlas and many others specific of berry, suggesting the specific role in berry ripening onset of the latter. Interestingly, most of these switch genes are transcription factors, thus supporting their regulative role in the immature-to-mature transition. Furthermore, some of these transcription factors have been identified as markers of the first transition of the onset of berry ripening (Fasoli et al., 2018), showing a rapid increase of expression level in the short-time lapse before *veraison*. This result indicates that

these genes may play a fundamental role as triggers of the maturation process in grapevine berry.

The selection of putative master regulators of berry ripening has been performed before analyzing the set transcription factors identified as switch genes of grapevine expression atlas and of both red and white berry transcriptomes and then analyzing their inclusion in the list of positive biomarkers of the first and second transitions at the onset of berry ripening (Table 1). Based on the above-mentioned criteria, *VviNAC33*, *VviNAC60*, *VviAGL15a*, *VviWRKY19* and *VvibHLH75* were selected for further characterization. *VviNAC33* and *VviNAC60* are switch genes of both expression atlas and red and white berry transcriptomes and markers of the first transition, *VviAGL15a* is a switch gene of red and white berry transcriptome, *VviWRKY19* is a switch gene of red and white berry transcriptome and a marker of both first and second transition, *VvibHLH75* is a switch gene of red and white berry transcriptome and a marker of the first transition. Furthermore, these transcription factors belong to large transcription factors gene families in plants, involved in many processes, such as plant development and growth, response to abiotic and biotic stress, flower and fruit development, hormone signaling and regulation of secondary metabolites production. Altogether, these collected information indicate that the selected transcription factors are promising candidates for functional characterization.

The expression profile of each transcription factors has been retrieved from previously released transcriptomic datasets. Regarding *VviNAC33*, its expression restricted to mature organs and tissues including ripening berry (Figure 1A, 2A) is consistent with the role of master regulator of the vegetative-to-mature transition in several grapevine organs. Furthermore, considering that in berry, it is preferential expressed at post-harvest and harvest (Figures 1A, 2A) phases seem to indicate that it may play a predominant role in the final phase of berry ripening. However, its strong induction before *veraison* (Figure 3A) indicate that it is a marker of the first transition (Table 1) performing a specific role also at the onset of berry ripening. The expression profile of *VviNAC60* (Figure 1B, 2B) shows that this gene is preferentially expressed in berry during post-harvest phase (Figure 1B) but in some varieties show a high expression during the end of *veraison* and harvest phases (Figure 2B), suggesting a specific role during the whole berry ripening process; however, *VviNAC60* is a marker of the first transition (Table 1): its expression starts before *veraison* (Figure 3B), suggesting a specific role also during this phase. Regarding

VviAGL15a, among the five selected genes, it is the only gene characterized by the absence of a rapid induction before *veraison* (Figure 3C): its high expression in mature berry especially during the post-harvest and harvest phases (Figures 1C, 2C) indicates that it may play a role of master regulator of the final phase of berry ripening. Finally, *VviWRKY19* and *VvibHLH75* are expressed only in mature berry especially during *veraison*, end of *veraison* and mid-ripening phases (Figures 1D, 1E, 2D, 2E), suggesting that they may have a specific function at the onset and during the first part of berry ripening. This observation is confirmed by their sudden increase of expression before *veraison* (Figures 3D, 3E): they are two marker genes of first transition of *veraison* (Table 1) and they may be involved in the regulation of specific processes during the onset of berry ripening. Furthermore, *VviWRKY19* is also a marker of the second transition (Table 1): as described by Fasoli et al., 2018, its expression could be regulated by specific markers of the first transition, suggesting the existence of a transcriptional hierarchy during the onset of berry ripening.

The final co-expression analysis using the global gene expression atlas, the most inclusive transcriptomic dataset of grapevine development plant and berry development, has shown that the selected transcription factors are co-expressed with other transcription factors or with genes involved in specific processes and metabolisms associated with ripening. This result represents a first indication of the processes/metabolisms controlled by each candidate, possibly in cooperation with other transcription factors. In detail, *VviNAC33* is co-expressed with two proteins *SENESCENCE-INDUCIBLE CHLOROPLAST STAY-GREEN PROTEIN 1* and *SENESCENCE-RELATED GENE 1*, involved in senescence process, a key event often associated to the maturation of grapevine organs; furthermore, the high expression of *VviNAC33* in senescent leaf (Figure 1A) is consistent with the results showing the co-expression with the two senescence-related proteins, indicating a role of *VviNAC33* in the regulation of this process. Other co-expressed gene are two *GALACTINOL SYNTHASE*, involved in sugar signaling, an important event during ripening, and *VviNAC36*, indicating a putative cooperation with other NAC genes to regulate specify process. Regarding *VviNAC60*, among the highly co-expressed genes are *LATERAL ORGAN BOUNDARIES DOMAIN 15*, a switch gene obtained from the expression atlas and from berry-specific transcriptomic datasets, and a marker of the first transition, suggesting a putative cooperation among the different switch genes during vegetative-to-mature transition, and *VviNAC61*, indicating the same consideration previously

described for *VviNAC33*. *VviAGL15a* is co-expressed with genes mainly involved in cell wall and carbohydrate metabolic processes, two typical events associated with ripening. Furthermore, the co-expression with a *MADS box INTERACTOR* and *VviNAC18* suggest a cooperation or a hierarchical transcriptional relationship with other transcription factors, including MADS-box proteins, to regulate specific processes. Regarding *VviWRKY19*, the highly co-expressed genes are represented by many genes involved in cell wall metabolism, indicating a direct regulation of this process by *VviWRKY19*, and some transcription factors, including NAC and WRKY transcription factors, suggesting the same considerations described for the others three genes. Finally, *VvibHLH75* is co-expressed with two ERF/AP2 genes and one SAUR, suggesting an involvement of this gene in the ethylene and auxin signaling, with other two *BASIC HELIX-LOOP-HELIX* genes, indicating a cooperation activity among genes of the same transcription factors family, and with many genes involved in cell wall metabolism, suggesting a role also in this process. Two genes involved in cell wall metabolism (one *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 32* and one *CELLULOSE SYNTHASE CSLG2*) and *VviWRKY14* are co-expressed with both *VviWRKY19* and *VvibHLH75*, suggesting that the regulation of many genes can be performed by more than one of the selected candidates.

The preliminary results of co-expression analysis have shown that the transcription factors selected seem to be mainly involved in the regulation of developmental process, cell wall metabolism, carbohydrate metabolic process and response to hormone stimulus; furthermore, the co-expression of many others transcription factors could indicate a specific cooperation or a hierarchical transcriptional relationship in the regulation of specific processes. Overall, these results support a role of the selected transcription factors as master regulators of grape berry ripening and confirm that they represent interesting candidates for functional characterization and, more in general, to the disentanglement of the molecular mechanism controlling the berry ripening process.

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Chapter 3

APPLICATION, IMPROVEMENT AND DEVELOPMENT OF GENE TRANSFER TECHNOLOGIES IN GRAPEVINE (*Vitis Vinifera* L.)

ABSTRACT

Stable genetic transformation and transient gene expression are the most important technologies used for gene functional analysis in grapevine (*Vitis Vinifera* L.). However, grapevine is a very recalcitrant plant and in some cases their application can be very difficult. To obtain an efficient experimental procedure, three different protocols of stable genetic transformation and three different grapevine varieties were used. Many parameters, including the embryogenic tissue and the *Agrobacterium* OD_{600} used for the transformation and different media for somatic embryogenesis and plant regeneration, were tested. The results showed that stable genetic transformation was cultivar dependent and the efficiency of transgenic somatic embryos and plants regeneration was higher when embryogenic calli were used. Regarding transient gene expression, two different approaches were tested: leaf agroinfiltration and berry agroinfiltration. The first one was performed using whole plants grown *in-vitro* of different cultivars; the time course analysis of YFP transient expression showed that the fluorescence signal is especially localized in the first and second leaves from apex but the day post infiltration of maximum YFP expression is different among the cultivars. Berry agroinfiltration was performed using berries derived from fruiting cuttings of Cabernet Sauvignon, the cultivar characterized by highest efficiency of inflorescence retaining and fruit production. The efficiency of this method is low but the visualization of YFP expression indicate that berry agroinfiltration can be performed and the analysis of fluorescence signal at different days post infiltration showed that it is higher in detached vacuum agroinfiltrated berries than attached agroinjected berries. Altogheter, these results indicate that both technologies can be applied, improved and subsequently used for functional studies in grapevine.

1. INTRODUCTION

Grapevine (*Vitis Vinifera* L.) is one of the most economically and valuable fruit crops in the world. Most of grape is processed into wine, but significant portions are also used for fresh fruit, dried fruit, and juice production. In the last years, the high economic value of grapevine has considerably increased the studies related with its genetic improvement. The clonal selection of spontaneous bud mutations and the conventional breeding represent the main techniques used for grapevine improvement (Dhekney et al., 2009). Clonal selection guarantees the uniformity of well determined yield and fruit quality of cultivars. Gene mutation does occur naturally over time and can contribute to improve yield or other characteristics of established cultivars. However, the random occurrence of bud mutations able to improve specific traits is very rare, thus limiting the possibility of efficient programs of improvements based solely on clonal selection. The genetic variability of grapevine can be increased by application of chemical mutagens or by irradiation, but these approaches present several constraints and have not been successful in producing improved clones or cultivars (Alleweldt and Possingham., 1988). Grapevine is highly heterozygous, and the vegetative propagation by cuttings ensures the preservation of the combination of genes responsible of important traits in the heterozygous genome (Torregrosa et al., 2015).

For this reason, the other approach for genetic improvement of grapevine, represented by conventional breeding, necessarily results in genotypes dissimilar to the parental varieties. The systematic use of breeding was initiated during the last half of 19th century when fungal diseases and phylloxera began to spread from North America to Europe and to decimate most of vineyards cultivated with *Vitis vinifera* cultivars. To protect the European vines against American fungal disease and pests and to incorporate biotic stress resistance in *Vitis vinifera* cultivars, breeding programs between phylloxera-resistant or tolerant native American species and European cultivars were initiated (Alleweldt and Possingham, 1988). These interspecific hybrids were used to prevent the fungal diseases, but the quality of their fruits was lower than wine-producers *Vitis vinifera* cultivars. The loss of important quality characteristic has reduced the production of phylloxera and fungal disease-resistant interspecific hybrids in Europe. They were replaced by the creation and utilization of phylloxera-resistant American rootstocks. Nowadays, most of European vineyards are cultivated with *Vitis vinifera* cultivars grafted on resistant rootstocks (Riaz et al., 2007), but the conventional breeding programs continue to be used

mainly to produce improved biotic and abiotic stress-resistant rootstocks, to produce table grapes varieties and, seldomly, to create new wine grape cultivars resistant to disease and pests (Gray et al., 2005). However, this approach is time consuming, laborious and expensive and its application has numerous constraints. The extreme heterozygosity of the *Vitis* genome and the inbreeding depression make backcrossing and selection very difficult. The fruit quality of progeny often cannot be assessed for several years because grapevine is a long-lived perennial plant and the juvenile period is relatively long. The resulting hybrid often possesses traits intermediate to each parent and the trait required is often at an unacceptably low level. In particular, for wine grape varieties, the use of breeding is greatly limited because it is impossible to introduce a useful trait without disrupting the desired phenotype and altering the quality of the final product.

An efficient and attractive alternative to traditional systems of genetic improvement of grapevine is represented by genetic engineering. The main aim of molecular grapevine breeding is the development and the application of technologies able to introduce genes in a specific targeted manner. The use of genetic transformation for the direct gene transfer is a very prominent approach for grapevine improvement: it is based on the transfer of DNA sequence into plant cell and its integration into the host genome and it allows the transmission of individual traits as single genes, with the minimum alteration of the original genome. This is particularly important for wine grape varieties: these cultivars must maintain the historic name but an improved genotype after the introduction of a specific gene maintaining the same essential characteristics could be accepted as a variant of the original cultivar. The genetic transformation can also be used for studying gene function and expression and for understanding biological processes in grapevine. The essential prerequisites to perform this approach are: the availability of a specific highly regenerative transformable tissue, a system to introduce foreign DNA and a protocol to select transformed cells and regenerate transgenic plants (Dhekney et al., 2012). The embryogenic culture represents the main target tissue used in genetic transformation of grapevine. Cultures can be obtained from young leaves of in-vitro plants or floral explants (immature anthers and pistils) and can be maintained in specific media for some years. There are two different types of embryogenic cultures used to introduce foreign DNA: a type I tissue consist of embryogenic undifferentiated calli and small globular embryos and a type II tissue deriving from type I calli and consists entirely of somatic embryos at different

developmental stages. Somatic embryos have been routinely used in grapevine genetic transformations (Scorza et al, 1996; Li et al, 2006, 2008; Dutt et al, 2008; Dhekney et al, 2009; Kandel et al, 2016;). Somatic embryos are ideal targets for transformation because the regenerative epidermal or sub-epidermal cells involved in secondary embryogenesis are accessible to the system used for introducing DNA and single cell origin should result in non-chimeric transformants. However, other reports of grapevine transformation (Franks et al., 1998; Iocco et al., 2001; Gambino et al., 2005) have used embryogenic calli as explants. The delivery of foreign DNA in embryogenic cells can be performed by biolistic bombardment (Franks et al., 1998; Vidal et al., 2003; Vidal et al., 2006) but the *Agrobacterium tumefaciens*-mediated transformation is the most used method to produce transgenic grapevine (Gambino et al., 2005; Li et al., 2006,2008; Dhekney et al., 2009; Franks et al., 1998; Iocco et al., 2001; Yamamoto et al., 2000; Kandel et al., 2016). The infection with *Agrobacterium* disarmed strains is followed by the transfer and the subsequent stable integration of T-DNA containing the gene of interest in the plant genome. However, the successful of a transformation system depends on the ability to recover positive transformants after cocultivation. Specific genes, incorporated into T-DNA with the gene(s) of interest, can be used to confirm the presence of T-DNA or to confer an advantage on the transformed cells compared to non-transformed cells (Dhekney et al., 2012). Very often, these genes are the reporter gene coding the green fluorescent protein (GFP) and the selectable marker genes as neomycin phosphotransferase II (*nptII*) that confer resistance to kanamycin antibiotic. Transgenic cells carrying these marker genes can be easily visualized and selectively grow on the culture medium containing selective antibiotic. This method has been largely used to test different protocols of transformation on different cultivars (Iocco et al., 2001; Li et al., 2008; Dhekney et al., 2009) but also to select transgenic plants overexpressing antimicrobial lytic peptides to protect grapevine plants from Pierce's disease (Li et al., 2015). Furthermore, the genetic transformation of grapevine has been used to evaluate its resistance to virus (Le Gall et al., 1994) and for the overexpression of antimicrobial and antifungal genes (Yamamoto et al., 2000; Vidal et al., 2003; Vidal et al., 2006) to confer bacterial and fungal disease resistance. In these reports, the selection of transgenic plants was performed using only the *nptII* gene. These data indicate that genetic transformation of grapevine can be performed, and it can be potentially used for the genetic improvement of grapevine, especially to confer biotic stress resistance. Despite these positive results, genetic transformation remains

a long and random process, the efficiency is low and only some varieties were successfully transformed. It is characterized by many limits, including the poor embryogenic potential of genotypes, wide variations among varieties in their response to genetic transformation, *Agrobacterium*-induced post-cocultivation necrosis of embryogenic cultures and poor plant recovery from transformed somatic embryo (Dhekney et al., 2009). These disadvantages limit the use of stable genetic transformation in grapevine for the studying of gene function.

An alternative strategy for gene functional analysis is represented by transient gene expression. This technology provides the most efficient way to study many genes in a very short time (Jelly et al., 2014). Transient expression methods are based on temporary, high-level transcription of DNA sequences that do not integrate into the plant genome. These assays involve direct transformation methods and *Agrobacterium tumefaciens*-mediated transformation method. The direct gene transfer can be performed by particle bombardment of intact plant cells or organs or by polyethylene glycol (PEG)-treatment or electroporation of protoplast cultures. The first approach is very difficult and expensive, while the use of protoplasts is very laborious especially for the preparation of protoplast culture and for maintaining of its vitality. The *Agrobacterium*-transformation method is simpler, and it is significantly cheaper than most other methods. The historic and the most used assay is represented by leaf-agroinfiltration. It is based on the forced infiltration of *Agrobacterium tumefaciens* suspension into the intercellular spaces of the leaf parenchyma, using a needleless syringe or a vacuum pump. The use of a needleless syringe (Zottini et al., 2008) is fast, but the gene expression is restricted to the infiltration zone; the vacuum infiltration is more complex, but it allows gene expression in the most of leaf (Bertazzon et al., 2012; Xu et al., 2010, 2014; Guan et al., 2011; Santos-Rosa et al., 2008; Visser et al., 2012). Leaf-agroinfiltration can be performed using both detached leaves or whole plants; it is usually carried out using tissue of *in-vitro* grown plants but it has been recently applied to leaves of greenhouse-grown plants (Ben-Amar et al., 2013). However, the use of agroinfiltration in grapevine is not limited to leaves but this approach has been recently tested in grape berries (Gao et al., 2018), using attached berries and syringe with needle, as performed in tomato (Orzaez et al., 2006) and strawberry fruits (Chai et al., 2011). The *Agrobacterium*-mediated gene transfer in grape berries was already tested (Kobayashi et al., 2005) but using a simple method of co-cultivation. The establishment and the evaluation of the efficiency of a transient gene expression protocol were performed using the *GUS* (Santos-Rosa

et al., 2008; Bertazzon et al., 2012), *GFP* (Zottini et al., 2008) and *LUC* reporter genes. After the set-up of an efficient protocol, transient expression methods can be used for the functional analysis of gene of interest, using both approaches of overexpression or gene silencing. Furthermore, these assays can be used for promoter sequence analysis or transcriptional studies. The applications of transient gene expression methods to grapevine are numerous and involving mainly studies related to the characterization of flavonoids biosynthesis and the tolerance to biotic and abiotic stress (Jelly et al., 2014).

In this chapter, both stable genetic transformation and transient gene expression approaches were applied using different protocols and methods. In order to establish a standardized procedure of stable transformation, in the first part of the chapter, different protocols of genetic transformation on different cultivars were tested. Different parameters that were evaluated included the type of embryogenic culture, the optical density (OD) of *Agrobacterium*, the strategy of recovery of transgenic somatic embryos and plants and the media used in the phases of pre- and post- transformation.

In the second part, a protocol of vacuum agroinfiltration of whole plants grown *in-vitro* of different cultivars of grapevine using the *YFP* reporter gene, are described. The analysis of YFP transient expression at different days post infiltration (d.p.i.) and in leaves at different positions has allowed to identify the d.p.i. of maximum expression and the leaves characterized by highest YFP expression. Moreover, different strategies to apply the transient gene expression in grape berry were tested.

2. MATERIALS AND METHODS

2.1 Plant material, source of explants and growth conditions

2.1.1 Embryogenic cultures: induction and maintenance

Embryogenic Garganega, Sangiovese and Shiraz callus was initiated from immature stamen cultures. Briefly, inflorescences were collected from plants of both cultivars growing in the same experimental vineyard in the province of Verona, Italy. The flowers were surface sterilized by immersing them in 100 mL of 7% $Ca(ClO)_2$ containing one/two drops of Tween-20 for 50 seconds with constant agitation, followed by three 5-min washes in sterile distilled water. Stamens (anthers with intact filaments) were carefully separated from the calyptra and pistil before

placing 50 stamens on plates containing PIV medium (Franks et al., 1998). After 2–3 months, embryogenic callus was transferred to C1P medium and subcultured in the same medium every 4 weeks (Iocco et al., 2000).

Embryogenic cultures of Thompson seedless cultivar were initiated from young unopened leaves of *in-vitro* shoot tip cultures (Li et al., 2009). Leaves were placed on NB2 medium and incubated in darkness at 28 °C for 5 to 7 weeks. Resulting embryogenic calli were transferred to $C1^P$ medium, maintained with the same conditions described above and subcultured at 4-week intervals.

2.1.2 *In-vitro* grown plants

Somatic embryos of Garganega, Sangiovese, Shiraz and Thompson seedless cultivars were obtained from embryogenic calli cultured in darkness on X6 medium (Li et al., 2006). Individual somatic embryos at late-cotyledonary stage of development were collected and transferred to MSB1 medium (Li et al., 2008) under light (60 µE and 16-h light/8-h dark photoperiod) to germinate. Regenerated plants of Thompson seedless were multiplied by clonal propagation on HB medium (Blaich., 1977) while plants of Garganega, Sangiovese and Shiraz were propagated by node cutting in three-quarter-strength MS medium (Kurth et al., 2012). Plants were subcultured every 4-5 weeks and maintained in a growth chamber (25 °C) under light (60 µE) and 16-h day length.

2.1.3 Fruiting cuttings

The fruiting cuttings were obtained following the method described by Mullins and Rajasekaran, 1980 and Baby et al., 2014. Dormant hardwood canes of 100 cm long and 1-1.5 cm in diameter were collected during the pruning time, sealed in a plastic bag and stored in a cold room (4 °C) for at least 4 weeks. Canes were cut to obtain cuttings of 40-50 cm long with 3-4 nodes but only the apical node was maintained in each cutting. Before the pre-rooting, cuttings were treated with 3000 ppm of rooting hormone indole butyric acid (IBA) at the basal cut; then, they were planted in different substrates (perlite and river sand) to a depth of 10 cm in a thermostatically controlled heated container (26°C at the bases of the cuttings) in a dark cold room (4°C). The rooting medium was kept moist with water at alternate days. After 5 weeks, rooted cuttings were planted in pots containing a mixture of perlite, vermiculite and peat (6:3:1) and were

transferred to a growth room (27°C day and 22°C night, 16 h photoperiod, humidity of 40% and 350 µE of light intensity at the plant level). At the bud burst, leaves of the single retained bud, proximal and adjacent to the inflorescence, were removed; then, also the shoot tip ix excised so that the inflorescence is in terminal position on the defoliated shoot. Then, one lateral shoot with five leaves is permitted to grow from one of the axillary buds proximal to the inflorescence. After that, one axillary shoot with four leaves was allowed to grow. The lateral shoot proximal to inflorescence and the axillary shoot provide the leaves to support the inflorescence and bunch development until fully ripe. After the transferring to the growth room, cuttings were irrigated on alternate days with half-strength Hoagland solution.

2.2 Transformation vectors and *Agrobacterium tumefaciens* strains

The transformation vectors used in stable genetic transformation and transient gene expression experiments were assembled using the GoldenBraid 2.0 (GB 2.0) system (Sarrion-Perdigones et al., 2013). The expression vectors used are of level 3 (based on an update version of pCAMBIA vector backbone). The vectors pEGB1α1-35S::*YFP*::TNOS, pEGB1α1R-TNOS::*NPTII*::Pnos and pEGB1α1-35S::*GFP*::TNOS, used for the construction of final vectors, were available from the GoldenBraid (GB) 2.0 toolkit. These vectors are GB expression vectors of level 1 (based on pGreenII backbone). The transition from level 1 to level 3 was performed following the strategy described by Sarrion-Perdigones et al., 2013. The vector pEGB1α1-35S::*YFP*::TNOS was recombined with pEGB1α2-SF (a "twister" plasmid described by Sarrion-Perdigones et al., 2011) in the destination vector pDGB3Ω1. The vectors pEGB1α1R-TNOS::*NPTII*::PNOS and pEGB1α1-35S::*GFP*::TNOS were recombined with pEGB1α2-SF in the destination vectors pDGB3Ω1 and pDGB3Ω2, respectively. Finally, the vectors pDGB3Ω1-TNOS::*NPTII*::PNOS-SF and pDGB3Ω2-35S::*GFP*::TNOS-SF were recombined in the destination vector pEGB3α1. The binary assembly between the vectors of the same level (alpha or omega) was performed following a detailed protocol generated using a software tool available at https://gbcloning.upv.es/. The final binary expression vectors pEGB3Ω1-35S::*YFP*::TNOS-SF and pEGB3α1-TNOS::*NPTII*::PNOS-SF-35S::*GFP*::TNOS-SF were introduced by electroporation into *Agrobacterium* strains C58C1 and EHA105 respectively. The *Agrobacterium* strain C58C1 harboring the vector pEGB3Ω1-35S::*YFP*::TNOS-SF was used in transient gene expression experiments while the *Agrobacterium* strain EHA105 containing the vector pEGB3α1-TNOS::*NPTII*::PNOS-SF-35S::*GFP*::TNOS-SF was

used in stable genetic transformation experiments. Bacterial cultures of C58C1 were grown in LB medium supplemented with tetracycline 5 mg/L and 50 mg/L of spectinomycin, while bacterial cultures of EHA105 were grown in MG/L medium supplemented with rifampicin 50 mg/L and kanamycin 50 mg/L.

2.3 Stable genetic transformation

Three different protocols of stable genetic transformation were tested.

2.3.1 Protocol 1

This protocol is based on the experimental procedure described by Cavallini., 2012, with some modifications. Embryogenic calli selected for the transformation were transferred from $C1^P$ medium to GS1CA medium and maintained in the same medium in darkness for 14 days. The *Agrobacterium* culture was prepared by inoculating 25 ml of selective MG/L liquid medium with a single colony. The suspension culture was grown overnight at 28 °C at 180 rpm. When the optical density at 600 nm (OD_{600}) reached a value of 0.8–1.0, bacterial suspension was centrifuged at 5000 rpm for 15 minutes and resuspended in 25 mL of modified liquid culture medium (modLCM) supplemented with acetosyringone 100 µM. The bacterial suspension was subsequently transferred to a 125 ml flask and cultured at 28 °C for an additional 3 h prior to use in transformation. For inoculation with Agrobacterium, the embryogenic calli 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. Then, embryogenic calli were 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 72 hours, the embryogenic calli were recovered and washed in liquid culture media (LCM) with timentin (1000 mg /L) and placed onto GS1CA medium supplemented with timentin for 7 days in the dark at 28°C. To select the transformed material, the calli was then moved into GS1CA supplemented with timentin and kanamycin 100 mg/mL; four weeks after the transformation calli 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 stereomicroscope. GFP positive embyros were collected on MS/2 medium and transferred in a growth room under light (80 µE) and 16-h of day lenght. After 3-4 weeks, cotyledons were excised to promote the shoot development. When

primary shoots emerged, the embryos were transferred firstly in the same media to help the shoot elongation and subsequently, plantlets were cut off and transferred into Magenta vessels containing three-quarter-strenght MS medium (Kurth et al., 2012) and cultured under the same conditions to allow further plant development. Vigorous transgenic plants with well-developed leaves and roots were then transplanted into soil in 7-cm plastic pots and acclimated in a growth room with high humidity for about 2 weeks before transfer to the greenhouse.

2.3.2 Protocol 2

This protocol was described by Torregrosa et al., 2015, with some modifications. Embryogenic calli were transferred from $C1^P$ medium to GS1CA medium and maintained in the same medium in darkness for 4 weeks. The *Agrobacterium* culture was prepared by inoculating one single colony overnight at 28 °C with shaking (180 rpm) in 50 mL of selective MG/L medium. The suspension was centrifuged at 4500 × g for 15 minutes, the pellet was resuspended in 100 mL induction medium, and incubated for a further 2 hours at 28 °C, with shaking at 100 rpm. The culture was centrifuged as above, and the pellet was resuspended in LCM medium. Finally, the concentration of the bacterial suspension was adjusted to an OD_{600} of 0.4. For inoculation with *Agrobacterium*, 20 mL of the bacterial suspension were added to each gram of embryogenic calli. After 10 minutes of incubation with gentle shaking, calli were separated from liquid phase and blotted on sterile Whatman filter paper. Then, embryogenic calli were transferred to a new Petri dish containing 3 pieces of Whatman filter paper saturated with liquid CM medium and incubated in the dark at 22°C for 48 °C. After cocultivation, wash the embryogenic calli was recovered, washed with 20 mL LCM medium plus 1,000 µg/mL Timentin and blotted briefly on Whatman filter paper. Then calli were distributed evenly onto GS1CA medium with 1,000 µg/mL Timentin and incubated in the dark at 28 °C for 2 weeks. Embryogenic calli were then transferred onto GS1CA medium with 1,000 µg/mL Timentin and 100 µg/mL kanamycin. After 1-month post inoculation, the calli were spreaded onto MG1 medium plus 150 µg/mL kanamycin and 1,000 µg/mL Timentin and incubate at 28 °C in the dark. Subculture GFP-positive kanamycin-resistant embryogenic cells onto fresh MG1 selective medium every 4 weeks. The selection of GFP-positive cali was performed by stereomicroscope. Well-developed GFP-positive somatic embryos were transferred to MG2 medium and incubate under light (45–60 µE) for further root and shoot development. After 4 weeks, roots were removed and cotyledons

3–5 mm from the base of embryos were excised to encourage the caulogenesis. Trimmed embryos were placed onto BFe2 medium plus 50 µg/mL kanamycin to stimulate growth of the shoot from the shoot meristem under light at 25 °C. Emerging shoots were subcultured 2–3 times on the same medium and the same conditions to promote axillary branching of the caulinar meristem. The regeneration of whole plants was carried out by transferring shoots onto root induction medium (RIM) in Magenta vessels. The transgenic plantlets were subcultured onto three-quarter-strenght MS medium (Kurth et al., 2012) for in vitro conservation (5 plants/line). Transgenic whole developed plants were finally transferred to potting soil in 10-cm pots and acclimatized in a growth chamber for 2-weeks before being moved to a greenhouse.

2.3.3 Protocol 3

This protocol is based on the experimental procedure described by Li et al., 2008, with some modifications. Embryogenic calli were transferred from $C1^P$ medium to X6 medium and maintained on the same medium for four weeks. The preparation of *Agrobacterium* culture was performed following the same procedure described for the protocol 1, with the only modification represented by the resuspension of the bacterial pellet in 25 mL of X2 medium. For inoculation with *Agrobacterium*, SE at mid-cotyledonary stage of development were collected in a Petri dish and submerged in 3 ml of bacterial suspension for 10 min. Bacterial suspension was withdrawn using a transfer pipette and any excess moisture was removed by blotting with sterilized Whatman 3MM filter paper. Then, SE were transferred into Petri dishes containing three layers of sterilized filter paper saturated with liquid DM medium. After 72 hours of co-cultivation, SE were transferred into a 125 ml flask containing 25 ml of liquid DMcc medium (DM medium supplemented with 200 mg/L each of cefotaxime and carbenicillin) and maintained on a rotary shaker (110 rpm) for24 h at 26°C. Liquid medium was then removed and replaced with the same amount of fresh DMcck50 medium (DMcc plus 50 mg l kanamycin) for 48 h. Then, SE were recovered and transferred onto a DMcck100 (100 mg/L kanamycin) callus induction medium. Each culture plate contained 30–40 separated SE. Cultures were kept in dark at 26°C for 30 days to induce transgenic calli. Afterwards, GFP-positive calli selected at the stereomicroscope were transferred to X6cck70 medium (X6 medium supplemented with 200 mg/L each of cefotaxime and carbenicillin and 70 mg/L kanamycin) for embryo induction from

transgenic embryogenic calli. Cultures were kept under the same conditions for 30 days. Transgenic SE were transferred onto fresh X6cck70 medium for embryo proliferation and development. Well-developed transgenic somatic embryos were plated on C2D4B medium (Li et al., 2014) under light (65 µE) and 16-h day length to stimulate the shoot development. Emerging shoot were then transferred into Magenta vessels containing three-quarter-strenght MS medium (Kurth et al., 2012) and cultured under the same conditions to allow further plant development. Vigorous transgenic plants were transferred to potting soil in 10-cm pots and acclimatized in a growth chamber for 2-weeks before being moved to a greenhouse.

2.4 Transient gene expression

Transient gene expression was performed using the *Agrobacterium tumefaciens*-mediated transformation (agroinfiltration) and two different grapevine tissue: leaves of plants grown *in-vitro* and grape berries obtained from fruiting cuttings.

2.4.1 Leaf agroinfiltration of whole plant grown *in-vitro*

Five mL of selective LB liquid medium was inoculated with one *Agrobacterium* fresh colony. The cultures were incubated for two days at 28°C. 200 ml of LB supplemented with antibiotics was subsequently inoculated with 5 mL of the bacterial culture and incubated overnight at 28°C at 200 rpm. The bacteria were collected by centrifugation and resuspended in the infiltration medium (10 mM MgSO4, 10 mM MES pH 5.5, 100 µM acetosyringone) to a final concentration of 0.5 OD_{600}. The bacterial suspension was then incubated at room temperature for about 3 h prior to infiltration. Agroinfiltration was conducted in non-sterile conditions. 6-weeks-old in vitro plantlets were immersed in bacterial suspension and vacuum infiltrated (90 kPa) for 2 min; then, vacuum was quickly released to let the bacterial suspension enter the leaf tissues. The procedure was repeated twice, until most of leaves appeared infiltrated. After infiltration, the plantlets were transferred in a growth chamber under standard growth conditions.

2.4.2 Berry agroinfiltration

The bacterial suspensions were prepared following the protocol described by Orzaez et al., 2006. One single colony of *Agrobacterium* was grown overnight at 28°C in 5 mL of YEB medium plus selective antibiotics. 50 mL of induction medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM MgSO4, 20 µM acetosyringone, 10 mM MES, pH 5.6) plus

antibiotics was inoculated with 1 mL of bacterial culture and grown again overnight. Next day, cultures were recovered by centrifugation, resuspended in infiltration medium (10 mM MgCl2, 10 mM MES, 200 µM acetosyringone, pH 5.6) to a final concentration of 1 OD_{600} and incubated at room temperature with gentle agitation (20 rpm) for a minimum of 2 h. Hard-green grape berries of Shiraz cultivar 20 days post anthesis (d.p.a.) were infiltrated both by syringe with needle and by vacuum. In syringe with needle experiments (Orzaez et al, 2006), detached berries were infiltrated using a 1-mL syringe with a … -mm needle; needle was introduced 3 to 4 mm in depth into the fruit tissue through the stylar apex, and the infiltration solution was gently injected into the fruit until the entire fruit surface has been infiltrated. In vacuum agroinfiltration experiments, detached berries were immersed in bacterial suspension and vacuum infiltrated (90 kPa) for 10 min; then, vacuum was quickly released to let the bacterial suspension enter the berry tissues. After both experiments of infiltration, detached berries were placed over a wet Whatman paper disc in a Petri dish and incubated in a growth chamber at 26 °C under a 16-h photoperiod at 60 µE cool-white light.

3. RESULTS

3.1 Stable genetic transformation

In this study, three different protocols of stable transformation have been tested in three different cultivars, Shiraz, Garganega and Sangiovese. Many parameters included type of embryogenic tissue, *Agrobacterium* OD_{600}, different media in pre- and post-transformation phases and different strategy of regeneration of transgenic somatic embryos and plants have been analyzed. Each protocol has been tested one single time for each cultivar and the results described are representative of one single experiment.

3.1.1 Effect of embryogenic tissue and *Agrobacterium* OD_{600}

Regarding the protocols 1 and 2 (see Materials and Methods, sections 2.3.1 and 2.3.2), embryogenic calli of each variety were transferred from $C1^P$ medium to GS1CA medium for two weeks and one month before *Agrobacterium* transformation, respectively. This phase is necessary to induce the formation and proliferation of somatic embryos (Franks et al, 1998) and different times of maintenance in this medium could affect the transformation efficiency. Furthermore, the gelling agent used for the solidification of this medium is different between

the two protocols: TC agar for the first and Bactoagar for the second protocol. Different brands of agar affect the embryogenic potential of cultures (Li et al, 2008) and consequently the transformation efficiency. Before transformations, embryogenic calli grown in GS1CA (solidified with TC agar) for two weeks (Figure 1A-B-C) are less white and more yellowish than the embryogenic calli grown in GS1CA (solidified with Bactoagar) for one month (Figure 1D-E-F); however, there are no differences in terms of structure among the embryogenic calli of the three cultivars maintained in GS1CA media with different gelling agents for different times. Regarding the protocol 3 (see Materials and Methods, section 2.3.3), embryogenic calli of Shiraz, Garganega and Sangiovese were transferred from $C1^P$ medium to X6 medium (solidified using TC Agar) to produce somatic embryos. After four weeks, each cultivar has produced somatic embryos at different developmental stages (Figure 1 G-H-I); however, only somatic embryos at mid cotyledonary stage of development (Li et al., 2008) were used for *Agrobacterium* transformation.

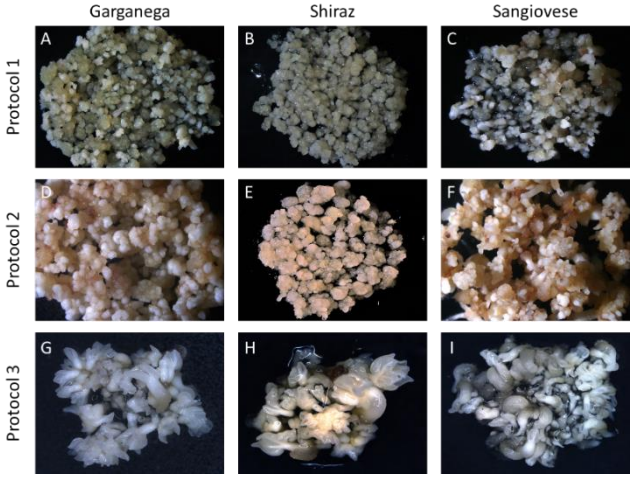

Figure 1: embryogenic tissues used in different stable genetic transformation. Embryogenic calli of Garganega (A), Shiraz (B) and Sangiovese (C) grown in GS1CA (solidified with TC agar) for two weeks. Embryogenic calli of Garganega (D), Shiraz (E) and Sangiovese (F) grown in GS1CA (solidified with Bactoagar) for one month. Somatic embryos at different developmental stages of Garganega (G), Shiraz (H) and Sangiovese (I) grown in X6 medium for one month.

The amount of material used for transformation was different from a protocol to another. Regarding protocol 1 and 2, about 1 and 2 grams of embryogenic calli have been used, respectively, while, regarding protocol 3, about 100 somatic embryos at mid cotyledonary stage

of development have been used. Genetic transformation has been performed using a Golden Braid vector (Sarrion-Perdigones et al., 2013), harboring a transcriptional unit for the constitutive expression of both *nptII* and *GFP*, and an overnight *Agrobacterium* culture at different OD_{600}: 0.8-1.0 for protocols 1 and 3 and 0.4 for protocol 2. EHA105 was the *Agrobacterium* strain used for each stable transformation protocol: it is the most efficient strain for *Agrobacterium* gene transfer into grapevine and it has been widely used in most of stable transformation experiments (Vidal et al., 2010). The necrosis of embryogenic culture is often *Agrobacterium*-induced: different OD_{600} value should show different necrosis effects. However, after 3- and 2-days post transformation (d.p.t.) for protocol 1 and 2, respectively, embryogenic calli of each variety show a slight browing. (Figure 2 A-F). Instead, 3 d.p.t. a more evident browning was found in somatic embryos of each cultivars (Figure 2 G-I). These results indicate that somatic embryos are a tissue more sensitive to *Agrobacterium* than embryogenic calli; moreover, the latter tissue has showed the same response to two different *Agrobacterium* OD_{600}.

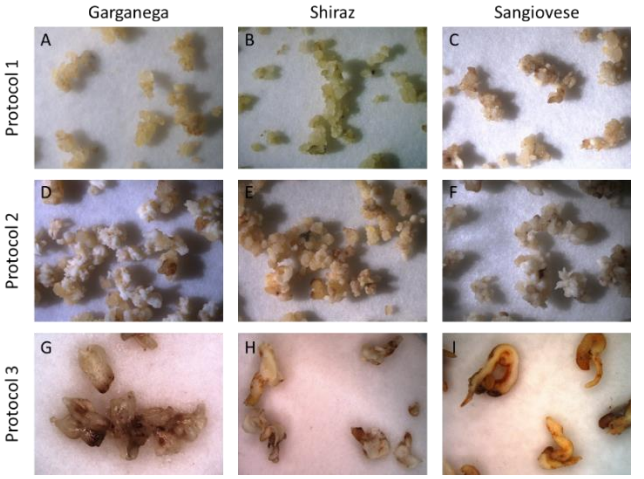

Figure 2: embryogenic tissues after *Agrobacterium* transformation. Embryogenic calli of Garganega (A), Shiraz (B) and Sangiovese (C) 3 d.p.t. (protocol 1). Embryogenic calli of Garganega (D), Shiraz (E) and Sangiovese (F) 2 d.p.t. (protocol 2). Somatic embryos at mid-cotyledonary stage of development of Garganega (G), Shiraz (H) and Sangiovese (I) 3 d.p.t. (protocol 3).

GFP transient expression has been analyzed in each embryogenic culture for each cultivar. After 3 and 2 d.p.t. for protocol 1 and 2, respectively, embryogenic calli of Garganega exhibited a good GFP transient expression with both protocol (Figure 3A, D), while Shiraz and Sangiovese

showed good GFP expression level (Figure 3B, E, C, F) only with one protocol (protocol 2 for Shiraz and protocol 1 for Sangiovese, Figure 3E, C, respectively). For these two cultivars, these results could be due to a too high *Agrobacterium* OD_{600} (protocol 1 with Shiraz, despite the absence of necrotic effects) or a too low *Agrobacterium* OD_{600} (protocol 2 with Sangiovese). However, the visualization of a specific GFP fluorescence signal in embryogenic calli of each cultivar indicate that this tissue can be used for genetic transformation. Regarding the protocol 3, 3 d.p.t. somatic embryos of Garganega and Shiraz showed a very high GFP transient expression (Figure 3G, H), while in somatic embryos of Sangiovese the GFP expression level is almost absent (Figure 3I). Furthermore, the GFP fluorescence intensity for the first two cultivars is higher than the signal visualized in embryogenic calli of the same varieties. Despite the browning caused by cocultivation with *Agrobacterium*, somatic embryos of Garganega and Shiraz seem to be a very suitable tissue for genetic transformation, while somatic embryos of Sangiovese are very recalcitrant to transformation.

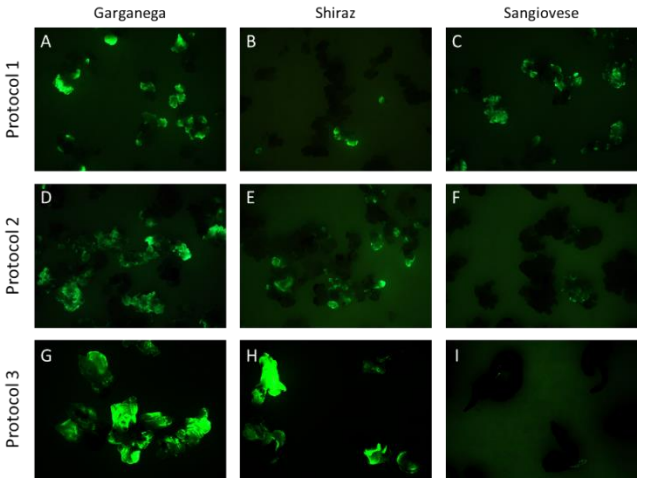

Figure 3: GFP transient expression analysis after *Agrobacterium* cocultivation. A, B, C: GFP expression (3 d.p.t) in embryogenic calli of three cultivars transformed with protocol 1. D, E, F: GFP expression (2 d.p.t) in embryogenic calli of three cultivars transformed with protocol 2. G, H, I: GFP expression (3 d.p.t) in somatic embryos of three cultivars transformed with protocol 3.

After cocultivation, embryogenic calli and somatic embryos of each cultivars have been washed with selective liquid medium to remove *Agrobacterium*, and then embryogenic calli (protocols 1 and 2) have been transferred in selective GS1CA medium to induce transgenic somatic embryos development while somatic embryos (protocol 3) have been transferred in selective

DM medium to induce transgenic calli formation. To evaluate the transformation efficiency, GFP transient expression has been constantly monitored in embryogenic tissue of each cultivar transformed with each protocol. Regarding protocol 1 and 2, 15 d.p.t. embryogenic calli of both Garganega and Shiraz cultivars were slightly browned (Figure 4A, G, B, H) and the fluorescence signal is reduced (Figure 4D, L, E, M) while embryogenic calli of Sangiovese transformed with both protocols were completely necrotized (Figure C, I) and the GFP expression is completely absent (Fig. F, N); despite the absence of browning in first phase post transformation, this result is well representative of the *Agrobacterium*-induced necrotic effect (Dhekney et al., 2009). Concerning protocol 3, 15 d.p.t. somatic embryos of each varieties showed a dark brown color (Figure 4O, P, Q); however, the fluorescence signal in both Garganega and Shiraz cultivars is still quite evident (Figure R, S), although it is lower than the first phase of transformation, while the GFP expression level in Sangiovese cultivar is completely absent (Figure 4T). The reduction of GFP expression in both embryogenic calli and somatic embryos of Garganega and Shiraz cultivars in the next phases of transformation is coherent with other results (Kandel et al., 2016; Dhekney et al., 2008).

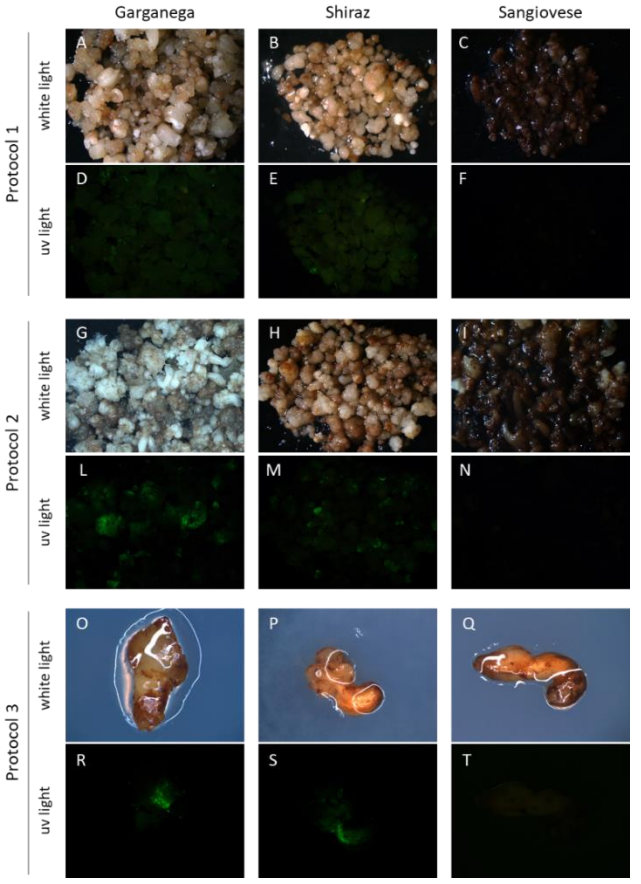

Figure 4: GFP expression analysis (15 d.p.t.) in embryogenic tissues of three cultivars. Embryogenic calli of Garganega, Shiraz and Sangiovese (A-C) transformed with protocol 1 and fluorescence signal emission (D-F). Embryogenic calli of Garganega, Shiraz and Sangiovese (G-H) transformed with protocol 2 and fluorescence signal emission (L-N). Somatic embryos of Garganega, Shiraz and Sangiovese (O-Q) and fluorescence signal emission (R-T).

These preliminary results of GFP transient expression showed that both embryogenic tissue of Garganega and Shiraz have a good response to stable transformation. Regarding Sangiovese, the browning of embryogenic tissues and the low or the complete absence of fluorescence signal in each transformation indicate that both embryogenic tissues of Sangiovese are very recalcitrant to *Agrobacterium* transformation and the next phases of each protocol haven't been performed and analyzed for this cultivar.

3.1.2 Regeneration of transgenic somatic embryos and shoot development

This phase has been performed using only embryogenic tissue of Garganega and Shiraz cultivars. Regarding protocol 1 and 2, 30 d.p.t. embryogenic calli were transferred to growth regulator-free medium for somatic embryos formation and proliferation. The GFP expression is considerably reduced than the preliminary phase of transformation; however, during the first cycle of regeneration, most of embryogenic calli were used to induce somatic embryos formation, excluding the necrotic tissue, while in next cycles, only cluster of GFP-expressing cells were transferred in fresh medium. First transgenic GFP-expressing somatic embryos of Shiraz (Figure 5) started to germinate 8 and 7 months after transformation for protocol 1 and 2, respectively, while the regeneration of the first transgenic GFP-expressing somatic embryos of Garganega (Figure 5) has begun 7 and 9 months after transformation for protocol 1 and 2, respectively.

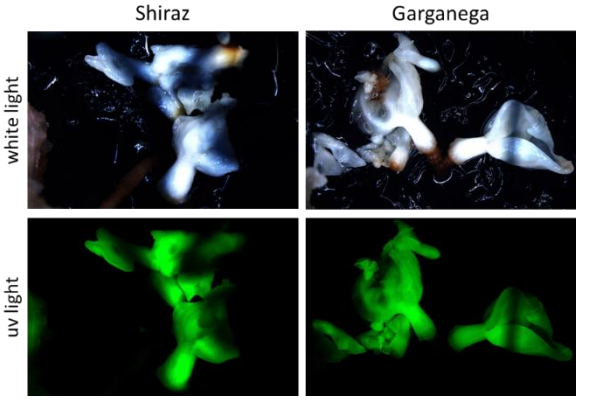

Figure 5: regenerated transgenic GFP-expressing somatic embryos of Shiraz and Garganega cultivars.

To further development, regenerated transgenic embryos of both cultivars of both protocols 1 and 2 have been maintained in the same growth regulator-free medium for another month before the transfer to shooting medium. Regarding the protocol 1, the number of well-developed regenerated transgenic embryos was 12 for Shiraz and 10 for Garganega (Table 1). To promote shoot formation, cotyledons of somatic embryos have been removed after 3 weeks in shooting medium. However, after this period, the shoots of 5 Garganega somatic embryos (Figure 6A) had already emerged while the removal of cotyledons from Shiraz somatic embryos and from remaining Garganega somatic embryos promoted the shooting after 1-2 weeks in only

6 Shiraz somatic embryos (Figure 6B). Regarding the protocol 2, the number of well-developed regenerated transgenic embryos was 3 for Shiraz and 19 for Garganega (Table 1). As for previous protocol, to encourage shoot formation, cotyledons and roots of somatic embryos have been removed after 4 weeks in MG2 medium supplemented with cytokinin. However, after 3 weeks in this medium, the shoots of 6 Garganega somatic embryos had already emerged (Figure 6C); the removal of cotyledons and roots has been performed in the remaining somatic embryos of both cultivars and then trimmed somatic embryos have been transferred to BFe2 medium to promote shooting and axillary branching. This medium is supplemented with 50 mg/L of kanamycin to favor the shooting of transformed somatic embryos. After 1-2 weeks in this medium, shoots of 1 Shiraz somatic embryo (Figure 6D) and 3 of Garganega somatic embryos (Figure 6E) emerged. The shooting of a limited number of somatic embryos seem to indicate the positive effect of selection of kanamycin; however, as described for the protocol 1, a similar number of shoots haven't emerged from somatic embryos transferred in a medium without kanamycin. As described by Iocco et al., 2001, the use of kanamycin to select only transgenic somatic embryos during the shooting phase is not effective.

Figure 6: shoot development from transgenic somatic embryos of both Shiraz and Garganega cultivars. Shoots of Garganega (A), regenerated in MS2 medium without cotyledons removal, and Shiraz (B), developed in MS2 medium after cotyledons removal, obtained with protocol 1. Shoots of Garganega (C), regenerated in MG2 medium without cotyledons removal and Shiraz (D) and Garganega (E) developed in BFe2 medium after cotyledons removal, obtained with protocol 2.

Regarding the protocol 3, somatic embryos of both Garganega and Shiraz cultivars have been maintained in DM medium to induce transgenic embryogenic calli. After 30 days in this medium (Li et al., 2008), somatic embryos of both varieties have generated transgenic but not embryogenic calli (Figures 7A, E, C, G). The same type of transgenic but not embryogenic calli has been viewed for both cultivars after 60 days in the same medium (Figures 7B, F, D, H).

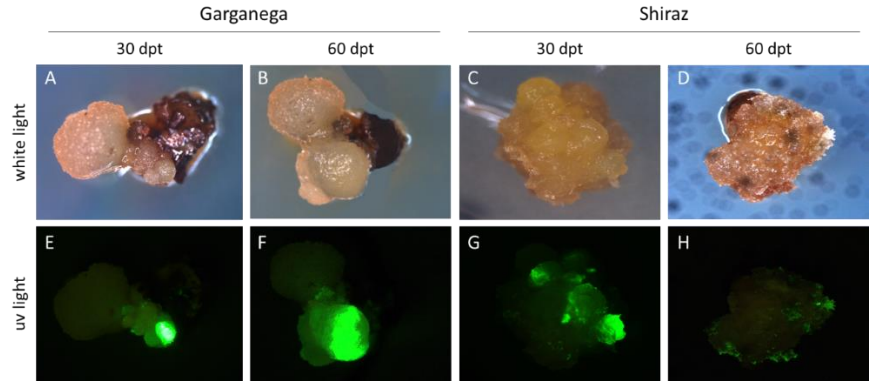

Figure 7: Regeneration of transgenic calli from somatic embryos of Garganega and Shiraz cultivars. At 30 and 60 d.p.t., somatic embryos of both varieties produced transgenic (E-H) but not embryogenic (A-D) calli.

These results indicate that the regeneration of embryogenic calli of these two cultivars from somatic embryos is a very difficult phase. Based on these results, the next phases (regeneration of transgenic somatic embryos from embryogenic calli and plant recovery) of this protocol haven't been performed.

Altogether, these results indicate that the regeneration of transgenic somatic embryos is more efficient when the *Agrobacterium* transformation is performed using embryogenic calli.

3.1.3 Transgenic plant recovery

The regeneration of transgenic whole plants has been performed using both Garganega and Shiraz transgenic somatic embryos obtained with protocols 1 and 2. Regarding the protocol 1, shoots of Shiraz and Garganega have been transferred in three-quarter-strength MS medium supplemented with indole-3-acetic acid (IAA; Kurth et al., 2012). After about 4 weeks, the number of well-developed transgenic grapevines (Figure 8) was 6 for Shiraz and 5 for Garganega (Table 1). However, the number of putatively independent transgenic line was 5 for Shiraz and 2 for Garganega (Table 1). Next genomic PCR and Real Time q-PCR analyzes should confirm the stable integration of the T-DNA region and its expression, respectively. Regarding the protocol 2, shoots of Shiraz and Garganega have been transferred to RIM to promote rooting and further plant development. After about 4 weeks, the number of well-developed transgenic grapevines (Figure 8) was 1 for Shiraz and 9 for Garganega (Table 1). However, the number of putatively independent transgenic line was 1 for Shiraz, the single regenerated plant, and 3 for Garganega

(Table 1). As for the previous protocol, next genomic PCR and Real Time q-PCR analyzes should confirm the stable integration of the T-DNA region and its expression, respectively. Furthermore, for the protocol 2, the propagation of nodal bud micro cuttings of well-developed putative transgenic grapevines into GNBC medium supplemented with kanamycin (Torregrosa et al., 2015) should discern the transgenic plants from chimeric plantlets, because, as described by Iocco et al., 2001 the use of kanamycin in this phase ensures the growth only of transgenic plants.

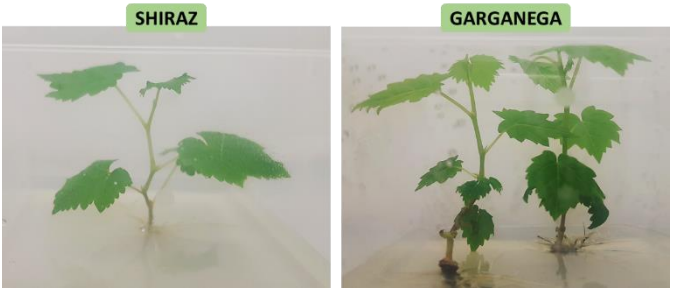

Figure 8: *In-vitro* regenerated transgenic GFP-expresssing plants of Shiraz and Garganega cultivars.

These results indicate that the protocol 1 is better for Shiraz *Agrobacterium*-mediated transformation (5 transgenic lines for protocol 1 versus 1 transgenic line for protocol 2) while there are no significative differences in the number of Garganega independent transgenic lines (2 independent lines for protocol 1 and 3 for protocol 2) between the two protocols.

Table 1: Summary of regenerated transgenic somatic embryos, shoots and plants of Shiraz and Garganega cultivars.

	Amount of initial transforming material (grams)		**N° of regenerated somatic embyos**		**N° of shoot produced**		**N° of regenerated transgenic plants**		**N° of putatively independent transgenic lines**	
Protocol	**1**	**2**	**1**	**2**	**1***	**2****	**1**	**2**	**1**	**2**
Shiraz	1	2	12	3	6	1	6	1	5	1
Garganega	1	2	10	19	5	9	5	9	2	3

* total number of shoots produced in MS2 medium with or without cotyledons removal.
** total number of shoots produced before and after the transfer to BFe2 medium with or without cotyledons removal.

After acclimation, vigorous regenerated transgenic plants of both Shiraz and Garganega cultivars obtained with both protocols 1 and 2 have been transferred to the greenhouse. Transgenic plant appeared to be healthy and grow vigorously (Figure 9); furthermore, their phenotype is comparable with that of wild type plants regenerated from embryogenic culture, indicating that *Agrobacterium*-mediated transformation and the stable integration of transgene don't alter the normal physiology of grapevine.

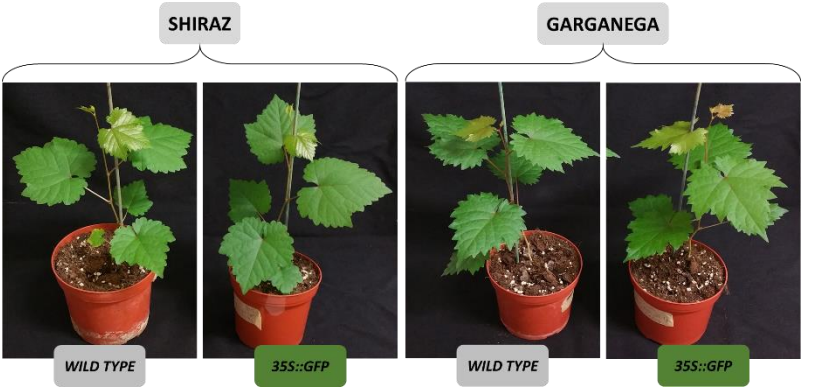

Figure 9: Regenerated transgenic GFP-expressing plants of both Shiraz and Garganega cultivars after transferring to the greenhouse.

3.2 Transient gene expression

3.2.1 Leaf agroinfiltration of whole plant grown *in-vitro*

3.2.1.1 Selection of plant material and agroinfiltration method

Most of grapevine leaf agroinfiltration experiments have been performed using plants grown *in-vitro* (Santos-Rosa et al., 2008; Bertazzon et al., 2012; Zottini et al., 2008). These types of plants grow in controlled environments and in sterile and specific conditions; they represent the optimal explant for agroinfiltration experiments. Furthermore, the very fine structure of leaf tissue should facilitate the entry of *Agrobacterium* suspension among the intercellular spaces. The agroinfiltration of plants grown *in-vitro* has been carried out using both detached (Santos-Rosa et al., 2008; Bertazzon et al., 2012) or attached (Zottini et al., 2008) leaves. In the first case, the infiltration has been performed by a vacuum system: leaves were completed infiltrated, but their removal from plants could damage or alter the expression of specific genes. In the second case, leaves have been infiltrated by syringe without needle: the infiltration is

very restricted, and the use of syringe can cause a mechanical damage. Grapevine leaf agroinfiltration has been performed using greenhouse-grown plants (Ben-Amar et al., 2015), but the growth in less controlled environments and the leaf tissue thicker could make more difficult the agroinfiltration. On the base of these considerations, the leaf agroinfiltration has been performed using whole plants grown *in-vitro* and a vacuum system.

3.2.1.2 Analysis of YFP transient expression in agroinfiltrated leaves

Whole plants grown in-vitro of 6 weeks old of Thompson seedless cultivar (Figure 10A) have been agroinfiltrated by vacuum system using the *Agrobacterium tumefaciens* strain C58C1 harboring a construct for YFP overexpression (Figure 10B, C). This *Agrobacterium* strain has been selected because it has been reported as very efficient for transient expression in different plant species (Wroblewski et al., 2005).), including grapevine (Santos-Rosa et al., 2008).

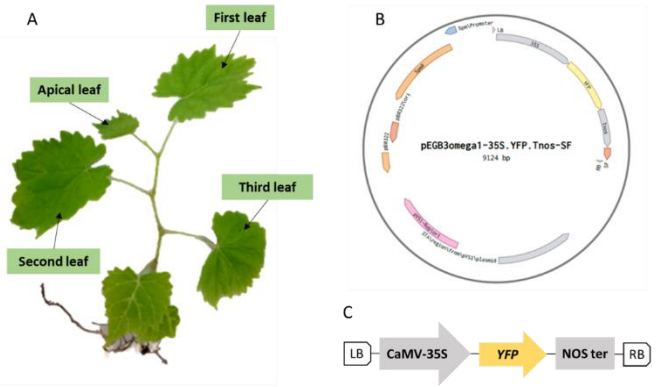

Figure 10: Grapevine plants, vector and transcriptional unit used in vacuum leaf agroinfiltration of whole plant grown *in-vitro*. A: 6 weeks old grown *in-vitro* plant of Thompson seedless cultivar. Schematic representation of the final vector (B) and the transcriptional unit (C) used for YFP transient overexpression.

To identify the day post infiltration (d.p.i.) of maximum expression and to identify the leaves with highest fluorescence emission, the analysis of YFP transient expression has been performed from 3 to 7 d.p.i. and in most of agroinfiltrated leaves, from the third to the apical, and in the stem (Figure 10A). The analysis of YFP fluorescence emission has been carried out by a stereomicroscope equipped with UV light. The results (Figure 11A) show that the YFP transient expression is observed starting from 3 d.p.i.: the fluorescence signal is especially localized in the first leaf and in the second, but in the third leaf is completely absent while in the apical leaf the

signal is very low. A similar trend of fluorescence can be observed at the 4, 5 and 6 d.p.i.: the highest YFP expression is localized in the first leaf while in the other leaves the fluorescence emission is very low or absent. The best YFP transient expression occurs at the 7 d.p.i.: the signal is again localized in the first leaf at high intensity, but it is also viewed in the second and in the third leaf, at higher level than the previous days. This qualitative analysis of the intensity of fluorescence emission seem to indicate that the d.p.i. of YFP maximum expression is the d.p.i. 7, with the highest expression in the first and in the second leaf from apex.

However, from 4 d.p.i., the YFP transient expression is also localized in the stem (Figure 11A), but the fluorescence signal is low and uneven: the expression in the stem can be considered negligible compared to the leaf. The complete absence of fluorescence signal at d.p.i. 7 in leaves and stem of plants agroinfiltrated with the same *Agrobacterium* strain harboring an empty vector (Figure 11B), confirms the YFP expression.

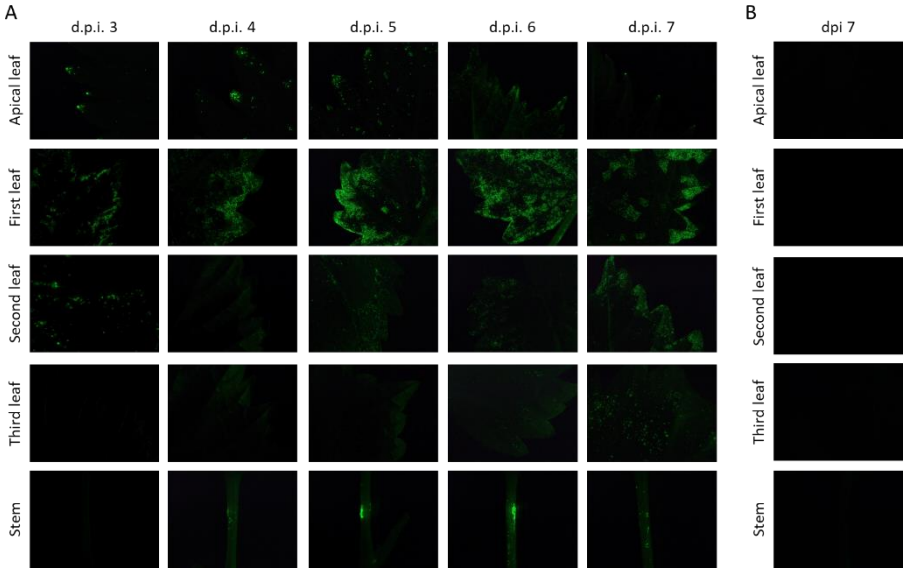

Figure 11: YFP transient expression analysis in vacuum agroinfiltrated Thompson seedless plants. A: UV light stereomicroscope images (x7 magnification) representative of the YFP expression from 3 to 7 d.p.i. in most of agroinfiltrated leaves (from third to the apical) and in the stem. B: UV light stereomicroscope images (x7 magnification) at 7 d.p.i. showing leaves and stem of Thompson seedless plants agroinfiltrated with an empty vector. Each picture is representative of two leaves/stems deriving from two independent plants. Experiment was repeated twice.

To confirm the results obtained with Thompson seedless cultivar, the same method has been tested with other two varieties, Shiraz and Garganega. The YFP transient expression has been again monitored from 3 to 7 d.p.i. but only in the first and in the second leaf from apex (Figure 12A). The results obtained for both cultivars are quite similar with Thomson seedless plants: the YFP expression starts from d.p.i. 3 and it is especially localized in the first leaf, but the expression in second leaf of both Garganega and Shiraz is quite high for the whole analysis, excluding the d.p.i. 7 for Shiraz, during which the fluorescence emission decrease considerably. This qualitative analysis of the intensity of fluorescence signal in both first and second leaf seem to indicate that the d.p.i. of maximum expression for Garganega is the d.p.i. 5 while for Shiraz is the d.p.i. 6. The absence of fluorescence signal at d.p.i. 7 in leaves of plants agroinfiltrated with *Agrobacterium* harboring the empty vector (Figure 12B), confirms again the YFP expression.

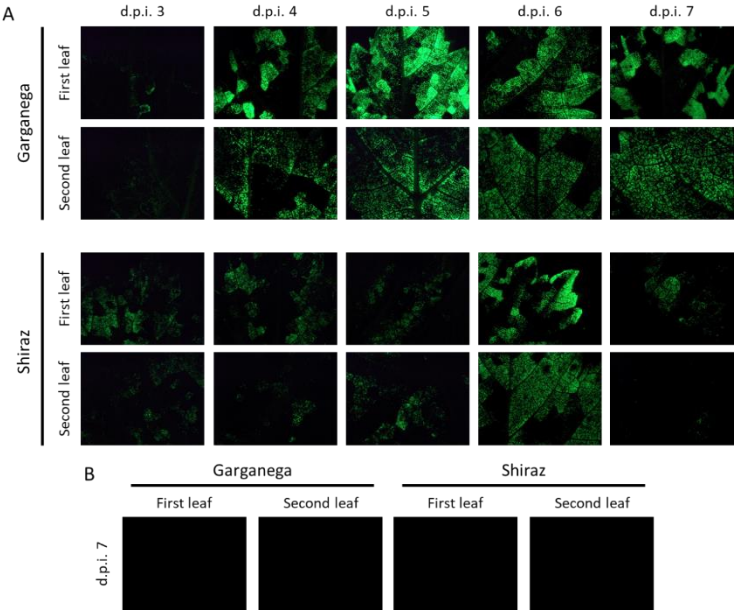

Figure 12: YFP transient expression analysis in vacuum agroinfiltrated Garganega and Shiraz plants. A: UV light stereomicroscope images (x7 magnification) representative of the YFP expression from 3 to 7 d.p.i. in first and second leaves to apex. B: UV light stereomicroscope images (x7 magnification) at 7 d.p.i. showing leaves Garganega and Shiraz plants agroinfiltrated with an empty vector. Each picture is representative of two leaves deriving from two independent plants. Experiment was repeated once.

3.2.2 Berry agroinfiltration

3.2.2.1 Fruiting cuttings as plant material selected

The application of leaf agroinfiltration in grapevine is a useful and rapid system for the characterization of specific genes involved in many processes (Jelly et al., 2014). However, many genes of interest make a specific function in grape berry and the study of their role by leaf agroinfiltration could be incomplete. The infiltration of *Agrobacterium* harboring a construct of interest directly in grapevine fruit can exceed this problem. However, the large sizes of field fruiting grapevine plants and the fruit production once a year, could make difficult the experimental design of grape berry agroinfiltration. To overcome this problem, an alternative is represented by using miniaturized fruiting cuttings. This fruiting test plants can be easily cultivated in growth chamber: they allow more than one fruits production per year, avoiding the dependence to use field-grown material and to natural season. The fruiting cuttings have been then selected for the grape berry agroinfiltration experiments.

The method described by Mullins and Rajasekaran, 1981 and illustrated in Figure 13, showed that the production of fruiting test plants is genotype dependent.

Figure 13: Main phases to produce fruiting cuttings. A: rooted cutting after five weeks in heating-bed. B: dormant bud in a rooted cutting. C: bud burst stage. D: rosette of leaf tips visible and ready for leaf

removal. E: inflorescence after basal leaves and apex removal. F: inflorescence and lateral shoot growth after three weeks of leaves removal. G: anthesis stage. H: Bunch at *veraison*. I: riped bunch. L: fruiting test plant with ripe bunch and lateral shoot with 10 leaves.

To identify the cultivar with highest capacity to produce fruit, this method has been tested in different varieties: Cabernet Sauvignon, Shiraz (two international varieties), Corvina, Corvinone, Rondinella and Sangiovese (four local varieties). Cabernet Sauvignon and Shiraz cultivars have been already tested (Mullins and Rajasekaran, 1981; Baby et al., 2015) while the method of fruiting cuttings has never been used for the four local varieties. One of the most important phases in this method is the pre-rooting; to optimize this phase, two different substrates were used: river sand and perlite. The results (Supplemental Figure 1) show that, after five weeks, the pre-rooting of each cultivar is better when river sand is used: the number of cuttings producing roots is higher and the roots generated are longer and in greater number than roots produced when perlite is used. However, cuttings of each pre-rooted cultivars with more than 5 roots > 5 cm in length (Table 2) have been used to produce fruit. The next phases of the method (removal of basal leaves at the bud-burst, excision of the shoot tip and development of lateral shoot) and the growth conditions are the same described in Mullins and Rajasekaran, 1981 and Baby et al., 2015. The critical phase of this procedure is the survival of inflorescence at the anthesis. The best result (Table 2) has been obtained for Cabernet Sauvignon and Sangiovese cultivars, followed by Shiraz. Conversely, at the anthesis, most of inflorescence of cuttings of Corvina, Corvinone and Rondinella are shriveled and died soon after anthesis. The inflorescences retained of each cuttings of each cultivars give rise to a bunch. The complete fruit ripening, excluding Corvinone, occurs after 4-5 month from the bud-burst; the size and the number of berries of every ripe fruit of every variety are similar each other (Supplemental Figure 2), except for Sangiovese, for which the sizes of bunch produced are very different. The results obtained show that the cultivars tested have a good ability to root in different substrates, but the survival of inflorescence at the anthesis and the mature bunch development of adequate sizes are cultivar dependent (Table 2). Based on the last two phases, the best results were obtained for Cabernet Sauvignon cultivar. Therefore, this variety has been used in berry agroinfiltration experiments.

Table 2: analysis of pre-rooting, inflorescence retaining and mature bunch production in fruiting test plants of different grapevine genotypes.

Cultivar	Number of cuttings pre-rooted		Number cuttings with retained inflorescence	Number of cuttings with a mature bunch
	River sand	Perlite		
Cabernet Sauvignon	6	6	12	12
Shiraz	6	3	9	9
Corvina	6	0	3	3
Corvinone	4	3	3	3
Rondinella	6	2	1	1
Sangiovese	6	6	12	12

3.2.2.2 Agroinfiltration methods

Grape berry agroinfiltration was performed only in one single case (Gao et al., 2018). In this report, berry agroinfiltration was performed using syringe with needle (agroinjection) and immature berries attached to the plant, the same procedure used for tomato fruits (Orzaez et al., 2006). Agroinjection is based on the introduction of needle in depth into the fruit tissue. The damage is minimal, the fruit remain attached to the plant and the *Agrobacterium* suspension reaches the entire fruit surface. An alternative method based on a vacuum system, the same used for leaf agroinfiltration, has never been used for grapevine. However, the vacuum agroinfiltration has been performed using detached tomato fruits (Fu et al., 2015). As described for leaf agroinfiltration, the removal of fruit from plants could damage the tissue but the positive results described are encouraging to test this method also in grapevine. Furthermore, the infiltration of detached fruits has been also performed in strawberry (Spolaore et al., 2001; Miyawaki et al., 2012), but using the method of agroinjection. The berry agroinfiltration experiments tested here are represented by agroinjection of attached berries and vacuum agroinfiltration of detached berries.

3.2.2.3 Analysis of YFP transient expression in agroinfiltrated berries

Hard-green berries (20 days post anthesis -DPA-; stage E-L 33; Figure 14A) obtained from Cabernet Sauvignon fruiting cuttings have been used for both agroinjection and vacuum agroinfiltration experiments. Berry infiltration has been performed using again the *Agrobacterium tumefaciens* strain C58C1 harboring a construct for YFP overexpression (Figure 14B, C).

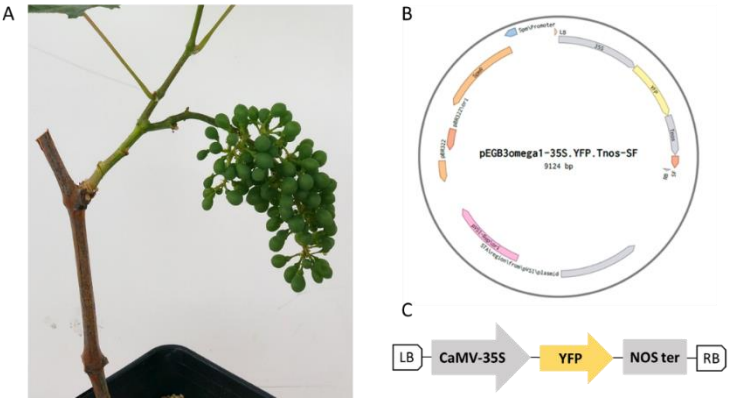

Figure 14: Grapevine berries, vector and transcriptional unit used in berry agroinfiltration experiments. A: bunch of Cabernet Sauvignon cultivar 20 d.p.a. obtained from fruiting test plants. Schematic representation of the final vector (B) and the transcriptional unit (C) used for YFP transient overexpression.

The YFP transient expression has been analyzed 3 and 6 d.p.i.. Upon dissection of infiltrated berries, the fluorescence signal was analyzed by a stereomicroscope equipped with UV light. 3 d.p.i. (Figure 15A) the YFP expression in agroinjected berries is very low and it is localized only in the inner flesh while the fluorescence emission in vacuum agroinfiltrated berries is higher, but it is again localized only in the inner flesh, especially around the seeds. 6 d.p.i. (Figure 15B), the fluorescence signal is completely absent in agroinjected berries while the YFP expression in vacuum agroinfiltrated berries is lower than 3 d.p.i. but it is always localized in the inner flesh around the seeds. Despite these positive and encouraging results, the efficiency of this method remains quite low: indeed, the highest YFP expression visualized in the vacuum agroinfiltrated berry showed in Figure 15A is representative of twenty in fifty agroinfiltrated berries. The absence of fluorescence signal in the negative control of both approaches confirms the YFP expression (Figure 15C).

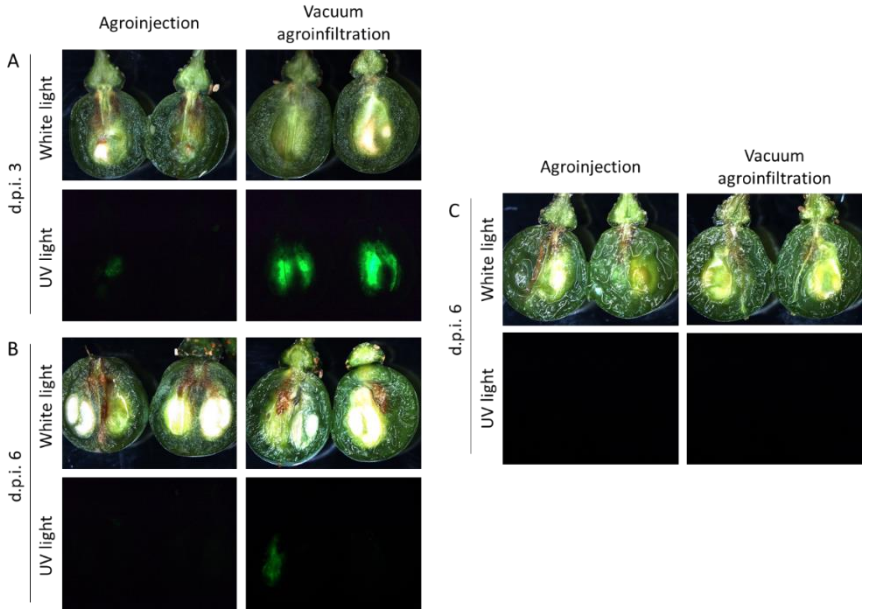

Figure 15: YFP transient expression analysis in attached agroinjected berries and in detached vacuum agroinfiltrated berries. A-B: White light and UV light stereomicroscope images (x7 magnification) representative of YFP expression in agroinjected and vacuum agroinfiltrated berries at 3 and 6 d.p.i. C: White light and UV light stereomicroscope images (x7 magnification) showing berries agroinjected and vacuum agroinfiltrated with an empty vector. Pictures are representative of two independent experiments.

4. DISCUSSION

4.1 Stable genetic transformation

To identify an optimized and standardized stable transformation method of *Vitis Vinifera*, three different protocols were tested in three different cultivars, Shiraz, Garganega and Sangiovese. The protocols have been especially compared in terms of number of regenerated transgenic plants, but the response to *Agrobacterium tumefaciens*, the number of regenerated transgenic somatic embryos, the capacity of shooting in different media and the plant recovery were also tested.

One of the most important factors for stable transformation is embryogenic tissue. Previous study (Franks et al., 1998) has reported that the best tissue for the stable transformation are embryogenic calli for their high efficiency to embryo regeneration. However, the proliferation

of somatic embryos of grapevine occurs by direct secondary embryogenesis from single epidermal or subepidermal cells; therefore, somatic embryos represent ideal targets for transformation, since the regenerative cells are accessible to *Agrobacterium* (Gray et al., 2005). In this work, both embryogenic calli and somatic embryos have been used.

The transformation of both embryogenic tissues has been performed using *Agrobacterium tumefaciens* strain EHA105 harboring the vector pEGB3α1-TNOS::*NPTII*::PNOS-SF-35S::*GFP*::TNOS-SF; in this way the identification and the selection of transformants can be performed by GFP analysis and use of a selective medium. The transformations have been carried out testing different *Agrobacterium* OD_{600}: this is another important factor to evaluate, because *Agrobacterium* is the main cause of embryogenic culture browning during the transformation and different *Agrobacterium* OD_{600} can have different effects on transformation efficiency.

Regarding protocols 1 and 2, the maintenance of embryogenic calli of each cultivar in GS1CA medium (solidified with different gelling agent) for different times before transformation haven't shown difference in the specific structure of calli (Figure 1). Furthermore, the use of different *Agrobacterium* OD_{600} (0.8-1.0 and 0.4) has caused some browning of embryogenic tissue (Figure 2), indicating that embryogenic calli have a good response to this factor; furthermore, excluding Shiraz and Sangiovese transformed using the protocols 1 and 2 respectively, an acceptable GFP expression level has been identified after cocultivation with *Agrobacterium* (Figure 3). However, different GFP transient expression in Shiraz and Sangiovese cultivars visualized in the first phase of the process using different *Agrobacterium* OD_{600}, suggests that this parameter is important for the transgene expression. These results are well representative of the wide variations of cultivars in response to genetic transformation (Dhekney et al, 2009). During the next phase of transformations, a considerably GFP expression reduction in each cultivar has occurred (Figure 4). This is probably caused by the *Agrobacterium* elimination and the long time necessary for the stable integration of the transgene in the embryogenic tissue. This decreasing of fluorescence signal has been particularly evident in Sangiovese cultivar (Figure 4); this result, associated with the complete browning of embryogenic culture, indicates that this variety is unsuitable to *Agrobacterium* transformation and it hasn't used in the next phases of transformations. Otherwise, both Shiraz and Garganega cultivars have regenerated both transgenic somatic embryos and plants. The regeneration of

transgenic GFP-expressing Shiraz somatic embryos has occurred 8 and 7 months after transformation for protocol 1 and 2, respectively (Figure 5); this time of regeneration is longer than the 12 weeks for Shiraz cultivar described by Iocco et al, 2001. The number of regenerated transgenic somatic embryos of Shiraz cultivar is 12 for protocol 1 and 3 for protocol 2, starting from 1 and 2 grams of embryogenic calli, respectively. This result seem to indicate that the quantity of initial transforming material it is not essential to obtain a higher number of transgenic somatic embryos; the highest number of regenerated somatic embryos of this cultivar obtained with protocol 1 may be due to the shorter maintenance time in GS1CA medium because a too long period in this medium could reduce the embryogenic potential of calli. Another consideration could be represented using different gelling agent: Bactoagar used in the protocol 2 may reduce the embryogenic potential, proving that TC agar is the best gelling agent to maintain high embryogenic capacity (Li et al., 2008). However, the number of regenerated transgenic somatic embryos of Shiraz is lower than those described by Iocco et al, 2001 (161 somatic embryos), but many transgenic somatic embryos produce a shoot (6/12 for protocol 1 and 1/3 for protocol 2; Figures 6B, D). Regarding this phase, an essential strategy for Shiraz shoot formation is represented by cotyledons excision, confirming the importance of this approach (Li et al., 2008; Dhekney et al., 2009). The number of putatively regenerated independent transgenic plants (5 for protocol 1 and 1 for protocol 2; Figure 8) of Shiraz cultivar is lower than those reported by Iocco et al, 2001 (45 transgenic plants). This result may be explained by the low quality of embryogenic calli and by their low potential to regenerate transgenic somatic embryos. As described by Iocco et al., 2001, these two parameters are essential to successfully regenerate transgenic plants. Regarding Garganega cultivar, the first transgenic GFP-expressing somatic embryos have started to germinate 7 and 9 months after transformation for protocol 1 and 2, respectively (Figure 5); this time of regeneration of somatic embryos is similar with the results obtained by Gambino et al., 2005 (9 months for the germination of the first embryos of Nebbiolo cultivar). The number of regenerated transgenic somatic embryos of Garganega is 10 and 19 for protocol 1 and 2 respectively. The lowest number of somatic embryos obtained with protocol 1 may be explained with opposed considerations than Shiraz cultivar: Garganega requires a longer maintenance time in GS1CA medium and the Bactoagar ensures a good embryogenic potential. Another explanation could be represented by the amount of initial transforming material: a higher quantity of embryogenic

calli (2 grams for protocol 2 and 1 gram for protocol 1) ensures a higher somatic embryos regeneration. The number of regenerated transgenic somatic embryos of Garganega with both protocols is like that reported for other varieties (Iocco et al., 2001; Gambino et al., 2005). Furthermore, shoots emerge from many Garganega somatic embryos (5 and 9 for protocol 1 and 2, respectively) and most of somatic embryos (5 and 6 for protocol 1 and 2, respectively) doesn't require the cotyledons removal to promote the emerging of shoots (Figure 6A, C), indicating that this strategy is only partially essential for this variety. The number of putatively regenerated independent transgenic plants of Garganega cultivar (Figure 8) was 2 for protocol 1 and 3 for protocol 2. As for Shiraz, the number of transgenic Garganega grapevine is low but is like the one reported for other cultivars (Iocco et al., 2001; Gambino et al., 2005). This could be due for the same considerations described for Shiraz; however, this is the first work describing the regeneration of transgenic somatic embryos of Garganega: since it is impossible to do a comparison with other works, this number or transgenic plants could be the effective potential of this variety.

Regarding the protocol 2, the use of kanamycin during the shooting phase is not effective to promote the shoot formation only from transgenic somatic embryos (see Results, section 3.1.1); the future transfer of putative transgenic plants in a medium supplemented with the same antibiotic should discerne among transgenic and not-transgenic plants, confirming the results described by Iocco et al., 2001. After the evaluation of this strategy, it could be tested during the rooting and plant development phases of the protocol 1. However, the stable integration and the expression of the transgene can be confirmed only after genomic PCR and real Time qPCR analyzes.

Concerning protocol 3, somatic embryos at mid-cotyledonary stage of development, excluding Sangiovese cultivar, are characterized by a high GFP transient expression in the first phase of the process, indicating that they represent a good tissue for stable transformation. Furthermore, the *Agrobacterium* OD_{600} (0.8-1.0) has cause only some browning, especially in the stem of somatic embryos, indicating that is suitable for both good transgene expression and low tissue necrosis. The next phase of this protocol is the induction of transgenic embryogenic calli from somatic embryos. During this phase, as for the previous 2 protocols, the fluorescence signal is considerably reduced for the same causes previously described. However, after 60 days in selective calli induction medium, both somatic embryos of Shiraz and Garganega have

generated transgenic but not embryogenic calli (Figures 7B, D, F, H), indicating that the transgene has been stably integrated, but the tissue doesn't have the potential to regenerate embryogenic calli. Consequently, the next phases of regeneration of transgenic somatic embryos and plant haven't been carried out. This result may be due to a selection of embryos at a wrong stage of development because the same protocol has been already tested with Shiraz cultivar (Li et al., 2008; Dhekney et al., 2009) with positive results in terms of regeneration of both transgenic somatic embryos and plants. Another factor could be represented by the embryogenic culture age: Dhekney et al., 2009 have reported that the regeneration of transgenic somatic embryos of Shiraz is efficient only using 4 months embryogenic culture while somatic embryos of Shiraz cultivar used in this study deriving from about 2 years embryogenic calli. Regarding Garganega, this procedure has never been tested with this variety: in this case the negative results may be due to the same causes described for Shiraz or to the composition of medium used to induce embryogenic calli. In fact, the generation of embryogenic calli from somatic embryos of a specific cultivar depends by composition of the medium (Dhekney et al., 2009). To improve this protocol of stable transformation, the response of Garganega and Shiraz somatic embryos at different calli induction medium and the use of younger embryogenic culture will be tested in the future. Regarding Sangiovese, the complete absence of GFP expression during all the phases of the process (Figures 3, 4) has suggested that the somatic embryos of this cultivar are inadequate to stable transformation. These results, added with those obtained with the others 2 protocols, indicate that Sangiovese cultivar is very recalcitrant to genetic transformation.

The results described in this work have shown that the regeneration of transgenic plants has been performed only using embryogenic calli of Shiraz and Garganega cultivars (Figures 8, 9). The regeneration of transgenic somatic embryos and plants is affected by many parameters, included maintenance time in GS1CA medium, the gelling agent, the Agrobacterium OD_{600} and the cotyledons excision. The best results in terms of number of transgenic plants of Shiraz has been obtained with protocol 1, while there haven't been differences for the number of Garganega transgenic grapevines between the two protocols. These results indicate that each cultivar has a specific response to different protocols of stable transformation; furthermore, to identify the best procedure, it is important to test some protocols with each selected variety. Despite the complexity and the long time necessary for its fulfillment, this approach can be

successfully used with grapevine and its application can be extended to the functional characterization of gene of interest.

4.2 Transient gene expression

The agroinfiltration, the historic and most useful approach among the *Agrobacterium*-mediated transient expression methods, has been performed in two different grapevine tissues: leaves of grapevine plantlets grown *in-vitro* and berries obtained from grapevine fruiting cuttings. Both agroinfiltration experiments have been carried out using the *Agrobacterium tumefaciens* C58C1 harboring the vector pEGB3Ω1-35S::*YFP*::TNOS-SF; in this way the transient expression in grapevine tissues has been monitored by YFP analysis.

Leaf agroinfiltration of whole plants grown *in-vitro* has been performed using a vacuum system; this approach has been already tested in other reports (Visser et al., 2012; Kurth et al., 2012) but in this work it has been improved analyzing the YFP transient expression at different days post infiltration (d.p.i.) and in different leaves. In this way it has been possible to identify the d.p.i. of maximum YFP expression and the leaves with highest protein expression. This system has been tested using three grapevine cultivars: Thompson seedless, Shiraz and Garganega. The fluorescence signal analysis showed that the YFP expression is especially localized in the first and in the second leaves from apex in each variety (Figures 10A, 11A). The highest YFP expression only in the youngest leaves is probably due to the lower thickness of these leaves with consequent increase of infiltration. The leaf position effect has already been documented (Santos-Rosa et al., 2008), indicating that the highest gene expression occurs only in the first fully expanded leaf. This study confirms this result, but it shows a high YFP transient expression also in the second fully expanded leaves. However, the results described by Santos-Rosa et al., 2008 deriving from vacuum agroinfiltration of detached leaves: the low or absent gene expression in the second leaf could be due by leaf physical damage. Furthermore, this study showed a very high YFP expression in each cultivar, excluding a varietal effect in the transient expression. Nevertheless, the qualitative analysis of fluorescence emission indicates that the d.p.i. of YFP maximum expression is different among the three cultivars (Figures 10A, 11A). Despite these differences in the YFP maximum expression among different cultivars, the vacuum agroinfiltration of grapevine whole plant grown *in-vitro* is an efficient and versatile method that it can be used for the expression of gene of interest in different cultivars.

Grapevine leaf agroinfiltration represents an efficient method to study gene function in a very shorth time. However, many genes of interest perform a specific function in grapevine fruit. The study of their role in leaf can be incomplete and the production of berries from transgenic plants is a very long and difficult process. The development of a rapid method for gene function study directly in berries is essential. Berry agroinfiltration is an approach that can satisfy this need. In this work, the agroinfiltration of grapevine fruit has been performed using berries derived from fruiting cuttings. This miniaturized fruiting test plants allow more than one fruits production per year and they represent a very useful material for berry agroinfiltration. The production of fruiting cuttings test plants has been tested using different genotypes (see Results) following the methods described by Mullins and Rajasekaran, 1981 and Baby et al., 2015. All cultivar tested have a good capacity to produce roots (Table 2, Supplemental Table 1), but the survival of inflorescence at the anthesis and the production of bunch of adequate sizes (Table 2, Supplemental Figure 2) only occur for some of them. The best results were obtained for Cabernet Sauvignon (Supplemental Figure 2) and the berries of this cultivar have been used in agroinfiltration experiments.

The agroinfiltration of fleshy fruits has already performed in tomato (Orzaez et al., 2004; Fu et al, 2005) and strawberry (Chai et al., 2011; Spolaore et al., 2001; Miyawaki et al., 2012); the fruit infiltration, is generally performed by using syringe with needle (Orzaez et al., 2004; Chai et al., 2011; Spolaore et al., 2001; Miyawaki et al., 2012). In grapevine, fruit infiltration has been tested only by Gao et al., 2018 using the same approach. However, Fu et al., 2005 have performed the vacuum agroinfiltration of detached tomato fruits. Based on these considerations, in this study both vacuum agroinfiltration of detached fruits and agroinjection of attached fruits have been tested. All experiments have been performed using hard-green berries 20 days post anthesis of Cabernet Sauvignon fruiting cuttings: this developmental stage was selected because the putative master regulators of berry ripening (see Chapters 2, 4 and 5) haven't expressed; in this way their overexpression should activate the molecular programs associated with them and promote an anticipation of ripening. Berry agroinfiltration was performed using again YFP as reporter gene and its transient expression was monitored at 3 and 6 d.p.i.. The analysis of fluorescence signal showed that the YFP expression in vacuum agroinfiltrated berries is higher than agroinjected berries and the highest YFP transient expression has been identified 3 d.p.i. in vacuum agroinfiltrated berries (Figure 14A); however, as described in Results section 3.2.2.3,

the efficiency of this method is very low: the YFP transient expression showed in Figure 15A occurs only in twenty in fifty agroinfiltrated berries. Nevertheless, in both methods, the YFP transient expression is higher in the first days after infiltration and this result is similar with those obtained in tomato (Orzaez et al., 2004). These results indicate that the method of vacuum agroinfiltration in grapevine is more efficient than agroinjection. Furthermore, the detached berries aren't damaged, and the presence of pedicel could favorite the entry of *Agrobacterium* suspension, as confirmed by the localization of YFP expression only in the central area of berry flesh. Altogether, these results indicate that, fruit agroinfiltration can also be performed in grapevine: some improvements are required but these encouraging results seem to indicate that the gene function will be studied directly in berries, avoiding to wait the long periods of time necessary for the regeneration of transgenic berries obtained by stable transformation methods.

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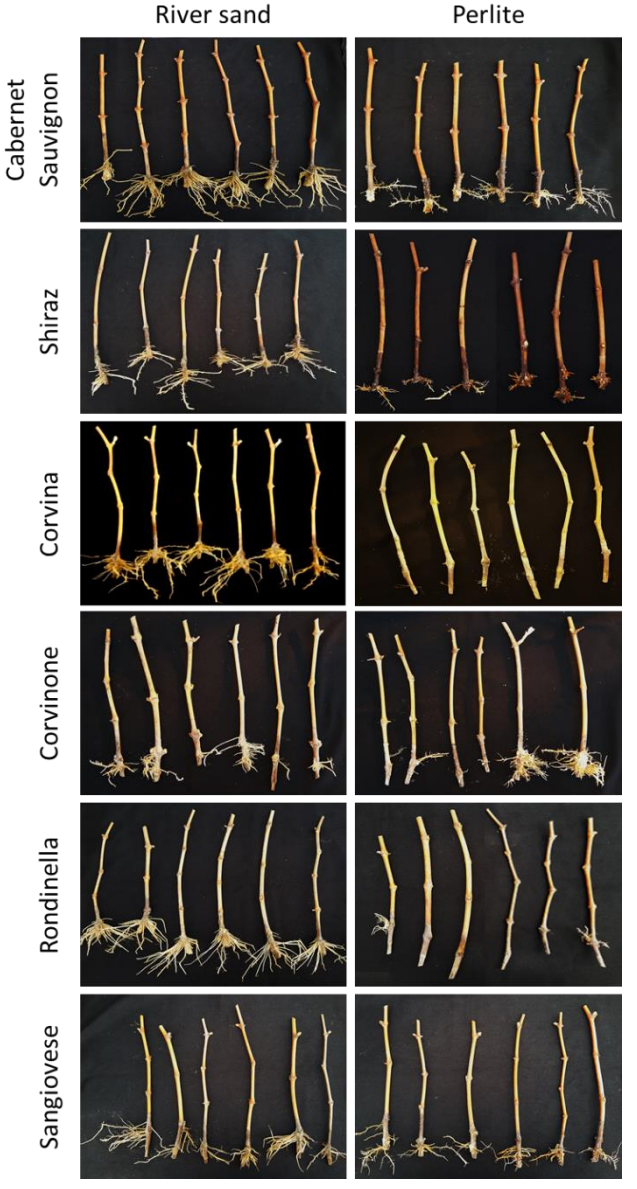
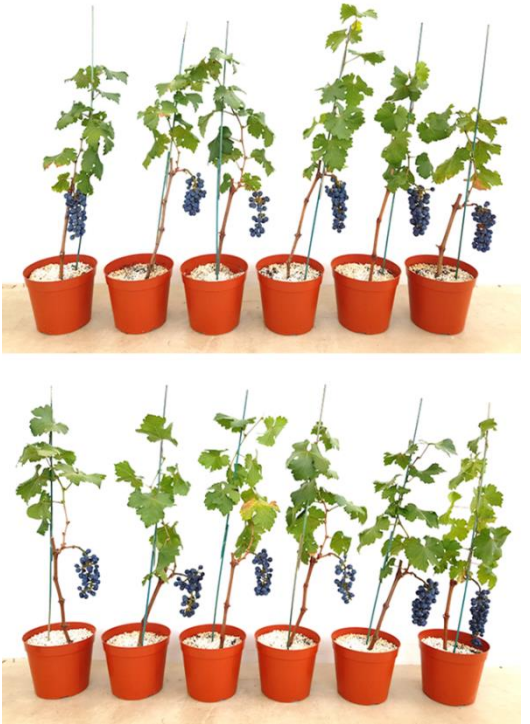
SUPPLEMENTAL DATA

Supplemental Figure 1: root development of different grapevine cultivars in two different substrates after five weeks in the heating bed (25°C).



Supplemental Figure 2: ripe bench development of different grapevine cultivars after 4-5 month from the bud-burst.

Cabernet Sauvignon



Shiraz

Sangiovese

Corvina

Corvinone

Rondinella

Chapter 4

ROLES OF *VviNAC33* AND *VviNAC60* IN GRAPEVINE DEVELOPMENT

ABSTRACT

NAC transcription factors represent one of the most important transcription factor family in plants. They are involved especially in the regulation of plant development and in biotic and abiotic stress responses. However, in grapevine, the specific functions of NAC genes are poorly known. Recent studies showed that some NAC transcription factors are induced in grapevine mature organs, suggesting they may have a role in the regulation of the maturation process. Two of them, *VviNAC33* and *VviNAC60* have been selected for functional analysis. In a previous work, the overexpression of each NAC gene in grapevine plants showed that *VviNAC33* affected the chlorophyll metabolism in leaves, while *VviNAC60* altered the plant growth and caused a higher accumulation of anthocyanins in leaves, suggesting an involvement of both NAC factors with different roles in the regulation of the processes associated to grapevine organ maturation/senescence. Furthermore, the molecular analysis of transgenic leaves showed an upregulation of many genes involved in the maturation process. Here, the coding sequence of these factors were fused to the EAR transcriptional repression motif, in order to turn them into strong repressors of transcription, and these constructs were used to transform grapevine under the control of their respective endogenous promoters. After the regeneration of transgenic plants, their phenotypic and molecular characterization showed a normal vegetative growth and the downregulation of some genes previously induced in the overexpressing plants. These results indicate that the addition of the EAR motif was successful in turning these factors into transcriptional repressor and confirm some of the preliminary data obtained from the overexpressing plants. Further molecular studies will allow to better define the function of these transcription factors and confirm their hypothesized role of master regulators of the vegetative-to-mature transition of grapevine.

1. INTRODUCTION

VviNAC33 and *VviNAC60* are transcription factors identified as candidates for functional analysis in grapevine. They have been identified as switch genes by the network analysis performed on the grapevine expression atlas dataset (Palumbo et al., 2014), suggesting they may play a specific role in the regulation of vegetative-to-mature transition in most grapevine organs. Furthermore, they emerged as switch genes from the analysis of berry specific transcriptomes (Palumbo et al., 2014; Massonnet et al., 2017) and they were found among the markers of the first transition representative of the onset of ripening described by Fasoli et al., 2018. These findings indicate they may have a general role in the processes associated to many maturing organs including ripening berry. The functional characterization of their roles could provide important information about the molecular mechanism involved in the regulation of the maturation-associated processes in grapevine.

NAC proteins are one of the largest families of plant-specific transcription factors. The NAC acronyms derived from NAM (No apical meristem), ATAF (Arabidopsis transcription activation factor) and CUC (Cup-shaped cotyledon), the first three characterized NAC proteins in Petunia and Arabidopsis (Zhu et al., 2012). NAC transcription factors are defined by the presence of the highly conserved NAC domain, at the N-terminal, which is divided into five conserved subdomains, involved in DNA-binding and responsible for protein-binding and dimerization. The C-terminal region of NAC proteins is more diverged, and it functions as a potential transcriptional regulatory domain which has either activator or repressor role and it may possess protein binding activity (Puranik et al., 2012). Proteins that contain the NAC domain are especially involved in the regulation of developmental processes, including embryonic, floral and vegetative development, lateral root formation and regulating senescence but they are also involved in auxin signaling, cell division and abiotic and biotic stress responses (Olsen et al., 2005; Zhu et al., 2012). NAC transcription factors have been identified and studied in many plant species, including *Arabidopsis* (105 NAC genes; Ooka et al., 2003), *Populus trichocarpa* (163 NAC genes, Hu et al., 2010) rice (151 NAC genes; Nuruzzaman et al., 2010) and tomato (104 NAC genes, Su et al., 2015). In grapevine, a genome-wide analysis has identified 74 NAC genes (Wang et al., 2013). The analysis of the expression profile of the entire NAC gene family revealed a wide differentiation in terms of specific pattern of expression of each NAC factor in in different tissues at different developmental stages and in response to abiotic and biotic stress conditions.

These results indicate that grapevine NAC transcription factors are especially involved in the regulation of development processes and in the stress responses; however, a complete and exhaustive functional characterization of these genes in grapevine has not yet been carried out. As previously described, among grapevine NAC genes, *VviNAC33* and *VviNAC60* seem to be key genes during the regulation of maturation process and they have been selected for functional analysis. In a previous work (D'Incà, 2017), the overexpression of *VviNAC33* and *VviNAC60* in grapevine by stable transformation approach (see Chapter 3 for a more detailed description of the method), has provide interesting information about the roles of these two genes during the vegetative-to-mature transition. Indeed, the overexpression of *VviNAC33* alters the chlorophyll metabolism and it causes an anticipated leaf chlorosis and senescence, a typical event during the maturation process, while the constitutive expression of *VviNAC60* impairs the normal plant development, with smaller leaves and stunted growth, indicating that its high expression could alter the standard developmental process; furthermore, the overexpression of *VviNAC60* causes an increase of red/purple coloration in transgenic leaves, suggesting an accumulation of anthocyanins, secondary metabolites responsible also of the red berry coloring starting at *veraison*. These preliminary results are consistent with a specific involvement of these two NAC genes in regulation of maturation processes in grapevine. To obtain more information about the role of *VviNAC33* and *VviNAC60* and on the molecular network in which they may take part, a microarray analysis on transgenic overexpressing leaves has been performed. The results have shown an upregulation of many genes involved in processes associated with ripening, suggesting that both *VviNAC33* and *VviNAC60* play mainly a role of transcriptional activators and confirming once more their putative involvement in the regulation of vegetative-to-mature transition in grapevine.

The functional characterization of genes can be also performed using loss-function approaches; among the most used methods in plant science, the RNA antisense approach should be mentioned, but in the last years an alternative method specific for the analysis of transcriptional activators has been tested. This approach is based on the use of transcriptional repression domains to convert the transcription factors into transcriptional repressors, inhibiting the expression of its target genes. In plants, one of the best characterized motives of transcriptional repression is the EAR (Ethylene-responsive element-binding factor-Associated amphiphilic Repression) motif (Kagale and Rozwadowski, 2011). EAR motif-mediated transcriptional

repression suppresses the expression of target genes through chromatin modification of regulatory regions by histone deacetylation via physically interacting with co-repressors. Chromatin modification, together with DNA methylation, are two key mechanisms involved in the epigenetic regulation of gene expression; a typical epigenetic pathway starts in response to intrinsic or external signals and it is coordinated by a complex network among transcriptional regulators, co-regulators and chromatin modifying factors. Transcriptional regulators can be both activators or repressors and they play an important role in perceiving and integrating internal or external signals to establish the correct epigenetic state and to obtain the appropriate phenotypic response. Proteins containing EAR motif are transcriptional repressors and EAR motif play a key role in the epigenetic reprogramming of gene expression during plant development and plant responses to stress and hormonal signals. The use of EAR motif to convert transcriptional activators into transcriptional repressors was tested in *Arabidopsis* (Hiratsu et al., 2003); the results of this work showed that the use of EAR motif allowed to convert specific transcription factors into strong transcriptional repressors and the chimeric repressors suppress the expression of specific target genes. Based on these considerations, it is possible to use the EAR motif to study the function of a specific transcription factor.

To gain information about the function of both NAC genes, in this chapter, *VviNAC33* and *VviNAC60* have been converted into transcriptional repressors by the fusion with the EAR motif. The chimeric repressors have been stably expressed in grapevine plants under the control of their respective endogenous promoters: in this way, the transcriptional repression of putative target genes will likely occur in the organs and at the developmental stages when endogenous genes are normally expressed.

2. MATERIALS AND METHODS

2.1 Gene cloning and bacterial transformation

The cloning of *VviNAC33* and *VviNAC60* fused with *EAR* motif at the C-terminal and under the control of their endogenous promoter was previously performed by Erica D'incà (University of Verona) using Getaway technology. The fusion of *EAR* motif and the isolation of *VviNAC33* and *VviNAC60* sequences was carried out by PCR from cDNA of *V. vinifera* cv. Corvina (obtained from RNA isolated from 200 mg of ground berries skin and pulp at *veraison*) using HiFi DNA

Polymerase (KAPA Biosystems), according to the manufacturer's instruction, and primers containing the CACC sequence (at 5'-end of primers for, underlined in the sequence) and EAR sequence (at 5'-end of primers rev, underlined in the sequence), showed in Table 1. Each generated PCR fragment was purified, directionally cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) and verified by sequencing. Before to transfer the sequences *VviNAC33EAR* and *VviNAC60EAR,* the final destination vector pK7WG2 (Laboratory of Plant Systems Biology, PSB; Ghent University, Belgium) was modified replacing the 35S promoter with the endogenous promoter (P) of each *VviNAC* gene and inserting the cassette P*UBQ10*::*eGFP*::T35S. Regulative regions of each *VviNAC* gene were isolated from genomic DNA of *Vitis vinifera* cv. Corvina (as described in section 2.3), using HiFi DNA Polymerase (KAPA Biosystems) and primers containing an *HindIII* (at 5'-end of primers for, underlined in the sequence) and a *SpeI* (at 5'-end of primers rev, underlined in the sequence) sites, showed int Table 1. Each PCR products was purified, directionally cloned into the pGEM®-T Vector (Promega), following the manufacturer's instruction, and verified by sequencing. After the digestion of pK7WG2 vector with *HindIII* and *SpeI* to remove 35S promoter, P*VviNAC33* and P*VviNAC60* (obtained from pGEM®-T-prom*VviNAC33* and pGEM®-T-prom*VviNAC60* digested with *HindIII* and *SpeI*) were directionally cloned in the linearized pK7WG2 vector using the same restriction enzymes. Subsequently, the vectors pK7WG2 containing the endogenous promoters of each *VviNAC* gene were again digested with *HindIII* and the cassette P*UBQ10*::*eGFP*::T35S (obtained from pH7WG2D - Laboratory of Plant Systems Biology, PSB; Ghent University, Belgium – after digestion with the same restriction enzyme) was inserted downstream than the endogenous promoter. Finally, *VviNAC33EAR* and *VviNAC60EAR* sequences were transferred from pENTR/D-TOPO to modified pK7WG2 vector by site specific recombination, according to the manufacturer's instruction. The final binary expression vectors pK7WG2-T*NOS*::*nptII*::P*NOS*-T35S::*VviNAC33EAR*::P*VviNAC33*-P*UBQ10*::*eGFP*::T35S and pK7WG2-T*NOS*::*nptII*::P*NOS*-T35S::*VviNAC60EAR*::P*VviNAC60*-P*UBQ10*::*eGFP*::T35S were introduced by electroporation into *Agrobacterium* strain EHA105. Bacterial cultures of EHA105 were grown in MG/L medium supplemented with rifampicin 50 mg/L, spectinomycin 100 mg/L and streptomycin 300 mg/L.

Table 1: Primer sequences used for cloning of *VviNAC33EAR*, *VviNAC60EAR*, p*VviNAC33* and p*VviNAC60*.

Gene	Primer name	Sequence (5'-3')
VviNAC33	NAC33 For	CACCATGGTTGAGTCAAGGTTGCCA
	NAC33EAR Rev	TTAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCGAGACAATAATG GTTCCAAATGG
	pNAC33 For	CCCAAGCTTGGGGTTGGATGGTAAGCATGAAA
	pNAC33 Rev	GGACTAGTCCCTCAATAATGCTCATTTTGA
VviNAC60	NAC60 For	CACCATGGACAACCCGCAATCCAC
	NAC60EAR Rev	TTAAGCGAAACCCAAACGGAGTTCTAGATCCAGATCGAGTCCTTGAAA TGGGAAATAAG
	pNAC60 For	CCCAAGCTTGGGTGTTGCCAATCGAATTGATGG
	pNAC60 Rev	GGACTAGTCCGGCTGTCGCTGAAAAATTATG

2.2 Embryogenic cultures and stable genetic transformation

The induction and maintenance of embryogenic cultures of Shiraz and Garganega cultivars was performed following the experimental procedure described in Chapter 3, section 2.1.1.

The stable transformation of embryogenic calli of Shiraz and Garganega cultivars was carried out using the protocol 1 described in Chapter 3, section 2.3.1.

2.3 Household genomic DNA extraction and genomic PCR analysis

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. Leaf tissue discs 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 minutes 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. The genomic DNA extracted was then used to perform a PCR analysis to evaluate the stable integration of the

transgene, using GoTaq DNA Polymerase (Promega), according to the manufacturer's instruction, and primers showed in Table 2.

Table 2: Primer sequences used for genomic PCR analysis to confirm the stable integration of T-DNA region of each NAC-EAR gene.

T-DNA region	Primer name	Sequence (5'-3')
nptII-VviNAC33EAR::PVviNAC33-eGFP	Kan For	AGAACCTGCGTGCAATCC
	NAC33 For	CACCATGGTTGAGTCAAGGTTGCCA
nptII-VviNAC60EAR::PVviNAC60-eGFP	Kan For	AGAACCTGCGTGCAATCC
	NAC60 For	CACCATGGACAACCCGCAATCCAC

2.4 Transcriptomic analyses

2.4.1 RNA extraction

Total RNA was isolated from approximately 100 mg of agroinfiltrated transgenic fully expanded leaves using Spectrum™ Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA quality and quantity were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4.2 Reverse transcription (RT) and RT-PCR

Two micrograms of extracted RNA were treated with 2 units (U) of Turbo DNase (TURBO DNA-free kit—Ambion) according to the instructions provided with the commercial kit. DNase-treated RNA was then used for cDNA synthesis using the SuperScriptIII Reverse Transcriptase kit (Invitrogen) following the producer's indications. To assess if the cDNA had been properly produced, an amplification with primers designed on VviUBIQUITIN gene (VIT_16s0098g01190) was performed. The cDNA correctly synthetized was then used to perform a RT-PCR analysis to evaluate the transgene expression, using GoTaq DNA Polymerase (Promega), according to the manufacturer's instruction, and primers showed in Table 3. Primers design was carried out using the cDNA sequence of each specific gene and the software Primer designing tool-NCBI–NIH (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), selecting a PCR product size of 100-120 base pairs (bp) and an optimal melting temperature (Tm) of 60 °C.

Table 3: Primer sequences used for RT-PCR analysis to confirm the expression of each NAC-EAR gene.

Gene	Primer name	Sequence (5'-3')
VviNAC33EAR	NAC33int For	CAATGTGGAAGAGTCACCAAGC
	EAR Rev	GCGAAACCCAAACGGAGTTCT
VviNAC60EAR	NAC60int For	TCAGTCAGACCTCCCGCAA
	EAR Rev	GCGAAACCCAAACGGAGTTCT

2.4.3 Real-Time qPCR analysis

The expression profiles were determined by Real-Time qPCR as described by Zenoni et al., 2011, using the SYBR Green PCR master mix (Applied Biosystems) and a Mx3000P real time PCR system (Stratagene). Each expression value, relative to VviUBIQUITIN, amplified with primers UBI FOR 5'-TCTGAGGCTTCGTGGTGGTA-3' and UBI REV 5'-AGGCGTGCATAACATTTGCG -3', was determined in triplicates. Non-specific PCR products were identified by the dissociation curves. Amplification efficiency was calculated from raw data using LingRegPCR software (Ramakers et al., 2003). The mean normalized expression (MNE)-value was calculated for each sample referred to the ubiquitin expression according to Simon equation (Simon, 2003). Standard error (SE)-values were calculated according to Pfaffl et al. (2001). The primer sequences used for Real-Time qPCR analysis are listed in Table 4. Primers design was performed as described in the previous section.

Table 4: Primer sequences used for Real-Time qPCR analysis.

Gene	Primer name	Sequence (5'-3')
NITRATE TRANSPORTER 3	Real Time Nitrtra For	GACGCCATGAGATGCCTACT
	Real Time Nitrtra Rev	GCTGAAATTGGATGGTTCGTT
VviNAC17	Real Time VviNAC17 For	AGAAGTCCAGAGCGGACTCA
	Real Time VviNAC17 Rev	CGAACGGGTCGAGTGAGTTA
VviWRKY16	Real Time VviWRKY16 For	ATAAGTGCACGAACCCAGGA

	Real Time VviWRKY16 Rev	CACATCATGGTTGTGCTTCC
VviNAC26	Real Time VviNAC26 For	CCGAACCAGCCTCTATTTGTGA
	Real Time VviNAC26 Rev	CATGCCCATCATGTCTAACCC
GALACTINOL SYNTHASE	Real Time Galsynt For	AATGTGTGAAGCTGGGCTT
	Real Time Galsynt Rev	CAAACTGCTCACTTAAACAGAT

3. RESULTS

3.1 Stable genetic transformation and regeneration of *VviNAC33EAR* and *VviNAC60EAR* transgenic plants

The isolation of NAC gene sequences and the fusion with EAR motif at the 3'-end have been performed by PCR using Corvina cultivar cDNA and specific pairs of primers (Table 1). Then, *VviNAC33EAR* and *VviNAC60EAR* sequences have been transferred in a modified pK7WG2 vector, containing the endogenous promoter of each gene (previously replaced to 35S promoter) and two specific cassettes for the constitutive expression of *eGFP* and *nptII* reporter genes. The endogenous promoter will allow the expression of chimeric repressors in the same tissue and at developmental stages of the endogenous NAC genes, while the constitutive expression of eGFP and nptII are essential to select only transgenic eGFP-expressing kanamycin-resistant somatic embryos. Finally, the resulting vectors pK7WG2-T*NOS*::*nptII*::P*NOS*-T35S::*VviNAC33EAR*::P*VviNAC33*-P*UBQ10*::*eGFP*::T35S and pK7WG2-T*NOS*::*nptII*::P*NOS*-T35S::*VviNAC60EAR*::P*VviNAC60*-P*UBQ10*::*eGFP*::T35S (Figure 1) were introduced in *A. tumefaciens* EHA105.

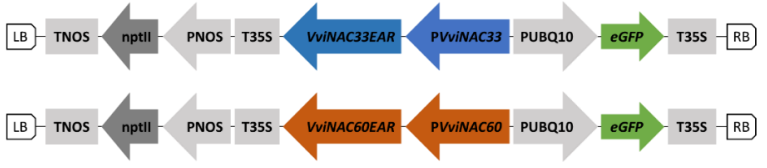


Figure 1: Physical map of T-DNA region of the modified pK7WG2 plasmid used for the stable expression of *VviNAC33EAR* and *VviNAC60EAR* in grapevine.

For both chimeric repressors, stable genetic transformation has been performed using both Shiraz and Garganega cultivars following the experimental procedure described in Chapter 2, section 2.3.1, the same protocol used for the overexpression of *VviNAC33* and *VviNAC60* (D'Incà, 2017). Based on eGFP expression analysis, the results obtained here follow the same progression described in Chapter 3: after high transient expression in the first phase of the process 3 days post transformation (d.p.t.), the fluorescence signal was completely absent after one month from transformation. After many regeneration cycles in growth regulator-free medium, 7-8 months post transformation first transgenic eGFP-expressing kanamycin-resistant somatic embryos of Shiraz and Garganega started to germinate. Regarding *VviNAC33EAR*, eight transgenic somatic embryos of Garganega and four transgenic somatic embryos of Shiraz have been regenerated, while, concerning *VviNAC60EAR*, three somatic embryos for Garganega and four for Shiraz have been regenerated. After another month of further development in growth regulator-free medium, transgenic somatic embryos for both chimeric repressors of both cultivars were transferred to shooting medium under light. As described in Chapter 3, the cotyledons removal was essential to promote shoot formation from somatic embryos of Shiraz, while the shoot formation in somatic embryos of Garganega happened before cotyledons excision. Regenerated transgenic shoots of both cultivars for both chimeric repressors were finally transferred to three-quarter-strength MS medium supplemented with indole-3-acetic acid (Kurth et al., 2012) to allow whole plant development. Well-developed transgenic plants were acclimated in a growth chamber and finally transferred to the greenhouse for phenotypic and molecular analysis. 3 Garganega and 1 Shiraz transgenic plants were regenerated for *VviNAC33EAR*, while, 1 Garganega and 1 Shiraz transgenic plants were regenerated for *VviNAC60EAR*. Genomic PCR analysis (Figure 2), using primers showed in Table 2, confirms the stable integration of the T-DNA region for both NAC-EAR genes.

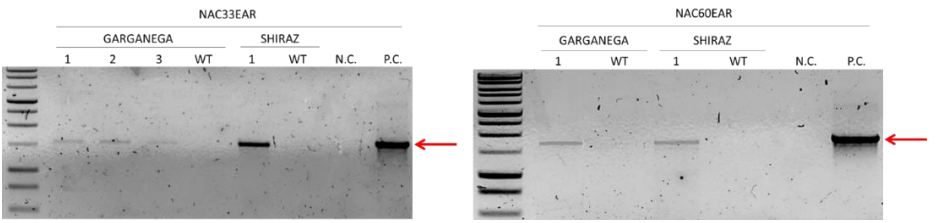


Figure 2: PCR analysis using genomic DNA of transgenic grapevines containing *VviNAC33EAR* and *VviNAC60EAR*. Both amplifications were obtained using *nptII*-, *VviNAC33*- and *VviNAC60*- specific primers, which successfully amplified the expected fragments (red rows). The numbers in the lanes indicate the

corresponding transgenic plants for each cultivar for each NAC-EAR gene. Abbreviations: WT: wild type plant of both cultivars; N.C.: negative control; P.C.: positive control represented by the modified pK7WG2 plasmid containing the T-DNA regions showed in Figure 1.

3.2 Phenotypic analysis and transgene expression analysis

After about two months from the transferring to the greenhouse, phenotypic analysis of Garganega (Figure 3A) and Shiraz (Figure 3B) transgenic plants showed a normal vegetative growth, with leaves of the same sizes and coloration of the WT plants, while the aberrant characteristic identified in the overexpressing plants (see Introduction) were completely absents.

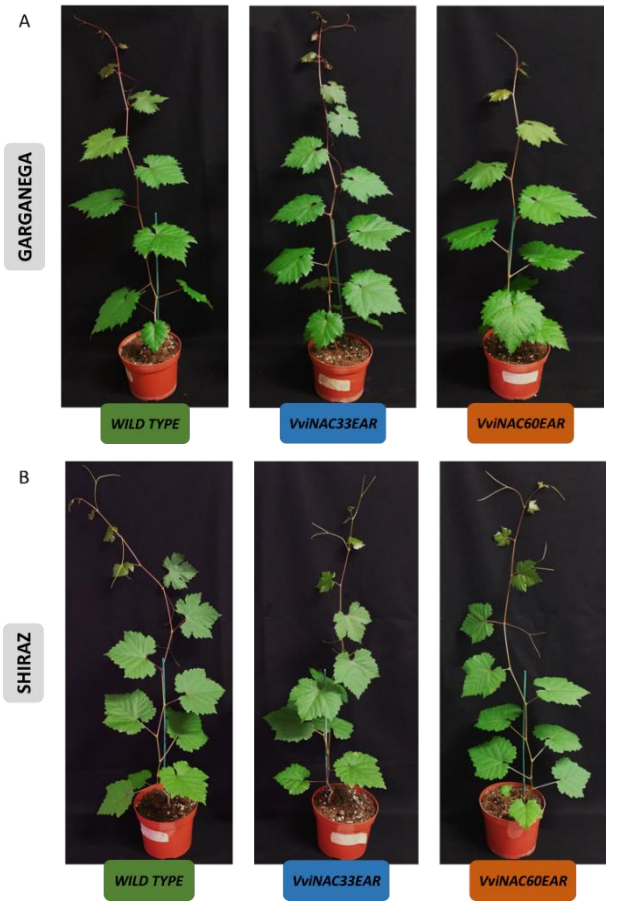


Figure 3: phenotypic analysis of greenhouse-grown Shiraz (A) and Garganega (B) transgenic plants. The picture related to Garganega expressing *VviNAC33EAR* is representative of three transgenic lines, while

the other pictures are representatives of the single transgenic line obtained for each NAC-EAR gene for both Garganega and Shiraz cultivars.

The confirmation of transgene expression was carried out by RT-PCR analysis. As previously described, the expression of chimeric repressors is under the control of endogenous promoter; consequently, their expression will occur in the same tissues and at developmental stages of the endogenous NAC genes. Considering only leaves, the analysis of expression profiles of each NAC genes (Figure 1A, 1B Chapter 2, section 3.2) retrieved from the grapevine expression atlas (Fasoli et al., 2012) showed that both genes are preferentially expressed in fully expanded leaves. The RT-PCR analysis using primers showed in Table 3, confirms the expression of *VviNAC33EAR* and *VviNAC60EAR* (Figure 4) fully expanded leaves of transgenic plants obtained of both cultivars.

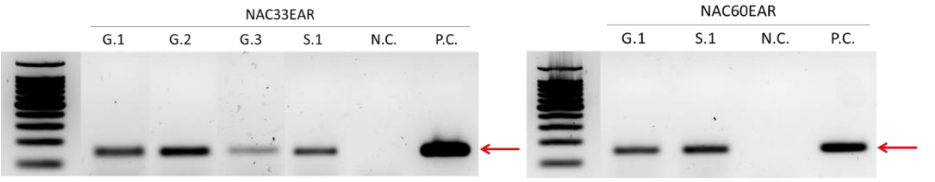


Figure 4: RT-PCR analysis performed on cDNA synthetized from RNA extracted from fully expanded leaves of *VviNAC33EAR* and *VviNAC60EAR* transgenic plants. Both amplifications were obtained using VviNAC33-, VviNAC60- and EAR motif- specific primers, which successfully amplified the expected fragments (red rows). The numbers in the lanes indicate the corresponding transgenic plants for each cultivar (G: Garganega, S: Shiraz) for each NAC-EAR gene. Abbreviations: N.C.: negative control; P.C.: positive control represented by the modified pK7WG2 plasmid containing the T-DNA regions showed in Figure 1.

3.3 Identification and validation of putative target genes of *VviNAC33* and *VviNAC60*

The previously performed microarray analysis of Shiraz transgenic plants overexpressing *VviNAC33* and *VviNAC60* showed that most up-regulated genes (fold change -FC- value > 2) are involved in processes closely associated with ripening, such as carbohydrate metabolic process, cell wall metabolism, secondary metabolic process, regulation of transcription factor activity and transport (D'Incà, 2017). These genes could be putative target of *VviNAC33* and *VviNAC60*; based on their FC value and biological role, some of these genes were selected and their up-regulation was analyzed by Real Time qPCR. The genes selected for *VviNAC33* were (i) *NITRATE TRANSPORTER 3* (VIT_12S0059G01240), a gene belonging to a nitrate transporters gene families, whose member are involved in root architecture, nutrient acquisition, vacuole nitrate,

protein storage, nutrient allocation from source to sink and sensing both abiotic and biotic stresses (Fan et al., 2017), and (ii) *VviNAC17* (VIT_19S0014G03290), another NAC gene, whose function during grapevine development and ripening is unkwown. Regarding *VviNAC60*, the genes selected were (i) *VviWRKY16* (VIT_06S0004G07500), belonging to WRKY transcription factor family, whose member are involved in many processes associated with developmental programs and responses to stress, (ii) *VviNAC26* (VIT_01S0026G02710), a NAC gene involved in the determination of the grape berry final size (Tello et al, 2015) and (iii) one *GALACTINOL SYNTHASE* (VIT_05S0077G00430), involved in the regulation of sugar signaling. The Real Time qPCR analysis confirmed the upregulation of the target genes in the leaves of the plants overexpressing *VviNAC33* (Figure 5A) or *VviNAC60* (Figure 5B), albeit the induction level was very different among the putative target genes. However, these preliminary results suggest that they act downstream the selected NAC transcription factors.

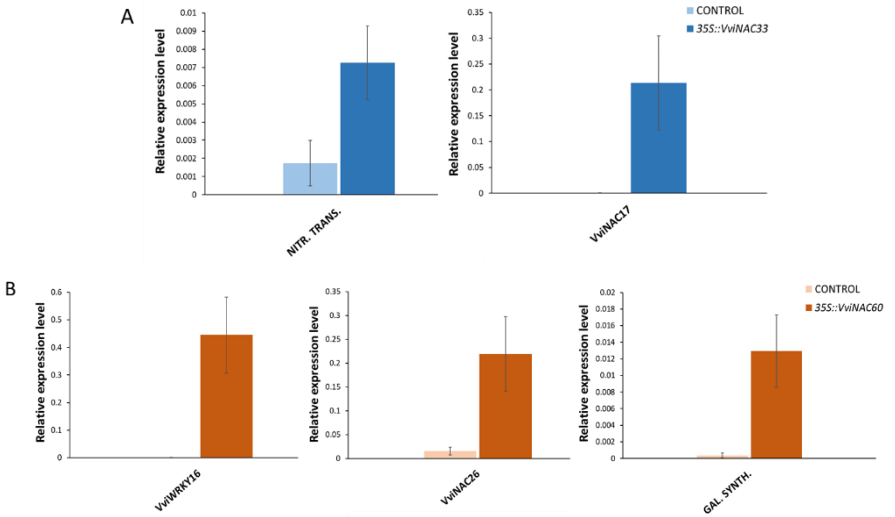


Figure 5: Real time qPCR analysis of target genes of *VviNAC33* (A) and *VviNAC60* (B) in the control and stably overexpressing lines of Shiraz plants. The expression level corresponds to the mean ± SE of three biological replicates for VviNAC33 and three technical replicates for VviNAC60, relative to the *VviUBIQUITIN* (VIT_16s0098g01190). Abbreviations correspond to: NITR. TRANS.: NITRATE TRANSPORTER 3; GAL. SYNTH.: GALACTINOL SYNTHASE.

The expression of the same putative target genes was then analyzed in Garganega transgenic plant expressing *VviNAC33EAR* and *VviNAC60EAR*. If these genes are specific target of *VviNA33* and *VviNAC60*, the inhibition of transcription by EAR motif should cause a descrease in their

expression level. In this way, it was possible to evaluate the role of the EAR motif as transcriptional repressor and confirm that the selected genes are target of NAC transcription factors. The Real Time qPCR analysis showed that the expression level of *VviNAC17* and *NITRATE TRANSPORTER 3* (Figure6A), and *VviNAC26*, *GALACTINOL SYNTHASE* and *VviWRKY16* (Figure 6B) was lower in the plants expressing *VviNAC33EAR* and *VviNAC60EAR* than WT plants, confirming both the EAR motif-mediated transcriptional repression and that the selected genes are specific target genes of *VviNAC33* and *VviNAC60*.

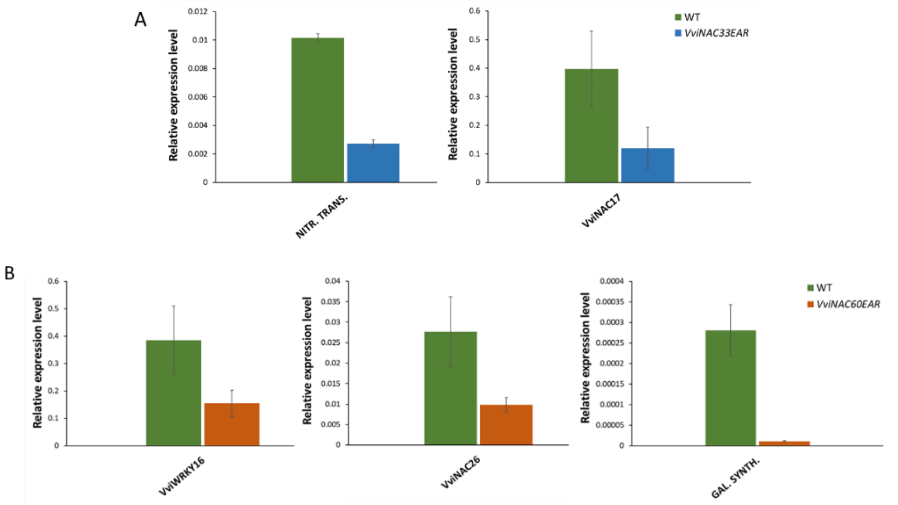


Figure 6: Real time qPCR analysis of target genes of *VviNAC33EAR* (A) and *VviNAC60EAR* (B) in the control and stably expressing lines of Garganega plants. The expression level corresponds to the mean ± SE of three biological replicates for *VviNAC33EAR* and three technical replicates for *VviNAC60EAR*, relative to the *VviUBIQUITIN* (VIT_16S0098G01190). Abbreviations correspond to: NITR. TRANS.: NITRATE TRANSPORTER 3; GAL. SYNTH.: GALACTINOL SYNTHASE.

4 DISCUSSION

VviNAC33 and *VviNAC60* transcription factors are the first two genes selected for functional characterization. They emerged as switch genes from the network analysis performed on the grapevine expression atlas and of berry transcriptomic datasets (Palumbo et al., 2014; Massonnet et al., 2017) and they were also found among markers of the first transition of berry development described by Fasoli et al., 2018. The identification of their specific roles during

grapevine development and ripening could provide important informations about the molecular programs controlling these processes.

Previous results (D'Incà, 2017) showed that the stable overexpression of *VviNAC33* in grapevine plants of Shiraz cultivar caused an alteration of chlorophyll metabolism with evident leaf chlorosis while the overexpression of *VviNAC60* in the same grapevine cultivar damaged the normal development and caused a weak anthocyanins accumulation in the leaves. Furthermore, the molecular analysis of overexpressing plants showed an upregulation of many genes involved in processes associated with plant development, ripening and senescence; these preliminary results seem to indicate an involvement of both NAC transcription factors in the regulation of vegetative-to-mature transition in grapevine. To complete the functional analysis of both *VviNAC33* and *VviNAC60* and to obtain more information about their role of master regulators during grapevine ripening, in this study they have been converted into transcriptional repressors by fusion with the EAR motif, one of the strongest transcriptional repression domain in plants, and stably expressed in grapevine plants. Each NAC-EAR gene was cloned under the control its endogenous promoter: in this way the chimeric repressor can inhibit the transcription of putative target genes in the same organs and at the developmental stage when the endogenous NAC genes are expressed.

VviNAC33EAR and *VviNAC60EAR* were stably expressed in two different grapevine cultivars, Garganega and Shiraz, using the experimental procedure described in Chapter 3, section section 2.3.1. This protocol allowed to obtain the highest number of transgenic Shiraz plants, the same cultivar used for overexpression of each NAC gene (D'Incà, 2017). The modified pK7WG2 vectors used for the stable transformation contained, in addition to each NAC-EAR expression cassette, two specific cassettes for the constitutive expression of *eGFP* and *nptII* reporter genes (Figure 1). So, the selection of putative transgenic somatic embyros was performed based on kanamycin resistance and eGFP expression. After many regeneration cycles, transgenic somatic embryos of both cultivars were emerged; the efficiency was quite low but the results obtained were similar to those described in Chapter 3, section 3.1.2 (Table 1): 11 somatic embryos of Garganega, 8 for *VviNAC33EAR* and 3 for *VviNAC60EAR* have been regenerated, while regarding Shiraz, 8 somatic embryos, 4 for *VviNAC33EAR* and 4 for *VviNAC60EAR*, have been regenerated. Among these transgenic somatic embryos, just 4 of Garganega, 3 for *VviNAC33EAR* and 1 for *VviNAC60EAR*, and just 2 of Shiraz, 1 for each NAC-EAR gene, produced a shoot. After

transferring to the plant development medium (Kurth et al., 2012), all regenerated transgenic shoots of both cultivars for both chimeric repressors produced a plant. These results are consistent with the results described in Chapter 3, section 3.1.3 (Table 1): many somatic embryos did not produce shoots, but all the regenerated shoots could develop a whole plant. Genomic PCR analysis (Figure 2) confirms the stable integration of the T-DNA region of both NAC-EAR genes: the number of regenerated transgenic plants was low, 4 for *VviNAC33EAR* (3 for Garganega and 1 for Shiraz), and 2 for *VviNAC60EAR*, (one for each cultivar). After acclimatization and transferring to the greenhouse, the phenotype of transgenic plants was analyzed, and the molecular analysis were performed.

Phenotypic analysis of transgenic plants of both cultivars (Figures 3A, B) showed a vegetative growth identical to WT plants. These results indicate that the expression of the chimeric repressors did not alter the normal development of transgenic plants. It should be remarked that the expression of each NAC-EAR gene is under the control of the endogenous promoter: considering only leaves, previous studies (Fasoli et al., 2012) showed that both *VviNAC33* and *VviNAC60* are preferentially expressed in fully expanded adult leaves. RT-PCR analysis (Figure 4) confirmed the higher expression of each chimeric repressor in the oldest leaves of transgenic plants of both cultivars. Despite the absence of phenotypic differences, the expression of *VviNAC33EAR* and *VviNAC60EAR* could inhibit the transcription of some target genes. This analysis was performed only using the 4 transgenic plants of Garganega, 3 expressing *VviNAC33EAR* and one expressing *VviNAC60EAR*. Supposing a role of transcriptional activators of these two regulators, the selection of these putative target genes was performed by inspecting the list of upregulated genes from the microarray data obtained from overexpressing plants of Shiraz cultivar produced in a previous project (D'Incà, 2017). The selection was performed considering both FC values and the biological function of the gene. Regarding *VviNAC33*, the genes selected were *VviNAC17* and *NITRATE TRANSPORTER 3*, while for *VviNAC60* the genes selected were *VviWRKY16*, *VviNAC26* and *GALACTINOL SYNTHASE*. Real Time qPCR analysis confirmed the upregulation of all genes (Figures 5A, B) and it showed a clear difference in the expression level between control (eGFP-overexpressing Shiraz plants) and NAC genes-overexpressing Shiraz plants. However, induction was very different among the target genes: this data could suggest that each NAC gene could have a preferential activation toward specific target genes. The expression of the same genes was analyzed in transgenic plants of

Garganega expressing NAC-EAR genes: real time qPCR analysis (Figures 6A, B) showed that all selected genes for both *VviNAC33* and *VviNAC60* are characterized by a lower expression than WT plants. These results strongly suggest that the fusion with the EAR motif was successful in turning the NAC candidates into transcriptional repressors and that the five above reported genes are targets of the NAC transcription factors. In particular, *VviNAC33* affect the expression of another NAC gene and seems to activate the transcription of a nitrate transporter, while *VviNAC60* regulates the expression of other two transcription factors and of one gene involved in the sugar signalling. To assess whether these are direct regulations, other experimental approaches, such as Dual Luciferase Reporter Assay or Chromatin ImmunoPrecipitation (ChIP) followed by sequencing of regulative regions, will be used. Moreover, many other putative target genes will be investigated to obtain more information about the roles of both *VviNAC33* and *VviNAC60*, but these preliminary results seem to indicate crucial roles of both NAC transcription factors in the regulation of grapevine development. Furthermore, the results described in this chapter indicate that the use of the EAR motif is a useful approach to study the function of a transcription factor, allowing to better define its putative target genes, and to complement the results obtained by overexpression.

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Chapter 5

FUNCTIONAL ANALYSIS OF *VviAGL15a*, *VviWRKY19* and *VvibHLH75*

ABSTRACT

The transcriptomic reprogramming during *veraison* showed an involvement of many transcription factors belonging to different families, suggesting the existence of a complex transcriptional regulatory network. The identification of the roles of these transcription factors could better define the molecular mechanisms controlling the onset of berry ripening. Among these transcription factors, *VviAGL15a*, *VviWRKY19* and *VviBHLH75* have been selected to be functionally characterized. They belong to three large transcription factors families in plant, involved especially in the regulation of reproductive development, responses to abiotic and biotic stress, senescence and hormone signalling. These transcription factors families have been described in grapevine, but the identification of the functions of the different members remain incomplete. The functional characterization of *VviAGL15a*, *VviWRKY19* and *VviBHLH75* was performed by transient overexpression in grapevine leaves through an improved protocol of leaf agroinfiltration. The exhaustive molecular analysis of transiently overexpressing leaves allowed to obtain preliminary information about the functions of these transcription factors. The results highlighted many putative target genes of *VviAGL15a*, *VviWRKY19* and *VviBHLH75* that are also possibly involved in processes associated with the berry ripening program, such as cell wall metabolism, carbohydrate metabolic process, secondary metabolic process, hormone signalling and regulation of transcription factor activity. This is consistent with the role of master regulators of the onset of berry ripening assumed for the three candidates under investigation.

1. INTRODUCTION

VviAGL15a, *VviWRKY19* and *VvibHLH75* are three transcription factors strongly activated at the onset of grape berry ripening, selected for functional analysis. They were identified as switch genes of both red and white berry transcriptomes (Palumbo et al., 2014; Massonnet et al., 2017) and *VviWRKY19 and VvibHLH75* were also identified among the markers of the first transition representative of the onset of ripening by Fasoli et al., 2018. The identification of their roles during the onset of berry ripening could better define the molecular mechanisms controlling the physiological transition occurring at *veraison*.

VviAGL15a is a member of MADS-box transcription factor family. The term MADS-box gene was coined after four subsequently characterized 'founding family members': MINICHROMOSOME MAINTENANCE 1 (MCM1) from *S. cerevisiae*, AGAMOUS (AG) from *Arabidopsis thaliana*, DEFICIENS (DEF) from *Antirrhinum majus* and SERUM RESPONSE FACTOR (SRF) from *Homo sapiens* (Gramzow and Theissen, 2010). These transcription factors are defined by the presence of a conserved domain, the MADS box, in the N-terminal region that is involved in DNA binding and dimerization with other MADS box proteins; they are involved in developmental control and signal transduction in eukaryotes. In plants, they are associated to numerous development processes most notably those related to reproductive development: flowering induction, specification of inflorescence and flower meristems, establishment of flower organ identity, as well as regulation of fruit, seed and embryo development (Grimplet et al., 2016). The MADS-box gene family can be divided into two main lineages, referred to as type I and type II, both of which are present in plants, animals and fungi (De Bodt et al., 2003). Type II group genes include MEF2-like genes of animals and yeast and MIKC type genes only found in plants. Regarding, MIKC-type genes received this name because, apart from the MADS (M) domain, they contain three additional conserved domains, the weakly conserved Intervening (I) domain, the conserved Keratin-like (K) domain and the highly variable C-terminal (C) domain. The I domain is responsible for specificity in the formation of DNA-binding dimers, the K domain mediates dimerization and the C domain functions in transcriptional activation and formation of higher order protein complexes. MIKC-type genes have been further divided in two subgroups, $MIKC^C$ and MIKC* based on divergence at the I and K domains (Diaz-Riquelme et al., 2009). $MIKC^C$-type MADS box genes are the best characterized group of MADS box genes; they are initially identified as floral organ identity genes, but they are further involved in essential and diverse

functions related to plant growth and development. In grapevine (*Vitis Vinifera* L.), the MADS-box transcription factors family has been described and 90 MADS-box genes have been identified (Grimplet et al., 2016); 42 of them are $MIKC^C$ –type II genes distributed in 13 subfamilies; *VviAGL15a* is $MIKC^C$ –type II gene of the subfamily of *VviAGL15*. The analysis of its expression profile (Grimplet et al., 2016; Diaz-Riquelme et al., 2009) confirms the results described in Chapter 2, section 3.2 (see Figure 1C): it is preferentially expressed in flowers and fruits while its expression level in vegetative organs and tissue is extremely low. There are no information about its specific role in grapevine, but previous studies in Arabidopsis thaliana have shown that its homologous, *AtAGL15*, is involved in the repression of floral transition (Adamczyk et al., 2007). Therefore, further characterization of the specific role of *VviAGL15a* in grapevine is necessary.

Regarding *VviWRKY19*, it belongs to WRKY transcription factors family. WRKY proteins represent an important class of transcriptional regulators in higher plants; most members of this multigene family are involved in the response to biotic stresses and they are central components of many aspects of the innate plant immune system. However, WRKY genes play a specific role in plant tolerance to a variety of abiotic stresses, including high salt, heat, osmotic stress, high CO_2 levels, high ozone concentrations, cold or drought and they have additional roles in other important plant processes, including seed dormancy, germination, plant development and leaf senescence (Wang et al., 2014). Moreover, WRKY transcription factors regulates the biosynthesis of secondary metabolites, including many phenylpropanoids, alkaloids, and terpenes (Schluttenhofer and Yuan, 2015). The name of this transcription factors family derived from the most prominent feature of these proteins, the WRKY domain, a 60 amino acid region that is highly conserved amongst family members and involved in DNA binding; the WRKY domain is defined by the conserved amino acid sequence WRKYGQK at its N-terminal end adjacent to an atypical zinc-finger-like motif at the C-terminus (Eulgem et al., 2000). WRKY genes have further been classified into three major groups based on the number of WRKY domains present. Group I members are characterized by two WRKY domains containing a C2H2 zinc-finger motif. Group II WRKY genes contain only one WRKY domain, characterized by a C2H2 zinc-finger motif. Group III consists of a small number of genes characterized by a single WRKY domain with a C2HC zinc-finger motif. In grapevine (*Vitis Vinifera* L.), 59 full-length genes encoding putative WRKY proteins were identified (Wang et al.,

2014). Some of grapevine WRKY transcription factors have been characterized: *VviWRKY26* is involved in the regulation of vacuolar acidification and flavonoid accumulation mechanisms during berry development (Amato et al., 2017), *VviWRKY1* increases the resistance of grapevine against the downy mildew regulating the jasmonic acid signaling pathway (Marchive et al., 2013), *VviWRKY33* is involved in the regulation of grapevine defense against *Plasmopara viticola* (Merz et al., 2015) while many other grapevine WRKYs (*VviWRKY03*, *VviWRKY24*, *VviWRKY43* and *VviWRKY53*) have a role in the regulation of the stilbene biosynthetic pathway (Vannozzi et al., 2018). These results are encouraging to proceed with the functional characterization of another WRKY gene, *VviWRKY19*, that is constitutively expressed during grape berry ripening but whose precise role remain unknown.

Finally, *VvibHLH75* is a member of basic helix-loop-helix transcription factors family, one of the largest families of transcription factors, widely distributed in all three eukaryotic kingdoms (Carretero-Paulet et al., 2010). This family is defined by the highly conserved bHLH signature domain, which consists of 60 amino acids with two functionally distinct regions. The basic region is involved in DNA binding, it consists of approximately 15 amino acids with a high number of basic residues and it is located at the N-terminal end of the domain, The HLH region, at the C-terminal end, functions as a dimerization domain and is constituted mainly of hydrophobic residues that form two amphipathic α-helices separated by a loop region of variable sequence and length (Toledo-Ortiz et al., 2003). bHLH genes are involved in many processes from regulation

of flavonoid biosynthesis, floral organogenesis and epidermal differentiation, to hormone responses, light signaling, responses to environmental factors and fruit dehiscence (Pires and Dolan, 2009; Hichri et al., 2010; Nicolas et al., 2013). In grapevine, the analysis of bHLH transcription factors family has identified 94 genes and the analysis of their expression has highlighted that many genes are induced by cold stress, suggesting their specific role in abiotic stress response (Wang et al., 2018). Furthermore, other studies related to functional analysis of grapevine bHLH genes have shown their involvement in the regulation of flavonoid biosynthesis (Hichri et al., 2010; Matus et al., 2010) and in the regulation of grape berry development (Nicolas et al., 2013). Most of grapevine bHLH transcriptional regulators, including *VvibHLH75*, have not been functionally characterized yet.

One of the best method to study *VviAGL15a*, *VviWRKY19* and *VvibHLH75* gene function would be the alteration of their expression by stable genetic transformation; however, as described in chapters 3 and 4, grapevine is very recalcitrant to this approach: the time of regeneration of transgenic plants is very long (about 10 months), the number of independent transgenic lines is low, and the production of transgenic fruits requires some years. Transient gene expression represents a valid alternative: it is an efficient and attractive method because of its simplicity and rapidity (Vidal et al., 2010). The main transient expression assays and their characteristics have already been described in Chapter 3. In grapevine, the most important and used method of transient gene expression is represented by leaf agroinfiltration (Jelly et al., 2014); it can be performed using both attached or detached leaves and syringe or vacuum pump, but the second system showed more applicability. Vacuum leaf agroinfiltration of whole plants has already been used for the functional characterization of transcription factors identified as switch genes (D'Inca, 2017) or involved in the regulation of the phenylpropanoid pathway and vacuolar transport (Cavallini et al., 2015; Amato et al., 2017). The positive results obtained in these works indicate that this method can be successfully used for the functional analysis of candidate genes. In this PhD thesis this protocol has been further implemented by using the YFP transient expression as marker of the transformation of agroinfiltrated tissues. A detailed experimental procedure related to the use of YFP is described in Chapter 3 section 3.2; this strategy allowed to identify both the post infiltration time of maximum expression and the agroinfiltrated leaves with the highest signal of transformation.

In this chapter, the functional analysis of *VviAGL15a*, *VviWRKY19* and *VvibHLH75* has been performed by using a transient gene expression approach. The transient overexpression of the selected transcription factors has been carried out by vacuum leaf agroinfiltration of whole plants (Thompson seedless cv) grown *in-vitro*, using the improved protocol based on YFP expression as reporter gene. The transient overexpression and the successive transcriptomic analysis of overexpressing leaves allowed to identify putative target genes of these transcription factors and to partially characterize their role during the onset of berry ripening in grapevine.

2. MATERIALS AND METHODS

2.1 Gene cloning and bacterial transformation

The cloning of *VviAGL15a* (VIT_13s0158g00100), *VviWRKY19* (VIT_07s0005g01710) and *VvibHLH75* (VIT_17s0000g00430) sequences (CDS + 3'-UTR), retrieved from Grape Genome Database (http://genomes.cribi.unipd.it/grape/index.php), was performed using the GoldenBraid 2.0 (GB 2.0) system (Sarrion-Perdigones et al., 2013). Briefly, the *VviAGL15a*, *VviWRKY19* and *VvibHLH075* sequences were amplified by PCR from cDNA of *V. vinifera* cv. Corvina (obtained from RNA isolated from 200 mg of ground berries skin and pulp at veraison) using Pfu DNA polymerase (Promega), according to the manufacturer's instruction, and GB adapted primers (Table 1). The sequences were then cloned in pUPD2 vector and verify by sequencing. Subsequently, the domesticated sequences were correctly assembled with 35S promoter and T-Nos terminator in pDGBα2 destination vector. Finally, the transcriptional units α2-35S::*VviAGL15a*::TNOS, α2-35S::*VviWRKY19*::TNOS and α2-35S::*VvibHLH075*::TNOS were assembled with the transcriptional unit α1-35S::*YFP*::TNOS (available from the GB 2.0 toolkit) in pDGBΩ1 destination vector. The domestication (including primers design), the multipartite assembly and the binary assembly were performed following a detailed protocol generated using a software tools available at https://gbcloning.upv.es/. The final binary expression vectors pEGB3Ω1-35S::*YFP*::TNOS-35S::*VviAGL15a*::TNOS, pEGB3Ω1-35S::*YFP*::TNOS-35S::*VviWRKY19*::TNOS and pEGB3Ω1-35S::*YFP*::TNOS-35S::*VvibHLH75*::TNOS were finally transferred to *Agrobacterium tumefaciens* strain C58C1 by electroporation. Bacterial cultures of C58C1 were grown in LB medium supplemented with tetracycline 5 mg/L and 50 mg/L of spectinomycin.

Table1: Primer sequences used for gene (CDS + 3'UTR) isolation.

Gene	Primer Name	Sequence (5'-3')
VviAGL15a	AGL15 For	GCGCCGTCTCGCTCGAATGGGACGTGGTAAGATTGAG
	AGL15 Rev	GCGCCGTCTCGCTCAAAGCTTAAAAATGCAACATCTACATTCTTC
VviWRKY19	WRKY19 Patch1 For	GCGCCGTCTCGCTCGAATGGAGAGGAGCGGGGTGAT
	WRKY19 Patch1 Rev	GCGCCGTCTCGTGATCTCTATGCAAAGCAGAAG

	WRKY19 Patch2 For	GCGCCGTCTCGATCATGGCCTTCTTCAAGATATTG
	WRKY19 Patch2 Rev	GCGCCGTCTCGCTCAAAGCTTAGCCAAAAAAAAAACATAATTAC CTAT
VvibHLH75	bHLH75 Patch1 For	GCGCCGTCTCGCTCGAATGGCAGCCTTTTCGTATCAA
	bHLH75 Patch1 Rev	GCGCCGTCTCGGCGACGTACTTTCATGAACC
	bHLH75 Patch2 For	GCGCCGTCTCGTCGCTTCAACACAGCTCAAAG
	bHLH75 Patch2 Rev	GCGCCGTCTCGCTCAAAGCTTAGGAGGGAATGTAACTGTAAAG

2.2 Grapevine leaf agroinfiltration

Leaf agroinfiltration of whole plant grown *in-vitro* of Thompson seedless cultivar was carried out using the same procedure described in Chapter 3, Section 2.4.1. Leaf agroinfiltration was performed using *A. tumefaciens* strain C58C1 harboring the vectors pEGB3Ω1-35S::*YFP*::TNOS-35S::*VviAGL15a*::TNOS, pEGB3Ω1-35S::*YFP*::TNOS-35S::*VviWRKY19*::TNOS and pEGB3Ω1-35S::*YFP*::TNOS-35S::*VvibHLH75*::TNOS for the transient overexpression of each gene of interest. As negative control, leaf agroinfiltration was performed using *Agrobacterium* strain C58C1 harboring the vector pEGB3Ω1-35S::*YFP*::TNOS-SF (Chapter 3, Section 2.2). 7 d.p.i. leaf material was collected for RNA extraction, Real-Time qPCR and transcriptomic analysis.

2.3 Transcriptomic analyses

2.3.1 RNA extraction

Total RNA was isolated from approximately 100 mg of agroinfiltrated YFP expressing young leaves using Spectrum™ Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. RNA quality and quantity were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and a Bioanalyzer Chip RNA 7500 series II (Agilent, Santa Clara, CA, USA).

2.3.2 Reverse Transcriptase (RT) and Real-Time qPCR analysis

Two micrograms of extracted RNA were treated with 2 units (U) of Turbo DNase (TURBO DNA-free kit—Ambion) according to the instructions provided with the commercial kit. DNase-treated RNA was then used for cDNA synthesis using the SuperScriptIII Reverse Transcriptase kit (Invitrogen) following the producer's indications. To assess if the cDNA had been properly

produced, an amplification with primers designed on *VviUBIQUITIN* gene (VIT_16s0098g01190) was performed. The expression profiles were determined by Real-Time qPCR as described by Zenoni et al., 2011, using the SYBR Green PCR master mix (Applied Biosystems) and a Mx3000P real time PCR system (Stratagene). Each expression value, relative to VviUBIQUITIN, amplified with primers UBI FOR 5'-TCTGAGGCTTCGTGGTGGTA-3' and UBI REV 5'-AGGCGTGCATAACATTTGCG -3', was determined in triplicates. Non-specific PCR products were identified by the dissociation curves. Amplification efficiency was calculated from raw data using LingRegPCR software (Ramakers et al., 2003). The mean normalized expression (MNE)-value was calculated for each sample referred to the ubiquitin expression according to Simon equation (Simon, 2003). Standard error (SE)-values were calculated according to Pfaffl et al. (2001). The primer sequences used for Real-Time qPCR analysis are listed in Table 2. Primers design was performed as described in Chapter 4, section 2.4.2.

Table 2: Primer sequences used for Real-Time qPCR analysis.

Gene	Primer name	Sequence (5'-3')
VviAGL15a	Real Time AGL15 For	TGCTCCTCTTCATGGTTTCTACT
	Real Time AGL15 Rev	AGATCAGACACTTGTTGGGTGA
VviWRKY19	Real Time WRKY19 For	CGGTGTAGACGGAAAAACCC
	Real Time WRKY19 Rev	TCTGTGTACAAAGGTGGAGGC
VvibHLH75	Real Time bHLH75 For	GGGCAGCAAAATCAATGGAGG
	Real Time bHLH75 Rev	TGCATGAGACTTTGGGAGTCA
XYLOGLUCAN ENDOTRANSGLUCOSYLASE/ HYDROLASE 23	Real Time xilogluc23 for	CACAGACACAAAGCGAGTCC
	Real Time xilogluc23 Rev	TGAAGGAAACTTCAGAAGCAAAC
VviERF045	Real Time ERF045 For	CTCTTGTGCCTGCTTGTTTGA
	Real Time ERF045 Rev	TCAACCCCATTTGAGCTGGT
VviNAC33	Real Time NAC33 For	TGCCCTGCTTCTCCGATATG
	Real Time NAC33 Rev	CTGGCATTCCTCCAAATATGG

VviNAC26	Real Time NAC26 For	CCGAACCAGCCTCTATTTGTGA
	Real Time NAC26 Rev	CATGCCCATCATGTCTAACCC
VviEXPA17	Real Time Exp17 For	GAAGGGGTCAGCAGTCAAGT
	Real Time Exp17 Rev	ACAAGGGAGACCAGAATCTACAC

2.3.3 Microarray analysis

The microarray analysis was performed according to the Agilent Microarray-Based Gene Expression Analysis Guide (V 6.5) and reviewed in Dal Santo et al, 2016. Agilent custom microarray 4-pack 44K format (Agilent Sure Print HD 4X44K 60-mer, G2514F-048771; Amato et al., 2016) were scanned using Agilent Scanner (Agilent Technologies, G2565CA) applying the instruction manual's settings. Feature extraction was evaluated by the QC report. The raw fluorescence intensities (gProcessedSignalvalues) were compared to the average negative signal (gNegCtrlAveNetSig) of all the samples. A gene was considered expressed only if at least two (out of three for *VviAGL15a*) or three (out of four for *VviWRKY19* and *VvibHLH75*) expression values exceeded the threshold in at least one condition (control or overexpressing plantlets). The filtered signals were then normalized by the 75th percentile of the overall signal intensity. Statistical analysis of the microarray data was conducted using TMeV v4.8 (http://mev.tm4.org). Differentially modulated genes were retrieved by performing a between-subjects (control vs. overexpressing plants) t-test ($\alpha = 0.05$), assuming equal variance among samples.

2.4 Co-expression analysis

The gene coexpression analyses of *VviAGL15a*, *VviWRKY19* and *VvibHLH75* with their putative targets was performed using CorTo software (http://www.usadellab.org/cms/index.php?page=corto) and setting Pearson's coefficient as correlation metric. The filtered and normalized 'Thompson Seedless' transcriptomic dataset (three overexpressing and three control leaf sample for *VviAGL15a* or four overexpressing and four control for *VviWRKY19* and *VvibHLH75*) was used.

2.5 Dual Luciferase Reporter Assay

2.5.1 Promoters (regulative regions) cloning and bacterial transformation

Genomic sequences of *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23* (VIT_11s0052g01330) and *VviERF045* (VIT_04S0008G06000) regulative regions were retrieved from the sequenced *Vitis vinifera* cv. Pinot Noir genome deposited at the Grape Genome Database - CRIBI website (http://genomes.cribi.unipd.it/grape/). The cloning was then performed using the GoldenBraid 2.0 (GB 2.0) system (Sarrion-Perdigones., 2013). The predicted *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23* and *VviERF045* regulative regions, indicated as promoter (p) XILO23 and promoter (p) ERF045, were amplified by PCR from genomic DNA of *Vitis vinifera* cv. Corvina (obtained as described in Materials and Methods section, Chapter 4) using PCRBIO HiFi Polymerase (PCRBIOSYSTEMS), according to the manufacturer's instruction, and GB adapted primers (Table 3). The regulative regions (pXILO23 and pERF045) were then cloned in pUPD2 vector and verify by sequencing. Subsequently, the domesticated promoters were correctly assembled with *FIREFLY LUCIFERASE* (LUC) coding sequence and TNos terminator (both available from the GB 2.0 toolkit) in pDGB3α1 destination vector. Then, the transcriptional units α1-pXILO23::LUC::TNos and α1-pERF045::LUC::TNos were recombined with α2-SF (stuffer fragment in pDGB1α2 vector, as described by Sarrion-Perdigones et al, 2011) in pDGB3Ω1 destination vector. Afterwards, the vectors pEGB3Ω1-pXILO23::*LUC*::TNos-SF and pEGB3Ω1-pERF045::*LUC*::TNos-SF were recombined with the vector pEGB3Ω2-35S::*Renilla Luciferase* (*REN*)::Tnos-35S::*p19*::TNos (both transcriptional units 35S::*REN*::TNos and 35S::*p19*::TNos available from the GB 2.0 toolkit and previously assembled from vectors pEGB1α1 and pEGB3α2, ripsectively, in the destination vector pDGB3Ω2) in the destination vector pDGB3α1. Finally, the vectors pEGB3α1-pXILO23::*LUC*::TNos-SF-35S::*REN*::Tnos-35S::*p19*::TNos and pDGB3α1-pERF045::*LUC*::TNos-SF-35S::*REN*::Tnos-35S::*p19*::TNos were recombined with pEGB3α2-35S::AGL15::TNos in the destination vector pDGB3Ω1. The domestication (including primers design), the multipartite assembly and the binary assembly were performed following a detailed protocol generated using a software tools available at https://gbcloning.upv.es/. The final binary expression vectors pEGB3Ω1-pXILO23::*LUC*::TNos-SF-35S::*REN*::Tnos-35S::*p19*::TNos-35S::*VviAGL15a*::TNos, pEGB3Ω1-pERF045::LUC::TNos-SF-35S::*REN*::Tnos-35S::*p19*::TNos-35S::*VviAGL15a*::TNos, pEGB3α1-pXILO23::*LUC*::TNos-SF-35S::*REN*::Tnos-35S::*p19*::TNos and pDGB3α1-pERF045::*LUC*::TNos-SF-

35S::*REN*::Tnos-35S::*p19*::TNos (the last two vectors used as negative controls of Dual Luciferase Reporter Assay) were introduced by electroporation into *Agrobacterium* strain EHA105. Bacterial cultures of EHA105 harboring the pEGB3α1 vectors were grown in LB medium supplemented with rifampicin 50 mg/L and kanamycin 50 mg/L, while bacterial cultures of EHA105 harboring the pEGB3Ω1 vectors were grown in LB medium supplemented with rifampicin 50 mg/L and 50 mg/L of spectinomycin.

Table 3: Primer sequences used for regulative regions cloning.

Gene	Primer Name	Sequence (5'-3')
XYLOGLUCAN ENDOTRANSGLUCOSYLASE/ HYDROLASE 23	promxilo23for	GCGCCGTCTCGCTCGGGAGAATCTCAGTGGTGATCCATAC
	promxilogluc23rev	GCGCCGTCTCGCTCACATTTAGTAGTTCATAAACCAAAATTTATTTTAAAT
VviERF045	promERF045for	GCGCCGTCTCGCTCGGGAGCCCCACATGGATTAAAAAAAAATTG
	promERF045rev	GCGCCGTCTCGCTCACATTCTTTTGGTTCCAGTTGGCTAA

2.5.2 *Nicotiana Benthamiana* transient expression

Five mL of selective LB liquid medium was inoculated with one *Agrobacterium* fresh colony. The cultures were incubated for two days at 28°C. 50 ml of LB supplemented with antibiotics was subsequently inoculated with 5 mL of the bacterial culture and incubated overnight at 28°C at 200 rpm. The bacteria were collected by centrifugation and resuspended in the infiltration medium (10 mM $MgCl_2$, 10 mM MES pH 5.5, 100 µM acetosyringone) to a final concentration of 0.3 OD_{600}. The bacterial suspension was then incubated at room temperature for about 3 h prior to infiltration. 5-6 weeks *Nicothiana Benthamiana* plants were infiltrated using a syringe without needle; for each of four constructs, three leaves of three plants were infiltrated.

2.5.3 Dual Luciferase Reporter Assay

Three days after the infiltration, 1 cm diameter leaf discs were excised from infiltrated leaves 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.

3. RESULTS

3.1 Transient overexpression of *VViAGL15*, *VViWRKY19* and *VVibHLH75* by leaf agroinfiltration

To elucidate the role of *VviAGL15a*, *VviWRKY19* and *VvibHLH75* as master regulators of immature-to-mature transition in berry ripening, a preliminary transient overexpression in *Vitis vinifera* cv. Thompson seedless of each transcription factor (TF) was performed.

The sequences (CDS + 3'UTR) of each TF were amplified by PCR from Corvina cultivar cDNA using adapted GB primers (Table 1) and cloned in pUPD2 vector. The sequencing of cloned regions (Supplemental Figure 1) and their alignment with Pinot sequences (retrieved from Grape Genome Database) showed the existence of some SNIP (Supplemental Figure 2); in particular, 3 SNIP were identified in the CDS of *VviAGL15a*, 3 SNIP, 2 in the CDS and 1 in the 3'UTR, were identified for *VvibHLH75* and 4 SNIP, 2 in the CDS and 2 in the 3'UTR were recognized for *VviWRKY19*. The next aminoacidic sequences alignment revealed that the Corvina isolated sequences and Pinot sequences share 99 % similarity (Supplemental Figure 3). Afterwards, the obtained DNA fragments were placed downstream the CaMV 35S promoter in the pDGB3α2 vector; then, the transcriptional unit (TU) α2-35S::CDS+3'UTR::TNOS for each TF were binarily assembled with the TU α1-35S::*YFP*::TNOS in the pDGB3Ω1 vector. Finally, the resulting vectors pEGB3Ω1-35S::*YFP*::TNOS-35S::CDS+3'UTR::TNOS (Figure 1A) for each TF were introduced in *A. tumefaciens* C58C1. The TU α1-35S::*YFP*::TNOS assembled in each final vector was used for the overexpression of YFP: in this way the positive results of transient expression have been visually identified by the specific YFP fluorescence analysis.

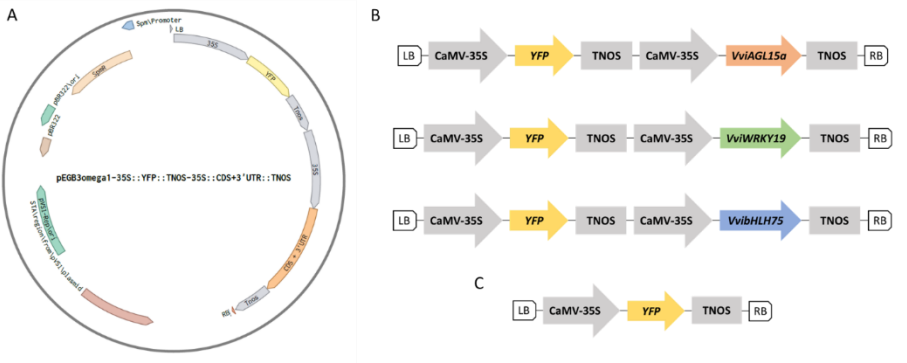

Figure 1: Vectors and transcriptional units used in leaf agroinfiltration experiments. A: schematic representation of the final vector (A), transcriptional units for the transient overexpression of *VviAGL15a*, *VviWRKY19* and *VvibHLH75* (B) and *YFP* (C) by leaf agroinfiltration of Thompson seedless plants.

Transient overexpression was performed by leaf agroinfiltration using the experimental procedure described in Chapter 3, section 2.4.1: 7 plants were used for the transient overexpression of each transcription factor (Ω1-35S::*YFP*::TNOS-35S::CDS+3'UTR::TNOS, Figure 1B) and others 7 plants were used for the overexpression of YFP (Ω1-35S::*YFP*::TNOS, Figure 1C) as negative controls. Based on YFP expression, 7 d.p.i., only YFP-expressing agroinfiltrated leaves were sampled. The YFP expression, and the associated candidate gene expression, occurred especially in the first and second leaves from apex (Figure 2), confirming the results previously described (Chapter 3, section 3.2.1.2).

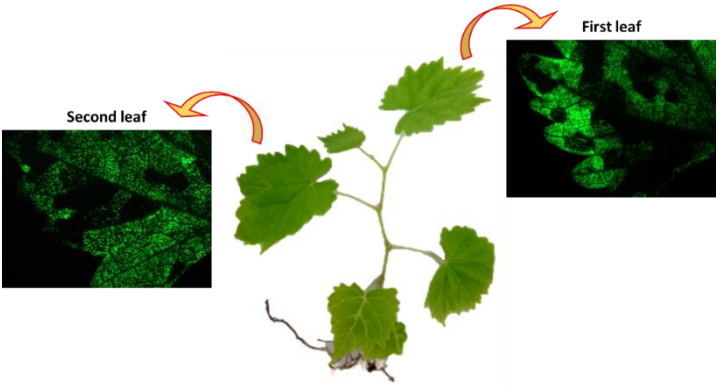

Figure 2: Transient overexpression of *VviAGL15a*, *VviWRKY19* and *VvibHLH75* by vacuum leaf agroinfiltration of whole plant grown *in-vitro* of Thompson seedless cultivar. The transcriptomic analysis was performed using only agroinfiltrated YFP expressing leaves (d.p.i. 7).

Agroinfiltrated plants were screened for *VviAGL15a*, *VviWRKY19* and *VvibHLH75* overexpression by Real-Time qPCR (data not shown) conducted on sampled leaves. The Real-Time qPCR analysis confirmed the overexpression of each target genes and it allowed the selection of the best overexpressing lines (3 lines for *VviAGL15a* and the 4 lines for *VviWRKY19* and *VvibHLH75*) for each transcription factors in comparison to their respective expression level in the control lines (Fig. 3).

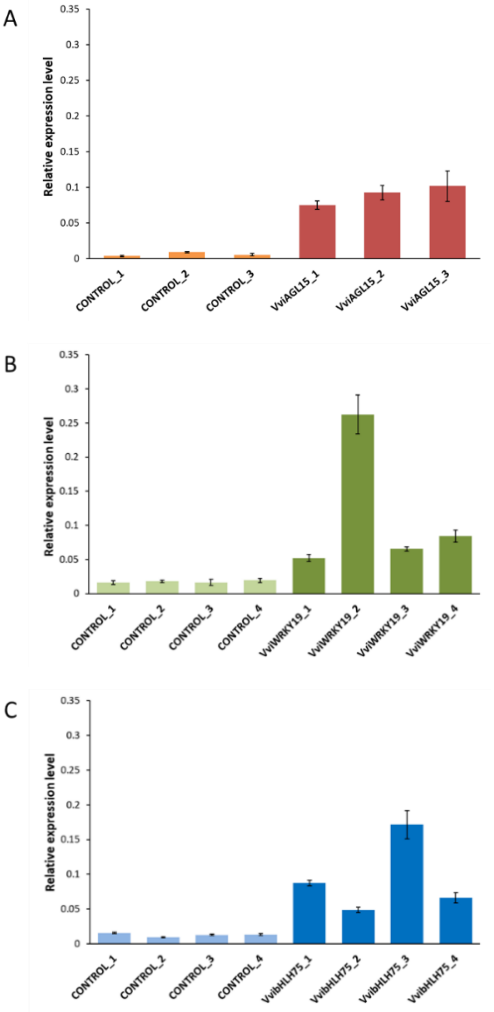

Figure 3: Real Time qPCR analysis of *VviAGL15a* (A), *VviWRKY19* (B) and *VvibHLH75* (C) expression level in leaves of overexpressing and control lines of Thompson seedless plants. Each expression value, relative to *UBIQUITIN* (VIT_16s0098g01190), was determined in triplicate ± S.E.

3.2 Microarray analysis on *VviAGL15a*, *VviWRKY19* and *VvibHLH75* overexpressing Thompson seedless leaves

To obtain more information about the role of these transcription factors and to identify their putative target genes, a microarray analysis was performed. Using an Agilent platform, the leaf transcriptomes of overexpressing and control lines selected for each transcription factors, were

compared. Filtered and normalized transcriptomic dataset (see Material and Methods, section 2.3.3) were used to perform two different analysis: a t-test and a co-expression analysis. Performing a t-test analysis with a Pearson's correlation value of 0.05, 758, 1070 and 2434 differential expressed genes (DEGs) were identified for *VviAGL15a*, *VviWRKY19* and *VvibHLH75*, respectively. Considering a fold change |FC| > 1.5, up- and down-regulated genes were identified for each transcription factor (Figure 4A). In parallel, the analysis of genes co-expressed with each transcription factors was performed using a specific correlation tool named CorTo and Pearson's coefficient as correlation metric. Differentially expressed and correlated genes were annotated using V1 version of the 12X draft annotation of the grapevine genome and distributed 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.

Based on their role of transcriptional activators, for each transcription factors selected, the analysis and identification of their putative target genes were performed by inspecting especially the list of up-regulated genes (|FC|>1.5) and the genes co-expressed with a Pearson's coefficient > 0.9 specifically involved in known metabolic processes and pathways clearly related to grapevine development and ripening. Finally, to obtain some information about the possible involvement of the putative target genes identified from transformed leaves in the berry ripening process, their expression was analyzed using the Corvina gene expression atlas (Fasoli et al., 2012).

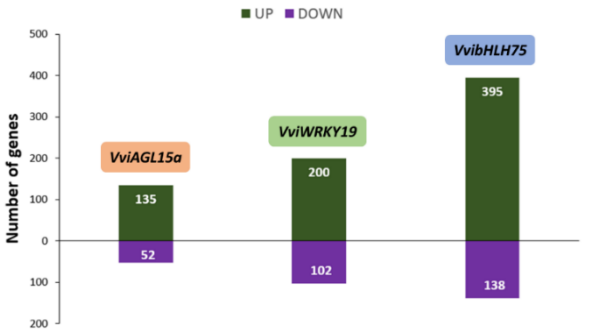

Figure 4: number of up- and down-regulated genes (|FC| > 1.5) in transiently *VviAGL15a*, *VviWRKY19 and VvibHLH75* overexpressing leaves compared to the controls.

3.2.1 *VviAGL15a*

Regarding *VviAGL15a*, 135 up-regulated genes and 52 down-regulated genes were identified (Fig. 4); among the up-regulated genes, the most represented functional categories were carbohydrate metabolic process, response to hormone stimulus, response to stress, signal transduction and transcription factor activity (Fig. 5).

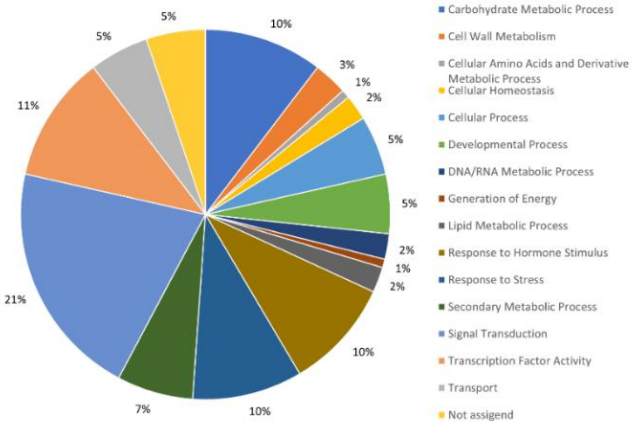

Fig. 5: Functional categories representatives of the up-regulated genes (FC > 1.5) in transiently *VviAGL15a* overexpressing Thompson seedless plants.

The first 10 genes with the highest modulation are shown in Table 4. *VviAGL15a* was detected as the most upregulated gene by the microarray probes, thus confirming the reliability of the entire experimental set-up. Furthermore, there are one *FERONIA RECEPTOR-LIKE KINASE* (VIT_01s0244g00090), whose function in signal transduction to regulate growth in response to internal or external mechanical forces is well studied in *Arabidopsis thaliana* (Shih et al., 2014), one *GLUTAREDOXIN* (VIT_05s0020g01750), a small enzyme mainly involved in oxidative stress responses in plants, whose role during the floral development in *Arabidopsis thaliana* has been documented (Li et al., 2009), three *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE* (VIT_11s0052g01260, VIT_11s0052g01190, VIT_11s0052g01330), enzymes involved in the modification of cell wall structure by cleaving and re-joining xyloglucan molecules, one *BETA-FRUCTOFURANOSIDASE* (VIT_05s0077g00510), an enzyme that catalyzes the hydrolysis of sucrose, and one *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_17s0000g08010 and VIT_14s0036g01210), involved in the biosynthesis of trehalose 6-phosphate, an important signaling metabolite, which plays an essential role in plant development (Ponnu et al., 2011).

Other genes with high modulation (FC > 2) are reported in Supplemental Data Set 1; among them, there are many genes belong to ERF/AP2 gene family, whose members are transcription factors involved in the regulation of many biological processes (Licausi et al., 2010), one *TRANS-RESVERATROL DI-O-METHYLTRANSFERASE* (VIT_10s0003g00470), involved in the biosynthesis of pterostilbene, a stilbenoid chemically related to resveratrol, and four *CIS-ZEATIN O-BETA-D-GLUCOSYLTRANSFERASE* (VIT_08s0007g08910, VIT_08s0007g08890, VIT_08s0007g08920, VIT_03s0017g01040), enzymes involved in the metabolism of cis-zeatin, a group of cytokinins, involved in the regulation of plant development and biotic stress responses.

Table 4: the 10 most up-regulated genes in *VviAGL15a* overexpressing Thompson seedless leaves.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	FC
VIT_13S0158G00100	putative MADS-box Agamous-like 15a (VviAGL15a)	Transcription Factor Activity	18.30
VIT_01S0244G00090	feronia receptor-like kinase	Signal Transduction	15.54
VIT_05S0020G01750	Glutaredoxin	Cellular Homeostasis	6.77
VIT_11S0052G01260	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	6.14
VIT_11S0052G01190	Xyloglucan endotransglucosylase-hydrolase XTH3	Cell Wall Metabolism	5.91
VIT_11S0052G01330	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	5.28
VIT_17S0119G00230	Trypsin and protease inhibitor Kunitz family	Response to stress	4.66
VIT_05S0020G02720	Aspartic Protease (VvAP11)	Cellular Process	3.93
VIT_05S0077G00510	Beta-fructofuranosidase	Carbohydrate Metabolic Process	3.84
VIT_17S0000G08010	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.53

Regarding the co-expression analysis, the correlated genes with a Pearson's coefficient > 0.9 are 199. The most representative functional category (Figure 6) are response to hormone stimulus and transcription factor activity, followed by cell wall metabolism, cellular process, response to stress and signal transduction.

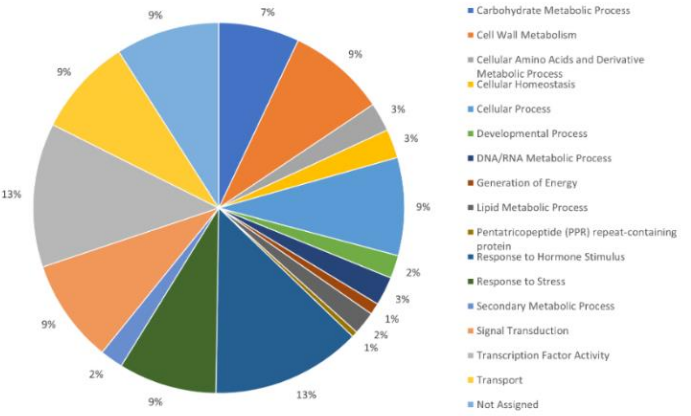

Figure 6: Functional categories representatives of genes (Pearson value > 0.9) correlated to *VviAGL15* in transiently *VviAGL15a* overexpressing Thompson seedless plants.

The first 10 genes with highest Pearson's coefficient are shown in Table 5. Among them, there are many genes involved in cell wall metabolism including three *XYLOGLUCAN ENDOTRANSGLUCOSYLASE* (VIT_11S0052G01260, VIT_11S0052G01300, VIT_11S0052G01340) and one EXPANSIN (VIT_03S0038G03430). Furthermore, there two transcription facors, one LATERAL ORGAN BOUNDARIES DOMAIN (VIT_13S0067G01880) and one *BTB/POZ DOMAIN-CONTAINING PROTEIN* (VIT_18S0122G01340), a transcription factor identified as switch gene of grapevine atlas (Palumbo et al., 2014) and one *LEUCINE-RICH REPEAT PROTEIN KINASE* (VIT_13S0067G03780), a transmembrane receptor-like kinases, involved in the regulation of a wide variety of developmental and defense-related processes. Other genes with high Pearson's coefficient (> 0.95) are reported in Supplemental Data Set 2; among them, there are many others *XYLOGLUCAN ENDOTRANSGLUCOSYLASE*, one ERF/AP2 gene, *VviERF045* (VIT_04S0008G06000), a switch gene emerged by the network analysis of berry transcriptome (Palumbo et al, 2014; Massonnet et al, 2017), upregulated during the berry ripening (Licausi et al, 2010), two *CIS-ZEATIN O-BETA-D-GLUCOSYLTRANSFERASE* (VIT_08s0007g08890, VIT_08s0007g08920), one *BETA-FRUCTOFURANOSIDASE* (VIT_05s0077g00510) and one *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_14s0036g01210).

Table 5: the 10 genes most correlated to *VviAGL15a* in overexpressing Thompson seedless leaves.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	Pearson
VIT_11S0052G01260	xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	0.997
VIT_18S0122G01340	BTB/POZ domain-containing protein	Transcription Factor Activity	0.996
VIT_11S0052G01300	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.993
VIT_06S0009G01930	5-AMP-activated protein kinase beta-2 subunit	Lipid Metabolic Process	0.992
VIT_11S0052G01340	Xyloglucan endo-transglycosylase, C-terminal	Cell Wall Metabolism	0.990
VIT_03S0038G03430	Expansin (VvEXLA1)	Cell Wall Metabolism	0.986
VIT_02S0012G00730	purine permease 10 PUP10	Transport	0.984
VIT_13S0067G01880	other LOB domain-containing protein ASL5	Transcription Factor Activity	0.982
VIT_13S0067G03780	Leucine-rich repeat protein kinase	Signal Transduction	0.982
VIT_17S0000G06370	Thioredoxin 2	Cellular Homeostasis	0.981

The comparison between the upregulated genes (FC > 2) and the most correlated genes (Pearson's coefficient > 0.95) showed that 16 genes are common (Table 6). In particular, there are four *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE* (VIT_11s0052g01260, VIT_11s0052g01330, VIT_11s0052g01250, VIT_11s0052g01190), two *CIS-ZEATIN O-BETA-D-GLUCOSYLTRANSFERASE* (VIT_08S0007G08890), one *BETA-FRUCTOFURANOSIDASE* (VIT_05S0077G00510), one *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_14s0036g01210) and one *LATERAL ORGAN BOUNDARIUES DOMAIN* (VIT_13S0067G01880).

Table 6: shared genes between upregulated (FC > 2) and correlated genes (Pearson's coefficient > 0.95) of *VviAGL15a*.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY
VIT_11S0052G01260	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism
VIT_11S0052G01190	Xyloglucan endotransglucosylase-hydrolase XTH3	Cell Wall Metabolism
VIT_11S0052G01330	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism
VIT_05S0077G00510	Beta-fructofuranosidase	Carbohydrate Metabolic Process
VIT_14S0036G01210	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process
VIT_18S0001G06180	Phosphate-induced protein 1	Cellular Process
VIT_08S0007G08890	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process
VIT_13S0067G01880	Other LOB domain-containing protein ASL5	Transcription Factor Activity
VIT_07S0005G01240	Triacylglycerol lipase	Lipid Metabolic Process

VIT_13S0067G03780	Leucine-rich repeat protein kinase	Response to Stress
VIT_18S0001G09850	Myb domain protein R1	Transcription Factor Activity
VIT_01S0026G00880	Transducin protein	#N/D
VIT_08S0007G08920	Zeatin O-glucosyltransferase	Secondary Metabolic Process
VIT_11S0052G01250	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism
VIT_11S0206G00090	Calmodulin-binding protein	Signal Transduction
VIT_19S0014G04650	Avr9/Cf-9 rapidly elicited protein 20	Response to Stress

Finally, the expression profiles of each gene with FC > 2 (Supplemental data Set 1) and with Pearson's coefficient > 0.95 (Supplemental data Set 2), excluding the common genes, were analyzed in Corvina berry development (Fasoli et al, 2012) and used to perform a hierarchical clustering analysis. The results show the existence of 10 clusters (Figure 7). Genes consistent with their supposed role in berry ripening as targets of *VviAGL15a* are grouped in cluster 1 and 2 (high expression in seed at *veraison* and mid ripening phases) and in clusters 3, 4, 5 and 6 (high expression during veraison, mid-ripening and ripening phases). In more detail, genes of cluster 3 are expressed during veraison/mid-ripening/ripening in both flesh and skin, genes of cluster 4 are preferentially actives during ripening phase while clusters 5 and 6 contain genes mainly expressed at veraison and mid-ripening in skin with low expression in flesh. Conversely, clusters 7, 8, 9 and 10 contain genes with preferential expression during fruit set phase in flesh, skin and seed, thus making unlikely their involvement in ripening processes driven by *VviAGL15a*.

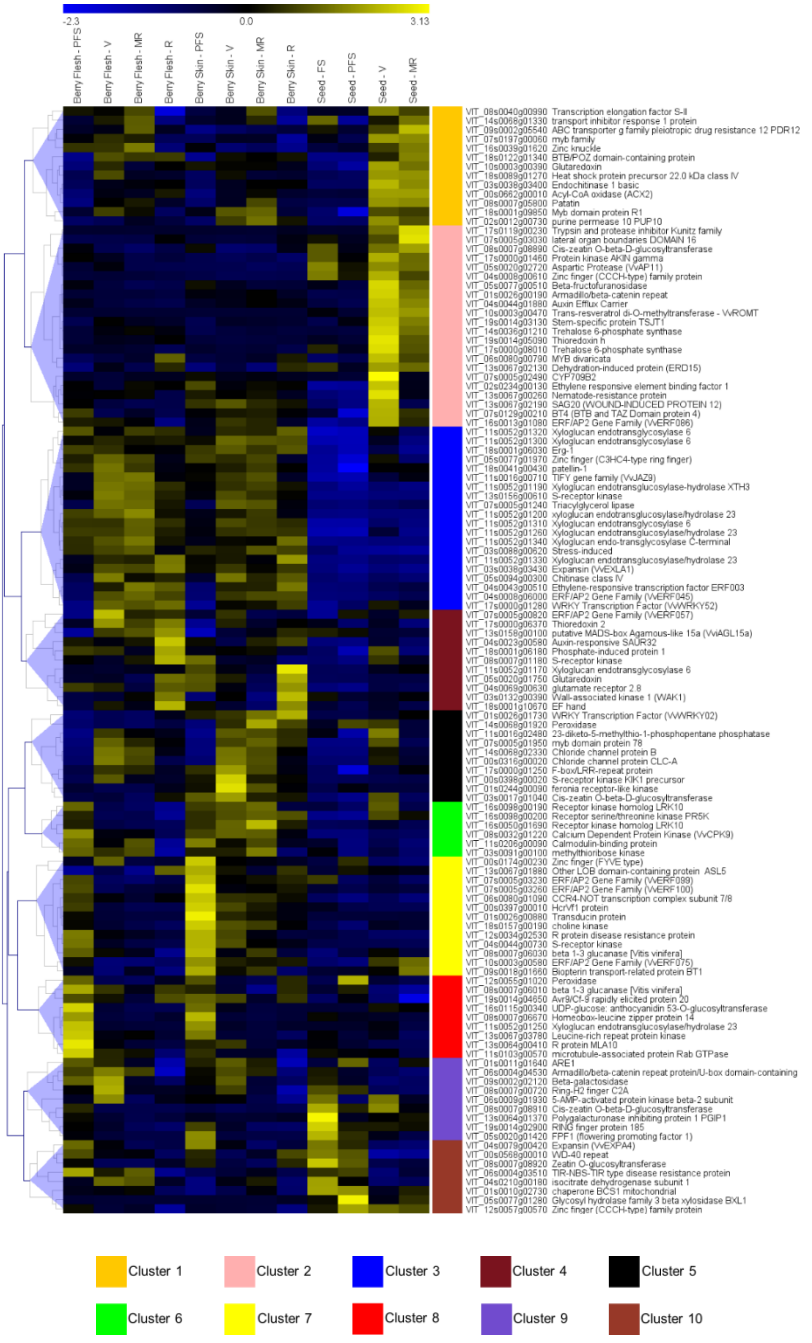
-2.3
0.0
3.13
Berry Flesh - PFS
Berry Flesh - V
Berry Flesh - MR
Berry Flesh - R
Berry Skin - PFS
Berry Skin - V
Berry Skin - MR
Berry Skin - R
Seed - FS
Seed - PFS
Seed - V
Seed - MR
VIT_08s0040g00990 Transcription elongation factor S-II
VIT_14s0068g01330 transport inhibitor response 1 protein
VIT_09s0002g05540 ABC transporter g family pleiotropic drug resistance 12 PDR12
VIT_07s0197g00060 myb family
VIT_16s0039g01620 Zinc knuckle
VIT_18s0122g01340 BTB/POZ domain-containing protein
VIT_10s0003g00390 Glutaredoxin
VIT_18s0089g01270 Heat shock protein precursor 22.0 kDa class IV
VIT_03s0038g03400 Endochitinase 1 basic
VIT_03s0062g00010 Acyl-CoA oxidase (ACX2)
VIT_08s0007g05800 Patatin
VIT_18s0001g09850 Myb domain protein R1
VIT_02s0012g00730 purine permease 10 PUP10
VIT_11s0118g00230 Trypsin and protease inhibitor Kunitz family
VIT_07s0005g03030 lateral organ boundaries DOMAIN 16
VIT_08s0007g08890 Cis-zeatin O-beta-D-glucosyltransferase
VIT_17s0000g01460 Protein kinase AKIN gamma
VIT_05s0020g02720 Aspartic Protease (VvAP11)
VIT_04s0008g00610 Zinc finger (CCCH-type) family protein
VIT_05s0077g00510 Beta-fructofuranosidase
VIT_01s0026g00190 Armadillo/beta-catenin repeat
VIT_04s0044g01880 Auxin Efflux Carrier
VIT_10s0003g00470 Trans-resveratrol di-O-methyltransferase - VvROMT
VIT_19s0014g03130 Stem-specific protein TSJT1
VIT_14s0036g01210 Trehalose 6-phosphate synthase
VIT_19s0014g05090 Thioredoxin h
VIT_17s0000g08010 Trehalose 6-phosphate synthase
VIT_06s0080g00790 MYB divaricata
VIT_13s0067g02130 Dehydration-induced protein (ERD15)
VIT_07s0005g02490 CYP709B2
VIT_02s0234g00130 Ethylene responsive element binding factor 1
VIT_13s0067g00260 Nematode-resistance protein
VIT_13s0067g02190 SAG20 (WOUND-INDUCED PROTEIN 12)
VIT_07s0129g00210 BT4 (BTB and TAZ Domain protein 4)
VIT_18s0013g01080 ERF/AP2 Gene Family (VvERF086)
VIT_11s0052g01320 Xyloglucan endotransglycosylase 6
VIT_11s0052g01300 Xyloglucan endotransglycosylase 6
VIT_18s0001g06030 Erg-1
VIT_05s0077g01970 Zinc finger (C3HC4-type ring finger)
VIT_18s0041g00430 patellin-1
VIT_11s0016g00710 TIFY gene family (VvJAZ9)
VIT_11s0052g01190 Xyloglucan endotransglucosylase-hydrolase XTH3
VIT_18s0156g00510 S-receptor kinase
VIT_07s0005g01240 Triacylglycerol lipase
VIT_11s0052g01200 xyloglucan endotransglucosylase/hydrolase 23
VIT_11s0052g01310 Xyloglucan endotransglycosylase 6
VIT_11s0052g01260 Xyloglucan endotransglucosylase/hydrolase 23
VIT_11s0052g01340 Xyloglucan endo-transglycosylase C-terminal
VIT_03s0088g00620 Stress-induced
VIT_11s0052g01330 Xyloglucan endotransglucosylase/hydrolase 23
VIT_03s0038g03430 Expansin (VvEXLA1)
VIT_05s0094g00300 Chitinase class IV
VIT_04s0043g00510 Ethylene-responsive transcription factor ERF003
VIT_04s0008g06000 ERF/AP2 Gene Family (VvERF045)
VIT_17s0000g01280 WRKY Transcription Factor (VvWRKY52)
VIT_07s0005g00820 ERF/AP2 Gene Family (VvERF057)
VIT_17s0000g06370 Thioredoxin 2
VIT_13s0158g00100 putative MADS-box Agamous-like 15a (VviAGL15a)
VIT_04s0023g00580 Auxin-responsive SAUR32
VIT_18s0001g06180 Phosphate-induced protein 1
VIT_08s0007g01180 S-receptor kinase
VIT_11s0052g01170 Xyloglucan endotransglycosylase 6
VIT_05s0020g01750 Glutaredoxin
VIT_04s0059g00630 glutamate receptor 2.8
VIT_03s0132g00390 Wall-associated kinase 1 (WAK1)
VIT_18s0001g10670 EF hand
VIT_01s0026g01730 WRKY Transcription Factor (VvWRKY02)
VIT_14s0068g01920 Peroxidase
VIT_11s0016g02480 2,3-diketo-5-methylthio-1-phosphopentane phosphatase
VIT_07s0005g01950 myb domain protein 78
VIT_14s0068g02330 Chloride channel protein B
VIT_03s0316g00020 Chloride channel protein CLC-A
VIT_17s0000g01250 F-box/LRR-repeat protein
VIT_06s0398g00020 S-receptor kinase KK1 precursor
VIT_01s0244g00090 feronia receptor-like kinase
VIT_03s0017g01040 Cis-zeatin O-beta-D-glucosyltransferase
VIT_18s0098g00190 Receptor kinase homolog LRK10
VIT_18s0098g00200 Receptor serine/threonine kinase PR5K
VIT_18s0050g01690 Receptor kinase homolog LRK10
VIT_08s0032g01220 Calcium Dependent Protein Kinase (VvCPK9)
VIT_11s0206g00090 Calmodulin-binding protein
VIT_03s0091g00100 methylthioribose kinase
VIT_09s0174g00230 Zinc finger (FYVE type)
VIT_13s0067g01880 Other LOB domain-containing protein ASL5
VIT_07s0005g03230 ERF/AP2 Gene Family (VvERF089)
VIT_07s0005g03260 ERF/AP2 Gene Family (VvERF100)
VIT_06s0080g01090 CCR4-NOT transcription complex subunit 7/8
VIT_03s0397g00010 Hcr/f1 protein
VIT_01s0026g00880 Transducin protein
VIT_18s0157g00190 choline kinase
VIT_12s0034g02530 R protein disease resistance protein
VIT_04s0044g00730 S-receptor kinase
VIT_08s0007g06030 beta 1-3 glucanase [Vitis vinifera]
VIT_08s0003g00580 ERF/AP2 Gene Family (VvERF075)
VIT_09s0018g01660 Bioperterin transport-related protein BT1
VIT_12s0055g01020 Peroxidase
VIT_08s0007g06010 beta 1-3 glucanase [Vitis vinifera]
VIT_19s0014g04650 AvrRpt2-like protein
VIT_16s0115g00340 UDP-glucose: anthocyanidin 5(3-O-glucosyltransferase
VIT_08s0007g06670 Homeobox-leucine zipper protein 14
VIT_11s0052g01250 Xyloglucan endotransglucosylase/hydrolase 23
VIT_13s0067g03780 Leucine-rich repeat protein kinase
VIT_13s0064g00410 R protein MLA10
VIT_11s0103g00570 microtubule-associated protein Rab GTPase
VIT_01s0011g01640 ARE1
VIT_05s0094g04530 Armadillo/beta-catenin repeat protein/U-box domain-containing
VIT_09s0002g02120 Beta-galactosidase
VIT_08s0007g00720 Ring-H2 finger C2A
VIT_05s0094g01930 5-AMP-activated protein kinase beta-2 subunit
VIT_08s0007g08910 Cis-zeatin O-beta-D-glucosyltransferase
VIT_13s0064g01370 Polygalacturonase inhibiting protein 1 PGIP1
VIT_19s0014g02900 RING finger protein 185
VIT_05s0020g01420 FPF1 (flowering promoting factor 1)
VIT_04s0079g00420 Expansin (VvEXPA4)
VIT_03s0568g00010 WD-40 repeat
VIT_08s0007g08920 Zeatin O-glucosyltransferase
VIT_08s0040g03510 TIR-NBS-TIR type disease resistance protein
VIT_04s0210g00180 isocitrate dehydrogenase subunit 1
VIT_01s0010g02730 chaperone BCS1 mitochondrial
VIT_05s0077g01280 Glycosyl hydrolase family 3 beta xylosidase BXL1
VIT_12s0057g00570 Zinc finger (CCCH-type) family protein
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10

Figure 7: Hierarchical clustering analysis of *VviAGL15a* putative target genes in Corvina berry development. Pearson's correlation and complete linkage were respectively chosen as distance metric and clustering method to create the transcriptional profile dendrogram (by rows). Samples (columns) are ordered by the progression of berry flesh, skin and seed development: FS, Fruit set; PFS, Post Fruit Set; V, Veraison; MR, Mid Ripening; R, Ripening. The normalized expression values range from low (blue) to high (yellow).

3.2.2 *VviWRKY19*

Regarding *VviWRKY19*, 200 up-regulated genes and 102 down-regulated genes were identified (Fig. 4); among the up-regulated genes, the most represented functional categories were carbohydrate metabolic process, cellular homeostasis, response to hormone stimulus, secondary metabolic process, signal transduction and transcription factor activity (Fig. 8).

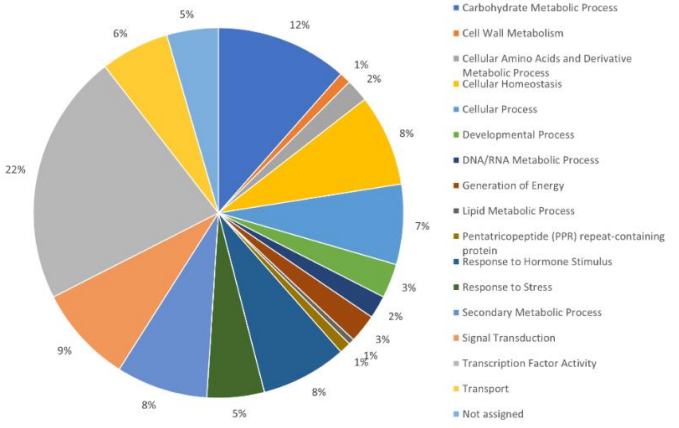

Fig. 8: Functional categories representatives of the up-regulated genes (FC > 1.5) in transiently *VviWRKY19* overexpressing Thompson seedless plants.

The first 10 genes with highest modulation are shown in Table 7. Among them, there are one *ALPHA-AMYLASE/SUBTILISIN INHIBITOR* (VIT_17s0119g00150): coding a protein characterized in both barley and rice (Nielsen et al, 2004; Yamasaki et al, 2006), involved in both regulation of α-amylase activity and in plant defense against microorganisms, by inhibiting subtilisin-type serine proteases. Furthermore, there are three *TRYPSIN AND PROTEASE INHIBITOR KUNITZ FAMILY* (VIT_17s0119g00230, VIT_17s0119g00160, VIT_00s1751g00010) proteins involved in defenses plant against herbivorous arthropods and microbial pests by inhibiting host protease activity (Rustgi et al., 2017), the NAC transcription factor, *VviNAC26* (VIT_01s0026g02710) and one ERF/AP2 Gene Family, *VvERF001* (VIT_19s0014g03180); finally there two *XYLOGLUCAN*

ENDOTRANSGLUCOSYLASE (VIT_11s0052g01220, VIT_11s0052g01300) and one *TRANS-RESVERATROL DI-O-METHYLTRANSFERASE* (VIT_10s0003g00470), whose functions were described above. Other genes with high modulation (FC > 2) are reported in Supplemental Data Set 3; among them, there are one *BETA-FRUCTOFURANOSIDASE* (VIT_05s0077g00510), two *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_17s0000g08010 and VIT_14s0036g01210), previously described for *VviAGL15,* and others two ERF/AP2 genes, *VviERF075* (VIT_10s0003g00580) and *VviERF055* (VIT_06s0004g08190). Many others upregulated genes belong to the functional category of secondary metabolic process; among these, four *CIS-ZEATIN O-BETA-D-GLUCOSYLTRANSFERASE* (VIT_08s0007g08910, VIT_08s0007g08920 and VIT_03s0017g01040*,* VIT_13s0019g03100), have been identified. Furthermore, two *ANTHOCYANIDIN GLUCOSYLTRANSFERASE* (VIT_16s0115g00340 and VIT_03s0017g02000), one *FLAVONOID 3-MONOOXYGENASE* (VIT_02s0109g00310), one *QUERCETIN 3-O-GLUCOSIDE-6''-O-MALONYLTRANSFERASE* (VIT_12s0134g00630), four genes involved in flavonoids biosynthesis, and one *TAXADIEN-5-ALPHA-OL-O-ACETYLTRANSFERASE* (VIT_06s0004g07650) and two *(-)-GERMACRENE D SYNTHASE* (VIT_19s0014g02550 and VIT_19s0014g02580), three genes involved in terpenoid biosynthesis, were identified. Concerning the transcription factor activity, the most representative are NAC transcription factors, including *VviNAC17* (VIT_19s0014g03290), *VviNAC61* (VIT_08s0007g07640), *VviNAC74* (VIT_06s0080g00780), *VviNAC39* (VIT_07s0031g02610), *VviNAC33* (VIT_19s0027g00230) and *VviNAC08* (VIT_18s0001g02300), and many members of zinc fingers transcription factors family. Furthermore, other two upregulated transcription factors are *LATERAL ORGAN BOUNDARIES PROTEIN 38* (VIT_03s0091g00670) and *LATERAL ORGAN BOUNDARIES PROTEIN 39* (VIT_07s0129g00330). Finally, there are three *GLUTAREDOXIN-LIKE* (VIT_01s0146g00220, VIT_10s0003g00390, VIT_14s0068g01570), three *KELCH REPEAT-CONTAINING F-BOX FAMILY PROTEINS* (VIT_09s0002g05210, VIT_09s0002g04930 and VIT_09s0002g05010), molecules involved in the protein degradation by ubiquitin-proteasome pathway, and two *PHENYLALANINE AMMONIA-LYASE* (VIT_11s0016g01520, VIT_11s0016g01640), the first enzyme in the phenylpropanoid pathway.

Table 7: the 10 most up-regulated genes in *VviWRKY19* overexpressing Thompson seedless leaves.

VIT	FUNCIONAL ANNOTATION	GENE ONTOLOGY	FC
VIT_17S0119G00150	Alpha-amylase/subtilisin inhibitor	Carbohydrate Metabolic Process	7.47
VIT_17S0119G00230	Trypsin and protease inhibitor Kunitz family	Response to Stress	7.29
VIT_19S0014G03180	ERF/AP2 Gene Family (VviERF001), Dehydration Responsive Element-Binding Transcription Factor (VvDREB33)	Response to Hormone Stimulus	7.09
VIT_17S0119G00160	Trypsin and protease inhibitor Kunitz family	Response to Stress	6.41
VIT_00S1751G00010	Trypsin and protease inhibitor family	Response to Stress	5.72
VIT_01S0026G02710	NAC domain-containing protein (VvNAC26)	Transcription Factor Activity	4.81
VIT_11S0052G01220	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	4.67
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	4.35
VIT_10S0003G00470	Trans-resveratrol di-O-methyltransferase - VvROMT	Secondary Metabolic Process	4.29
VIT_11S0052G01300	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	4.04

Regarding the co-expression analysis, the correlated genes with a Pearson's coefficient > 0.9 are 45. The most representative functional categories (Figure 9) are DNA/RNA metabolic process, response to hormone stimulus, signal transduction and transcription factor activity.

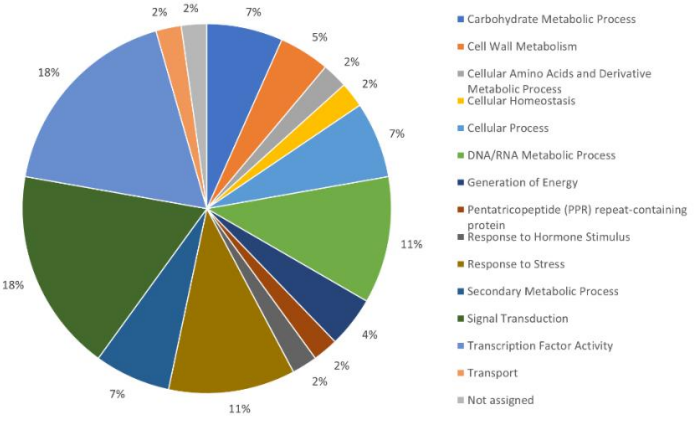

Figure 9: Functional categories representatives of genes (Pearson value > 0.9) correlated to *VviWRKY19* in transiently *VviWRKY19* overexpressing Thompson seedless plants.

The first 10 genes with highest Pearson's coefficient are shown in Table 8. Among them, there are one *NAD+ ADP-RIBOSYLTRANSFERASE*, a protein that catalyzed the covalent attachment of ADP-ribose to a target protein, involved in plant immunity (Feng et al., 2016), one gene involved in sugar transport (VIT_18S0001G08210), one *XYLOGLUCAN ENDOTRANSGLUCOSYLASE* (VIT_11S0052G01210) and two transcription factors, one zinc fingers (VIT_08S0007G08210) and one *GT2-LIKE TRIHELIX DNA-BINDING PROTEIN* (VIT_04S0044G00510). Other genes with high Pearson's coefficient (> 0.9) are reported in Supplemental Data Set 4; among them, there are two genes involved in sugar transport (VIT_00S0181G00010, VIT_13S0019G01480), one *XYLOGLUCAN ENDOTRANSGLUCOSYLASE* (VIT_11S0052G01220), many protein kinases involved in signal transduction, and, among the transcription factors, there are two zinc fingers (VIT_08S0105G00290, VIT_08S0007G03880), one *LATERAL ORGAN BOUNDARIES DOMAIN* (VIT_13S0019G03700), one *BASIC HELIX LOOP HELIX* (VIT_05S0124G00240) and the NAC transcription factor, *VviNAC08* (VIT_18S0001G02300).

Table 8: the 10 genes most correlated to *VviWRKY19* in overexpressing Thompson seedless leaves.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	0.989
VIT_14S0068G02130	fidgetin-like 1	Transport	0.976
VIT_06S0004G03920	Pto serine/threonine kinase	Signal Transduction	0.960
VIT_18S0001G08210	SUT4 (sucrose transporter 4)	Carbohydrate Metabolic Process	0.959
VIT_12S0035G01280	R protein disease resistance protein	Response to Stress	0.954
VIT_06S0004G05500	CHLORORESPIRATORY REDUCTION 2 (CRR2)	Generation of Energy	0.948
VIT_11S0052G01210	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.944
VIT_04S0044G00510	GT2-like trihelix DNA-binding protein	Transcription Factor Activity	0.941
VIT_08S0007G08210	Zinc finger (CCCH-type) family protein	Transcription Factor Activity	0.941
VIT_17S0053G00360	pentatricopeptide (PPR) repeat	Pentatricopeptide (PPR) repeat-containing protein	0.937

The comparison between the upregulated genes (FC > 2) and the most correlated genes (Pearson's coefficient > 0.9) showed that only 3 genes are common (Table 9), one *XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6* (VIT_11S0052G01220), *NAD+ ADP-RIBOSYLTRANSFERASE* (VIT_07S0005G00630) and one *HOMOGENTISATE 1,2-DIOXYGENASE* (VIT_19s0014g01800).

Table 9: shared genes between upregulated (FC > 2) and correlated genes (Pearson's coefficient > 0.9) of *VviWRK19*.

VIT	FUNCIONAL ANNOTATION	GENE ONTOLOGY
VIT_11S0052G01220	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process
VIT_19S0014G01800	Homogentisate 1,2-dioxygenase	Cellular Amino Acids and Derivative Metabolic Process

The hierarchical clustering analysis of *VviWRKY19* was performed using putative target genes with FC > 2 (Supplemental data Set 3) and with Pearson's coefficient > 0.9 (Supplemental data Set 4); their expression profiles were analyzed in Corvina berry development (Fasoli et al., 2012) and the results show the existence of 10 clusters (Figure 10). As described for *VviAGL15a*, many genes show a preferential expression in seed, during the fruit set and post fruit set phases (clusters 1, 2 and 3) and during *veraison* and mid-ripening phases (cluster 5). Cluster 10 contain genes characterized by high expression during fruit set phase in both flesh and skin; genes of cluster 8 show a preferential expression during ripening phase in both flesh and skin while genes of 9 cluster are mainly expressed in skin (all phases) and in seed at *veraison*. Finally, cluster 4 contain genes preferentially expressed at *veraison*, mid-ripening and ripening in flesh, skin and seed and genes with high expression only in seed at *veraison* and mid-ripening while genes grouped in clusters 6 and 7 show genes mainly expressed in flesh and skin during *veraison*, mid-ripening and ripening.

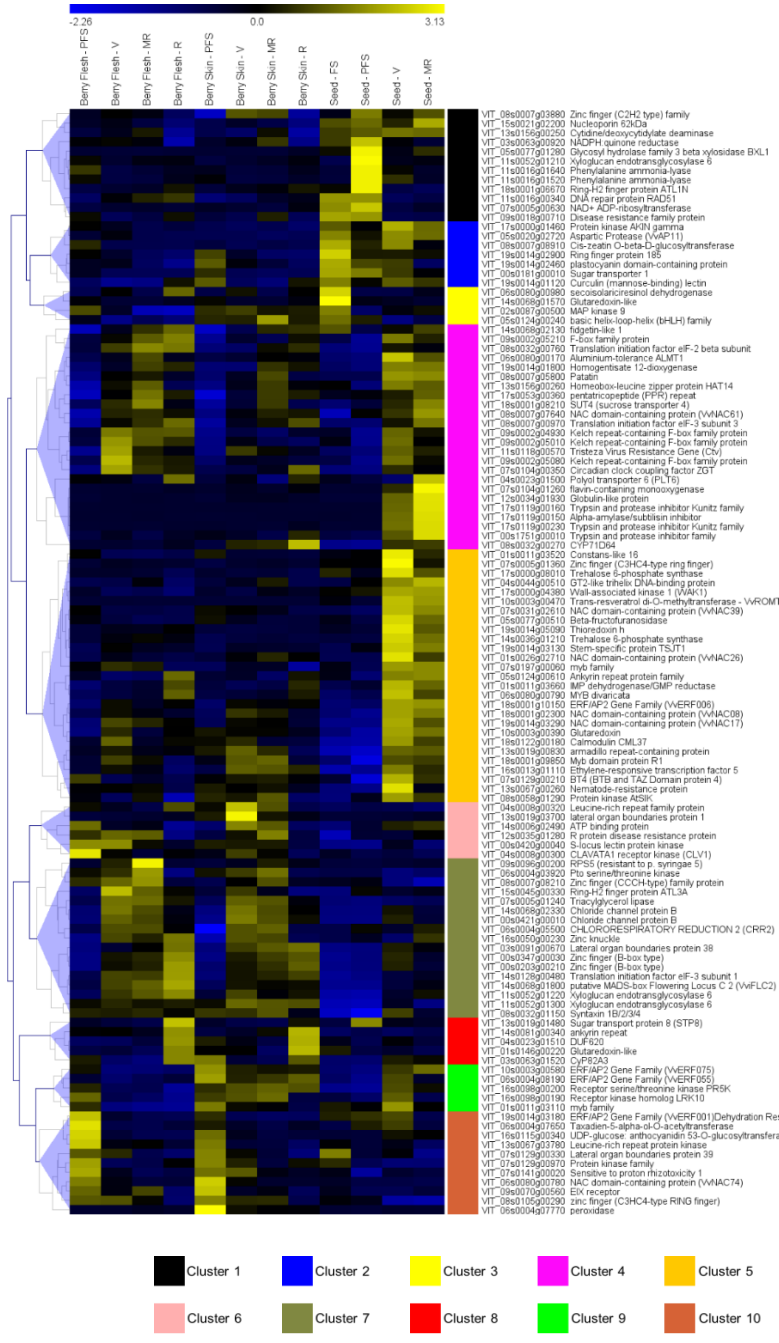
-2.26
0.0
3.13
Berry Flesh - PFS
Berry Flesh - V
Berry Flesh - MR
Berry Flesh - R
Berry Skin - PFS
Berry Skin - V
Berry Skin - MR
Berry Skin - R
Seed - FS
Seed - PFS
Seed - V
Seed - MR
VIT_08s0007g03880 Zinc finger (C2H2 type) family
VIT_15s0021g02200 Nucleoporin 62kDa
VIT_13s0156g00250 Cytidinedeoxycytidylate deaminase
VIT_03s0063g00920 NADPH:quinone reductase
VIT_05s0077g01280 Glycosyl hydrolase family 3 beta xylosidase BXL1
VIT_11s0052g01210 Xyloglucan endotransglycosylase 6
VIT_11s0016g01640 Phenylalanine ammonia-lyase
VIT_11s0016g01520 Phenylalanine ammonia-lyase
VIT_18s0001g06670 Ring-H2 finger protein ATL1N
VIT_11s0016g00340 DNA repair protein RAD51
VIT_07s0005g00630 NAD+ ADP-ribosyltransferase
VIT_08s0018g00710 Disease resistance family protein
VIT_17s0000g01460 Protein kinase AKIN gamma
VIT_05s0020g02720 Aspartic Protease (VvAP11)
VIT_08s0007g08910 Cis-zeatin O-beta-D-glucosyltransferase
VIT_19s0014g02900 Ring finger protein 185
VIT_19s0014g02460 plastocyanin domain-containing protein
VIT_03s0181g00010 Sugar transporter 1
VIT_19s0014g01120 Curculin (mannose-binding) lectin
VIT_06s0080g00980 secoisolariciresinol dehydrogenase
VIT_14s0068g01570 Glutaredoxin-like
VIT_02s0087g00500 MAP kinase 9
VIT_05s0124g00240 basic helix-loop-helix (bHLH) family
VIT_14s0068g02130 fidgetin-like 1
VIT_09s0002g05210 F-box family protein
VIT_08s0032g00760 Translation initiation factor eIF-2 beta subunit
VIT_06s0080g00170 Aluminium-tolerance ALMT1
VIT_19s0014g01800 Homogentisate 12-dioxygenase
VIT_08s0007g05800 Patatin
VIT_13s0156g00260 Homeobox-leucine zipper protein HAT14
VIT_17s0053g00360 pentatricopeptide (PPR) repeat
VIT_18s0001g08210 SUT4 (sucrose transporter 4)
VIT_08s0007g07640 NAC domain-containing protein (VvNAC61)
VIT_08s0007g00970 Translation initiation factor eIF-3 subunit 3
VIT_09s0002g04930 Kelch repeat-containing F-box family protein
VIT_09s0002g05010 Kelch repeat-containing F-box family protein
VIT_11s0118g00570 Tristeza Virus Resistance Gene (Ctv)
VIT_09s0002g05080 Kelch repeat-containing F-box family protein
VIT_07s0104g00350 Circadian clock coupling factor ZGT
VIT_04s0023g01500 Polyol transporter 6 (PLT6)
VIT_07s0104g01260 flavin-containing monooxygenase
VIT_12s0034g01830 Globulin-like protein
VIT_17s0119g00160 Trypsin and protease inhibitor Kunitz family
VIT_17s0119g00150 Alpha-amylase/subtilisin inhibitor
VIT_17s0119g00230 Trypsin and protease inhibitor Kunitz family
VIT_00s1751g00010 Trypsin and protease inhibitor family
VIT_08s0032g00270 CYP71D64
VIT_01s0011g03520 Constans-like 16
VIT_07s0005g01360 Zinc finger (C3HC4-type ring finger)
VIT_17s0000g08010 Trehalose 6-phosphate synthase
VIT_04s0044g00510 GT2-like trihelix DNA-binding protein
VIT_17s0000g04380 Wall-associated kinase 1 (WAK1)
VIT_10s0003g03470 Trans-resveratrol di-O-methyltransferase - VvROMT
VIT_07s0031g02610 NAC domain-containing protein (VvNAC39)
VIT_05s0077g00510 Beta-fructofuranosidase
VIT_19s0014g03090 Thioredoxin h
VIT_14s0036g01210 Trehalose 6-phosphate synthase
VIT_19s0014g03130 Stem-specific protein TSJT1
VIT_01s0026g02710 NAC domain-containing protein (VvNAC26)
VIT_07s0187g00060 myb family
VIT_05s0124g00610 Ankyrin repeat protein family
VIT_01s0011g03660 IMP dehydrogenase/GMP reductase
VIT_06s0080g00790 MYB divaricata
VIT_18s0001g10150 ERF/AP2 Gene Family (VvERF006)
VIT_18s0001g02300 NAC domain-containing protein (VvNAC08)
VIT_19s0014g03290 NAC domain-containing protein (VvNAC17)
VIT_10s0003g03990 Glutaredoxin
VIT_18s0122g00180 Calmodulin CML37
VIT_13s0019g00830 armadillo repeat-containing protein
VIT_18s0001g09850 Myb domain protein R1
VIT_16s0013g01110 Ethylene-responsive transcription factor 5
VIT_07s0129g00210 BTB (BTB and TAZ Domain protein 4)
VIT_13s0067g00260 Nematode-resistance protein
VIT_08s0058g01290 Protein kinase AtSIK
VIT_04s0008g00320 Leucine-rich repeat family protein
VIT_13s0019g03700 lateral organ boundaries protein 1
VIT_14s0006g02490 ATP binding protein
VIT_12s0035g01280 R protein disease resistance protein
VIT_00s0420g00040 S-locus lectin protein kinase
VIT_04s0008g00300 CLAVATA1 receptor kinase (CLV1)
VIT_09s0096g01200 RPS5 (resistant to p. syringae 5)
VIT_06s0004g03920 Pto serine/threonine kinase
VIT_08s0007g08210 Zinc finger (CCCH-type) family protein
VIT_15s0045g01330 Ring-H2 finger protein ATL3A
VIT_07s0005g01240 Triacylglycerol lipase
VIT_14s0068g02330 Chloride channel protein B
VIT_09s0421g00010 Chloride channel protein B
VIT_06s0004g05500 CHLORORESPIRATORY REDUCTION 2 (CRR2)
VIT_16s0050g00230 Zinc knuckle
VIT_03s0091g00670 Lateral organ boundaries protein 38
VIT_03s0347g00030 Zinc finger (B-box type)
VIT_00s0203g00210 Zinc finger (B-box type)
VIT_14s0128g00480 Translation initiation factor eIF-3 subunit 1
VIT_14s0068g01800 putative MADS-box Flowering Locus C 2 (VviFLC2)
VIT_11s0052g01220 Xyloglucan endotransglycosylase 6
VIT_11s0052g01300 Xyloglucan endotransglycosylase 6
VIT_08s0032g01150 Syntaxin 1B/2/3/4
VIT_13s0019g01480 Sugar transport protein 8 (STP8)
VIT_14s0081g03940 ankyrin repeat
VIT_04s0023g01510 DUF620
VIT_01s0146g00220 Glutaredoxin-like
VIT_03s0063g01570 CyP82A3
VIT_10s0003g03580 ERF/AP2 Gene Family (VvERF075)
VIT_06s0004g08190 ERF/AP2 Gene Family (VvERF055)
VIT_16s0098g00200 Receptor serine/threonine kinase PR5K
VIT_16s0098g00190 Receptor kinase homolog LRK10
VIT_01s0011g03110 myb family
VIT_19s0014g03180 ERF/AP2 Gene Family (VvERF001)Dehydration Responsive Element-Binding Transcription Factor (
VIT_06s0004g07650 Taxadien-5-alpha-ol-O-acetyltransferase
VIT_16s0115g00340 UDP-glucose: anthocyanidin 53-O-glucosyltransferase
VIT_13s0067g03780 Leucine-rich repeat protein kinase
VIT_07s0129g00330 Lateral organ boundaries protein 39
VIT_07s0129g00970 Protein kinase family
VIT_07s0141g00020 Sensitive to proton rhizotoxicity 1
VIT_06s0080g00780 NAC domain-containing protein (VvNAC74)
VIT_09s0070g00560 EIX receptor
VIT_08s0105g00290 zinc finger (C3HC4-type RING finger)
VIT_06s0004g07770 peroxidase
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8
Cluster 9
Cluster 10

Figure 10: Hierarchical clustering analysis of *VviWRKY19* putative target genes in Corvina berry development. Pearson's correlation and complete linkage were respectively chosen as distance metric and clustering method to create the transcriptional profile dendrogram (by rows). Samples (columns) are ordered by the progression of berry flesh, skin and seed development: FS, Fruit set; PFS, Post Fruit Set; V, Veraison; MR, Mid Ripening; R, Ripening. The normalized expression values range from low (blue) to high (yellow).

3.2.3 *VvibHLH75*

Finally, concerning *VvibHLH75*, 395 up-regulated genes and 138 down-regulated genes were identified (Fig. 4); among the up-regulated genes, the over represented functional categories were secondary metabolic process, signal transduction, transcription factor activity and transport (Fig. 11).

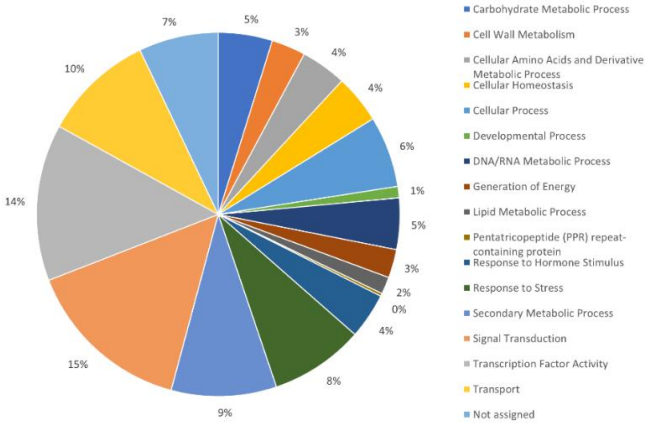

Figure 11: Functional categories representatives of the up-regulated genes (FC > 1.5) in transiently *VvibHLH75* overexpressing Thompson seedless plants.

The first 10 genes with highest FC value are shown in Table 10. Among them, there are one *CHAPERONE BCS1 MITOCHONDRIAL* (VIT_01s0010g02730), a mitochondrial protein involved in the biogenesis of the respiratory chain (Kolli et al., 2018), one *POLYGALACTURONASE GH28* (VIT_14s0066g01060), an enzyme involved int the pectin remodeling during plant development and the basic helix-loop-helix family (*VvibHLH75*, VIT_17s0000g00430), the gene in question in this study, whose overexpression is also confirmed by microarray analysis, following the introduction of 3'UTR region, containing the microarray probe, in the overexpression constructs. Furthermore, there are one *NAD+ ADP-RIBOSYLTRANSFERASE* (VIT_07S0005G00630), one *GERMIN-LIKE PROTEIN 3*, involved in plant development, osmotic

regulation, response to stress and programmed cell death (Dunwell et al., 2008), three gene coding enzymes of the cytochrome P450s (CYPs) family (VIT_18s0001g11540, VIT_18s0001g09660, VIT_18s0001g11450) and one *WAK1* (VIT_17s0000g04380), a Wall-Associated Kinase protein that functions as pectin receptors, performing role in both pathogen response and cell expansion during plant development (Kohorn and Kohorn., 2012). Other genes with high modulation (FC > 2) are reported in Supplemental Data Set 5; among them and others genes with FC > 1.5 (data not shown), there are many genes involved in secondary metabolic process, including four *CIS-ZEATIN O-BETA-D-GLUCOSYLTRANSFERASE* (VIT_08s0007g08910, VIT_08s0007g08890, VIT_03s0017g01040, VIT_13s0019g03100) and the *TRANS-RESVERATROL DI-O-METHYLTRANSFERASE* (VIT_10s0003g00470); furthermore, two *CINNAMYL ALCOHOL DEHYDROGENASE* (VIT_00s0615g00020 and VIT_00s0218g00010), one *SINAPYL ALCOHOL DEHYDROGENASE*, (VIT_00s0346g00080), one *CONIFERYL-ALCOHOL GLUCOSYLTRANSFERASE* (VIT_18s0001g12040), one *CAFFEIC ACID 3-O-METHYLTRANSFERASE* (VIT_02s0025g02920), one *CINNAMATE 4-HYDROXYLASE* (VIT_11s0078g00290), six enzymes involved in phenylpropanoid biosynthesis, and one *QUERCETIN 3-O-GLUCOSIDE-6''-O-MALONYLTRANSFERASE* (VIT_12s0134g00630), one *LEUCOANTHOCYANIDIN DIOXYGENASE* (VIT_13s0067g01020), one *ANTHOCYANIDIN 3-O-GLUCOSIDE-6''-O-MALONYLTRANSFERASE* (VIT_12s0134g00590) and one *ANTHOCYANIDIN 3-O-GLUCOSYLTRANSFERASE* (VIT_04s0023g01240), four genes involved in flavonoids biosynthesis, were identified. Finally, there are five *STILBENE SYNTHASEs* (VIT_10s0042g00840, VIT_16s0100g00960, VIT_16s0100g01060, VIT_10s0042g00860, VIT_10s0042g00930, VIT_16s0100g00920, VIT_16s0100g00840 and VIT_16s0100g00800), enzymes involved in the biosynthesis of resveratrol and stilbenes, molecules highly produced in response to abiotic and biotic stress (Vannozzi et al., 2012). Among the transcription factors, the most representative are WRKY transcription factors, including *VviWRKY07* (VIT_04s0008g05750), *VviWRKY43* (VIT_14s0068g01770), *VviWRKY03* (VIT_01s0010g03930), *VviWRKY20* (VIT_07s0005g02570), *VviWRKY30* (VIT_10s0003g01600), *VviWRKY02* (VIT_01s0026g01730), *VviWRKY19* (VIT_07s0005g01710), *VviWRKY14* (VIT_05s0077g00730), and NAC transcription factors, with 8 members, *VviNAC39* (VIT_07s0031g02610), *VviNAC26* (VIT_01s0026g02710), *VviNAC60bis* (VIT_08s0007g07660), *VviNAC17* (VIT_19s0014g03290), *VviNAC05* (VIT_17s0000g06400), *VviNAC50* (VIT_15s0048g02340), *VviNAC30* (VIT_19s0027g00870) and *VviNAC33*

(VIT_19s0027g00230). Furthermore, others upregulated transcriptions are *LATERAL ORGAN BOUNDARIES DOMAIN 15* (VIT_06s0004g07790), *LATERAL ORGAN BOUNDARIES PROTEIN 39* (VIT_07s0129g00330), *LATERAL ORGAN BOUNDARIES DOMAIN 16* (VIT_07s0005g03030), *LATERAL ORGAN BOUNDARIES PROTEIN 38* (VIT_03s0091g00670) and many zinc fingers transcription factors. Other upregulated genes involved in carbohydrate metabolic process and cell wall metabolism are two *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_17s0000g08010, VIT_14s0036g01210), one *POLYOL TRANSPORTER* (VIT_04s0023g01500) involved in sugar transport and other enzymes involved in the metabolism of many sugars, one *PECTINESTERASE* (VIT_07s0005g01930) and one *PECTATE LYASE* (VIT_17s0000g09810), enzymes involved int the pectin remodeling during plant development, two expansin, *VviEXPA17* (VIT_17s0000g06360) and *VviEXPB3* (VIT_15s0021g02670), proteins involved in the regulation of cell wall expansion and cell enlargement (Dal Santo et al., 2013) and two *β 1-3 GLUCANASE* (VIT_08s0007g06060, VIT_08s0007g06030), enzymes that catalyze the hydrolysis of β 1-3- glycosidic bond of glucans and they perform many roles from regulation of cell division to abiotic stresses resistance. Furthermore, there are many ERF/AP2 genes, involved in response to hormone stimulus, and many protein kinases, involved in signal transduction.

Table 10: the 10 most up-regulated genes in *VvibHLH75* overexpressing Thompson seedless leaves.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	FC
VIT_01S0010G02730	Chaperone BCS1 mitochondrial	Cellular Homeostasis	8.04
VIT_14S0066G01060	Polygalacturonase GH28	Cell Wall Metabolism	7.54
VIT_17S0000G00430	basic helix-loop-helix family (VviBHLH75))	Transcription Factor Activity	6.07
VIT_14S0128G00670	Germin-like protein 3 [Vitis vinifera]	Cellular Process	5.94
VIT_05S0077G00500	myb domain protein 108	Transcription Factor Activity	5.36
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	5.02
VIT_18S0001G11540	CYPLXXXII	Cellular Process	4.64
VIT_17S0000G04380	Wall-associated kinase 1 (WAK1)	Signal Transduction	4.39
VIT_18S0001G09660	CYP81D2	Cellular Process	4.18
VIT_18S0001G11450	CYP82C1p	Cellular Process	4.08

Regarding co-expression analysis, the correlated genes with a Pearson's coefficient > 0.9 are 114. The most representative functional category (Figure 12) are transcription factor activity

and transport, followed by response to stress, cellular homeostasis, cellular amino acids and derivative metabolic process and response to hormone stimulus.

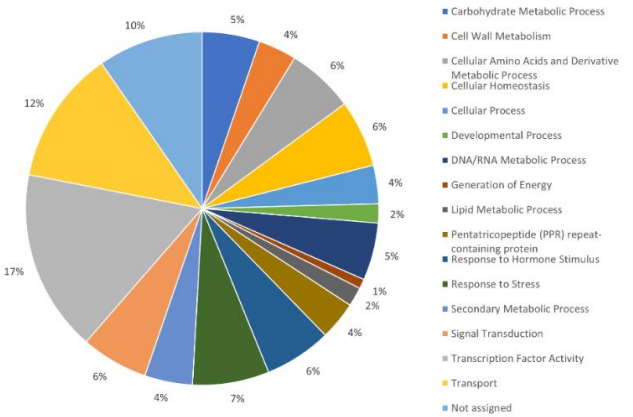

Figure 12: Functional categories representatives of genes (Pearson value > 0.9) correlated to *VvibHLH75* in transiently *VvibHLH75* overexpressing Thompson seedless plants.

The first 10 genes with highest Pearson's coefficient are shown in Table 11. Among them, there are one *URIDYLATE KINASE* (VIT_08S0007G04160), an enzyme involved in pyrimidine metabolism, one *S-ADENOSYL-L-METHIONINE: SALICYLIC ACID CARBOXYL METHYLTRANSFERASE* (VIT_04S0023G02200), an enzyme involved in the biosynthesis of Methylsalicylate, a molecule produced during plant defense responses mediated by salicylic acid (Ross et al., 1999), one ERF/AP2 Gene Family, *VviAP2-13* (VIT_07S0031G00220), and two transcription factors, one NAC gene, *VviNAC05* (VIT_17S0000G06400) and one zinc finger (VIT_11S0016G04980). Other genes with high Pearson's coefficient (> 0.92) are reported in Supplemental Data Set 6; among them, there are one *EXPANSIN*, *VviEXPB3* (VIT_15s0021g02670), one *NAD+ ADP-RIBOSYLTRANSFERASE* (VIT_07S0005G00630), one *CHAPERONE BCS1 MITOCHONDRIAL* (VIT_01s0010g02730), two WRKY transcription factors, *VviWRKY19* (VIT_07S0005G01710) and *VviWRKY17* (VIT_07S0141G00680) and one NAC transcription factor, *VviNAC08* (VIT_18S0001G02300).

Table 11: the 10 genes most correlated to *VvibHLH75* in overexpressing Thompson seedless leaves.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON
VIT_12S0057G00800	Receptor Like Protein 27	Signal Transduction	0.983
VIT_08S0007G04160	Uridylate kinase	DNA/RNA Metabolic Process	0.980
VIT_08S0007G06760	cation efflux family protein MTPc3	Transport	0.979
VIT_04S0023G02200	S-adenosyl-L-methionine: salicylic acid carboxyl methyltransferase	Cellular Amino Acids and Derivative Metabolic Process	0.977
VIT_17S0000G06400	NAC domain-containing protein (VvNAC05)	Transcription Factor Activity	0.977
VIT_17S0000G00400	phosphate carrier protein	Transport	0.976
VIT_04S0044G01300	DNA cross-link repair protein	DNA/RNA Metabolic Process	0.973
VIT_00S0984G00010	Phosphoglycerate mutase	Carbohydrate Metabolic Process	0.969
VIT_07S0031G00220	ERF/AP2 Gene Family (VvAP2-13)	Response to Hormone Stimulus	0.964
VIT_11S0016G04980	Zinc finger (CCCH-type) family protein	Transcription Factor Activity	0.964

The comparison between the upregulated genes (FC > 2) and the most correlated genes (Pearson's coefficient > 0.92) showed that 8 genes are common (Table 12); among them there are *CHAPERONE BCS1 MITOCHONDRIAL* (VIT_01s0010g02730), the *NAD+ ADP-RIBOSYLTRANSFERASE* (VIT_07S0005G00630) and one Expansin, *VviEXPB3* (VIT_15s0021g02670).

Table 12: shared genes between upregulated (FC > 2) and correlated genes (Pearson's coefficient > 0.92) of *VvibHLH75*.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY
VIT_01S0010G02730	Chaperone BCS1 mitochondrial	Cellular Homeostasis
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process
VIT_13S0067G02130	Dehydration-induced protein (ERD15)	Response to Hormone Stimulus
VIT_07S0104G00430	Endo-1,3;1,4-beta-D-glucanase precursor	#N/D
VIT_04S0044G01300	DNA cross-link repair protein	DNA/RNA Metabolic Process
VIT_14S0068G02330	Chloride channel protein B	Transport
VIT_15S0021G02670	Expansin (VvEXPB3)	Cell Wall Metabolism
VIT_00S0316G00020	Chloride channel protein CLC-A	Transport

The expression profiles of putative target genes of *VvibHLH75* with FC > 2 (Supplemental data Set 5) and with Pearson's coefficient > 0.92 (Supplemental data Set 6) were analyzed in Corvina

berry development (Fasoli et al, 2012); the hierarchical clustering analysis of *VvibHLH75* grouped genes in 8 clusters (Figure 13). Genes of clusters 1 and 2 are characterized by a high expression during ripening phase in both flesh and skin. Clusters 3 and 4 contain genes preferentially expressed during fruit set phase in skin and flesh, respectively. Genes of clusters 5 and 6 contain genes with preferential expression during fruit set and post fruit set phases in seed, while genes of cluster 7 show a high expression during *veraison* and mid-ripening phases in seed. Finally, cluster 8 contain genes preferentially expresses during *veraison*, mid-ripening and ripening phases in flesh, skin and seed.

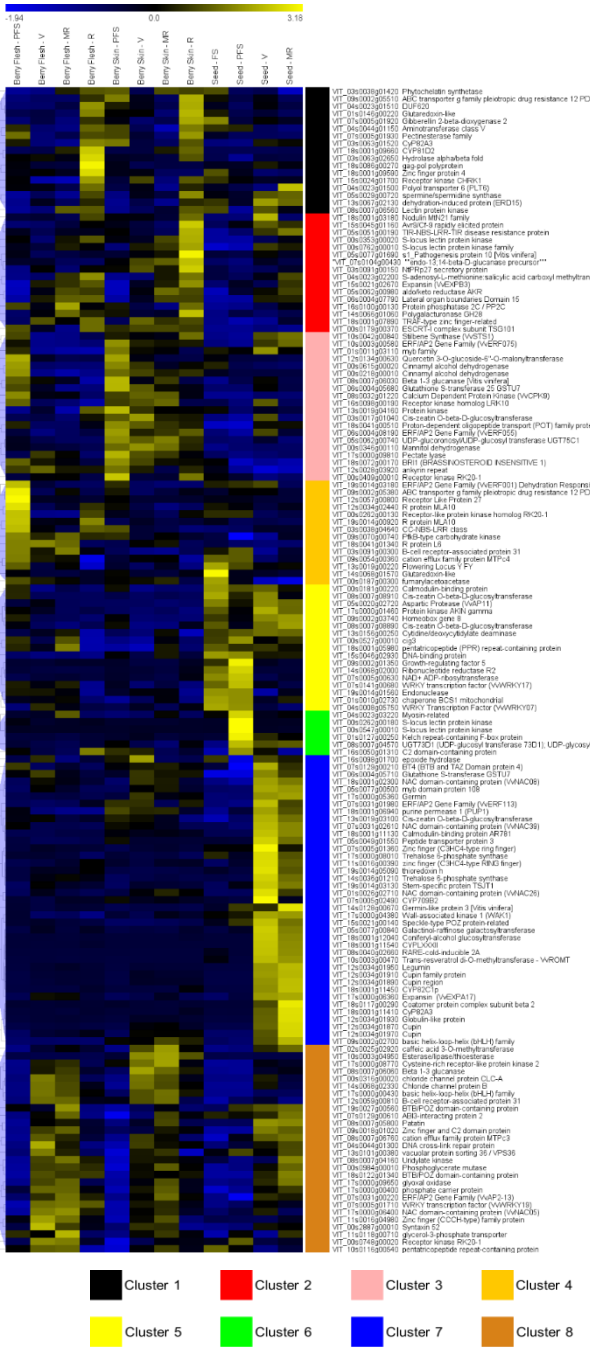
-1.94
0.0
3.18
Berry Flesh . PFS
Berry Flesh . V
Berry Flesh . MR
Berry Flesh . R
Berry Skin . PFS
Berry Skin . V
Berry Skin . MR
Berry Skin . R
Seed . FS
Seed . PFS
Seed . V
Seed . MR
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Cluster 7
Cluster 8

Figure 13: Hierarchical clustering analysis of *VvibHLH75* putative target genes in Corvina berry development. Pearson's correlation and complete linkage were respectively chosen as distance metric and clustering method to create the transcriptional profile dendrogram (by rows). Samples (columns) are ordered by the progression of berry flesh, skin and seed development: FS, Fruit set; PFS, Post Fruit Set; V, Veraison; MR, Mid Ripening; R, Ripening. The normalized expression values range from low (blue) to high (yellow).

3.2.4 Shared up-regulated genes among *VviAGL15*, *VviWRKY19* and *VvibHLH75* overexpressing Thompson seedless leaves

The comparison of up-regulated genes (FC > 2) of *VviAGL15a*, *VviWRKY19* and *VvibHLH75* showed that 12 genes (Table 13) are common among the three transcription factors. There are two *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_17s0000g08010, VIT_14s0036g01210), one ERF/AP2 Gene, *VviERF075* (VIT_10s0003g00580), one *TRANS-RESVERATROL DI-O-METHYLTRANSFERASE* (VIT_10s0003g00470) and one *CIS-ZEATIN O-BETA-D-GLUCOSYLTRANSFERASE* (VIT_08s0007g08910). Shared genes between *VviAGL15a* and *VviWRKY19* are 10, 5 genes are in common between *VviAGL15a* and *VvibHLH75* and 12 genes are shared by *VviWRKY19* and *VvibHLH75*. These results indicate that genes belonging to functional categories such as carbohydrate metabolic process, response to hormone stimulus, secondary metabolic process and transcription factor activity, are putative targets or belong to metabolisms controlled by the three transcription factors.

Table 13: up-regulated genes by overexpression of *VviAGL15a*, *VviWRKY19* and *VvibHLH75*.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	FC *VviAGL15a*	FC *VviWRKY19*	FC *VvibHLH75*
VIT_05S0020G02720	Aspartic Protease (VvAP11)	Cellular Process	3.93	3.21	2.39
VIT_05S0077G00510	Beta-fructofuranosidase	Carbohydrate Metabolic Process	3.84	2.94	-
VIT_07S0129G00210	BT4 (BTB and TAZ Domain protein 4)	Transcription Factor Activity	2.07	2.16	2.33
VIT_08S0032G01220	Calcium Dependent Protein Kinase (VvCPK9)	Carbohydrate Metabolic Process	2.58	-	2.56
VIT_14S0068G02330	Chloride channel protein B	Transport	3.07	2.29	2.20
VIT_00S0316G00020	Chloride channel protein CLC-A	Transport	2.87	-	2.09
VIT_08S0007G08890	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	2.78	-	2.06

VIT_08S0007G08910	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	3.06	2.29	2.41
VIT_07S0005G02490	CYP709B2	Cellular Process	2.79	-	2.29
VIT_03S0063G01520	CyP82A3	Cellular Process	-	2.58	2.53
VIT_13S0067G02130	Dehydration-induced protein (ERD15)	Response to Hormone Stimulus	2.48	-	2.62
VIT_04S0023G01510	DUF620	Cellular Process	-	2.97	2.66
VIT_19S0014G03180	ERF/AP2 Gene Family (VvERF001), Dehydration Responsive Element-Binding Transcription Factor (VvDREB33)	Response to Hormone Stimulus	-	7.09	3.91
VIT_10S0003G00580	ERF/AP2 Gene Family (VvERF075)	Response to Hormone Stimulus	2.95	2.99	2.40
VIT_12S0034G01930	Globulin-like protein	Cellular Process	-	3.37	2.84
VIT_10S0003G00390	Glutaredoxin	Cellular Homeostasis	2.80	3.80	-
VIT_14S0068G01570	Glutaredoxin-like	Cellular Homeostasis	-	3.51	2.14
VIT_01S0146G00220	Glutaredoxin-like	Cellular Homeostasis	-	3.90	2.63
VIT_05S0077G01280	Glycosyl hydrolase family 3 beta xylosidase BXL1	Carbohydrate Metabolic Process	2.94	2.64	-
VIT_13S0067G03780	Leucine-rich repeat protein kinase	Response to Stress	2.65	2.28	-
VIT_18S0001G09850	Myb domain protein R1	Transcription Factor Activity	2.61	2.06	-
VIT_01S0026G02710	NAC domain-containing protein (VvNAC26)	Transcription Factor Activity	-	4.81	3.51
VIT_07S0031G02610	NAC domain-containing protein (VvNAC39)	Transcription Factor Activity	-	2.81	3.68
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	-	4.35	5.02
VIT_13S0067G00260	Nematode-resistance protein	Response to Stress	3.33	2.65	-
VIT_08S0007G05800	Patatin	Cellular Process	2.01	2.03	2.11

VIT_04S0023G01500	Polyol transporter 6 (PLT6)	Carbohydrate Metabolic Process	-	3.12	3.75
VIT_17S0000G01460	Protein kinase AKIN gamma	Signal Transduction	2.70	2.07	2.48
VIT_16S0098G00190	Receptor kinase homolog LRK10	Signal Transduction	2.35	2.14	2.10
VIT_16S0098G00200	Receptor serine/threonine kinase PR5K	Signal Transduction	2.11	2.12	-
VIT_19S0014G03130	Stem-specific protein TSJT1	Developmental Process	3.25	3.11	2.36
VIT_10S0003G00470	Trans-resveratrol di-O-methyltransferase - VvROMT	Secondary Metabolic Process	3.48	4.29	2.72
VIT_14S0036G01210	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.34	3.26	2.93
VIT_17S0000G08010	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.53	3.17	3.24
VIT_07S0005G01240	Triacylglycerol lipase	Lipid Metabolic Process	2.68	2.54	-
VIT_17S0119G00230	Trypsin and protease inhibitor Kunitz family	Response to Stress	4.66	7.29	-
VIT_16S0115G00340	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase	Secondary Metabolic Process	2.22	2.08	-
VIT_17S0000G04380	Wall-associated kinase 1 (WAK1)	Signal Transduction	-	3.08	4.39
VIT_07S0005G01360	Zinc finger (C3HC4-type ring finger)	Transcription Factor Activity	-	3.58	2.97

3.3 Identification and validation of putative target genes of *VviAGL15*, *VviWRK19* and *VvibHLH75*

The up-regulated and the highly correlated genes obtained from microarray and co-expression analysis, respectively, could act downstream the selected transcription factors, representing their putative target genes. A selection for further validation of putative target genes of *VviAGL15a, VviWRK19 and VvibHLH75* was performed on the basis on their FC and Pearson's coefficient values and considering their biological role.

Regarding *VviAGL15a*, the selected genes are *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23* (VIT_11S0052G01330), with a high FC value, and

VviERF045 (VIT_04S0008G06000), with a high Pearson's coefficient value; furthermore, the first gene plays an important in the structure modification of cell wall while the function of *VviERF045* during the berry ripening has been already described (Leida et al, 2016). Regarding *VviWRK19*, two NAC transcription factors, *VviNAC33* (VIT_19s0027g00230) and *VviNAC26* (VIT_01s0026g02710), were selected specifically for their biological role: the first is a switch gene emerged from the network analysis of the grapevine expression atlas and berry specific transcriptomes (Palumbo et al, 2014; Massonnet et al, 2017) while the function of the second in the determination of the grape berry final size has been recently proposed (Tello et al, 2015). Finally, the genes selected for *VvibHLH75* are *VviWRK19* (VIT_07S0005G01710), for its high correlation with *VvibHLH75* and because it was included in the list of switch genes (Palumbo et al, 2014; Massonnet et al, 2017), and *VviEXPA17*, for its high FC value and for the role of this class of protein in the remodeling of cell wall (Dal Santo et al., 2013).

The up-regulation of the selected putative targets was confirmed by Real-Time qPCR analysis. The results show that the expression level of each target genes of *VviAGL15a* (Figure 14A), *VviWRK19* (Figure 14B) and *VvibHLH75* (Figure 14C) is higher in the overexpressing lines than the control lines.

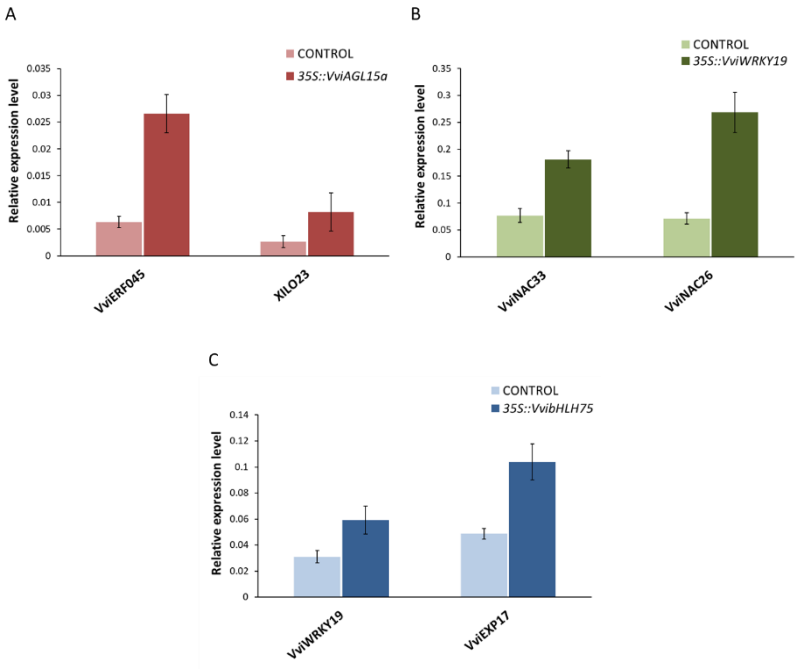

Figure 14: Real time qPCR analysis of target genes of *VviAGL15a* (A), *VviWRK19* (B) and *VvibHLH75* (C) in the control and overexpressing lines of Thompson seedless plants. The expression level corresponds to the mean ± SE of three biological replicates relative to the *VviUBIQUITIN* (VIT_16s0098g01190). Abbreviations correspond to: XILO23, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23*.

Moreover, regarding *VviAGL15a*, a dual luciferase reporter assay was performed to verify the expression of luciferase reporter gene following the activation by the transcription factor of the regulative regions of the putative target genes above mentioned. The genomic sequence (1500 base pairs up-stream ATG codon) of *VviERF045* and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23*, virtually including their respective promoters (P) was isolated from genomic DNA of *Vitis Vinifera* (cultivar Corvina) using adapted GB primers (Table 3) and cloned up-stream the *LUCIFERASE FIREFLY* (*LUC*) gene in the pDGB3α1 vector; subsequently, the transcriptional unit (TU) α1-PROM::*LUC*::TNos of each promoter was binarily assembled with the TU Ω2-35S::*RENILLA LUCIFERASE* (*REN*):TNos-35S::*p19*::TNos and the TU α2-35S::*VviAGL15a*::TNOS in the pDGB3Ω1 vector. Finally, the resulting vectors pEGB3α1-P::*LUC*::TNos-35S::*REN*:TNOS-35S::*p19*::TNOS (used as negative control in the dual luciferase reporter assay, Figure 11A) and the vectors pEGB3Ω1-P::*LUC*::TNOS-35S::*REN*:TNOS-35S::*p19*::TNOS-35S::*VviAGL15a*::TNOS (used to study the putative activation in the dual luciferase reporter assay, Figure 15B) of each promoters were introduced into *Agrobacterium* strain EHA105 by electroporation.

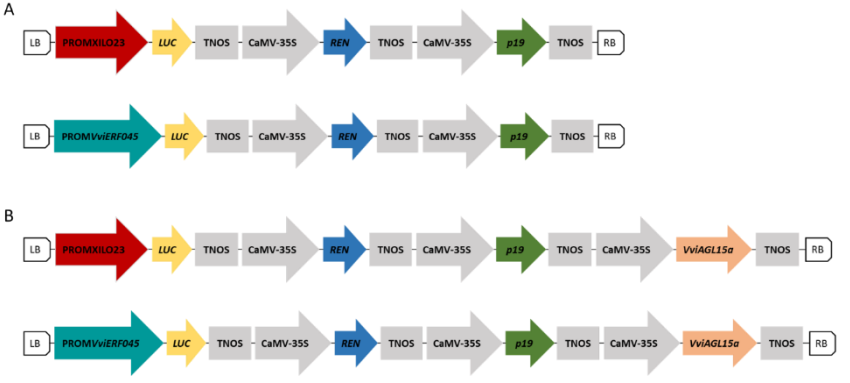

Figure 15: transcriptional units used in Dual Luciferase Reporter Assay. A: transcriptional unit without *VviAGL15a* used as negative control. B: transcriptional unit with *VviAGL15a* to test the putative regulative regions activation.

The putative activation was performed using *Nicotiana benthamiana* as heterologous expression system. Leaves were agroinfiltrated with *Agrobacterium* harboring each of four constructs (Figure 16). The dual luciferase reporter assay was performed 3 d.p.i. using leaf discs from agroinfiltrated leaves with each construct. The results of the trans-activation (Figure 12) showed a direct activation of *VviERF045* ($p < 0.05$) and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23* ($p < 0.01$) regulative regions by *VviAGL15a*, indicating that both target genes are under the transcriptional control of *VviAGL15a*.

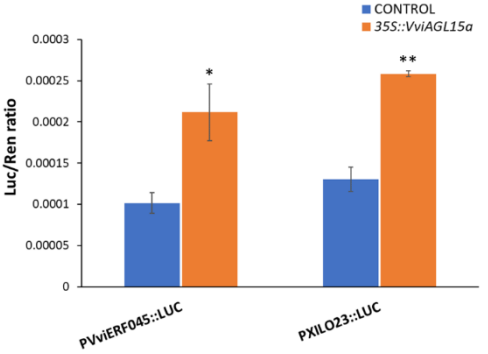

Figure 16: Trans-activation analysis of regulative regions of putative target genes of *VviAGL15a* by Dual Luciferase Reporter Assay. Each value of Luc/Ren ratio represents the mean ± S.E. of three biological replicates. * and ** indicate significant differences ($p < 0.05$ and $p < 0.01$, respectively) in promoter activation compared with the negative control. Abbreviations correspond to: XILO23, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23*.

4. DISCUSSION

VviAGL15a, *VviWRKY19* and *VvibHLH75* are the transcription factors selected in this study to be functionally characterized. They all emerged as switch genes from the network analysis performed on the berry transcriptomes (Palumbo et al., 2014; Massonnet et al., 2017) whereas only *VviWRK19* and *VvibHLH75* were included in the list of markers of the first transition of berry development (Fasoli et al., 2018). For these properties their putative role of master regulators of vegetative-to-mature transition during berry development deserve further investigation.

The functional characterization of these three transcription factors was performed by using a transient gene expression approach. This technology allows the temporary high expression of gene(s) of interest in relatively low time-consuming experiments. Transient overexpression of *VviAGL15a*, *VviWRK19* and *VvibHLH75* was performed by vacuum grapevine leaf agroinfiltration

of whole plant grown *in-vitro* of Thompson seedless cultivar. The transcriptional units used for the transient overexpression were obtained using the GoldenBraid 2.0 system (Sarrion-Perdigones et al., 2013); each transcriptional unit was formed by two parts (Figure 1B), one for the overexpression of gene on interest and one for the overexpression of YFP. The use of *YFP* as reporter genes allowed to monitor over time the expression of transcriptional unit, to verify the successful of the agroinfiltration and to select and sample only the agroinfiltrated YFP-expressing leaves (Figure 2).

The overexpression of each transcription factor has been confirmed by Real time qPCR analysis; furthermore, this analysis allowed to select the best overexpressing lines (Figure 3), three for *VviAGL15a* and four for *VviWRKY19* and *VvibHLH75*, and the respective control lines (agroinfiltrated only with a transcriptional unit for YFP overexpression, Figure 1C). To obtain a global view of grapevine transcriptome post overexpression and to identify putative target genes of each transcription factors, the overexpressing and the control lines were then used to perform a microarray analysis. Data obtained were filtered, normalized and used to identify DEGs and to perform a co-expression analysis. DEGs were obtained using a p-value cut-off of 0.05: this broad range value could have generated some false positives. Further analysis will be necessary to confirm the up- or down-regulation of specific target genes. Regarding the analysis of differential genes ($|FC\ value| > 1.5$), for each transcription factors the number of upregulated genes was higher than the downregulated genes; these results seem to indicate a direct activation of putative target genes or an activation mediated by other genes in turn regulated by the transcription factors selected. However, the up-regulation of many genes may be caused by the down-regulation of some specific genes, but, considering the putative role of transcriptional activators of *VviAGL15a*, *VviWRKY19* and *VvibHLH75*, a detailed analysis has been performed only using the up-regulated genes.

Overall, among the upregulated genes (FC > 1.5) of each transcription factors, the functional categories overrepresented are carbohydrate metabolic process, cellular homeostasis, response to hormone stimulus, secondary metabolic process, signal transduction and transcription factor activity. Regarding the genes highly correlated with *VviAGL15a*, *VviWRK19* and *VvibHLH75*, the most represented functional categories are the same reported for the genes resulted upregulated by t-test analysis. However, for each transcription factors, the number of genes shared between the two different analysis was very low (Tables 6, 9 and 12).

Therefore, both analyses have been used in a complementary way to identify putative target genes of each transcription factors. To obtain more information about the putative target genes emerged from the transcriptomic analysis of infiltrated leaves, the analysis of their expression profile in different tissues (flesh, skin and seed) during berry development retrieved from the expression atlas (Fasoli et al., 2012) has been performed. The expression of these genes during *veraison* or post-*veraison* phases would be a necessary condition to be included among the putative targets induced by the selected transcription factors during berry development.

Regarding *VviAGL15*, the most up-regulated genes (Table 4 and Supplemental Data Set 1) include many *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASEs*, enzymes involved in the modification of cell wall structure; the same genes and many others *XYLOGLUCAN ENDOTRANSGLUCOSYLASEs* have been identified using the co-expression analysis (Tables 5-6 and Supplemental Data set 2). Furthermore, the up-regulation of one specific *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE* 23 (VIT_11s0052g01330) has been confirmed by Real Time qPCR analysis (Figure 14A) and the activation of its regulative region by *VviAGL15a* has been demonstrated by Dual Luciferase Reporter Assay (Figure 16). This result indicates that this gene is a specific target of *VviAGL15a*. As described for rice (Hara et al., 2014), also in grapevine, the xyloglucan endotransglucosylase/hydrolases belong to a gene family and they are characterized by very high gene similarity/sequence homology. The up-regulation of a high number of these genes suggest that they are a specific target of *VviAGL15a*, suggesting a role of this transcription in the cell wall modification, events associated with berry ripening. Another putative target of *VviAGL15a* is represented by *VviERF045*, belonging to the ERF/AP2 gene family, a family of transcription factors involved in many processes (Licausi et al., 2010); this gene resulted highly co-expressed with *VviAGL15a* in agroinfiltrated leaves (Supplemental Data Set 2), and the activation of its regulative region have been confirmed (Figure 14A). These results suggest that *VviERF045* is a target of *VviAGL15a,* which, therefore, may play a role in ethylene-mediated responses. Other genes upregulated upon the ectopic expression of *VviAGL15a* (Tables 4, 5 and 6 and Supplemental Data Sets 1 and 2) are some *TREHALOSE 6-PHOSPHATE SYNTHASEs* and one *BETA-FRUCTOFURANOSIDASE*, involved in the biosynthesis of the signaling metabolite threalose and in sucrose hydrolysis, respectively. Sugars-mediated signaling and sugars metabolism are two important events during ripening: the high modulation and correlation of these genes could indicate an involvement of *VviAGL15a* in the regulation of

these processes. Finally, other putative targets are *FERONIA RECEPTOR-LIKE KINASE*, the second most up-regulated gene, and *BTB/POZ DOMAIN-CONTAINING PROTEIN*, indicating that *VviAGL15a* could be involved in signal transduction and in the regulation of other transcription factors. The analysis of the expression of these putative targets during berry development revealed that they are preferentially expressed during *veraison* or post-*veraison* phases (Figure 7). The clustering hierarchical analysis showed that most of the *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASEs*, *VviERF045* and the *FERONIA RECEPTOR-LIKE KINASE* grouped with genes with high expression at *veraison* and ripening phases in both flesh and skin (clusters 3, 4 and 5; Figure 7), while *TREHALOSE 6-PHOSPHATE SYNTHASE*, *BETA-FRUCTOFURANOSIDASE* and *BTB/POZ DOMAIN-CONTAINING PROTEIN* are preferentially expressed is seed during *veraison* and mid-ripening (clusters 1 and 2; Figure 7), indicating that these genes may play a role during ripening process, in different parts of the berry. These results indicated that these genes are putative targets of *VviAGL15a* and that their regulation may be controlled by this transcription factor during berry ripening.

The most up-regulated genes of *VviWRKY19* include one *ALPHA-AMYLASE/SUBTILISIN INHIBITOR* and some *TRYPSIN AND PROTEASE INHIBITOR KUNITZ FAMILY*, genes involved in plant response to microorganism attacks, consistently with the role of many members of the WRKY transcription factors family in the biotic stress responses (Amato et al., 2017; Wang et al., 2014). However, the function of many others up-regulated and highly correlated genes (Tables 7, 8 and Supplemental Data Set 3) such as two *XYLOGLUCAN ENDOTRANSGLUCOSYLASEs* (VIT_11s0052g01220, VIT_11s0052g01300), one *BETA-FRUCTOFURANOSIDASE* and two *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_17s0000g08010 and VIT_14s0036g01210), suggests a role of *VviWRKY19* in the regulation of cell wall structure, sugars signaling and metabolism, important events occurring during berry ripening. Interestingly, many others up-regulated genes (see Results, section 3.2.2 and Supplemental Data Set 3) are involved in flavonoids and terpenoids biosynthesis. Flavonoids play important functions in disease resistance, protection from UV radiation, and coloration of flowers and fruits (Bogs et al., 2006), while terpenoids are involved in plant defense against biotic and abiotic stresses or they play a role as signal molecules to attract the pollinating insects (Singh and Sharma, 2015). These secondary metabolites are produced during many phases of grapevine development, including flowering and berry ripening, indicating a role of *VviWRKY19*

in the regulation of these important processes. Others up-regulated genes are represented by many transcription factors; among them, there are many NAC transcription factors (see Results, section 3.2.2) and three *LATERAL ORGAN BOUNDARIES* proteins. The up-regulation of *VviNAC33* and *VviNAC26* has been confirmed by Real-Time qPCR analysis (Figure 14B), indicating a possible regulation of their transcription by *VviWRKY19*. Finally, others up-regulated and highly correlated genes (Tables 7, 8 and Supplemental Data Sets 3, 4) are some *GLUTAREDOXIN-LIKE* and some *KELCH REPEAT-CONTAINING F-BOX FAMILY PROTEINS*, involved in cellular homeostasis, the SUCROSE TRANSPORTER, *SUT4*, and one *NAD+ ADP-RIBOSYLTRANSFERASE*, involved in sugar transport and plant immunity, respectively (see Results, section 3.2.2), suggesting a putative role of *VviWRKY19* in the regulation of these processes. The hierarchical clustering analysis of *VviWRKY19* targets grouped most of the above cited genes in clusters preferentially expressed during *veraison* and post-*veraison* phases (Figure 10). In particular, *ALPHA-AMYLASE/SUBTILISIN INHIBITOR*, members of *TRYPSIN AND PROTEASE INHIBITOR KUNITZ FAMILY* and *KELCH REPEAT-CONTAINING F-BOX FAMILY PROTEINS*, *SUT4* and *VviNAC61* are expressed during *veraison*, mid-ripening and ripening in flesh, skin and seed (cluster 4, Figure 10); many NAC transcription factors (*VviNAC26*, *VviNAC17*, *VviNAC39* and *VviNAC08*), *BETA-FRUCTOFURANOSIDASE* and two *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_17s0000g08010 and VIT_14s0036g01210) have an high expression during *veraison* and mid-ripening in seed (cluster 5, Figure 10); finally, *XYLOGLUCAN ENDOTRANSGLUCOSYLASE* (VIT_11s0052g01220, VIT_11s0052g01300), *VviLOB38* and one *GLUTAREDOXIN-LIKE* (VIT_01s0146g00220) are preferentially expressed during *veraison*, mid-ripening and ripening phases (clusters 7 and 8, Figure 10). Instead, *NAD+ ADP-RIBOSYLTRANSFERASE*, one *GLUTAREDOXIN-LIKE* (VIT_14s0068g01570), *VviNAC74* and *VviLOB39*, are mainly expressed during fruit set phase in seed (cluster 1, Figure 10), flesh and skin (cluster 10, Figure 10), thus representing unlikely players in the vegetative-to-mature transition of grape berry.

Finally, regarding *VvibHLH75*, by the analysis of the most up-regulated genes (Table 10), there are one *CHAPERONE BCS1 MITOCHONDRIAL*, three enzymes of the cytochrome P450s (CYPs) family (VIT_18s0001g09660, VIT_18s0001g11540, VIT_18s0001g11450), one Wall-associated kinase 1 (*WAK1*) and one *GERMIN-LIKE PROTEIN 3*, suggesting a specific role of *VvibHLH75* in the regulation of cellular homeostasis, cellular process and signal transduction. Another up-regulated gene is *NAD+ ADP-RIBOSYLTRANSFERASE*, involved in plant immunity. Furthermore,

many other up-regulated genes (Table 10 and Supplemental Data Set 5) encode enzymes involved in the modification, structure regulation and remodeling of components of cell wall such as the *POLYGALACTURONASE GH28*, one *PECTINESTERASE* (VIT_07s0005g01930), one *PECTATE LYASE* (VIT_17s0000g09810), the expansins *VviEXPA17* and *VviEXPB3* and two *β 1-3 GLUCANASEs* (VIT_08s0007g06060 and VIT_08s0007g06030), whereas two *TREHALOSE 6-PHOSPHATE SYNTHASE* (VIT_17s0000g08010, VIT_14s0036g01210) and one *POLYOL TRANSPORTER* (VIT_04s0023g01500) are involved in sugar signaling and transport. These results indicate that *VvibHLH75* may regulate these specific processes, playing roles partially overlapping with *VviAGL15a* and *VviWRKY19*. Among the up-regulated genes, there are many genes involved in phenylpropanoid and flavonoids biosynthesis and many *STILBENE SYNTHASEs* (see Results, section 3.2.3); moreover, the ectopic expression of *VvibHLH75* affects the expression of numerous transcription factors (see Results, section 3.2.3); among which there are many members of WRKY and NAC transcription factors families, including *VviWRKY19* and *VviNAC33*, and some *LATERAL ORGAN BOUNDARIES DOMAIN* proteins, including *VviLOB15* and *VviLOB38*. The up-regulation of *VviWRKY19* has been confirmed by Real-Time qPCR (Figure 14C), suggesting that this transcription factor play a role downstream *VvibHLH75*. The hierarchical clustering analysis (Figure 13) of *VvibHLH75* targets allowed to group genes by their expression profile during berry development. Most of them are expressed during *veraison* or post-*veraison* phases, suggesting a regulation by *VvibHLH75*. However, two of the most up-regulated genes, *CHAPERONE BCS1 MITOCHONDRIAL* and *NAD+ ADP-RIBOSYLTRANSFERASE*, many genes involved in phenylpropanoid biosynthesis, one *β 1-3 GLUCANASE* (VIT_08s0007g06030), the *PECTATE LYASE* and one *VviWRKY17* are expressed during fruit set phase in seed (cluster 5, Figure 13), flesh and skin (cluster 3, Figure 13) suggesting that they don't play a specific role during ripening process. Other genes above cited, including the *POLYGALACTURONASE GH28*, the PECTINESTERASE, one *CYP* (VIT_18s0001g09660) *VviEXPB3*, the *POLYOL TRANSPORTER* and *VviLOB15* are preferentially expressed during ripening phase in both flesh and skin (clusters 1 and 2, Figure 13); interestingly, one *S-ADENOSYL-L-METHIONINE: SALICYLIC ACID CARBOXYL METHYLTRANSFERASE* (VIT_04S0023G02200), a high correlated gene (Table 11), has a high expression at ripening in skin (cluster 2, Figure). Then, the two *TREHALOSE 6-PHOSPHATE SYNTHASE*, *VviEXPA17*, many NAC transcription factors, including *VviNAC26*, *VviNAC39* and *VviNAC08*, two CYPs (VIT_18s0001g11540, VIT_18s0001g11450), *WAK1* and the

GERMIN-LIKE PROTEIN 3 show a high expression during *veraison* and mid-ripening phases in seed (cluster 7, Figure 13). Finally, *VviWRKY19*, *VviNAC05*, one *β 1-3 GLUCANASE* (VIT_08s0007g06060) show a preferential expression during *veraison* and mid-ripening phases in flesh, skin and seed (cluster 8, Figure 13). These results indicate that genes play a specific role during berry ripening and their regulation could be mediated by *VvibHLH75*.

Altogether, these results allowed to select those genes, up-regulated upon the transient ectopic expression of each transcription factors in leaves, that are characterized by a preferential expression during *veraison*, mid-ripening and ripening phases in flesh, skin and seed. This indicates that they play a specific role during the ripening phase in these tissues and that their expression may be regulated by *VviAGL15a*, *VviWRKY19* and *VvibHLH75*. Moreover, as showed in table 13, many upregulated genes are common among the three transcription factors such as the *TRANS-RESVERATROL DI-O-METHYLTRANSFERASE*, two *TREHALOSE 6-PHOSPHATE SYNTHASE*, one *PATATIN*, expressed preferentially during *veraison* and mid-ripening in seed, and one *CHLORIDE CHANNEL PROTEIN B*, with high expression during *veraison* and mid-ripening in flesh and skin. Moreover, *VviNAC26* and *VviNAC39*, two commo targets between *VviWRKY19* and *VvibHLH75*, show a high expression during *veraison* and post-*veraison* phases in seed, while one *TRYPSIN AND PROTEASE INHIBITOR KUNITZ FAMILY*, common between *VviAGL15a* and *VviWRKY19*, show a preferential expression during *veraison*, mid-ripening and ripening in flesh, skin and seed. Overall these results indicate that *VviAGL15a*, *VviWRKY19* and *VvibHLH75* may take part to the same processes. Some of the above described putative hierarchical transcriptional relationships deserve further attention and need to be confirmed, using, for example, the Dual Luciferase Reporter Assay as described for the two genes that were confirmed as targets of *VviAGL15a* (see Results, section 3.3). Overall, these preliminary results have highlighted important information about the complex transcriptional regulatory network controlling the processes associated with berry ripening.

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SUPPLEMENTAL DATA

Supplemental figure 1: Sequencing of Corvina *VviAGL15*, *VviWRKY19* and *VvibHLH75* cloned regions. Stop codons are indicated in yellow, while 3'UTRs are indicated in grey.

VviAGL15

ATGGGACGTGGTAAGATTGAGATAAAGAAGATCGAGAATGCTAATAGCAGGCAAGTGACATTCTCCAAACGCCGTGTTGGGCTGCTC
AAGAAGGCTTCCGAACTGGCGATTTTGTGCGATGCTCAAGTTGGTGTTATTATTTTCTCAAATACCGGCAAACTTTTTGAGTTTTCCAGT
ACTAGCATGAAGCGAATAATTTCAAGATACAACAAGTTAGATTCATCAGAGGGTGCTCTAGTAGAATACAAGGCAGAGGAGCCTAAA
GAGGTGGACATTCTGAAAGATGAAATTCGAAAGCTACAAACGAGACAGTTACAGCTGTTGGGTAAGGACCTATCCGGCTTGAGCTTA
AAAGAGTTACAAAATCTAGAACAGCAATTAAATGAAAGTTTATTATCAGTCAAAGAGAGGAAGGAGCAAGTACTGATGGAGCAGCTT
GAGCAATCGAGAGTACAGGAACAGCGAGCTGTACTGGAGAATGAGACTTTGCGAAGACAGGTTGAGGAGCTTCGAGGTTTGGTTCC
ATCATCTGACTGCTTGGTGCCACCTTTTCTTGAGTACCATCCCTTAGAAAGGAAAGATTCCATCACAAAATCTGTTGTAATAAGTCCAG
ACGTCTGTGATTTTGCAGTTGAGAGGGAAGAATCAGATACTACCTTGCAGTTAGGGCTTCCCACTGAGATTAGTCGCAAGAGGAAGG
CACCTGCCAAGATGGAAACCCGCTCCAACAACTCTGGCAGTTAAATACATCTACTGTAATGCTCCTCTTCATGGTTTCTACTTAGAAACT
GAAACAACTTAAAAAAATTGTAACTAATGGTCAAGGTTTAGCAGTTTTTTGTAAATGGGAATCATTTTCACCCAACAAGTGTCTGATCT
GCGGGTTAGACTCTTGAGAAATCATTATAATGAAGAATGTAGATGTTGCATTTTTAA

VviWRKY19

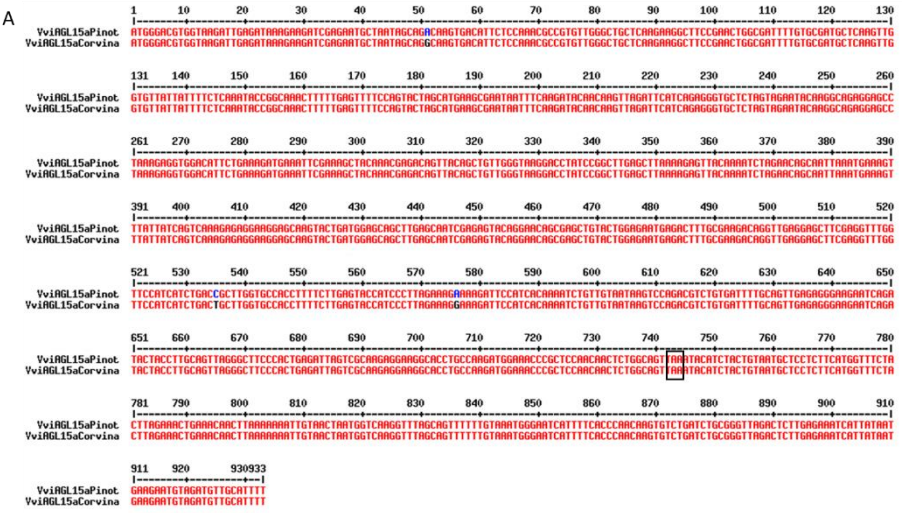
ATGGAGAGGAGCGGGGTGATGAAAATGGAGGATCCAGTTGGTTCGTGGTCATTTTCAGATCACGCTCCGGAGATTCCGGCCGTGTTT
GACTTCTCCGACGAAGGCGAGAAGGGAACGCTAGGGTTCATGGAGTTACTGAGTATTCAAAATTATGGGCCGCCGTTGTTTGATTTG
GTGCAGCTGCCGTCTTTGGAGAAACCGACGGGTGCAGCTCCGGCAGTGCCGGCGTCTTCTGACGTGGTGAATCCACCGGCCACGCCG
AACTCGTCGTCGGTGTCGTCGGCGTCCAGTCATGAGCAAGGCAGCAAAGCAGTTGAAGAAGAGGAGGAGGAGGATGAGGAAGAGAAGA
AGACTAAGAAAGAGTTGAAGCCCAAGAAGACAACAAGTCAAAAGAGGCAGAGAGAACCGAGATTCGCCTTCATGACAAAGAGCGA
GGTTGATCATCTGGAAGATGGGTACAGATGGAGAAAGTACGGCCAAAAAGCTGTGAAAAACAGCCCCTTTCCTAGGAGTTACTATCG
TTGCACCAGTGCATCATGTAATGTGAAGAAACGAGTGGAGAGGTGTTTCAAGGATCCGGCCATTGTCGTAACCACCTACGAAGGTCA
ACACACCCACCCAAGCCCAATCATGCCTCGAGCAAACCCATCCTCCATTGCCACCACCTTTGCTGGCCCGAGGCTTCAAACGACGCCGT
TTCAACACCTACACCTCCACCACCACCAGCAACAGCAACATCCCTCTTTCAACGATGTTCCACTCTTGAATTATGGCCATGGCAGCAGCT
TTGGGAGCGCCGCAAGGTCTGTTCAAGAGAGGCGTTCTTGCGCACCAACTTCTGCTTTGCATAGAGATCATGGCCTTCTTCAAGATATT
GTGCCCTCCCATATGAGAAAGGAAGAGGAGATATGACGAAACGCGACTCCATTACTGTTTTGAGAGAGGAGGTGCGGACTGCGGTG
TAGACGGAAAAACCCAGTTCTGGCATGTAATTCGCTTGCTGACTAGTACTGATAGAGAGCCTTCTTTCTCTCACGGATAAACCCCCCTC
CACCTTTGTACACAGACCCTCATGAATATCACTGTAACAATATGGGGTTTTTGATCCAATGGAGGGACTAGTTTCTTTGTAAGTTGGGC
GCCTTGGTATTTTTCCTTTTTTGATGTGTTTCTTTCTTTCTGGGTATGTAGAAGTTATACAACATACAGAATAGGTAATTATGTTTTTTTTT
TGGCT

VvibHLH75

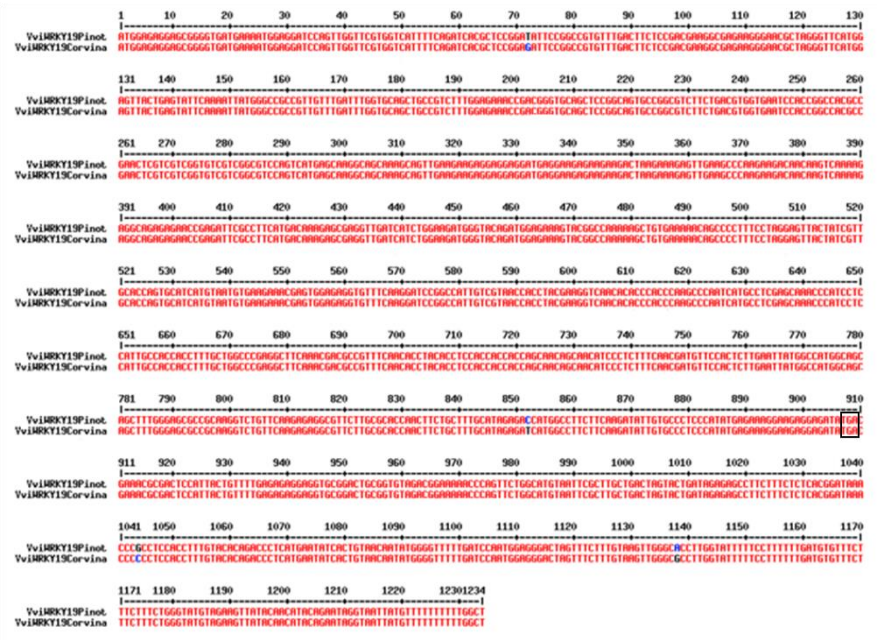
ATGGCAGCCTTTTCGTATCAACACCCACCTTTTCTTCTTGACTCAGTTTTCTTGCCGAGTACTCCCATTAAGATGTCTGGTTTTATGGAGG
AAGGGAACATCACCACTTGTTTCTCTCAGTTTTTCCCTTCTGAATCTCTTCATGAGGTTCCTGCTGATGCTAGGGTTCATGAAAGTACGT
CGCTTCAACACAGCTCAAAGGTCACTCTCAGTGACAATGAGCCTTGTGTGACCCAGAAACTGAGCACAGACTCTTCGTCAGTGGTGGA
TAGGCTTGAACTTGGTGAACAGGTCACCCAGAAGGTGGCTCCCATAGAGAGGGAGAGGAAGAGGAAGAGCAGAGATGGGTCTTCCT
TGACTTCTGCTCAATCGAAGGATGCAAGAGAAGGGAAAGGAAAGAAGGCAAAGAAAGGCAGTGGTCTGGTGAAGGATGGAGAAGA
GGAGCAGCTCAAAGCAGACAAGAAGGATCAGAAGAAAGCCTCTGAAGAGCCTCCAACCGGCTACATTCATGTAAGAGCAAGGAGGG
GCCAAGCAACAGACAGCCACAGCCTTGCAGAGAGGGTAAGAAGAGAGAAAATCAGTGAGAGGATGAAGCTCTTGCAAGCACTTGTT
CCTGGTTGTGACAAGGTTACTGGAAAGGCCCTTATGTTGGATGAAATAATCAACTATGTCCAGTCCCTACAGAATCAAGTAGAGTTCC
TCTCTATGAAGCTTGCTTCTGTGAATCCTATGTTCTATGACTTTGGCATGGACCTAGATGCACTCATGGTGAGGCCAGAGAGATTGAGT
GCCTTGACATCACCACTGCCATCTCTGCAACAATGCAGTCCTTCCCAGCCCACAGCTTATGCTGATACAACCACCACCTTCACTGCAACA
AATAACTATCCTGTTATGGACACTTCAGCTTCAATTTTATTTCACCAGGGGCAAAGGCTAAATGTCTTCTCACAGGATAATGGTAGTCT
ATTGTGGGATGTGGATGATCAAAGACAGAAGTTCATTAATCCATCTGGACTCATCAGCAACAACTTGTGTTCTTTCAATTAAAATAATA
AACTGAGCTGCCCTACCAACATCTGTGTGTGTGTGAGGAGGATTTGGAAGAGAACTTGGGAGAAAAGAAACCTTAGAAGACATGGA
ATTGTTTTGTTCTTAGGATGAGTTCCAATGGAGGGGGCTTCTTCTTCTCAAAAGCTATGATTCCATCATTTCCAAGTCCAGTTGTTTAAA
GGAGAAGGGTATCTGAACAATGAATAAAGGGGCAGCAAAATCAATGGAGGAAGAACAGACACAAAAGGACAGTTATAACCGACCA
AATAAGACCAGATCTTTTATAGATCCCTTGTTCCTCTCCAATTTGACTCCCAAAGTCTCATGCACAAATTCCAAGAGCCTGTGGTGGATC
AACCCTAGTCCTACAAACATTACCATTCTGAACAAATATTTATATATATGTAATGTATGTAAAAAAAATAATCCCATGTATGTCCTTCCA
TGCATTATAAATTTCATTCATTATGGTTCTGTTATTACAGTTATCTTTACAGTTACATTCCCTCCT

Supplemental Figure 2: Alignment between predicted Pinot Noir nucleotide sequences of *VviAGL15* (A), *VviWRKY19* (B) and *VvibHLH75* (C) and the Corvina cloned regions. SNIPs are indicated in blue/black, while stop codons are indicated with empty boxes.

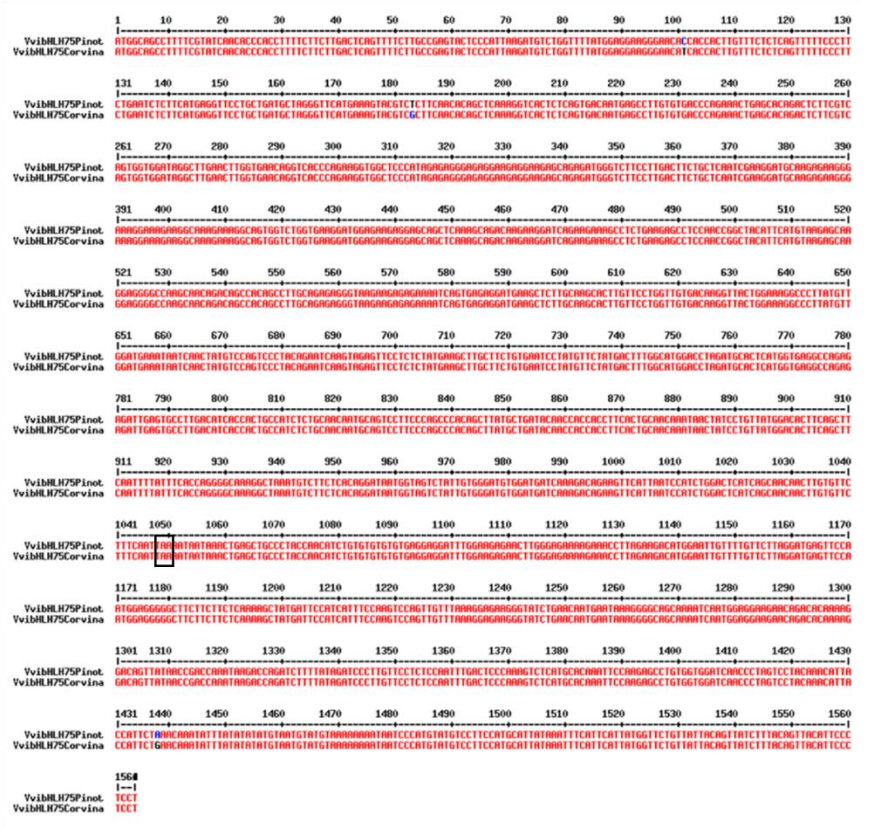
A



B



C



Supplemental Figure 3: Alignment of *VviAGL15* (A), *VviWRKY19* (B) and *VvibHLH75* (C) amino acidic sequences from Pinot Noir and 'Corvina cultivars. Different amino acids are indicated with a dot.

A

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Pinot          1 MGRGKIEIKKIENANSRQVTFSKRRVGLLKKASELAILCDAQVGVIIFSNTGKLFEFSST
Corvina        1 MGRGKIEIKKIENANSRQVTFSKRRVGLLKKASELAILCDAQVGVIIFSNTGKLFEFSST
                 ************************************************************

Pinot         61 SMKRIISRYNKLDSSEGALVEYKAEEPKEVDILKDEIRKLQTRQLQLLGKDLSGLSLKEL
Corvina       61 SMKRIISRYNKLDSSEGALVEYKAEEPKEVDILKDEIRKLQTRQLQLLGKDLSGLSLKEL
                 ************************************************************

Pinot        121 QNLEQQLNESLLSVKERKEQVLMEQLEQSRVQEQRAVLENETLRRQVEELRGLVPSSDRL
Corvina      121 QNLEQQLNESLLSVKERKEQVLMEQLEQSRVQEQRAVLENETLRRQVEELRGLVPSSDCL
                 ************************************************************·*

Pinot        181 VPPFLEYHPLERKDSITKSVVISPDVCDFAVEREESDTTLQLGLPTEISRKRKAPAKMET
Corvina      181 VPPFLEYHPLERKDSITKSVVISPDVCDFAVEREESDTTLQLGLPTEISRKRKAPAKMET
                 ************************************************************

Pinot        241 RSNNSGS-
Corvina      241 RSNNSGS-
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B

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Pinot          1 MERSGVMKMEDPVGSWSFSDHAPDIPAVFDFSDEGEKGTLGFMELLSIQNYGPPLFDLVQ
Corvina        1 MERSGVMKMEDPVGSWSFSDHAPEIPAVFDFSDEGEKGTLGFMELLSIQNYGPPLFDLVQ
                 **********************·*************************************

Pinot         61 LPSLEKPTGAAPAVPASSDVVNPPATPNSSSVSSASSHEQGSKAVEEEEEDEEEKKTKKE
Corvina       61 LPSLEKPTGAAPAVPASSDVVNPPATPNSSSVSSASSHEQGSKAVEEEEEDEEEKKTKKE
                 ************************************************************

Pinot        121 LKPKKTTSQKRQREPRFAFMTKSEVDHLEDGYRWRKYGQKAVKNSPFPRSYYRCTSASCN
Corvina      121 LKPKKTTSQKRQREPRFAFMTKSEVDHLEDGYRWRKYGQKAVKNSPFPRSYYRCTSASCN
                 ************************************************************

Pinot        181 VKKRVERCFKDPAIVVTTYEGQHTHPSPIMPRANPSSIATTFAGPRLQTTPFQHLHLHHH
Corvina      181 VKKRVERCFKDPAIVVTTYEGQHTHPSPIMPRANPSSIATTFAGPRLQTTPFQHLHLHHH
                 ************************************************************

Pinot        241 QQQQHPSFNDVPLLNYGHGSSFGSAARSVQERRSCAPTSALHRDHGLLQDIVPSHMRKEE
Corvina      241 QQQQHPSFNDVPLLNYGHGSSFGSAARSVQERRSCAPTSALHRDHGLLQDIVPSHMRKEE
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Pinot        301 EI-
Corvina      301 EI-
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C

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Pinot          1 MAAFSYQHPPFLLDSVFLPSTPIKMSGFMEEGNTTTCFSQFFPSESLHEVPADARVHEST
Corvina        1 MAAFSYQHPPFLLDSVFLPSTPIKMSGFMEEGNITTCFSQFFPSESLHEVPADARVHEST
                 ********************************.***********************

Pinot         61 SLQHSSKVTLSDNEPCVTQKLSTDSSSVVDRLELGEQVTQKVAPIERERKRKSRDGSSLT
Corvina       61 SLQHSSKVTLSDNEPCVTQKLSTDSSSVVDRLELGEQVTQKVAPIERERKRKSRDGSSLT
                 ************************************************************

Pinot        121 SAQSKDAREGKGKKAKKGSGLVKDGEEEQLKADKKDQKKASEEPPTGYIHVRARRGQATD
Corvina      121 SAQSKDAREGKGKKAKKGSGLVKDGEEEQLKADKKDQKKASEEPPTGYIHVRARRGQATD
                 ************************************************************

Pinot        181 SHSLAERVRREKISERMKLLQALVPGCDKVTGKALMLDEIINYVQSLQNQVEFLSMKLAS
Corvina      181 SHSLAERVRREKISERMKLLQALVPGCDKVTGKALMLDEIINYVQSLQNQVEFLSMKLAS
                 ************************************************************

Pinot        241 VNPMFYDFGMDLDALMVRPERLSALTSPLPSLQQCSPSQPTAYADTTTTFTATNNYPVMD
Corvina      241 VNPMFYDFGMDLDALMVRPERLSALTSPLPSLQQCSPSQPTAYADTTTTFTATNNYPVMD
                 ************************************************************

Pinot        301 TSASILFHQGQRLNVFSQDNGSLLWDVDDQRQKFINPSGLISNNLCSFN -
Corvina      301 TSASILFHQGQRLNVFSQDNGSLLWDVDDQRQKFINPSGLISNNLCSFN -
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Supplemental data set 1: list of upregulated genes (FC > 2) in *VviAGL15a* transiently overexpressing Thompson seedless leaves compared to the control lines.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	FC
VIT_13S0158G00100	putative MADS-box Agamous-like 15a (VviAGL15a)	Transcription Factor Activity	18.30
VIT_01S0244G00090	feronia receptor-like kinase	Signal Transduction	15.54
VIT_05S0020G01750	Glutaredoxin	Cellular Homeostasis	6.77
VIT_11S0052G01260	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	6.14
VIT_11S0052G01190	Xyloglucan endotransglucosylase-hydrolase XTH3	Cell Wall Metabolism	5.91
VIT_11S0052G01330	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	5.28
VIT_17S0119G00230	Trypsin and protease inhibitor Kunitz family	Response to Stress	4.66
VIT_05S0020G02720	Aspartic Protease (VvAP11)	Cellular Process	3.93
VIT_05S0077G00510	Beta-fructofuranosidase	Carbohydrate Metabolic Process	3.84
VIT_17S0000G08010	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.53
VIT_10S0003G00470	Trans-resveratrol di-O-methyltransferase - VvROMT	Secondary Metabolic Process	3.48
VIT_09S0002G05540	ABC transporter g family pleiotropic drug resistance 12 PDR12	Transport	3.39
VIT_14S0036G01210	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.34
VIT_13S0067G00260	Nematode-resistance protein	Response to Stress	3.33
VIT_19S0014G03130	Stem-specific protein TSJT1	Developmental Process	3.25
VIT_18S0001G06180	Phosphate-induced protein 1	Cellular Process	3.08

VIT_14S0068G02330	Chloride channel protein B	Transport	3.07
VIT_08S0007G08910	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	3.06
VIT_07S0197G00060	myb family	Transcription Factor Activity	3.01
VIT_04S0023G00580	Auxin-responsive SAUR32	Response to Hormone Stimulus	2.97
VIT_10S0003G00580	ERF/AP2 Gene Family (VvERF075)	Response to Hormone Stimulus	2.95
VIT_05S0077G01280	Glycosyl hydrolase family 3 beta xylosidase BXL1	Carbohydrate Metabolic Process	2.94
VIT_12S0055G01020	Peroxidase	Response to Stress	2.94
VIT_00S0316G00020	Chloride channel protein CLC-A	Transport	2.87
VIT_05S0077G01970	Zinc finger (C3HC4-type ring finger)	Transcription Factor Activity	2.85
VIT_10S0003G00390	Glutaredoxin	Cellular Homeostasis	2.80
VIT_07S0005G02490	CYP709B2	Cellular Process	2.79
VIT_08S0007G08890	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	2.78
VIT_17S0000G01460	Protein kinase AKIN gamma	Signal Transduction	2.70
VIT_13S0067G01880	Other LOB domain-containing protein ASL5	Transcription Factor Activity	2.69
VIT_07S0005G01240	Triacylglycerol lipase	Lipid Metabolic Process	2.68
VIT_13S0067G03780	Leucine-rich repeat protein kinase	Response to Stress	2.65
VIT_18S0001G09850	Myb domain protein R1	Transcription Factor Activity	2.61
VIT_08S0032G01220	Calcium Dependent Protein Kinase (VvCPK9)	Carbohydrate Metabolic Process	2.58
VIT_19S0014G05090	Thioredoxin h	Cellular Homeostasis	2.51
VIT_03S0132G00390	Wall-associated kinase 1 (WAK1)	Signal Transduction	2.49
VIT_13S0067G02130	Dehydration-induced protein (ERD15)	Response to Hormone Stimulus	2.48
VIT_06S0080G01090	CCR4-NOT transcription complex subunit 7/8	DNA/RNA Metabolic Process	2.42
VIT_04S0008G00610	Zinc finger (CCCH-type) family protein	Transcription Factor Activity	2.41
VIT_01S0026G00880	Transducin protein	#N/D	2.38
VIT_17S0000G01280	WRKY Transcription Factor (VvWRKY52)	Transcription Factor Activity	2.38
VIT_16S0098G00190	Receptor kinase homolog LRK10	Signal Transduction	2.35
VIT_03S0038G03400	Endochitinase 1, basic	Carbohydrate Metabolic Process	2.35
VIT_18S0001G10670	EF hand	Signal Transduction	2.32
VIT_07S0005G01950	myb domain protein 78	Transcription Factor Activity	2.32
VIT_05S0020G01420	FPF1 (flowering promoting factor 1)	Developmental Process	2.32
VIT_13S0064G01370	Polygalacturonase inhibiting protein 1 PGIP1	Response to Stress	2.32
VIT_07S0005G03260	ERF/AP2 Gene Family (VvERF100)	Response to Hormone Stimulus	2.32
VIT_08S0007G08920	Zeatin O-glucosyltransferase	Secondary Metabolic Process	2.30
VIT_11S0052G01250	Xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	2.29

VIT_03S0017G01040	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	2.29
VIT_09S0002G02120	Beta-galactosidase	Carbohydrate Metabolic Process	2.27
VIT_12S0034G02530	R protein disease resistance protein	Response to Stress	2.25
VIT_16S0115G00340	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase	Secondary Metabolic Process	2.22
VIT_16S0013G01080	ERF/AP2 Gene Family (VvERF086)	Response to Hormone Stimulus	2.22
VIT_11S0206G00090	Calmodulin-binding protein	Signal Transduction	2.22
VIT_02S0234G00130	Ethylene responsive element binding factor 1	Response to Hormone Stimulus	2.18
VIT_14S0068G01920	Peroxidase	Response to Stress	2.18
VIT_01S0026G00190	Armadillo/beta-catenin repeat	Signal Transduction	2.13
VIT_07S0005G03230	ERF/AP2 Gene Family (VvERF099)	Response to Hormone Stimulus	2.12
VIT_16S0098G00200	Receptor serine/threonine kinase PR5K	Signal Transduction	2.11
VIT_11S0016G00710	TIFY gene family (VvJAZ9)	Response to Hormone Stimulus	2.10
VIT_08S0007G06670	Homeobox-leucine zipper protein 14	Transcription Factor Activity	2.09
VIT_13S0156G00610	S-receptor kinase	Signal Transduction	2.07
VIT_07S0129G00210	BT4 (BTB and TAZ Domain protein 4)	Transcription Factor Activity	2.07
VIT_06S0080G00790	MYB divaricata	Developmental Process	2.06
VIT_01S0026G01730	WRKY Transcription Factor (VvWRKY02)	Transcription Factor Activity	2.04
VIT_19S0014G04650	Avr9/Cf-9 rapidly elicited protein 20	Response to Stress	2.02
VIT_08S0007G05800	Patatin	Cellular Process	2.01

Supplemental data set 2: list of correlated genes (Pearson's coefficient > 0.95) to *VviAGL15a* in transiently *VviAGL15* overexpressing Thompson seedless leaves compared to the control lines.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_11S0052G01260	xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	0.997
VIT_18S0122G01340	BTB/POZ domain-containing protein	Transcription Factor Activity	0.996
VIT_11S0052G01300	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.993
VIT_06S0009G01930	5-AMP-activated protein kinase beta-2 subunit	Lipid Metabolic Process	0.992
VIT_11S0052G01340	Xyloglucan endo-transglycosylase, C-terminal	Cell Wall Metabolism	0.990
VIT_03S0038G03430	Expansin (VvEXLA1)	Cell Wall Metabolism	0.986
VIT_02S0012G00730	purine permease 10 PUP10	Transport	0.984
VIT_13S0067G03780	Leucine-rich repeat protein kinase	Signal Transduction	0.982
VIT_13S0067G01880	other LOB domain-containing protein ASL5	Transcription Factor Activity	0.982
VIT_17S0000G06370	Thioredoxin 2	Cellular Homeostasis	0.981

VIT_11S0052G01200	xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	0.979
VIT_11S0052G01320	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.979
VIT_04S0008G06000	ERF/AP2 Gene Family (VvERF045)	Response to Hormone Stimulus	0.979
VIT_18S0089G01270	Heat shock protein precursor 22.0 kDa class IV	Response to Stress	0.979
VIT_08S0007G08890	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	0.979
VIT_08S0040G00990	Transcription elongation factor S-II	Transcription Factor Activity	0.979
VIT_01S0010G02730	chaperone BCS1 mitochondrial	Cellular Homeostasis	0.978
VIT_14S0068G01330	transport inhibitor response 1 protein	Response to Hormone Stimulus	0.977
VIT_06S0004G04530	Armadillo/beta-catenin repeat protein/U-box domain-containing	Signal Transduction	0.976
VIT_07S0005G03030	lateral organ boundaries DOMAIN 16	Transcription Factor Activity	0.976
VIT_01S0026G00880	transducin protein	#N/D	0.976
VIT_08S0007G06030	beta 1-3 glucanase [Vitis vinifera]	Cell Wall Metabolism	0.975
VIT_11S0052G01310	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.975
VIT_05S0094G00300	Chitinase class IV	Carbohydrate Metabolic Process	0.974
VIT_13S0064G00410	R protein MLA10	Response to Stress	0.973
VIT_11S0052G01170	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.972
VIT_08S0007G06010	beta 1-3 glucanase [Vitis vinifera]	Cell Wall Metabolism	0.971
VIT_05S0077G00510	Beta-fructofuranosidase	Carbohydrate Metabolic Process	0.970
VIT_16S0050G01690	Receptor kinase homolog LRK10	Signal Transduction	0.970
VIT_04S0079G00420	Expansin (VvEXPA4)	Cell Wall Metabolism	0.969
VIT_17S0000G01250	F-box/LRR-repeat protein	#N/D	0.969
VIT_03S0091G00100	methylthioribose kinase	Cellular Amino Acids and Derivative Metabolic Process	0.967
VIT_00S0397G00010	HcrVf1 protein	Response to Stress	0.967
VIT_09S0018G01660	Biopterin transport-related protein BT1	Transport	0.966
VIT_11S0103G00570	microtubule-associated protein Rab GTPase	Cellular Process	0.965
VIT_11S0206G00090	calmodulin-binding protein	Signal Transduction	0.965
VIT_18S0001G09850	myb domain protein R1	Transcription Factor Activity	0.965
VIT_14S0036G01210	trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	0.964
VIT_00S0174G00230	Zinc finger (FYVE type)	Developmental Process	0.964
VIT_18S0001G06030	Erg-1	Response to Hormone Stimulus	0.964
VIT_19S0014G04650	Avr9/Cf-9 rapidly elicited protein 20	Response to Stress	0.964
VIT_18S0001G06180	Phosphate-induced protein 1	Cellular Process	0.963
VIT_11S0052G01330	xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	0.962
VIT_08S0007G08920	zeatin O-glucosyltransferase	Secondary Metabolic Process	0.962

VIT_00S0662G00010	Acyl-CoA oxidase (ACX2)	Lipid Metabolic Process	0.961
VIT_04S0043G00510	Ethylene-responsive transcription factor ERF003	Response to Hormone Stimulus	0.961
VIT_18S0041G00430	patellin-1	#N/D	0.961
VIT_11S0016G02480	2,3-diketo-5-methylthio-1-phosphopentane phosphatase	Cellular Process	0.960
VIT_13S0067G02190	SAG20 (WOUND-INDUCED PROTEIN 12)	#N/D	0.960
VIT_12S0057G00570	Zinc finger (CCCH-type) family protein	Transcription Factor Activity	0.958
VIT_19S0014G02900	RING finger protein 185	#N/D	0.956
VIT_04S0210G00180	isocitrate dehydrogenase subunit 1	Carbohydrate Metabolic Process	0.955
VIT_16S0039G01620	Zinc knuckle	Response to Stress	0.955
VIT_18S0157G00190	choline kinase	Cellular Amino Acids and Derivative Metabolic Process	0.954
VIT_07S0005G01240	triacylglycerol lipase	Lipid Metabolic Process	0.954
VIT_07S0005G00820	ERF/AP2 Gene Family (VvERF057)	Response to Hormone Stimulus	0.954
VIT_03S0088G00620	Stress-induced	Response to Stress	0.954
VIT_08S0007G00720	Ring-H2 finger C2A	Transcription Factor Activity	0.954
VIT_01S0011G01640	ARE1	Transport	0.954
VIT_11S0052G01250	xyloglucan endotransglucosylase/hydrolase 23	Cell Wall Metabolism	0.953
VIT_00S0568G00010	WD-40 repeat	Cellular Process	0.953
VIT_04S0044G01880	Auxin Efflux Carrier	Response to Hormone Stimulus	0.953
VIT_11S0052G01190	xyloglucan endotransglucosylase-hydrolase XTH3	Cell Wall Metabolism	0.952
VIT_06S0004G03510	TIR-NBS-TIR type disease resistance protein	Signal Transduction	0.952
VIT_00S0398G00020	S-receptor kinase KIK1 precursor	Signal Transduction	0.952
VIT_08S0007G01180	S-receptor kinase	Signal Transduction	0.951
VIT_04S0069G00630	glutamate receptor 2.8	Transport	0.951
VIT_04S0044G00730	S-receptor kinase	#N/D	0.950

Supplemental data set 3: list of upregulated genes (FC > 2) in *VviWRKY19* transiently overexpressing Thompson seedless leaves compared to the control lines.

VIT	FUNCIONAL ANNOTATION	GENE ONTOLOGY	FC
VIT_17S0119G00150	Alpha-amylase/subtilisin inhibitor	Carbohydrate Metabolic Process	7.47
VIT_17S0119G00230	Trypsin and protease inhibitor Kunitz family	Response to Stress	7.29
VIT_19S0014G03180	ERF/AP2 Gene Family (VvERF001), Dehydration Responsive Element-Binding Transcription Factor (VvDREB33)	Response to Hormone Stimulus	7.09
VIT_17S0119G00160	Trypsin and protease inhibitor Kunitz family	Response to Stress	6.41
VIT_00S1751G00010	Trypsin and protease inhibitor family	Response to Stress	5.72
VIT_01S0026G02710	NAC domain-containing protein (VvNAC26)	Transcription Factor Activity	4.81

VIT_11S0052G01220	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	4.67
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	4.35
VIT_10S0003G00470	Trans-resveratrol di-O-methyltransferase - VvROMT	Secondary Metabolic Process	4.29
VIT_11S0052G01300	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	4.04
VIT_01S0146G00220	Glutaredoxin-like	Cellular Homeostasis	3.90
VIT_10S0003G00390	Glutaredoxin	Cellular Homeostasis	3.80
VIT_07S0005G01360	Zinc finger (C3HC4-type ring finger)	Transcription Factor Activity	3.58
VIT_07S0104G01260	flavin-containing monooxygenase	Secondary Metabolic Process	3.55
VIT_14S0068G01570	Glutaredoxin-like	Cellular Homeostasis	3.51
VIT_12S0034G01930	Globulin-like protein	Cellular Process	3.37
VIT_14S0036G01210	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.26
VIT_05S0020G02720	Aspartic Protease (VvAP11)	Cellular Process	3.21
VIT_17S0000G08010	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.17
VIT_19S0014G03290	NAC domain-containing protein (VvNAC17)	Transcription Factor Activity	3.16
VIT_04S0023G01500	Polyol transporter 6 (PLT6)	Carbohydrate Metabolic Process	3.12
VIT_19S0014G03130	Stem-specific protein TSJT1	Developmental Process	3.11
VIT_18S0122G00180	Calmodulin CML37	Signal Transduction	3.10
VIT_17S0000G04380	Wall-associated kinase 1 (WAK1)	Signal Transduction	3.08
VIT_10S0003G00580	ERF/AP2 Gene Family (VvERF075)	Response to Hormone Stimulus	2.99
VIT_04S0023G01510	DUF620	Cellular Process	2.97
VIT_06S0080G00170	Aluminium-tolerance ALMT1	Transport	2.95
VIT_05S0077G00510	Beta-fructofuranosidase	Carbohydrate Metabolic Process	2.94
VIT_08S0007G07640	NAC domain-containing protein (VvNAC61)	Transcription Factor Activity	2.92
VIT_01S0011G03660	IMP dehydrogenase/GMP reductase	DNA/RNA Metabolic Process	2.90
VIT_07S0104G00350	Circadian clock coupling factor ZGT	#N/D	2.89
VIT_06S0080G00780	NAC domain-containing protein (VvNAC74)	Transcription Factor Activity	2.87
VIT_07S0031G02610	NAC domain-containing protein (VvNAC39)	Transcription Factor Activity	2.81
VIT_07S0197G00060	myb family	Transcription Factor Activity	2.74
VIT_05S0124G00610	Ankyrin repeat protein family	Cellular Process	2.73
VIT_13S0067G00260	Nematode-resistance protein	Response to Stress	2.65
VIT_05S0077G01280	Glycosyl hydrolase family 3 beta xylosidase BXL1	Carbohydrate Metabolic Process	2.64
VIT_03S0063G01520	CyP82A3	Cellular Process	2.58
VIT_07S0005G01240	Triacylglycerol lipase	Lipid Metabolic Process	2.54
VIT_19S0014G01800	Homogentisate 1,2-dioxygenase	Cellular Amino Acids and Derivative Metabolic Process	2.54

VIT_19S0014G05090	Thioredoxin h	Cellular Homeostasis	2.52
VIT_19S0014G01120	Curculin (mannose-binding) lectin	Cellular Process	2.52
VIT_09S0002G05210	F-box family protein	Cellular Homeostasis	2.50
VIT_09S0018G00710	Disease resistance family protein	Response to Stress	2.41
VIT_06S0004G07650	Taxadien-5-alpha-ol-O-acetyltransferase	Secondary Metabolic Process	2.37
VIT_14S0068G02330	Chloride channel protein B	Transport	2.29
VIT_08S0007G08910	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	2.29
VIT_13S0067G03780	Leucine-rich repeat protein kinase	Response to Stress	2.28
VIT_08S0032G01150	Syntaxin 1B/2/3/4	Transport	2.27
VIT_19S0014G02900	Ring finger protein 185	#N/D	2.26
VIT_01S0011G03110	myb family	Transcription Factor Activity	2.21
VIT_13S0156G00260	Homeobox-leucine zipper protein HAT14	Transcription Factor Activity	2.20
VIT_00S0203G00210	Zinc finger (B-box type)	Transcription Factor Activity	2.20
VIT_00S0347G00030	Zinc finger (B-box type)	Transcription Factor Activity	2.19
VIT_18S0001G06670	Ring-H2 finger protein ATL1N	#N/D	2.18
VIT_03S0091G00670	Lateral organ boundaries protein 38	Transcription Factor Activity	2.18
VIT_01S0011G03520	Constans-like 16	Developmental Process	2.17
VIT_07S0129G00210	BT4 (BTB and TAZ Domain protein 4)	Transcription Factor Activity	2.16
VIT_00S0421G00010	Chloride channel protein B	Transport	2.15
VIT_16S0098G00190	Receptor kinase homolog LRK10	Signal Transduction	2.14
VIT_09S0002G05080	Kelch repeat-containing F-box family protein	Cellular Homeostasis	2.13
VIT_06S0004G08190	ERF/AP2 Gene Family (VvERF055)	Response to Hormone Stimulus	2.13
VIT_16S0098G00200	Receptor serine/threonine kinase PR5K	Signal Transduction	2.12
VIT_08S0032G00760	Translation initiation factor eIF-2 beta subunit	DNA/RNA Metabolic Process	2.11
VIT_11S0016G01520	Phenylalanine ammonia-lyase	Generation of Energy	2.09
VIT_16S0115G00340	UDP-glucose: anthocyanidin 5,3-O-glucosyltransferase	Secondary Metabolic Process	2.08
VIT_07S0129G00330	Lateral organ boundaries protein 39	Transcription Factor Activity	2.07
VIT_17S0000G01460	Protein kinase AKIN gamma	Signal Transduction	2.07
VIT_09S0002G04930	Kelch repeat-containing F-box family protein	Cellular Homeostasis	2.06
VIT_18S0001G09850	Myb domain protein R1	Transcription Factor Activity	2.06
VIT_11S0016G01640	Phenylalanine ammonia-lyase	Generation of Energy	2.06
VIT_14S0068G01800	putative MADS-box Flowering Locus C 2 (VviFLC2)	Transcription Factor Activity	2.04
VIT_16S0013G01110	Ethylene-responsive transcription factor 5	Response to Hormone Stimulus	2.04
VIT_08S0007G05800	Patatin	Cellular Process	2.03

VIT_06S0080G00790	MYB divaricata	Developmental Process	2.01
VIT_09S0002G05010	Kelch repeat-containing F-box family protein	Cellular Homeostasis	2.00

Supplemental data set 4: list of correlated genes (Pearson's coefficient > 0.9) to *VviWRKY19* in transiently *VviWRKY19* overexpressing Thompson seedless leaves compared to the control lines.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	0.989
VIT_14S0068G02130	fidgetin-like 1	Transport	0.976
VIT_06S0004G03920	Pto serine/threonine kinase	Signal Transduction	0.960
VIT_18S0001G08210	SUT4 (sucrose transporter 4)	Carbohydrate Metabolic Process	0.959
VIT_12S0035G01280	R protein disease resistance protein	Response to Stress	0.954
VIT_06S0004G05500	CHLORORESPIRATORY REDUCTION 2 (CRR2)	Generation of Energy	0.948
VIT_11S0052G01210	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.944
VIT_04S0044G00510	GT2-like trihelix DNA-binding protein	Transcription Factor Activity	0.941
VIT_08S0007G08210	Zinc finger (CCCH-type) family protein	Transcription Factor Activity	0.941
VIT_17S0053G00360	pentatricopeptide (PPR) repeat	Pentatricopeptide (PPR) repeat-containing protein	0.937
VIT_09S0070G00560	EIX receptor	Response to Stress	0.936
VIT_00S0181G00010	Sugar transporter 1	Carbohydrate Metabolic Process	0.935
VIT_14S0081G00340	ankyrin repeat	Cellular Process	0.935
VIT_11S0118G00570	Tristeza Virus Resistance Gene (Ctv)	Response to Stress	0.934
VIT_08S0105G00290	zinc finger (C3HC4-type RING finger)	Transcription Factor Activity	0.934
VIT_06S0004G07770	peroxidase	Response to Stress	0.932
VIT_08S0007G03880	Zinc finger (C2H2 type) family	Transcription Factor Activity	0.93
VIT_19S0014G01800	Homogentisate 1,2-dioxygenase	Cellular Amino Acids and Derivative Metabolic Process	0.929
VIT_04S0008G00320	Leucine-rich repeat family protein	Signal Transduction	0.927
VIT_08S0032G00270	CYP71D64	Cellular Process	0.926
VIT_13S0019G01480	Sugar transport protein 8 (STP8)	Carbohydrate Metabolic Process	0.925
VIT_11S0016G00340	DNA repair protein RAD51	DNA/RNA Metabolic Process	0.92
VIT_11S0052G01220	Xyloglucan endotransglycosylase 6	Cell Wall Metabolism	0.918
VIT_08S0007G00970	Translation initiation factor eIF-3 subunit 3	DNA/RNA Metabolic Process	0.918
VIT_03S0063G00920	NADPH:quinone reductase	Secondary Metabolic Process	0.917
VIT_13S0019G00830	armadillo repeat-containing protein	Signal Transduction	0.917

VIT_02S0087G00500	MAP kinase 9	Signal Transduction	0.915
VIT_13S0019G03700	lateral organ boundaries protein 1	Transcription Factor Activity	0.909
VIT_13S0156G00250	Cytidine/deoxycytidylate deaminase	DNA/RNA Metabolic Process	0.908
VIT_07S0141G00020	Sensitive to proton rhizotoxicity 1	Transcription Factor Activity	0.908
VIT_05S0124G00240	basic helix-loop-helix (bHLH) family	Transcription Factor Activity	0.907
VIT_18S0001G02300	NAC domain-containing protein (VvNAC08)	Transcription Factor Activity	0.906
VIT_14S0128G00480	Translation initiation factor eIF-3 subunit 1	DNA/RNA Metabolic Process	0.905
VIT_09S0096G00200	RPS5 (resistant to p. syringae 5)	Response to Stress	0.905
VIT_16S0050G00230	Zinc knuckle	#N/D	0.905
VIT_15S0021G02200	Nucleoporin 62kDa	DNA/RNA Metabolic Process	0.904
VIT_19S0014G02460	plastocyanin domain-containing protein	Generation of Energy	0.904
VIT_08S0058G01290	Protein kinase AtSIK	Signal Transduction	0.902
VIT_00S0420G00040	S-locus lectin protein kinase	Signal Transduction	0.902
VIT_04S0008G00300	CLAVATA1 receptor kinase (CLV1)	Signal Transduction	0.902
VIT_18S0001G10150	ERF/AP2 Gene Family (VvERF006)	Response to Hormone Stimulus	0.901
VIT_06S0080G00980	secoisolariciresinol dehydrogenase	Secondary Metabolic Process	0.901
VIT_07S0129G00970	Protein kinase family	Signal Transduction	0.901
VIT_15S0045G00330	Ring-H2 finger protein ATL3A	Cellular Homeostasis	0.90
VIT_14S0006G02490	ATP binding protein	Cellular Process	0.90

Supplemental data set 5: list of upregulated genes (FC > 2) in *VvibHLH75* transiently overexpressing Thompson seedless leaves compared to the control lines.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	FC
VIT_01S0010G02730	Chaperone BCS1 mitochondrial	Cellular Homeostasis	8.04
VIT_14S0066G01060	Polygalacturonase GH28	Cell Wall Metabolism	7.54
VIT_17S0000G00430	basic helix-loop-helix (bHLH) family	Transcription Factor Activity	6.07
VIT_14S0128G00670	Germin-like protein 3 [Vitis vinifera]	Cellular Process	5.94
VIT_05S0077G00500	myb domain protein 108	Transcription Factor Activity	5.36
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	5.02
VIT_18S0001G11540	CYPLXXXII	Cellular Process	4.64
VIT_17S0000G04380	Wall-associated kinase 1 (WAK1)	Signal Transduction	4.39
VIT_18S0001G09660	CYP81D2	Cellular Process	4.18
VIT_18S0001G11450	CYP82C1p	Cellular Process	4.08
VIT_05S0077G01690	s1_Pathogenesis protein 10 [Vitis vinifera]	Response to Stress	4.02

VIT_19S0014G03180	ERF/AP2 Gene Family (VvERF001), Dehydration Responsive Element-Binding Transcription Factor (VvDREB33)	Response to Hormone Stimulus	3.91
VIT_04S0023G01500	Polyol transporter 6 (PLT6)	Carbohydrate Metabolic Process	3.75
VIT_07S0031G02610	NAC domain-containing protein (VvNAC39)	Transcription Factor Activity	3.68
VIT_01S0026G02710	NAC domain-containing protein (VvNAC26)	Transcription Factor Activity	3.51
VIT_18S0001G03180	Nodulin MtN21 family	Transport	3.39
VIT_12S0034G01910	Cupin family protein	Cellular Process	3.29
VIT_17S0000G08010	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	3.24
VIT_05S0077G00840	Galactinol-raffinose galactosyltransferase	Carbohydrate Metabolic Process	2.99
VIT_12S0034G01950	Legumin	Cellular Process	2.98
VIT_07S0005G01360	Zinc finger (C3HC4-type ring finger)	Transcription Factor Activity	2.97
VIT_07S0005G01930	Pectinesterase family	Cell Wall Metabolism	2.96
VIT_14S0036G01210	Trehalose 6-phosphate synthase	Carbohydrate Metabolic Process	2.93
VIT_14S0068G02000	Ribonucleotide reductase R2	DNA/RNA Metabolic Process	2.87
VIT_12S0034G01870	Cupin	Response to Stress	2.84
VIT_12S0034G01930	Globulin-like protein	Cellular Process	2.84
VIT_17S0000G06360	Expansin (VvEXPA17)	Cell Wall Metabolism	2.81
VIT_15S0024G01700	Receptor kinase CHRK1	Signal Transduction	2.79
VIT_00S0748G00020	Receptor kinase RK20-1	Signal Transduction	2.77
VIT_08S0007G06060	Beta 1-3 glucanase	Cell Wall Metabolism	2.74
VIT_13S0019G04160	Protein kinase	Signal Transduction	2.74
VIT_12S0034G01890	Cupin region	Response to Stress	2.72
VIT_10S0003G00470	Trans-resveratrol di-O-methyltransferase - VvROMT	Secondary Metabolic Process	2.72
VIT_04S0023G01510	DUF620	Cellular Process	2.66
VIT_05S0062G00740	UDP-glucoronosyl/UDP-glucosyl transferase UGT75C1	Secondary Metabolic Process	2.65
VIT_01S0146G00220	Glutaredoxin-like	Cellular Homeostasis	2.63
VIT_06S0004G07790	Lateral organ boundaries Domain 15	Transcription Factor Activity	2.63
VIT_03S0038G01420	Phytochelatin synthetase	Cellular Amino Acids and Derivative Metabolic Process	2.62
VIT_13S0067G02130	Dehydration-induced protein (ERD15)	Response to Hormone Stimulus	2.62
VIT_00S0262G00130	Receptor-like protein kinase homolog RK20-1	Signal Transduction	2.61
VIT_12S0034G01970	Cupin	Response to Stress	2.58
VIT_08S0032G01220	Calcium Dependent Protein Kinase (VvCPK9)	Carbohydrate Metabolic Process	2.56
VIT_03S0063G01520	CyP82A3	Cellular Process	2.53
VIT_08S0007G06560	Lectin protein kinase	Signal Transduction	2.50
VIT_00S0762G00010	S-locus lectin protein kinase family	Signal Transduction	2.49

VIT_17S0000G01460	Protein kinase AKIN gamma	Signal Transduction	2.48
VIT_07S0104G00430	Endo-1,3;1,4-beta-D-glucanase precursor	#N/D	2.46
VIT_10S0003G04950	Esterase/lipase/thioesterase	#N/D	2.44
VIT_09S0002G01350	Growth-regulating factor 5	Transcription Factor Activity	2.44
VIT_08S0007G08910	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	2.41
VIT_09S0002G05510	ABC transporter g family pleiotropic drug resistance 12 PDR12	Transport	2.41
VIT_10S0003G00580	ERF/AP2 Gene Family (VvERF075)	Response to Hormone Stimulus	2.40
VIT_05S0020G02720	Aspartic Protease (VvAP11)	Cellular Process	2.39
VIT_04S0044G01300	DNA cross-link repair protein	DNA/RNA Metabolic Process	2.38
VIT_18S0001G09590	Zinc finger protein 4	Transcription Factor Activity	2.36
VIT_19S0014G03130	Stem-specific protein TSJT1	Developmental Process	2.36
VIT_00S0181G00220	Calmodulin-binding protein	Signal Transduction	2.35
VIT_00S0615G00020	Cinnamyl alcohol dehydrogenase	Secondary Metabolic Process	2.34
VIT_07S0129G00210	BT4 (BTB and TAZ Domain protein 4)	Transcription Factor Activity	2.33
VIT_08S0007G04570	UGT73D1 (UDP-glucosyl transferase 73D1); UDP-glycosyltransferase	#N/D	2.33
VIT_09S0002G02700	basic helix-loop-helix (bHLH) family	Transcription Factor Activity	2.31
VIT_18S0041G01340	R protein L6	Response to Stress	2.30
VIT_16S0100G00130	Protein phosphatase 2C / PP2C	Signal Transduction	2.30
VIT_09S0002G05380	ABC transporter g family pleiotropic drug resistance 12 PDR12	Transport	2.29
VIT_07S0005G02490	CYP709B2	Cellular Process	2.29
VIT_07S0005G01920	Gibberellin 2-beta-dioxygenase 2	Response to Hormone Stimulus	2.28
VIT_00S0346G00110	Mannitol dehydrogenase	Carbohydrate Metabolic Process	2.27
VIT_00S0547G00010	S-locus lectin protein kinase	Signal Transduction	2.27
VIT_18S0041G00510	Proton-dependent oligopeptide transport (POT) family protein	Transport	2.26
VIT_18S0001G12040	Coniferyl-alcohol glucosyltransferase	Secondary Metabolic Process	2.21
VIT_14S0068G02330	Chloride channel protein B	Transport	2.20
VIT_17S0000G05360	Germin	Cellular Process	2.18
VIT_17S0000G09810	Pectate lyase	Cell Wall Metabolism	2.17
VIT_16S0050G01310	C2 domain-containing protein	Signal Transduction	2.17
VIT_03S0063G02650	Hydrolase, alpha/beta fold	#N/D	2.15
VIT_07S0031G01980	ERF/AP2 Gene Family (VvERF113)	Response to Hormone Stimulus	2.15
VIT_14S0068G01570	Glutaredoxin-like	Cellular Homeostasis	2.14
VIT_02S0025G02920	caffeic acid 3-O-methyltransferase	Secondary Metabolic Process	2.14
VIT_18S0001G11410	CyP82A3	Cellular Process	2.12

VIT_08S0007G05800	Patatin	Cellular Process	2.11
VIT_18S0122G01340	BTB/POZ domain-containing protein	Transcription Factor Activity	2.11
VIT_15S0021G02670	Expansin (VvEXPB3)	Cell Wall Metabolism	2.10
VIT_16S0098G00190	Receptor kinase homolog LRK10	Signal Transduction	2.10
VIT_00S0316G00020	Chloride channel protein CLC-A	Transport	2.09
VIT_04S0008G05750	WRKY Transcription Factor (VvWRKY07)	Transcription Factor Activity	2.08
VIT_09S0070G00740	PfkB-type carbohydrate kinase	#N/D	2.08
VIT_08S0040G02660	RARE-cold-inducible 2A	Transport	2.08
VIT_00S0179G00370	ESCRT-I complex subunit TSG101	Transport	2.08
VIT_00S0218G00010	Cinnamyl alcohol dehydrogenase	Secondary Metabolic Process	2.06
VIT_08S0007G08890	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	2.06
VIT_10S0042G00840	Stilbene Synthase (VvSTS1)	Secondary Metabolic Process	2.03
VIT_00S0409G00010	Receptor kinase RK20-1	Signal Transduction	2.03
VIT_03S0017G01040	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	2.03
VIT_15S0045G01160	Avr9/Cf-9 rapidly elicited protein	Response to Stress	2.02
VIT_18S0117G00290	Coatomer protein complex, subunit beta 2	Transport	2.02
VIT_04S0044G01150	Aminotransferase, class V	Cellular Homeostasis	2.02
VIT_12S0134G00630	Quercetin 3-O-glucoside-6''-O-malonyltransferase	Secondary Metabolic Process	2.02
VIT_03S0038G04640	CC-NBS-LRR class	Response to Stress	2.02
VIT_00S0262G00180	S-locus lectin protein kinase	Signal Transduction	2.02
VIT_08S0007G06030	Beta 1-3 glucanase [Vitis vinifera]	Cell Wall Metabolism	2.02
VIT_19S0014G01560	Endonuclease	#N/D	2.02
VIT_18S0001G11130	Calmodulin-binding protein AR781	Signal Transduction	2.01

Supplemental data set 6: list of correlated genes (Pearson's coefficient > 0.92) to *VvibHLH75* in transiently *VvibHLH75* overexpressing Thompson seedless leaves compared to the control lines.

VIT	FUNCTIONAL ANNOTATION	GENE ONTOLOGY	PEARSON'S COEFFICIENT
VIT_12S0057G00800	Receptor Like Protein 27	Signal Transduction	0.983
VIT_08S0007G04160	Uridylate kinase	DNA/RNA Metabolic Process	0.98
VIT_08S0007G06760	cation efflux family protein MTPc3	Transport	0.979
VIT_04S0023G02200	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase	Cellular Amino Acids and Derivative Metabolic Process	0.977
VIT_17S0000G06400	NAC domain-containing protein (VvNAC05)	Transcription Factor Activity	0.977
VIT_17S0000G00400	phosphate carrier protein	Transport	0.976

VIT_04S0044G01300	DNA cross-link repair protein	DNA/RNA Metabolic Process	0.973
VIT_00S0984G00010	Phosphoglycerate mutase	Carbohydrate Metabolic Process	0.969
VIT_07S0031G00220	ERF/AP2 Gene Family (VvAP2-13)	Response to Hormone Stimulus	0.964
VIT_11S0016G04980	Zinc finger (CCCH-type) family protein	Transcription Factor Activity	0.964
VIT_19S0014G00920	R protein MLA10	Response to Stress	0.963
VIT_05S0062G00980	aldo/keto reductase AKR	Carbohydrate Metabolic Process	0.961
VIT_15S0021G02670	Expansin (VvEXPB3)	Cell Wall Metabolism	0.961
VIT_07S0129G00610	ABI3-interacting protein 2	Response to Hormone Stimulus	0.961
VIT_13S0101G00380	vacuolar protein sorting 36 / VPS36	Transport	0.96
VIT_07S0005G00630	NAD+ ADP-ribosyltransferase	Secondary Metabolic Process	0.955
VIT_09S0018G01020	Zinc finger and C2 domain protein	Transcription Factor Activity	0.952
VIT_00S0187G00300	fumarylacetoacetase	Cellular Amino Acids and Derivative Metabolic Process	0.951
VIT_01S0010G02730	chaperone BCS1 mitochondrial	Cellular Homeostasis	0.950
VIT_11S0016G00390	zinc finger (C3HC4-type RING finger)	Transcription Factor Activity	0.950
VIT_15S0046G02930	DNA-binding protein	DNA/RNA Metabolic Process	0.949
VIT_03S0091G00300	B-cell receptor-associated protein 31	Cellular Homeostasis	0.947
VIT_13S0067G02130	dehydration-induced protein (ERD15)	Response to Hormone Stimulus	0.944
VIT_04S0023G03220	Myosin-related	Cellular Process	0.943
VIT_06S0004G05710	Glutathione S-transferase GSTU7	Secondary Metabolic Process	0.943
VIT_07S0005G01710	WRKY transcription factor (VvWRKY19)	Transcription Factor Activity	0.943
VIT_09S0002G03740	Homeobox gene 8	Transcription Factor Activity	0.942
VIT_16S0098G01700	epoxide hydrolase	#N/D	0.942
VIT_12S0028G03920	ankyrin repeat	Cellular Process	0.94
VIT_00S0527G00010	cig3	DNA/RNA Metabolic Process	0.939
VIT_10S0116G00540	pentatricopeptide repeat-containing protein	Pentatricopeptide (PPR) repeat-containing protein	0.939
VIT_17S0000G08770	Cysteine-rich receptor-like protein kinase 2	Signal Transduction	0.939
VIT_01S0011G03110	myb family	Transcription Factor Activity	0.939
VIT_15S0021G00140	Speckle-type POZ protein-related	Transcription Factor Activity	0.939
VIT_00S0353G00020	S-locus lectin protein kinase	Signal Transduction	0.938
VIT_14S0068G02330	Chloride channel protein B	Transport	0.938
VIT_18S0001G02300	NAC domain-containing protein (VvNAC08)	Transcription Factor Activity	0.936
VIT_18S0086G00270	gag-pol polyprotein	Cellular Process	0.935
VIT_13S0019G03100	Cis-zeatin O-beta-D-glucosyltransferase	Secondary Metabolic Process	0.935
VIT_07S0104G00430	"endo-1,3;1,4-beta-D-glucanase precursor"	#N/D	0.935

VIT_13S0019G00220	Flowering Locus Y FY	Developmental Process	0.934
VIT_05S0029G00720	spermine/spermidine synthase	Cellular Amino Acids and Derivative Metabolic Process	0.933
VIT_09S0054G00360	cation efflux family protein MTPc4	Transport	0.933
VIT_03S0091G00150	NtPRp27 secretory protein	Response to Stress	0.932
VIT_18S0001G07890	TRAF-type zinc finger-related	#N/D	0.932
VIT_18S0001G05980	pentatricopeptide (PPR) repeat-containing protein	Pentatricopeptide (PPR) repeat-containing protein	0.931
VIT_13S0156G00250	Cytidine/deoxycytidylate deaminase	DNA/RNA Metabolic Process	0.930
VIT_06S0004G08190	ERF/AP2 Gene Family (VvERF055)	Response to Hormone Stimulus	0.930
VIT_18S0072G00170	BRI1 (BRASSINOSTEROID INSENSITIVE 1)	Response to Hormone Stimulus	0.930
VIT_00S0316G00020	chloride channel protein CLC-A	Transport	0.930
VIT_19S0014G05090	thioredoxin h	Cellular Homeostasis	0.929
VIT_06S0004G05680	Glutathione S-transferase 25 GSTU7	Secondary Metabolic Process	0.929
VIT_00S2887G00010	Syntaxin 52	Transport	0.929
VIT_17S0000G09650	glyoxal oxidase	Carbohydrate Metabolic Process	0.927
VIT_11S0118G00710	glycerol-3-phosphate transporter	Carbohydrate Metabolic Process	0.927
VIT_12S0034G02440	R protein MLA10	Response to Stress	0.926
VIT_05S0049G01550	Peptide transporter protein 3	Transport	0.926
VIT_01S0127G00250	Kelch repeat-containing F-box protein	Cellular Homeostasis	0.925
VIT_07S0141G00680	WRKY transcription factor (VvWRKY17)	Transcription Factor Activity	0.924
VIT_12S0059G00810	B-cell receptor-associated protein 31	Cellular Homeostasis	0.923
VIT_05S0051G00190	TIR-NBS-LRR-TIR disease resistance protein	Response to Stress	0.920
VIT_18S0001G06940	purine permease 1 (PUP1)	Secondary Metabolic Process	0.920
VIT_19S0027G00560	BTB/POZ domain-containing protein	Transcription Factor Activity	0.920

Chapter 6

PLANT REGENERATION FROM PROTOPLASTS: A NEW PERSPECTIVE FOR GENOME EDITING APPLICATION IN GRAPEVINE

ABSTRACT

Plant protoplasts represent a useful tool for basic research and biotechnological approaches. Protoplasts can be exploited for physiological, biochemical and molecular studies, from functional analysis of gene and characterization of metabolic pathways to recent applications of genome editing. However, most of these studies require the regeneration of the entire plants from protoplasts. This phase represents the bottleneck of this technology, because, most agronomically important plant species, including grapevine, are recalcitrant to regeneration. Grapevine (*Vitis vinifera L.*) protoplasts were obtained from many sources of plant material (leaves, stems, roots, mesocarp) and used for many studies, but the regeneration of plants was successfully performed only from protoplasts isolated from embryogenic tissue. Here, the application of a modified previously reported protocol for protoplasts isolation and plant regeneration of two Italian cultivars, Garganega and Sangiovese, is described. Protoplasts of both varieties were obtained from stamen-derived embryogenic calli. After isolation, protoplasts were cultivated in solid Nitsch's medium, supplemented with sugars, auxin and cytochinin. Within four months from the initiation of culture, well developed protoplasts-derived torpedo somatic embryos were transferred into medium supplemented with cytochinin under light in order to induce germination. Subsequently, germinated somatic embryos were moved in a rooting medium. Regenerated plants were transferred to the greenhouse and showed a normal morphology. Finally, protoplasts PEG-mediated transfection has been tested using a plasmid carrying YFP as marker gene. Fluorescence microscopic analysis showed that the YFP expression was initially low, but it took place after 24 hours and continued after 48 and 72 hours from the transfection. These results indicate that this system represents a useful tool for numerous applications in grapevine, including the genome editing.

1. INTRODUCTION

Plant protoplasts, naked plant cells lacking cell walls, are a useful tool for basic research and biotechnological approaches. Protoplasts can be exploited for physiological, biochemical and molecular studies, from characterization of metabolic pathways and transport studies to gene functional analysis and isolation of subcellular fractions. Furthermore, protoplasts are an excellent tool to introduce foreign genes to plant cells due to the removed cell wall. Polyethylene glycol (PEG)-mediated or electroporation are the mostly used methods, but the genetic transformation can be also performed by *Agrobacterium tumefaciens* or biolistics. Finally, another application of plant protoplasts is somatic hybridization by protoplast fusion (Papadakis et al., 2009).

Recently, plant protoplasts have been used in application of genome editing (Xie et al., 2013; Subburaj et al., 2016; Woo et al., 2015). Site directed mutagenesis of genome is carried out using sequence-specific nucleases and it represents the new frontier of plant breeding to improve plant with novel traits. The emerging tool used for genome editing is represented by the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) system. This system has been tested in many species, including tomato (Pan et al., 2016), wheat (Zhang et al., 2016; Liang et al., 2017), rice (Xie et al., 2013), petunia (Subburaj et al., 2016), maize (Svitashev et al., 2016) and grapevine (Ren et al., 2016). It requires only a common protein Cas9 and a single guide RNA (sgRNA); the sgRNA guides Cas9 to recognize and cleave target DNA. Type-II CRISPR/Cas9 system is widespread in bacteria and archea. It mainly acts as a defense system against invading DNAs viral and plasmid using RNA-guided endonuclease (RGEN) activity. The genome editing in plants by CRISPR/Cas9 system can be performed using *Agrobacterium tumefaciens* as delivery vector (Ren et al., 2016; Pan et al., 2016), using non-integrating plasmids transfected into plant cells to deliver nucleases (Xie et al., 2013; Zhang et al., 2016;) and by delivery of preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) (Woo et al., 2015; Subburaj et al., 2016; Svitashev et al., 2016; Zhang et al., 2016). The use of *Agrobacterium* causes the presence of foreign DNA in edited genome while the non-integrating plasmids can be digested by endogenous nuclease with insertion of fragments in plant genome. Only with RNP, the recombinant DNA is completely absent from edited genome. In this way, the genome-edited plant might be excluded from genetically modified organism (GMO) regulations in plants because no foreign DNA is introduced. The

delivery of RNP can be performed by particle bombardment of embryogenic tissue (Zhang et al., 2016; Svitashev et al., 2016) or by PEG-mediated transfection of protoplast (Woo et al., 2015; Subburaj et al., 2016); however, the application of protoplasts avoids the regeneration of chimeric structure. The use of protoplasts and RNP seems to represent one of the best solutions to apply genome editing in plants.

Nevertheless, most of these studies require the regeneration of entire plants from protoplasts. Plant cells are totipotents: from fully differentiated, non-dividing cells, protoplasts or somatic cells are able to dedifferentiate, re-enter the cell cycle and proliferate until to regenerate the whole plant. The plant regeneration process from a protoplast can be divided into 4 main phases: reformation of new cell wall, cell elongation and first cell divisions, continued cell divisions to micro- and macrocallus callus formation, and plant regeneration by direct organogenesis or somatic embryogenesis (Papadakis et al., 2009). These processes are very difficult, the efficiency is very low and most of agronomically important plants, including grapevine, are recalcitrant to regeneration. Grapevine (*Vitis vinifera L.*) protoplasts have been obtained from many sources of plant material (leaves, roots, mesocarp) and used for many studies, but the regeneration of plants has been successfully performed only in two cultivars Seyval Blanc and Koshusanjaku; the protoplasts of these varieties were isolated from embryogenic tissue (Reustle et al., 1995; Zhu et al., 1997). Therefore, it is necessary to identify an efficient system of plant regeneration from grapevine protoplast.

In this chapter, we describe the plant regeneration from embryogenic calli-derived protoplasts of two grapevine Italian cultivars, Garganega and Sangiovese, and the application of a protocol of PEG-mediated transfection. The results obtained provide the possibility to perform many studies in grapevine, including genome editing. This technology has been successfully applied in *Vitis Vinifera*, using both protoplast transfection (Malnoy et al., 2016) and *Agrobacterium*-mediated transformation of embryogenic calli (Ren et al., 2016), but the regeneration of edited plants was obtained only in the second case, with the disadvantages previously described. The application of genome editing using protoplasts with plant regeneration has so far been performed only in other plants (Woo et al., 2015).

2. MATERIALS AND METHODS

2.1 Plant material and induction of embryogenic culture

Embryogenic cultures of Garganega and Sangiovese cultivars were initiated and maintained as described in Chapter 3, section 2.1.1.

2.2 Protoplast isolation and culture

The isolation and cultivation of protoplasts were performed as described in Zhu et al., 1997. Briefly, protoplasts were isolated from embryogenic calli of 7-10 days of subculture in $C1^P$ medium. Embryogenic calli were incubated in filter-sterilized digestion solution (10 mL for 1 gram of embryogenic material) containing 2% w/v Cellulase Onozuka, 1% w/v Macerozyme R-10, 0.05% w/v Pectolyase Y-23, 10 mM $CaCl_2*2H_2O$, 5 mM MES and 0.5 M mannitol [pH=5.7] on a gyratory shaker. After six hours of incubation, the mixture was filtered on nylon sieve (60 µm) and then protoplasts were washed twice with washing solution (10 mM CaCl2*2H2O and 0.5 M mannitol). The viability of the protoplasts was tested under UV light after staining with 0.5 mg/mL fluorescein diacetate (FDA). Then, isolated protoplasts were cultivated at $1*10^5$ protoplasts/mL with disc-culture method; in this method, droplets (800 µL) containing protoplasts in solid Nitsch's medium supplemented with 2 mg/L 1-naphthaleneacetic acid (NAA), 0.5 mg/L 6-benzylaminopurine (6-BAP), 0.3 M glucose, 0.09 M sucrose and 2 g/L gellan gum, pH=5.7, were poured in Petri dishes. After solidification, liquid Nitsch's medium with the same composition but supplemented with 0.3% activated charcoal was added as a reservoir. The liquid medium was replaced every two weeks by fresh medium described above but lacking glucose. Cultured protoplasts were maintained at 28°C.

2.3 Somatic embryogenesis

After three-four 4 months of culture, protoplast-derived cotyledonal somatic embryos were transferred in solid Nitsch's medium supplemented with 30 g/L sucrose and 2 g/L gellan gum [pH=5.7] and maintained in the dark for 4 weeks to allow complete germination.

2.4 Embryo development and regeneration of whole plants

Embryo development and plant recovery were performed following the method described in Li et al., 2014. Germinated somatic embryos were transferred to C2D4B medium (C2D medium supplemented with 30 g/L sucrose, 4 µM 6-BAP and 7 g/L TC agar, [pH=5.8]) and maintained under light (65 µE, 16-h photoperiod and 25°C) for 3-4 weeks. Plantlets were transferred to MSN medium (MS medium containing 30 g/L sucrose, 0.5 µM NAA and 7g/L TC agar, [pH=5.8]) in order to promote elongation of roots and development of the whole plant. Vigorous plants were transplanted to potting soil and acclimated in a growth room for about 2 weeks before transfer to the greenhouse.

2.5 Protoplast PEG-mediated transfection and YFP fluorescence analysis

Protoplast transfection was performed as described in Woo et al., 2015. A mixture of 5×10^5 protoplasts of Garganega cv re-suspended in 200 µl MMG solution (0.4 M mannitol, 15 mM MgCl2, 4 mM MES [pH 5.7]) was gently mixed with 50 µg of a vector carrying the *yfp* marker gene (pEGB3Ω1-35S::YFP::Tnos; Sarrion-Perdigones et al., 2013) and 210 µl of freshly prepared PEG solution (40% [w/v] PEG 4000 (SIGMA) 0.2 M mannitol and 0.1 M $CaCl_2*2H_2O$), and then incubated at 25°C for 20 min in darkness. After incubation, 950 µL W5 solution (2 mM MES [pH 5.7], 154 mM NaCl, 125 mM $CaCl_2*2H_2O$ and 5 mM KCl) was added slowly. The resulting solution was mixed well by pipetting. Protoplasts were pelleted by centrifugation at 100g for 3 min and re-suspended gently in 1 mL WI solution (0.5 M mannitol, 20 mM KCl and 4 mM MES [pH 5.7]). Finally, the protoplasts were transferred into multi-well plates and cultured under dark conditions at 25°C. The YFP expression in transfected protoplasts was monitored 24, 48 and 72 hours post transfection by using a LEICA stereomicroscope (MZ 16 F) equipped with a LEICA light source (CLS 150 X) and YFP filter set composed of an excitation filter (500/20 nm) and a barrier filter (535/30 nm).

3. RESULTS

3.1 Protoplast isolation

Protoplasts of both Garganega and Sangiovese cultivars were isolated from embryogenic calli of 7-10 days of subculture in $C1^P$ medium (Figure 1A). The quantity of embryogenic material

used was different between the cultivars: 0.5 g for Garganega and 0.2 g for Sangiovese. During the subculture, many embryogenic calli of Sangiovese browned and they haven't been used in protoplast isolation. Consequently, also the yields obtained were different: $5*10^6$ protoplasts for Garganega and $2*10^6$ protoplasts for Sangiovese. The viability of isolated protoplasts of both cultivars was more than 80% as evaluated with FDA staining (Figure 1B).

3.2 Cell division, microcolonies formation and somatic embryogenesis

Isolated protoplasts were cultivated at $1*10^5$ protoplast/mL by using the disc-culture medium. The droplets of solid culture medium containing protoplast are surrounded by liquid culture medium supplemented with activated charcoal, which was essential to avoid the browning of the culture and to allow the cell division and the colonies formation. The first cell division of protoplast of both cultivars occurred after ten days from isolation (Figure 1C, D). Further cellular divisions occured after 3-4 weeks of culture (Figure 1E, F) while microcolonies formation (Fig. 1G, H) of both Garganega and Sangiovese cultivars occur after 8 weeks from protoplast isolation, suggesting that embryogenesis was not induced directly from protoplasts but rather from the protoplast-derived callus. Mature cotyledonary embryos (Figure 1I) were formed starting 3-4 months after the protoplast culture. These results are similar with the results described by Zhu et al., 1997 and Reustle et al., 1995. After 4-5 months, the number of regenerated torpedo embryos was 87 for Garganega and 78 for Sangiovese.

3.3 SE germination and plant regeneration

To complete germination, cotyledonary embryos were maintained in the dark in Nitsh's medium supplemented with sucrose to further development. The direct transferring of small embryos from culture medium to Nitsh's medium under light has caused browning and finally their death. Germinated somatic embryos (Fig. 1L) were then transferred in C2D4B medium under light to germinate. The number of germinated embryos obtained was 55 for Garganega and 33 for Sangiovese. The remaining somatic embryos weren't developed enough, or they were completely deformed. Shoot elongation (Figure 1M) from germinated embryos generally occurred within 5 weeks from the transferring under light, but in some cases the time necessary is longer (8-10 weeks). Then, 21 plantlets of Garganega and 9 for Sangiovese were transferred to MSN medium to allow root elongation and further plant development. The germinated embryos developed into whole plants with expanded leaves and roots (Fig. 1N) after 4 weeks.

Finally, protoplast-derived regenerated plants (Fig. 1N) were 21 for Garganega and 7 for Sangiovese. After acclimation, regenerated plants were transferred to the greenhouse. Plants of both cultivars showed normal growth and morphology (Fig. 1O).

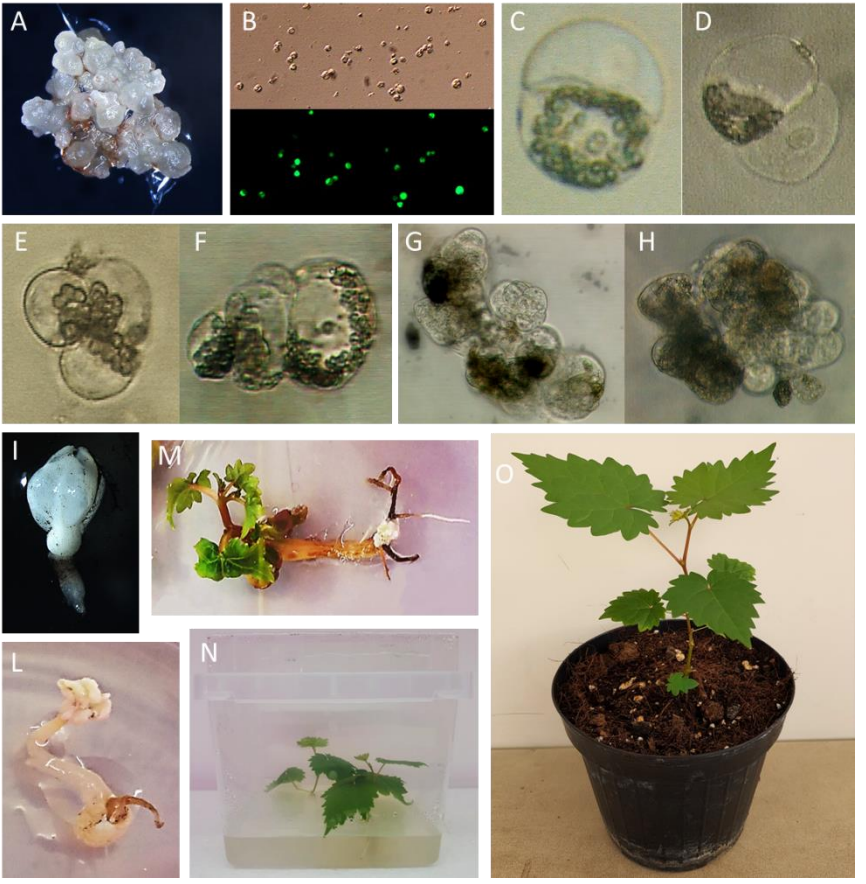

Figure 1: plant regeneration from embryogenic calli-derived protoplasts. A: embryogenic calli of 7-10 days of subculture; B: isolated protoplasts labeled with FDA and observed under visible light and UV light; C-D: first cellular division 10 days from isolation; E-F: further cellular division; G-H: microcolonies formation 8 weeks after the isolation; I: regenerated torpedo somatic embryo; L: mature well-developed somatic embryo; M: germinated somatic embryo; N: in-vitro regenerated plant; N: regenerated protoplast-derived plant after acclimation and transferring in the greenhouse.

3.4 Protoplats PEG-mediated transfection and YFP expression

The PEG-mediated transfection was performed only using protoplasts of Garganega, the variety characterized by higher efficiency of somatic embryos and plant regeneration. The efficiency of

protoplast transfection was validated with *YFP* marker gene. The transfection was carried out using 50 µg of a plasmid (pEGB3Ω1-35S::*YFP*::TNOS) containing a cassette for YFP overexpression, 40% PEG 4000 and $1*10^5$ protoplasts. The YFP expression was analyzed 24, 48 and 72 hours post transfection. The fluorescence microscopic analysis (Figure 2) shows that the YFP expression takes place after 24 hours and continues also after 48 and 72 hours post PEG-mediated transfection. Transfected protoplasts show a homogeneous YFP expression and there is no increase in fluorescence emission from 24 to 72 hours post transfection. The absence of fluorescence signal in negative control (protoplasts transfected with empty vector) confirms the successful of the transfection (Figure 2).

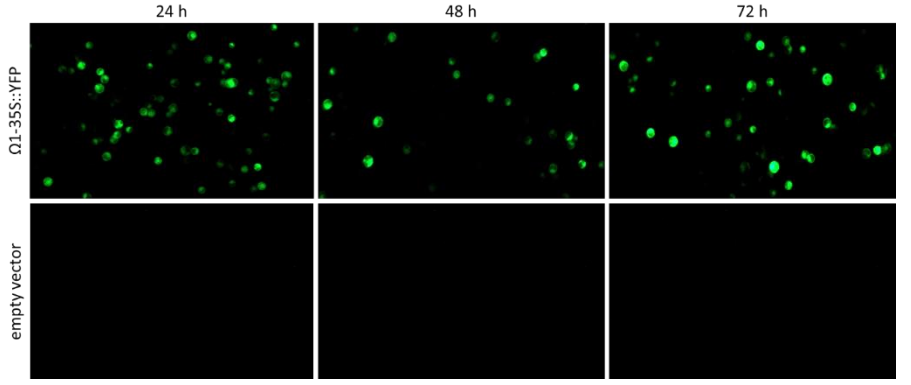

Figure 2: protoplast PEG-mediated transfection. The microscopic fluorescence analysis was performed 24, 48 and 72 hours post transfection. Transfected protoplasts show a homogenous YFP expression.

4. DISCUSSION

Plant protoplasts represent a versatile system used for many studies, from cell structure and subcellular localization to gene functional analysis and somatic hybridization by protoplast fusion. Furthermore, protoplasts have been tested in recent applications of genome editing by using the CRISPR/Cas system (Xie et al., 2013; Subburaj et al., 2016; Woo et al., 2015). This genome editing technology represent the emerging tool of plant breeding to improve traits in plants. One of the main advantages of this method is the possibility to delivery in plant ribonucleoproteins (RNP, preassembled Cas protein and guide RNA; Woo et al., 2015; Subburaj et al., 2016; Svitashev et al., 2016; Zhang et al., 2016); in this way the foreign DNA is completely absent, and the edited plant obtained could avoid the current GM regulations. Moreover, the

use of protoplasts ensures the absence of chimeric plants. The only disadvantage is the recalcitrance to many plant species, including grapevine, to regenerate plants from protoplast. Grapevine protoplasts have been isolated from various organs, but the regeneration of whole plants has been achieved in only a handful of cases and only when the protoplasts were isolated from embryogenic tissue (Reustle et al., 1995; Zhu et al., 1997). These regeneration protocols have not been widely adopted because they tend to be inefficient and highly genotype dependent, which is challenging in a species renowned for its huge range of cultivars.

The results described in this chapter show how whole plants can be regenerated from protoplasts of Garganega and Sangiovese, two Italian cultivars of grapevine. The method is based on the protocol described by Zhu et al, 1997. Protoplasts of both cultivars were isolated from embryogenic calli of 7-10 days of subculture; isolated protoplasts thrive and are able to divide and form microcolonies. The cultivation in solid medium, by disc culture method, and the presence of activated charcoal to adsorb contaminants released from plant cell, have been essential to ensure the survival of the protoplasts and their regeneration into somatic embryos. The number of regenerated cotyledonary embryos are similar for both cultivars, but the development of germinated somatic embryos, shoot elongation and plant regeneration were more efficient in Garganega. The protocol of plant regeneration from somatic embryos was based on the method described by Li et al., 2014. The presence of 6-BAP in C2D4B medium was essential for shoot elongation of germinated somatic embryos, while the presence of NAA in MSN medium was indispensable to induce root elongation and further plant development. The regenerated protoplast-derived plants show a normal growth. The efficiency is not very high, but the results obtained are prominent and these methods could be applied to other grapevine cultivars. Furthermore, the protoplasts of Garganega have been used to test PEG-mediated transfection. Most transfected protoplasts show high and uniform YFP expression also after 72 hours post transfection. However, there are no differences in the number of protoplasts transfected and in the YFP expression from 24 to 72 hours post transfection.

These results indicate that grapevine protoplast technology is a prominent approach and it can be used for many biotechnological applications, including genome editing for the introduction of targeted genetic changes with unprecedented control and accuracy. Genome editing has been successfully applied in grapevine by the transfection of protoplasts with standard guide RNA/Cas9 vectors, by the direct introduction of guide RNA/Cas9 ribonucleoproteins (Malnoy et

al., 2016; Osakabe et al., 2018) and by the transformation of embryogenic callus with A. tumefaciens (Ren et al., 2016). These studies have shown that the protoplast transfection (with standard vectors or ribonucleoproteins) is likely to be the most effective genome editing approach in grapevine because regenerating plants from a single transformed or edited cell avoids the formation of chimeric regenerants, which is a common problem when the target is a multicellular tissue such as callus. However, the regeneration of genome-edited plants from transformed protoplasts has yet to be reported. Therefore, this improved protocol for the regeneration of grapevine plants from protoplasts through embryogenesis may address the limitations encountered in previous attempts to generate genome-edited plants from protoplasts.

5. REFERENCES

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Chapter 7

GENERAL DISCUSSION

In the last years, climate changes, characterized especially by temperature increase which determines the anticipation of ripening and consequent negative effects on grape quality, e.g. excess in sugar levels, reduction of berry size and coloration, loss of aroma and flavor compounds, represent the main concern of viticulturists and global wine industry. Specific agronomic practices developed to mitigate such negative effects have been proposed (Palliotti et al., 2012), but their complexity and their high costs, make difficult their application on a large scale. The identification of the molecular mechanisms controlling the ripening process could allow the set-up of more specific intervention strategies. Recent molecular studies have highlighted a profound transcriptomic shift during the immature-to-mature transition in grapevine (Fasoli et al., 2012) and the existence of a specific group of genes, highly expressed only in mature organs/tissues, named switch genes, represented especially by transcription factors, that could perform a specific role during this phase transition (Palumbo et al., 2014; Massonnet et al., 2017). Furthermore, some of these transcription factors have been identified as markers of two rapid transitions (i.e. the fast upregulation/downregulation profiles of two groups of genes) during *veraison* (Fasoli et al., 2018), suggesting that they could act as master regulators of the onset of berry ripening, and/or of specific developmental/metabolic features of the ripening process in grapevine. The interpretation of their specific roles could better define the molecular mechanisms underlying the maturation process.

Five of these transcription factors, *VviNAC33*, *VviNAC60*, *VviAGL15*, *VviWRKY19* and *VvibHLH75*, have been selected for functional characterization. Preliminary information about the roles of these genes has been obtained by analyzing their expression profiles, using both the grapevine expression atlas (Fasoli et al., 2012), a transcriptome dataset of both red and white varieties (Massonnet et al, 2017) and the very detailed transcriptomic map of Cabernet Sauvignon and Pinot Noir berry ripening recently published by Fasoli et al. (2018). A co-expression analysis was also performed on the grapevine expression atlas dataset. The expression analysis showed that these genes are mainly transcribed in mature organs, consistently with their proposed role of

switch genes. Furthermore, excluding *VviAGL15*, the selected transcription factors are characterized by a strong induction just before *veraison*, suggesting their involvement in the regulation of the onset of berry ripening. The co-expression analysis have highlighted that many of the genes co-expressed with each candidate are involved in processes associated with ripening, including hormone and sugars signaling, cell wall metabolism and senescence. Among the co-expressed genes were many transcription factors. These preliminary results suggest an involvement of the selected genes in the regulation of the onset of ripening and the existence of a complex transcriptional regulatory network controlling the whole ripening process; however, they do not provide information about the precise role of each of these candidates in the control of the onset of ripening or details about the specific relationships with their putative target genes. Therefore, a more in-depth functional analysis of the selected transcription factors was initiated.

The functional characterization of the five selected transcription factors has been performed using stable genetic transformation and leaf agroinfiltration approaches. The first system has been used for *VviNAC33* and *VviNAC60*, while the second method has been used for *VviAGL15*, *VviWRKY19* and *VvibHLH75.* Regarding both NAC genes, in a previous work they have been overexpressed in Shiraz plants by stable transformation (D'incà, 2017). *VviNAC33* overexpressing plants showed altered chlorophyll metabolism and anticipated leaf senescence, while the overexpression of *VviNAC60* impaired the normal plant development, with smaller leaves and stunted growth. To obtain more information about their functions, in this work both NAC genes have been converted into transcriptional repressors by fusion with EAR motif and stably expressed in grapevine plants of both Shiraz and Garganega cultivars under the control of their endogenous promoter. Regenerated transgenic plants showed a normal development and the absence of aberrant characteristic found in the overexpressing plants. Each NAC-EAR chimeric repressor driven by the gene native promoter should be expressed only in the organs and at the developmental stage when the endogenous NAC gene is expressed: excluding berries, another organ characterized by high expression of both NAC genes is represented by the fully expanded leaf. After the confirmation of the expression of each NAC-EAR gene in fully expanded leaves, the analysis of the expression of putative target genes of both NAC genes has been performed. Real time qPCR showed that *VviNAC17* and *NITRATE TRANSPORTER 3,* putative targets of *VviNAC33,* and *VviWRKY16*, *VviNAC26* and *GALACTINOL SYNTHASE* putative targets

of *VviNAC60*, are characterized by an expression level lower in each respective transgenics than WT plants. These results suggest that the fusion with the EAR motif converted the NAC (putative) transcriptional activators into transcriptional repressors and allowed to confirm putative target genes of both NAC transcription factors. These preliminary results are consistent with a role of *VviNAC33* and *VviNAC60* in the hierarchical network controlling the vegetative-to-mature transition in grapevine.

Regarding *VviAGL15*, *VviWRKY19* and *VvibHLH75*, they have been functionally analyzed by transient overexpression in Thompson Seedless leaves followed by microarray analysis of overexpressing leaf tissues. The results of this analysis showed that for all three transformations most of DEGs are up-regulated. Thus, assuming that the three factors act primarily as transcriptional activators, the following analyses were performed using exclusively the up-regulated genes. Most of the genes up-regulated upon the overexpression of each factor in the leaf belong to functional categories such as carbohydrate metabolic process, cellular homeostasis, response to hormone stimulus, secondary metabolic process, signal transduction and transcription factor activity, suggesting an involvement of the selected transcription factors in the regulation of these processes. The expression profile of these genes was evaluated in different tissues (flesh, skin and seed) during berry development to select those that may represent target of each factor in the berry. Some *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASEs*, involved in the rearrangement of cell wall, one ERF/AP2 gene (*VviERF045*), involved in the ethylene signaling, and some genes involved in the carbohydrate metabolic processes/sugar signaling, were upregulated by *VviAGL15.* The analysis of the expression of these genes during berry development showed that they are preferentially expressed during *veraison* or ripening, strongly suggesting that their expression is truly regulated by *VviAGL15*. The direct activation of one *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 23* (VIT_11s0052g01330) and *VviERF045* by *VviAGL15* has been confirmed by Dual Luciferase Reporter Assay. Regarding genes upregulated by *VviWRKY19,* two encoded XYLOGLUCAN ENDOTRANSGLUCOSYLASEs, several belonged to flavonoid and terpenoid biosynthesis category and some were involved in carbohydrate metabolic processes/sugar signaling and cellular homeostasis categories. Moreover, unlike for *VviAGL15*, many transcription factors, including many NAC genes and three LATERAL ORGAN BOUNDARIES proteins, turned out to be up-regulated by *VviWRKY19,* some of which were

classified as switch genes of berry vegetative-to-mature transition (Palumbo et al., 2014). Many genes upregulated by *VviWRKY19* in leaves are normally expressed in berry during *veraison* or ripening. These data strongly suggest that among the genes up-regulated by *VviWRKY19* in the leaf, many targets are involved in the regulation of specific processes associated with berry ripening. Finally, the overexpression of *VvibHLH75* induced genes mainly involved in cell wall metabolism, cellular homeostasis and sugar signaling. Furthermore, many upregulated genes were involved in flavonoid and stilbene biosynthesis. Among the transcription factors there were two NACs, *VviNAC26* and *VviNAC33*, two LATERAL ORGAN BOUNDARIES DOMAINs, *VviLOB15* and *VviLOB38*, and, very interestingly, *VviWRKY19*. Many *VvibHLH75* targets are normally expressed during berry ripening and some were classified as switch genes by Palumbo et al. (2014), suggesting that they participate in the regulation of processes associated with berry ripening. Moreover, *VvibHLH75* seems to play a particularly important role in the ripening transcriptional regulatory network, regulating the expression of many other transcription factors. Altogether, these interesting preliminary results obtained in leaves seem to indicate a direct involvement of *VviAGL15*, *VviWRKY19* and *VvibHLH75* in the regulation of many processes related to maturation, consistent with their proposed role of master regulators of the onset of berry ripening. Furthermore, these data have provided important information about the hierarchical aspects of the different transcription factors during the transcriptional regulatory network of berry ripening.

In grapevine, the most important methodologies for gene functional analysis are stable genetic transformation and transient gene expression. However, grapevine is a very recalcitrant species and the transformation efficiency is relatively low. Consequently, the development, the application and the improvement of these methods has paralleled and supported the functional characterization of the selected candidates. Regarding stable genetic transformation, three different protocols based on those described in Cavallini (2012), Torregrosa et al., (2015) and Li et al., (2008) have been tested in three different cultivars, Garganega, Sangiovese and Shiraz, using EHA105 as Agrobacterium strain and a plasmid carrying a cassette for GFP overexpression. The main difference among these protocols is the embryogenic material used for transformation (embryogenic calli for protocols 1 and 2 and somatic embryos for protocol 3), but some other parameters, including different Agrobacterium OD600 and media, were analyzed. The results showed that during the first phases of transformation for each protocol

there was a high GFP expression, but the fluorescence signal considerably decreased after many days post transformation for all the cultivars with the exception of Sangiovese, which was characterized by the complete necrosis of both tissue tested and by the complete absence of GFP expression. For this reason, Sangiovese has not been used in the following phases of transformation. These preliminary results indicate that in grapevine the success of stable transformation is cultivar-dependent. Regarding Garganega and Shiraz, after many months post-transformation, stably transformed GFP-expressing somatic embryos have been regenerated only using two out of the three tested protocols, suggesting that this system is strongly affected by the embryogenic tissue used. However, the recovery of transgenic plants has been obtained only from some somatic embryos: the number of putatively independent transgenic lines was 5 and 1 for Shiraz transformed with protocol 1 and 2, respectively, and 2 and 3 for Garganega transformed with protocol 1 and 2, respectively. To confirm the stable integration of the transgene, further molecular analysis will be performed. These results indicate that the efficiency of transformation, in terms of number of regenerated transgenic plants, is quite low but they indicated that two different cultivars can be transformed with different protocols.

Regarding transient gene expression, two different approaches have been tested: leaf agroinfiltration of whole plants grown in vitro, and berry agroinfiltration, using fruiting cuttings-derived berries. Grapevine leaf agroinfiltration has been already tested in grapevine (Santos-Rosa et al., 2008; Bertazzon et al., 2012; Zottini et al., 2008; Kurth et al., 2012) while one single report (Gao et al., 2018) describes fruit agroinfiltration in grapevine. Both agroinfiltration methods have been performed using an Agrobacterium strain carrying a cassette for YFP overexpression. The leaf agroinfiltration approach has been tested using different grapevine cultivars (Thompson seedless, Garganega and Shiraz) and a vacuum system. The qualitative analysis of YFP expression showed that it is especially localized in the first and second leaves from apex in each cultivar but that the day-post-infiltation (d.p.i.) of maximum expression is different from one cultivar to another. Further molecular analysis will be performed to identify and confirm the d.p.i. of maximum expression. Overall, these results showed that leaf agroinfiltration is a very useful and rapid method and that it could be successfully used for functional analysis of genes of interest. Regarding berry agroinfiltration, this approach has been tested because many genes of interest are expressed only in berry and their functional analysis

performed in the expression tissue could give more reliable information. The analysis of YFP transient expression in berries obtained from fruiting cuttings of Cabernet Sauvignon showed that the fluorescence signal is higher at 3 d.p.i. than at 6, and higher in detached vacuum agroinfiltrated berries than in berries infiltrated using a syringe with needle. However, the efficiency of this method remains quite low: only 20 out of 50 berries showed YFP expression. These results are encouraging to further improve this approach, testing for example different cultivars or using different Agrobacterium strains. After the identification of the best parameters, this method could be used for the functional analysis of genes of interest directly in the berry.

Finally, an efficient protocol for the regeneration of whole plants from protoplasts isolated from embryogenic callus has been developed for two Italian wine grapevine cultivars The protoplasts were cultivated using the disc-culture method (Zhu et al., 1997) at a density of 1x105 cells/mL and were regenerated by first encouraging them to form somatic embryos. The first cell division occurred ~10 days after protoplast isolation, microcolonies appeared after ~1 month, and cotyledonal somatic embryos were observed after ~3 months. A critical step was the maintenance of cotyledonary embryos in the dark for 1 month before transfer to shoot elongation medium because this allowed the embryos to complete germination and thus to become competent for further development. Germinated somatic embryos were transferred to the light for shoot elongation followed by root elongation and growth, resulting in the recovery of whole plants ~6 months after protoplast isolation. The protoplasts were amenable to PEG-mediated transfection, indicating that the combination of transfection and our new regeneration procedure can be used for the application of biotechnological approaches such as genome editing in a wider range of grapevine cultivars than previously envisaged.

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