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TRANSCRIPTIONAL REGULATION OF STOMATAL RESPONSES TO STRESS: FROM A MODEL SYSTEM TO *Vitis vinifera*

SUPERVISOR. PROF. OSVALDO FAILLA

CO-SUPERVISOR. DOTT. MASSIMO GALBIATI

Ph.D. Thesis of:

FABIO SIMEONI

MATRICOLA R09732

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ABSTRACT

Grapevine (*Vitis spp*.) is a fruit crop traditionally subjected to moderate or severe water stress. Vitis species adapt well to drought conditions due to good osmotic adjustment, but the strength and timing of these responses varies between different cultivars and major differences in water stress tolerance can be found among species or hybrids from the Vitis genus. These genotype-related variations involve different aspects of the physiology of the plant, including differences in stomatal conductance (g_s, mmol H_2O $m^{-2}s^{-1}$). Stomatal conductance is a key trait in grapevine, as it directly determines the isohydric/anisohydric behaviour displayed by different genotypes.

Stomata are small pores on the surfaces of leaves and stems, surrounded by a pair of guard cells that control the exchange of gases between the atmosphere and the interior of the leaf. This allows the plant to cope with the conflicting needs of ensuring adequate uptake of CO_2 for photosynthesis and preventing water loss by transpiration. Moreover stomata play an active role in plant defence, restricting bacterial invasion as part of the plant innate immune system.

Goal of this work is to investigate the molecular basis of stomatal responses, through the analysis of the transcriptional changes occurring in guard cells (GCs) in response to biotic and abiotic stress.

Among GC related genes, the transcription factor *AtMYB60* has been shown to play a pivotal role in stomata opening. Arabidopsis loss of function *atmyb60-1* lines are characterised by closer stomata and enhanced survival when subjected to lethal drought conditions. Most importantly, *AtMYB60* from Arabidopsis and its grape counterpart, *VvMYB60*, display an exceptionally high degree of sequence identity and a conserved function in GCs.

We focused on the plant model system Arabidopsis in order to gain more insights into the transcriptional mechanisms that regulate stomatal activity. Next, we analysed the transcriptional changes occurring in GCs in response to water stress in different grapevine genotypes and different grafted combination.

In the first part of this thesis we focused on technological aspects of stomata analysis. In order to improve the current methods employed to investigate stomata activity, usually performed with epidermal peel, we developed a semi-automatic confocal microscopy technique that allows measuring stomatal opening in intact leaf samples over extended periods of time. We successfully confirmed the sensibility of this approach in Arabidopsis, testing light induced stomatal aperture and ABA induced stomatal closure. The same approach was used to investigate the role of *AtMYB60* in response to pathogen associated molecular pattern (PAMPs). The treatment with flg22 and LPS on WT and *atmyb60-1* revealed that *AtMYB60* is not involved in stomatal closure in response to these PAMPs.

In order to improve the accuracy of transcriptional analyses in GCs, two different approaches have been employed for the purification of RNAs specifically from GCs of both Arabidopsis and grapevine. Laser micro-dissection (LMD) is the most accurate technique to obtain RNA samples from pure preparation of single cell types. This approach allowed us to compare GCs transcript with mesophyll cells transcript. As an alternative to LMD, we have adopted a mechanical disruption protocol of the leaf tissues to obtain epidermal preparations enriched in GCs. Both these approaches revealed enhanced expression of GC marker genes proving that a GC-enrichment occurred. LMD guaranteed the high purity of GCs sample, whereas the blender method allowed obtaining intact GCs in a short period of time and in relatively large amounts.

We first focused on the analysis of the Arabidopsis *atmyb60-1* mutant to gain more insights into the mechanisms by which *AtMYB60* mediates stomatal activity. Metabolomic analysis of lipids accumulation in whole leaves or in purified GCs, revealed an increased accumulation of oxylipins in *atmyb60-1* mutant plants compared with the WT. Moreover the accumulation was higher in GCs

of the mutant compared to WT GCs. Interestingly, oxylipins have been recently shown to directly promote stomata closure in response to both drought and pathogen attack.
qPCR analysis of the expression of genes involved in oxylipin biosynthesis did not uncovered significant differences in transcript levels between WT and mutant plants. This suggests a possible indirect and more complex role of AtMYB60 transcription factor in regulating oxylipins accumulation in GCs.
Transcriptomic analysis suggested a possible involvement of *AtMYB60* in the salicylic acid (SA)-mediated innate immune response in GC. Indeed we demonstrated that SA-induced stomatal closure was impaired in the *atmyb60-1* mutant. Consistently, analysis of gene expression in laser micro-dissected GCs, revealed that SA-regulated genes, as *Pathoghenesis Related Protein*s *1*, were less activated in the mutant compared to WT.
VvMYB60 was already shown to represent a true ortholog of *AtMYB60*, mainly based on experiments performed in an heterologous system. Expression profiling of LMD-purified samples confirmed its guard cell specificity in grapevine as well. Analysis of *VvMYB60* expression in different rootstocks highlighted a positive correlation between the level of gene expression and the regulation of stomatal conductance in response to water stress. Comparative analysis of M4 and 101.14 disclosed interestingly results; data showed a strong down-regulation of *VvMYB60* expression in 101.14 leaves compared to M4. This correlated with an increased stomatal closure of 101.14 under drought stress. To get more insight into guard cell gene regulation, the genes involved in the regulation of the ABA pathway have been analyzed. More into details, genes involved in ABA-synthesis and ABA-mediated responses to drought have been assessed, including the PYL/RCAR receptor gene family, the PP2C protein phosphatases, the SnRK protein kinases and guard cell-related downstream targets, in different rootstocks under normal and water stress conditions.
Analysis of the ABA synthesis marker gene *VvNCED1*, showed a general up-regulation in response to water stress. Extensive analysis of ABA receptor genes in grape revealed a high degree of variability among *VvRCARs* under drought stress. However, the *VvRCAR* family showed a general down-regulation in most genotypes analysed, with the exception of *VvRCAR3*, whose expression resulted mostly up-regulated. Most PP2C genes were generally up-regulated under drought stress. *VvPP2C24*, putative ortholog of *AtABI1,* was strongly up-regulated in almost all the genotypes. We found a positive correlation with ABA synthesis, through the analysis of *VvNCED1*, and ABA perception, in particular with *VvPP2C24* gene expression.
Contrary to the expected, *VvERA1,* a positive regulator of ABA signalling, showed little variations under drought stress. In particular, *VvERA1* resulted more up-regulated in K5BB and in M4 compared to 1103P and 101.14. This together to *VvMYB60* expression, *VvERA1* behaviour explains the enhanced stomatal responses disclosed by 1103P and 101.14 compared to K5BB and M4.
The positive correlation of the guard cell-specific genes (*VvMYB60* and *VvSIRK*) with ABA related genes could contribute to the different phenotypic responses shown by different genotypes under water stress. Moreover, some genotypes disclosed an ABA independent response. However these genotypes showed a down-regulation of *VvMYB60* and decrease of g_s. Thus, like in Arabidopsis, the expression of *VvMYB60* in grape could be modulated by both ABA-dependent and ABA-independent pathways.
Grapevine rootstocks can confer resistance to various pathogens and tolerance to abiotic stresses. The transport from the root to the scion through the xylem of chemical signals (including ABA) in the early stages of water-deficit reduces leaf transpiration and restrains leaf growth. Little is known about the influence of the rootstocks on the regulation of the expression of GC-related genes in the scion.
Both M4 and 101.14 showed a constitutive up-regulation of ABA biosynthetic and signalling genes in leaves from grafted plants (M4/M4; 101.14/101.14) compared with ungrafted plants. This could suggest a possible stress condition induced by the graft itself.

Moreover the comparison of CS grafted on M4 and 101.14 showed that the regulation of ABA synthesis (*VvNCED1*) is independent by the rootstocks, in agreement with the existence of a cell-autonomous ABA biosynthetic pathway in GC. However, ABA perception was affected by the genotype of the rootstock. *VvPP2C24* was more up-regulated in GC from CS/M4 and M4/M4 compared to CS/101.14 and 101.14/101.14. Moreover *VvMYB60* displayed a similar rootstock-dependent response.
Finally, in order to analyze the role of *VvMYB60* in biotic response we performed a SA treatment on grapevines plants. Preliminary analysis showed a down-regulation of *VvMYB60* suggesting a similar behavior for both the Arabidopsis and grape *MYB60* genes.

Introduction

Grapevine is grown widely throughout the world and grape production is in the top ranking agriculture crop in many countries (Bisson *et al.*, 2002). According to FAOSTAT there were 67,067,128.92 tonnes of grapes produced on 6,969,373.10 ha in 2012 (http://faostat.fao.org; latest available).

Grapevine is generally a stress tolerant plant due to its deep root system and its physiological mechanism of drought avoidance (Chaves *et al.*, 2010). However the combined effects of intensive illumination, high temperatures and low atmospheric water pressure tension could presumably act as major constraints for leaf photosynthesis, particularly under conditions of severe soil water deficits that are usually encountered by this crop (Pavlousek, 2011). Moreover the frequency of extreme events such as heat waves or heavy rains is also predicted to increase, with negative effects on yield and quality of grapes (Chaves *et al.*, 2010). With an increase in aridity predicted in the near future according to global climate models, water deficits may become a limiting factor in wine production and quality (Chaves *et al.*, 2010). The strength and timing of drought responses varies between different cultivars and major differences in water stress tolerance can be found when compared to other species or hybrids from the Vitis genus (Flexas *et al.*, 2009). Although these genotype-related variations involve different aspects of the physiology of the plant, they are largely linked to differences in stomatal conductance (g_s, mmol H_2O $m^{-2}s^{-1}$). The role of stomata in drought response is of preventing water loss by transpiration and, at the same time, ensuring adequate uptake of CO_2 for photosynthesis. Stomata are microscopic pores formed by a pair of specialized cells, namely guard cells (GCs), spread on the surface of aerial parts of most higher plants. Guard cell controls stomatal movement (opening and closure) in response to external (e.g. light, temperature, relative humidity) and internal (e.g. endogenous hormones) stimuli. Guard cells coordinate membrane transport within a complex network of intracellular signals (Willmer and Fricker, 1996; Hetherington, 2001; Shimazaki *et al.*, 2007) to regulate fluxes, mainly of K^+, Cl^2, and malate, driving cell turgor and stomatal aperture (Wang *et al.*, 2014).

One of the earliest responses to drought is the stomatal closure in response to alteration of plant water balance in the roots on in the leaves (Tran *et al.*, 2004). In response to drought, plants can exhibit either drought escape or drought avoidance (Price *et al.*, 2002). Drought escape is described as the ability of plants to complete the life cycle before severe stress sets in. Drought avoidance is ability to increase water uptake and reduce water loss. This behaviour includes reduction of water loss reducing epidermal (stomatal and cuticolar) conductance, reducing radiation absorption, and

reducing evaporative surface (leaf area) (Price *et al.*, 2002). The ecological classification of drought avoidance or drought escape is analogous to the physiological classification into isohydric and anisohydric plants (Schultz, 2003). The determination of the isohydric/anisohydric behaviour displayed by different genotypes is directly linked to stomatal conductance. These differences are due to stomatal control over evaporative demand rather than stomatal density in vegetative tissues (Rogiers *et al.*, 2009).

Guard cells gas regulation has a central role for ecological and biotechnological applications. It has been shown in Arabidopsis that the manipulation of stomatal response, through modifications in guard cell signal transduction elements can reduce transpiration water loss and desiccation during drought periods (Gosti *et al.*, 1999; Hugouvieux *et al.*, 2014; Pei *et al.*, 1998). Moreover, guard cells are a well-developed model system for understanding how components interact within a signalling network in a single cell. They are well suited for dissecting the functions of genes and proteins in signalling cascades. (Schroeder, Kwak, *et al.*, 2001). The complex network of transport and metabolism in guard cells that drives stomatal movements presents a formidable barrier to targeted genetic engineering (Wang *et al.*, 2014). Guard cells respond cell-autonomously to well-known plant physiological signals, including red and blue light, CO_2, plant pathogens, abscisic acid (ABA), auxin, cytokinin and gibberellins, and other environmental signals (Schroeder *et al.*, 2001). Thus many specific receptors and early signalling mechanisms function at the single-cell level in guard cells. Analysis of stomatal opening and closing in response to various stimuli, is a useful strategy to understand mechanisms affecting signal transduction (Schroeder *et al.*, 2001).

ABA is associated with the regulation of many developmental and physiological processes in plants including responses to a number of stresses (Zeevaart and Creelman, 1988). Abiotic stress conditions such as drought-induced ABA biosynthesis initiating the signalling pathways that lead to a number of molecular and cellular responses, such as the expression of stress-related genes and stomatal closure (Lee and Luan, 2012). ABA-induced stomatal closing is mediated by a reduction in the turgor pressure of guard cells, which requires an efflux of potassium and anions, sucrose removal and the conversion of malate to osmotically inactive starch (Schroeder *et al.*, 2001). The ABA signal in guard cells is transduced through a complex network of signalling events, including the production of compounds such as nitric oxide (NO) and H_2O_2, signalling intermediaries such as the guard cell-specific Open stomata 1 (OST1) kinase, changes in cytosolic Ca_2^+ levels, and Ca_2^+ oscillations (Schroeder *et al.*, 2001; Mustilli *et al.*, 2002). Ion channels are responsive at different ABA concentration, this allows a modulation of ion efflux from the guard cells (Hosy *et al.*, 2003). The closure of the stomatal pore is due to ion efflux from the guard cells that drives the efflux of water and results in a change in guard cell turgor (Schroeder *et al.*, 2001). It has been well

established that reductions in soil water availability increase levels of ABA in the xylem and apoplastic sap which, in turn, are associated with decreasing stomatal conductance (g_s) (Dodd, 2003).

In grape, cultivar-specific differences in the plant adaptation to water stress, including stomatal closure, have been ascribed to differences in abscisic acid metabolism and signalling. ABA perception is the main player involved in stomatal control of isohydric and anisohydric plants (Tardieu *et al.*, 2104). It has been suggested that isohydric behaviour is linked to an interaction between hydraulic and chemical information, while anisohydric behaviour is linked to an absence of interaction (Lovisolo *et al.*, 2010).

The completion in 2007 of the grapevine genome by two international groups opened countless possibilities for genomic studies in this species (Jaillon *et al.*, 2007; Velasco *et al.*, 2007). A first GeneChip® *Vitis vinifera* Genome Array has been released (Affymetrix), which provides comprehensive coverage of the *V. vinifera* genome, for the purpose of transcriptomic analyses. Over 1,750,000 SNPs polymorphisms were mapped, with at least one SNP present in nearly 90% of the genes anchored to chromosomes. This allows for high-throughput analyses of the genetic natural variation present in wild species or in selected synthetic populations, together with the identification of molecular markers for breeding programs. Moreover the assembly of a reference genome also allowed for Next Generation Sequencing approaches for whole genome gene profiling experiments (RNAseq).

The selection of plants that partially close their stomata in response to water deficit can be a useful strategy to increase water-use efficiency (WUE, i.e. biomass gain as a function of water use), but avoiding decrease the yield (Flexas *et al.*, 2010). The maintenance of yield in conditions of water shortage has also been identified as an important target in grapevine breeding programmes (Flexas *et al.*, 2010). In addition to biochemical modifications (epicuticular wax composition, lipid composition, mesophyll thickness, etc.), the genotypic variation in WUE is largely linked to diversity in stomatal conductance, under both well-watered and water-deficit conditions (Escalona *et al.*, 2003; Chaves and Oliveira, 2004). A comprehensive understanding of the molecular mechanisms of the genes affecting WUE and stomatal activity in grape, represent a key step toward the full exploitation of genomic approaches for the selection of novel genotypes with improved stress-tolerance traits.

Arabidopsis thaliana: a model system for the functional characterization of grapevine genes

Molecular studies, '-omics' technologies, such as transcriptomics and proteomics, and mainly the availability of the grapevine genome sequence have provided substantial support to the study of water stress in grapevine (Jaillon *et al.*, 2007; Zharkikh *et al.*, 2008).

Several candidate genes involved in the regulation of the grape response to both biotic and abiotic stresses have been identified. Yet, the direct analysis of their functional role in grape is often hindered by the lack of appropriate genetic and biotechnological tools. For instance, the generation of gain-of-function or loss-of-function lines via the generation of stable transgenic lines or large-scale mutagenesis programs in grapevine, are extremely laborious and require a long time. The ectopic expression of grape genes in heterologous systems, such as *Arabidopsis thaliana*, can, at least in part, overcome these difficulties. Interestingly, the function of several genes identified in different grape species and involved in the plant response to different environmental or biotic stresses, has been characterized using Arabidopsis. This approach was successfully employed in the characterization of the *Shaker-Like* K^+ *Channel* (*VvSIRK*), the first guard cell-specific gene from grapevine (Pratelli *et al.*, 2002). Other genes, related to cold and drought stress, include *Vitis rupestris C-repeat Binding Factor 1* (*VrCBF1*) (Tillett *et al.*, 2012), *Vitis vinifera Zinc Finger Protein Like* (*VvZFPL*) *and Vitis rupestris C-repeat Binding Factor 4* (*VrCBF4*) (Tillett *et al.*, 2012), *Vitis vinifera C-repeat Binding Factor 2* (*VvCBF2*) (Kobayashi *et al.*, 2012), *Vitis amurensis Basic helix-loop-helix 1* (*VabHLH1*) (Xu *et al.*, 2014). While *Vitis vinifera Acyl-CoA-Binding Protein* (*VvACBP*) (Takato *et al.*, 2013), *Vitis vinifera uridine diphosphate glucose-dependent glycosyltransferase* (*VvUGT72B1*) (Xu *et al.*, 2013) and *Vitis pseudoreticulata RING-finger protein 1* (*VpRFP1*) (Xu *et al.*, 2011) were identified for their role in biotic defence.

Arabidopsis thaliana is a small dicotyledonous species, a member of the Brassicaceae or mustard family. Despite Arabidopsis is not an economically important plant, it has been the focus of intense genetic research, because Arabidopsis has a number of natural attributes that collectively make it well suited for molecular genetic studies on flowering plants (Wixon, 2001). First, the *Arabidopsis* plant is very small; the maximum diameter of the rosette is 4 to 6 cm, and the inflorescence reaches 30 to 40 cm in height. Second, relatively to other seed plants, the generation time of *Arabidopsis* is remarkably short. Under standard conditions of light and temperature (100 µE $m^{-2}s^{-1}$; 20°C) it will flower within a month and produce mature seeds within two months. Third, the self-fertility and the large number of progeny (>1000 seeds/plant) simplify the analyses of inheritance and the maintenance of genetic stocks. Last, the small diploid genome (125 megabase pairs) reduces the

amount of labour and time needed to clone and manipulate genes (Bennett *et al.*, 2003). Moreover, many methods and resource materials were developed in *Arabidopsis*. These include simple procedures for chemical and insertional mutagenesis, efficient methods for performing crosses and introducing DNA through plant transformation, extensive collections of mutants with diverse phenotypes, and a variety of chromosome maps of mutant genes and molecular markers (Meinke *et al.*, 1998).

Insertional mutagenesis with transferred DNA (T-DNA) from *Agrobacterium tumefaciens* has become routine through development of whole-plant transformation methods (Clough and Bent, 1998) that avoid the pitfalls associated with plant regeneration in culture. Thousands of transgenic lines carrying random T-DNA insertions throughout the genome have been deposited in public stock centers (Clough and Bent, 1998). T-DNA insertion approach has been used also in grapevine (Gambino *et al.*, 2009).

The role of *AtMYB60* and *VvMYB60* in the regulation of guard cell activity

ABA, like other hormones, functions through a complex network of signalling pathways that begins by ABA perception which triggers downstream signalling cascades to induce the final physiological effects. Numerous downstream components involved in ABA signal transduction have been identified by genetic approaches (Himmelbach *et al.*, 2003; Shinozaki and Yamaguchi-Shinozaki, 2006). Signalling pathways are usually composed of regulatory networks of transcription factors (TFs) which specifically bind short DNA sequences (*cis*-elements) in the regulatory regions (promoters) of their target genes to regulate their expression levels in response to hormonal/environmental signals (Nakabayashi *et al.*, 2005). This suggests that various transcriptional regulatory mechanisms function in the drought-stress response. In plants, transcription factor families are usually very large, as compared to microorganisms and animals (Qu and Zhu, 2006).

AtMYB60 was the first transcription factors characterized in stomatal opening in Arabidopsis (Cominelli *et al.*, 2005). The *AtMYB60* gene was identified through the analysis of a large collection of T-DNA insertional mutants of Arabidopsis (Galbiati *et al.*, 2000). *AtMYB60* has been shown to be differentially expressed in guard cells in response to ABA, and the loss-of function

atmyb60-1 mutant displays constitutive reduction of light-induced stomatal opening and enhanced tolerance to dehydration (Cominelli *et al.*, 2005). Leaves from the *atmyb60-1* knock-out mutant displayed a decrease in the light-induced aperture of stomatal pores of approximately 30% compared to wild-type leaves (Cominelli *et al.*, 2005). Indeed, *AtMYB60* is a positive regulator of stomatal opening that is silenced in stress conditions. It has been clearly shown that the constitutively reduced opening in these plants helps to limit water loss during drought thus enhancing plant tolerance (Cominelli *et al.*, 2005). Moreover the effects of the *AtMYB60* mutation on global gene expression revealed alteration also in genes involved in biotic stress. Indeed expression of some genes involved in pathogen attack are impaired in *atmyb60-1* (Cominelli *et al.*, 2005). *AtMYB60* is a transcriptional regulator likely involved in stomatal movements that could integrate multiple signal transduction processes by modulating the expression of genes involved in guard cell responses to light and to biotic and abiotic stresses (Cominelli *et al.*, 2010). A full and precise comprehension of the transcriptional factor and gene response in guard cells is central to understanding plant mechanisms involved in stress responses and to improve crop tolerance to abiotic and biotic stresses.

Three other Arabidopsis R2R3MYB genes have been described for their involvement in guard cell movement: *AtMYB61, AtMYB44* and *AtMYB15* (Liang *et al.*, 2005; Jung *et al.*, 2008; Ding *et al.*, 2009). In contrast to *AtMYB60*, the *AtMYB61* gene is mainly expressed in guard cells in the darkness, when stomata are closed (Liang *et al.*, 2005). Similarly, *AtMYB44* and *AtMYB15* are involved in the regulation of stomatal closure (Jung *et al.*, 2008; Ding *et al.*, 2009).

Guard cell-specific MYB genes are focal points in understanding stomatal regulation in plants and represent molecular targets to modulate guard cell activity to improve crop survival and productivity during drought (Rusconi *et al.*, 2013).

The MYB superfamily constitutes the most abundant group of transcription factors described in plants. Besides stomatal aperture and drought response (Cominelli *et al.*, 2005; Abe *et al.*, 1997) (Liang *et al.*, 2005), members of this family participate in key processes such as epidermal cell differentiation which includes pigmentation (Nesi *et al.*, 2001) and formation of trichomes (Oppenheimer *et al.*, 2014) flavonoid synthesis (Deluc *et al.*, 2006), response to cold (J., Zhu *et al.*, 2005), pathogen resistance (Lee *et al.*, 2001; Mengiste *et al.*, 2003), seed development (Romano *et al.*, 2012), phytochrome A-dependent light-sensing responses (Ballesteros *et al.*, 2001) and sucrose related responses (Teng *et al.*, 2005). MYB DNA binding domains are 100–160 aminoacid residues in length, depending on the number of imperfect repeats (named R) in the N-terminal region. Among the different classes identified, the R2R3 subfamily is the most abundant in plants (Stracke *et al.*, 2010). In Arabidopsis 126 R2R3 subfamily members are estimated, although only some of

them have been functionally characterised (Yanhui *et al.*, 2006). In grapes, most of the *MYB* genes characterised to date are involved in the control of flavonoid synthesis such as *MYBA* (Kobayashi *et al.*, 2002), *MYB5a* (*MYBCS-1*) (Deluc *et al.*, 2006), *MYB5b* (Deluc *et al.*, 2008), and *MYBPA1* (Bogs *et al.*, 2007). More in details, most studies focused on the MYB factors involved in the anthocyanin and proanthocyanidin synthesis. These compounds affect quality parameters in grapes and wines such as colour, bitterness and astringency (Matus *et al.*, 2008). Despite the well-established role for several MYB proteins in the regulation of stress responses in many plant species, very little is known about the involvement of grape MYBs adaptation to stress and in the regulation of stomatal activity.

VvMYB60 gene is recognized as a functional ortholog of the Arabidopsis *AtMYB60* gene (Galbiati *et al.*, 2011). The VvMYB60 protein was identified based on the strong conservation of its aminoacidic sequence in comparison with AtMYB60. Strikingly, the Arabidopsis and grape MYB60 proteins resulted more similar to each other than to any other MYBs in the grape or Arabidopsis genomes (Galbiati *et al.*, 2011). Moreover, the *VvMYB60* and *AtMYB60* genes show very similar expression profiles, both in terms of tissue- and cell-specificity and response to ABA (Galbiati *et al.*, 2011). The Arabidopsis *AtMYB60* gene is expressed in seedlings, rosette leaves, stems and flowers and its level of expression is rapidly down-regulated by the stress hormone ABA (Cominelli *et al.*, 2005). *VvMYB60* is preferentially expressed in leaves, berry skin and seeds. Similarly to *AtMYB60*, expression of *VvMYB60* in seeds is down-regulated during seed development. In berry skin, before veraison *VvMYB60* expression is higher, when stomata are functional, then its expression decrease. Moreover *VvMYB60* is down-regulated by ABA (Galbiati *et al.*, 2011). Ectopic expression of *VvMYB60* in Arabidopsis showed that the promoter of *VvMYB60* drives expression of reporter genes exclusively in guard cells like the promoter of *AtMYB60* (Galbiati *et al.*, 2011). Finally, Galbiati and co-worker showed that the expression of *VvMYB60* in the *atmyb60-1* mutant background completely rescued the loss of the *AtMYB60* function (Galbiati *et al.*, 2011).

Several studies of gene expression analysis of grapevine under different stresses underline the importance to increase the comprehension of transcriptional regulation in stress response. The dehydration responsive element-binding (DREB) proteins play a critical role in plant development and abiotic stress-mediated gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994). It has been proved that they are major transcription factors (TFs) that respond to abiotic stresses by regulating downstream genes that contain a core DRE sequence (50 -A/GCCGAC-30) cis-acting element in their promoters (Yamaguchi-Shinozaki and Shinozaki, 2006). It has been shown that in grape *VvDREBs* are involved in long-term water and salt stress response, ABA treatment,

perception of photoperiod berry development and ripening (Zhao *et al.*, 2014). The transcriptional regulation of ABA related genes is affected by the type of stress (salinity, drought and cold stress) and the duration of stress (Tattersall *et al.*, 2007). Moreover, transcriptional analysis of grapevine in the recovery phase after drought stress showed that, in petiole, genes involved in ABA perception were up-regulated but not those involved in ABA synthesis (Perrone *et al.*, 2012). This suggests that the increase of ABA in petiole under recovery is due to a passive transport of ABA from the root (Perrone *et al.*, 2012).

The role of the rootstocks in the response to drought stress

Cultivated grapevines are generally grown as a scion grafted onto a rootstock, because of the devastating effect of phylloxera (*Daktulosphaira vitifoliae*), a soil borne aphid pest introduced into Europe in the middle of the 19th century. Every study aimed at investigating the stress adaptation mechanism in grape cannot disregard the fact that the final behaviour and the physiological performance of the plant is influenced by the interaction between the rootstock and the scion (Cortell *et al.*, 2008; Gambetta *et al.*, 2010). Indeed, in addition to their ability to help the scion to cope with biotic stresses, rootstocks can confer also tolerance to a large range of abiotic stresses. Grapevine rootstocks have been shown to play an important role in adaptation to water deficit in pots (Carbonneau, 1985) and, most importantly, in the vineyard (Soar *et al.*, 2006). In this context, rootstocks may play an important role in limiting crop loss by improving water use efficiency, potential for survival, growth capacity and scion adaptability to stress conditions (Marguerit *et al.*, 2012).

Genetic variability of grapevine rootstocks undoubtedly plays a fundamental role in the adaptation to future climate changes, especially to drought (Tsegay *et al.*, 2014). Nevertheless rootstocks used in Europe were selected just for a few traits (rooting ability, phylloxera resistance and scion-induced vigour) reducing their genetic variation (Serra *et al.*, 2013). Currently most of the world viticulture uses no more than 5-6 different rootstocks (Porro *et al.*, 2013).

The SERRES project (Selection of new grape rootstocks resistant to abiotic stresses through the development and validation of physiological and molecular markers), founded by the AGER (Agroalimentare e Ricerca) program, aimed at producing new rootstocks able to withstand drought as well as high levels of limestone in the soil (Porro *et al.*, 2013). This project involved researchers from the University of Milan, Padua, Turin and Piacenza, CRA Vite (Vine) of Conegliano

(Treviso), and FEM of S. Michele all'Adige (Trento). The project analysed four innovative root stocks (series M) in different areas, and evaluated them in different climatic and economic scenarios (Porro *et al.*, 2013). In addition, the SERRES project analysed, under drought stress, several rootstocks including commercial rootstocks and new hybrids produced at the Edmund Mach Foundation, S. Michele all'Adige (Trento). Many of the genotypes analysed in the SERRES project have been used in this thesis.

Root to shoot signalling under conditions of both mild and severe drought is an important area for research because of its implications for agricultural production and water use efficiency (WUE) of plants (Schachtman and Goodger, 2008). Rootstocks can affect vegetative, reproductive and physiological parameters of the plant. Under drought stress, it has been shown that the rootstock can have an impact on the gas exchange and the water status of the scion (Iacono *et al.*, 1998). It has been shown that different rootstocks have different capacities to extract water from the soil and transfer it to the scion (SPEIRS *et al.*, 2013). It has been shown that many differences in stress responses mediated by the rootstock are linked to differences in ABA metabolism. Indeed, ABA is the main chemical signal coming from the roots and the most likely candidate for root-to-shoot signalling in stomatal control (Davies and J., Zhang, 1991).

The ability of the rootstock to confer high tolerance to water stress depend on several factors, of which vigour is one of the most important (Corso and Bonghi, 2014). Aquaporin proteins play a pivotal role in the control of water use during drought. It has been shown that PLASMA MEMBRANE INTRINSIC PROTEINS (VvPIPs) are directly involved in the control of grape vigour mediated by rootstocks (Gambetta *et al.*, 2013).

Within the SERRES project a detailed assessment of the performance of novel rootstocks to water stress was performed (Meggio *et al.*, 2014). The rootstock M4 [(*V. vinifera* x *V. berlandieri*) x *V. berlandieri* x cv Resseguier n.1], produced in 1985 by the Agricultural and Environmental Sciences - Production, Landscape, Agroenergy research group operating at the Milan University, was selected for its high tolerance to osmotic stresses. In comparison with the 101.14 commercial genotype, considered susceptible to drought (Alsina *et al.*, 2011), M4 ungrafted plants subjected to water and salt stress showed a greater capacity to tolerate water stress. In the early phase of drought stress both genotypes showed a concurrent decrease of stomatal conductance (g_s) and net assimilation (A_n). On the contrary, at later time points, an almost complete inhibition of both assimilation and transpiration rates was observed in 101.14 but not in M4 (Meggio *et al.*, 2014). Thus, M4 maintains higher transpiration and net assimilation rates in comparison to the susceptible genotype, suggesting a less drought-avoiding behaviour of M4 compared with that of 101.14. Taken

together, these results showed that the higher capacity of M4 to respond to WS conditions appears to be dependent on root responses (Meggio *et al.*, 2014).

Stomata are active players in the innate immune system

Besides the control of stomatal movements in response to external and internal stimuli, guard cells can sense and respond to pathogens and protect the leaf against microbial invasion by closing the stomatal pore (Gudesblat *et al.*, 2009; Melotto *et al.*, 2006; Schellenberg *et al.*, 2010). This phenomenon has been defined as stomatal immunity, as it requires well-known molecular components of the plant innate immune system (Zeng and He, 2010).

Melotto and colleagues noted that stomata close in response to the plant pathogen, *Pseudomonas syringae* pv. tomato (Pst) DC3000, and the human pathogen, *Escherichia coli* O157:H7 (Melotto *et al.*, 2006). Interestingly, this response can also be triggered by well-characterized pathogen/microbe-associated molecular patterns (PAMPs or MAMPs), such as flg22 (a peptide derived from bacterial flagellin) and lipopolysaccharide (LPS) (Melotto *et al.*, 2006).

MAMPs are recognized by pattern-recognition receptors (PRRs) located in the plant plasma membrane (Boller and Felix, 2009). For example, flg22 is perceived by its cognate PRR FLS2, which is required for flg22 to trigger stomatal closure (Melotto *et al.*, 2006). Because each pathogen can potentially release multiple MAMPs, it is of interest to determine the relative importance of different PRRs in controlling stomatal closure during actual infection (Zeng and He, 2010).

Some of the downstream molecular processes in the guard cell after microbe recognition are somewhat overlapping with the ones associated with abiotic stress. For instance, synthesis and signalling of the (ABA) in response to drought stress (Schroeder, Allen, *et al.*, 2001) is also linked to stomatal immunity (Melotto *et al.*, 2006).

Moreover it has been proposed that it is the combinatorial action of Salicylic acid (SA) and ABA signalling pathways that mediates stomatal closure in response to bacterial pathogens (Melotto *et al.*, 2006).

Salicylic acid is a phenolic compound with key roles in pathogen defence (Vlot *et al.*, 2009). It has been reported that SA plays a positive regulatory role in stomatal closure in Arabidopsis (Khokon *et al.*, 2011) in *Vicia faba* and *Commelina communis* (Joon-Sang, 1998), (Mori *et al.*, 2001). Stomatal closure in response to bacterial pathogens is compromised in transgenic NahG plants (deficient in

SA) and in the SA biosynthetic mutant *ateds16-2*, indicating that SA is required for stomatal defence (Melotto *et al.*, 2008).

Long-distance signals initiated at the infection site lead to the induction of specific *pathogenesis-related* (*PR*) genes in uninfected parts of the plant, a process termed ‘‘systemic acquired resistance’’ or SAR (Durrant and Dong, 2004). SAR is accompanied by an increase in levels of salicylic acid (SA) and its derivative SA-glucoside (SAG), and elevated expression of SA-responsive genes in the pathogen-free organs. Elevated expression of the SA-responsive *PR1* gene has routinely been used as a molecular marker of SAR. SA accumulation and signalling in these organs are primed to further increase to higher levels upon challenge with a pathogen (Jung *et al.*, 2009). Besides SA, methyl-jasmonate, ethylene, hydrogen peroxide, and superoxide radicals have been proposed to be involved in the induction and coordination of these plant responses (Durrant and Dong, 2004; Hammond-Kosack and Jones, 1996). Jasmonic acid is an oxylipins (lipid-derived) plant hormone regulating vegetative and reproductive growth, and defence responses against abiotic stresses (UV light and ozone), insects and necrotrophic pathogens (Katsir *et al.*, 2008; Ballaré, 2011). Moreover, it has been proposed that JA could be an important player for stomatal closure during drought stress based on its accumulation during drought (Creelman and Mullet, 1997) and its positive regulatory role in stomatal closure (Munemasa, *et al.,* 2007a). Crosstalk between the salicylic acid and the jasmonic acid-ethylene (JA/ ET) is one of the best-studied examples of defence-related signal in response pathways (Thaler *et al.*, 2012).

Biotrophic and hemi-biotrophic pathogens are generally more sensitive to SA-dependent responses, whereas necrotrophic pathogens and herbivorous insects are commonly deterred by JA/ ET-dependent defence (Campos *et al.*, 2014). Intriguingly, some pathogens can induce multiple plant signal molecules and hormones, such as SA and JA. In such cases, crosstalk between these signalling pathways may be the mechanism that allows the plant to prioritize one response over the other (Spoel and Dong, 2008). SA can suppress both JA biosynthesis and sensitivity (Spoel *et al.*, 2003). In other cases, antagonism between pathways allows the defence response to be controlled in a focused manner. For instance, plants that are infected by SAR-inducing pathogens have been shown to suppress JA-dependent defences against certain herbivorous insects or necrotrophic pathogens (Spoel *et al.*, 2003) thereby prioritizing SA-dependent defence responses over JA dependent responses. The protein NONEXPRESSOR OF PR1 (NPR1) plays an important role in mediating the suppressive effect of SA downstream of JA (Spoel *et al.*, 2003). NPR1, is a possible modulator of crosstalk between the SA and JA signals (Dong, 2004). The cytosolic function of the NPR1 protein is important during SA-JA crosstalk while the nuclear function of NPR1 is important during the activation of SA-responsive genes (Spoel *et al.*, 2003). It has been shown that SA-

mediated suppression of the JA-responsive genes *lipoxigenase 2* (*AtLOX2*)*, vegetative storage protein* (*AtVSP*)*, and plant defensine* (*AtPDF1.2*), normally observed in WT plants, is abolished in mutant *atnpr1* plants, suggesting that *AtNPR1* is essential for the inhibition of JA-responsive gene expression by SA (Spoel *et al.*, 2003).

Stomatal function in immune response has also been reported in grapevine. *Plasmopara viticola* is the causal agent of grapevine downy mildew, a major disease that can cause severe losses in yield and quality (Dai *et al.*, 1995). This obligate biotrophic oomycete enters the plant via stomata and develops an intercellular mycelial network inside the mesophyll tissue, causing the 'oil spot' symptom visible on the adaxial side of the leaf (Dai *et al.*, 1995). It has been shown that *P. viticola* can induce an abnormal opening of grapevine stomata and it has suggested that this facilitates the emergence of sporangiophores and pathogen dissemination (Allègre *et al.*, 2007).

Materials and Methods

Plant material

Arabidopsis

atmyb60-1 mutant plants and GFP marker line *p60:GFP/atmyb60,* were published by Cominelli et al., 2005. *atnpr1* seeds were obtained from Nottingham Arabidopsis Stock Centre (NASC) seed stock centre. *atnpr1/atmyb60-1* double mutants and GFP marker lines *p60:GFP/atnpr1-1* and *p60:GFP/atmyb60-1/atnpr1-1* were obtained by cross breeding.

Grapevine

Two-year-old grapevines of commercial rootstocks 101.14 and M4 and grafted combinations (CS/CS CS/M4, CS/101.14, M4/M4 and 101.14/101.14 were provided by professor L. Espen (Dipartimento di Scienze Agrarie e Ambientali – Produzione, Territorio, Agroenergia (DISAA), Università degli Studi di Milano, Via Celoria, 2-20133 Milano, Italy).

Additional rootstocks (genus *Vitis*), were obtained by the SERRES project (M1, M2, M3 M4, 101.14, K5BB, 1103P, SO4, SM24, SM79, SM82 and SM93).

Growing conditions

Arabidopsis

Arabidopsis thaliana (L.) Heyhn. Ecotype Col-0 plants were grown in Arabasket pots plus Araflat (BETATECH) filled with a blend (4:1, v/v) of loam sandy soil and peat (Vigorplant, Italia), under long day conditions (16 hours light/ 8 hours dark), in a controlled growth chamber with 70% air humidity, 22 degrees Celsius temperature, and 100 µmol $m^{-2}s^{-1}$ light intensity.

Grapevine

Two-year-old plants were grown in pots filled with a sand:peat mixture (7:3 in volume). For the first year the experiment was conducted in a greenhouse sited in Milan (Italy) equipped with supplementary light and a cooling system, with a 16 h light (~PPFD of µmol $m^{-2}s^{-1}$) and an 8-h dark photoperiod. Plants were grown in 3-L pots fertilised monthly with 100 mL of solution containing 0.54 g KNO_3, 0.084 g NH_4HPO_4, 0.42 g $MgSO_4$ and 0.01 g of a microelement mixture (OligoGreen, GREEN Italia, Canale d'Alba, Italy). For the second and the third year grapevines plant were grown in the same condition as above and the experiment was performed in a greenhouse of Fondazione Filarete, sited in Milan (Italy) equipped with supplementary light and a cooling system, with a 16 h light [~PPFD of 600 µmol $m^{-2}s^{-1}$] and an 8-h dark photoperiod.

Metabolic analysis of lipid composition

All rosette leaves were detached from four 4-week-old WT, *atmyb60.1* plants. Whole leaves were frozen in liquid nitrogen and completely ground while leaves for guard cell purification were whisked with blender method. All sample were lyophilized. Metabolic analyses were performed and processed by professor Patrick Galivasco (Max Planck Institute).

Salicylic acid treatment

Arabidopsis

4-week-old WT, *atmyb60.1, atnpr1-1* and *atmyb60-1/atnpr1-1* plants were treated with SA 100 μM. Leaves from random plants were harvested at 0h, 4h, 12h and 24h. The samples for whole RNA extraction were frozen in liquid nitrogen and stored at −80°C. The sample for guard cells RNA extraction were plunged in RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA) and stored as 4°C for a few minutes, waiting to be processed.

Grapevine

Three grapevine plants for each genotype (CS/M4, CS/101.14, M4/M4 and 101.14/101.14) grown as previous described were treated with SA 10μM. Leaves from random plants were harvested at 0h, 12h and 24. The samples for whole RNA extraction were frozen in liquid nitrogen and stored at −80°C. The sample for guard cells RNA extraction were plunged in RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA) and stored at 4°C for a few minutes, waiting to be processed.

Drought stress treatment

The leaves were sampled from five plant for each genotype (CS/M4, CS/101, M4/M4 and 101.14/101.14 at 90%, 60% and 30% of field capacity at the same time in the morning. The sample for total RNA extraction were frozen in liquid nitrogen and stored at −80°C. The sample for guard cells RNA extraction were plunged in RNAlater solution (Sigma-Aldrich, St. Louis, MO, USA) and stored at 4°C for a few minutes, waiting to be processed.

6 plants for each genotype of ungrafted grapevines (genus *Vitis*), from the SERRES collection, were grown in semi-controlled condition in a greenhouse sited in Tavazzano (LO) (Italy) without supplementary light or cooling system. Plants were grown in pots as described above. For each genotypes 3 plants were kept at 90% of field capacity (control plants) while for 3 plants for each genotype water supply was progressively reduced until 30% of field capacity (stressed plants), weighing the pots. Stomatal conductance was measured using AP4 leaf porometer (Delta-T Devices, UK).

Bioinformatic analysis

MapMan categories were performed using PageMan (http://mapman.mpimp-golm.mpg.de/index.shtml) with default setting.

Measurement of Stomatal Density and Index

Epidermal pieces of grapevine leaves were obtained by blending of leaf tissues. Images of epidermal pieces were digitally recorded with a Leica DM2500 optical microscope (Leica Microsystems GmbH, Wetzlar, Germany), at 20X magnification.

Guard cell purification

Blender method

The main vain from Arabidopsis or grape leaves were removed with a scalpel blade then the leaves were whisked with a blender in ice-cold deionised water with crushed ice for 1 minute and then filtered through a 210 µm nylon net. After two further rounds of whisking, the materials remaining on the nylon net and containing the light green epidermal fraction, enriched in guard cells, was frozen in liquid nitrogen and stored at −80°C.

Sample preparation for LMD

Arabidopsis harvested leaves were stored in bio-box and transferred to ice cold Ethanol:Acetic Acid 3:1 (v/v) and vacuum-infiltrated at 4°C, followed by three changes of the same solution 1 h each. Sample were then transferred to a fresh fixative solution over night at 4°C. Samples were washed with ice-cold EtOH:H_2O dilutions for six times (70:30, 80:20, 90:10 v/v) and three times with EtOH 100% for 1h each. The samples in the last solution were stored o/n. Then samples were washed with ice-cold EtOH:Xilol (3:1, 1:1, 1:3 v/v) and three times with xylol 100%) for 1h each. Finally ~ 100g of paraffin (paraplast) were added to the samples in xylol and stored o/n at 4°C. The sample were embedded in a liquid paraplast solution for 4 hours, in microtome moulds and stored at 4°C.

The same protocol was performed on grapevine leaves with little modifications. The five main veins of the leaves were removed with a scalpel blade. The pieces of leaves were packed in multiple layer so that they remained as flat as possible. Washings steps were prolonged for 2 hours.

From the paraplast samples describe above, 10 µm sections were cut with a Leica RM2265 microtome (Leica Microsystems, GmbH, Wetzlar, Germany). Sections were mounted on PET-membrane-coated glass slides (Leica Microsystems, GmbH, Wetzlar, Germany). Post-fixation included three steps for 10 min in xylol, immediately air-dried, and microdissected. Microdissection was performed using a Leica Laser Microdissection 6000 system (Leica Microsystems, GmbH,

Wetzlar, Germany). On average, approximately ~ 3000 stomata and ~ 1mm^2 of mesophyll were dissected in each experiment.

Guard cell opening measurements

WT, p60:GFP*/myb60, p60:GFP/atnpr1-1 and p60:GFP/atmyb60-1/npr1-1* plants were kept in 100 µmol $m^{-2}s^{-1}$ light intensity 2-3h. Measurements were performed with a Confocal Leica TCS SP5 (Leica Microsystems, GmbH, Wetzlar, Germany). As a light source for GFP excitation an argon laser was used. Two detached leaves for each experiment of 3 -week-old Arabidopsis plants were submerged in 25mM MES pH=6.15, 10mM KCl buffer in a custom made polydimethylsiloxane PDMS chamber. During the confocal analysis an extra source of light was provided by two cold white LEDs. Ten x,y position were setted with 30 Z plane each 2 µm apart from each other. Images were digitally recorded. For the analysis of light-induced stomata opening plants were kept in the dark for 8 hours then leaves were transferred in the physiological buffer. For chemical and elicitors treatment, Arabidopsis plants were kept under light (200 µmol $m^{-2}s^{-1}$) for 2 hours. Then detached leaves were submerged in MES buffer and chemicals were added. ABA 10 µM, LPS 1µg/ml, SA 100 µM or flg22 10 µM were added to the buffer after the first acquisition.

qPCR analyses

Arabidopsis and grape leaf samples for total RNA extraction were immediately frozen in liquid nitrogen and stored at −80°C. RNA from whole leaf samples and GC-enriched epidermal fragments was isolated using the Spectrum™ Plant Total RNA (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions. RNA from LMD sample was isolated using the PicoPure® RNA Isolation Kit (Life Technology) in Arabidopsis and PureLink® RNA Mini Kit and RNAqueous® Kit in grapevine. A total of 1 mg of total RNA was used for complementary DNA synthesis with the SuperScript VILO cDNA Synthesis Kit (Invitrogen).

Prior to qPCR experiments the quality of the cDNAs was assessed by RT-PCR analysis of housekeeping genes (*AtACT* and *VvACT*). RT-PCR were performed in a total volume of 20 µl reaction mixture, containing 1-2 microl of cDNA, 0,2µl of 1 × reaction buffer 2.5 mM MgCl2, 0.5µl of 0.2 mM dNTP, 1 U of Taq DNA polymerase and 0.6µl of 0.15 µM of each primer. PCR was performed in a thermocycler (Eppendorf Mastercycler) with a programme consisting of 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 40 s at 56 °C, 2min at 72 °C, and 5 min extension at 72 °C.

Quantitative real-time PCR was performed with Fast SYBR Green Master Mix (Applied Biosystems), and amplification was real-time monitored on a 7900 HT Fast Real-Time PCR system (Applied Biosystems). Changes in gene expression were calculated relative to selected

housekeeping genes (*AtACT2* o *AteIF4a* for Arabidopsis and *VvG3PDH* for grapevine), using the ΔΔCt method (Livak and Schmittgen, 2001). qPCR primer sequences are reported in Table 1.

Table 1 List of primers used in this work

Gene	Gene ID	Forward (5'-3')	Reverse (5'-3')	Reference
AtActin2	AT3G18780	CTCTCCCGCTATGTATGTCGCCA	CTCTCCCGCTATGTATGTCGCCA	(Riboni *et al.*, 2013)
AtMYB60	AT1G08810	CATGAAGATGGTGATCATGAGG	TTCCATTTGACCCCCAGTAG	(Cominelli *et al.*, 2011)
AteIF4a	AT3G13920	TGACCACACAGTCTCTGCAA	ACCAGGGAGACTTGTTGGAC	{Seo:2010cb}
AtPR1	AT2G14610	TCAGTGAGACTCGGATGTG	TCAGTGAGACTCGGATGTG	(Roberts *et al.*, 2007)
AtPR2	AT3G57260	TTATCACCGCTGCAAAGTCCT	TGGCGCTCGGTTCACAGTA	
AtPR5	AT1G75040	GACTCCAGGTGCTTCCCGACAG	ACTCCGCCGCCGTTACATCTT	
AtSID2	AT1G74710	GAGACTTACGAAGGAAGATGATGAG	GAGACTTACGAAGGAAGATGATGAG	(Chen *et al.*, n.d.)
AtIFL1	AT5G60690	CCAAGCTGTGAATCTGTGGTC	CGATCTTTGAGGATCTCTGCA	(Zhong and Ye, 1999)
AtPHO1	AT4G26530	ACACCATTCCAGGCATCCTCCTC	ACGGTGAGCAAACAATCTTCCGC	{Zimmerli:2012ku}
AtNPR1	AT1G64280	AGCA TTCTCTCAAAGGCCGAC	AGCATTCTCTCAAAGGCCGAC	(X., Zhang *et al.*, n.d.)
AtFAD2	AT3G12120	CCAAAGCAGAAATCAGCAATCA	GCAGCAGCGTAACGG TAAAGAC	(Keunen *et al.*, 2013)
AtFAD3	AT2G29980	TTTATTGGGCCGCCCAAG	TGTCTGAGAAACTCCCATGTCCA	(Keunen *et al.*, 2013)
AtFAD7	AT3G11170	CCATGTTTTGGGCTCTCTTTGTT	CCACACTGTTCAACTTCGGATCA	(Keunen *et al.*, 2013)
AtDAD1	AT2G44810	TCGGTAAGGAGCTTCGGCTGAG	CTGAATGGACACGTGGAGCTCAC	(Keunen *et al.*, 2013)
AtLOX1	AT1G55020	ACTCTTCGTCTTGTAAGCTCTG	GTGACCTTGAAAGCGGATTCG	(Keunen *et al.*, 2013)
AtLOX2	AT3G45140	TTTGCTCGCCAGACACTTG	GGGATCACCATAAACGGCC	(Keunen *et al.*, 2013)
AtLOX3	AT1G17420	ACGTTGTCGTACTGGTCGCC	GTCTCGTGGCACATACATAGGTAATG	(Keunen *et al.*, 2013)
AtLOX4	AT1G72520	AAGGTCTCCCTGCTGATCTCAT	AAGCCCATGTGGTTGTGTTG	(Keunen *et al.*, 2013)
AtLOX5	AT3G22400	GGCAAAACCGGCCGTAAAT	CGTCCCTTGGCACGTATATGTT	(Keunen *et al.*, 2013)
AtLOX6	AT1G67560	GGCGATTTGACATGGAAGGA	ACAAGCCTCACGCCACATTC	(Keunen *et al.*, 2013)
AtAOS	AT5G42650	CGGGCGGGTCATCAAGTTC	GCTCCCATCGTGAGTTCTCC	(Keunen *et al.*, 2013)
AtAOC1	AT3G25760	CCCAGACCAAGCAAAGTTCAAG	TCTCCGAGACCAAACCTAAAGC	(Shih *et al.*, 2014)

AtAOC2	AT1G13280	ATCACTCACCACATAAAGTAAAAGTCTC	CGATGAAATTGTTGATTACATGAAAGATTG	Shih:2014ci}
AtAOC3	AT3G25780	CTTTCTTCTGGAATTGGGGC	CTTTCTTCTGGAATTGGGGC	Shih:2014ci}
AtAOC4	AT1G13280	AATGTGTCCCGTCCCTATAAGC	AATGTGTCCCGTCCCTATAAGC	Shih:2014ci}
AtOPR1	AT1G76680	ATCCAGGAGCATTAGGGC	ATCCAGGAGCATTAGGGC	(Beynon *et al.*, 2009)
AtOPR2	AT1G76690	GTGTCCCTCTTCTCACCCCTTA	GTGTCCCTCTTCTCACCCCTTA	(Beynon *et al.*, 2009)
AtOPR3	AT2G06050	GGACGCAACTGATTCTGACCCAC	GGACGCAACTGATTCTGACCCAC	(Beynon *et al.*, 2009)
AtPKT1	AT1G04710	ACCCTTTCCGGACTTGCAAA	ACCCTTTCCGGACTTGCAAA	(Keunen *et al.*, 2013)
AtPKT2	AT2G33150	ATGGCCATAGGCCATCCTTT	ATGGCCATAGGCCATCCTTT	(Keunen *et al.*, 2013)
AtKAT5	AT5G48880	GGGGAGATAATCGAGGGCTG	GGGGAGATAATCGAGGGCTG	(Feng *et al.*, 2014)
AtJMT	AT1G19640	AGCTCTTATGTCCATGGCCAAA	CTCGCAGCATAGTAAGGAGCGT	(Keunen *et al.*, 2013)
AtACX1	AT4G16760	ACGGATGTAACTGAAAGACTGGC	GTGGTGGTGAGAGACTTCAATCCTG	(Keunen *et al.*, 2013)
AtACX2	AT5G65110	TGTTCACGCCTTCATTGTTCCG	TCAACGCACCATTATCCACTCCATT	(Keunen *et al.*, 2013)
AtMPF	AT3G06860	ACACTCATCAATCCTCCCGTCA	CATCATTCCTGCTCAAGGCCT	(Keunen *et al.*, 2013)
AtOPCL1	AT1G20510	TTTATTGTTTGTGCTGTAGACCTGTAG	GCCATTTATGTGTGTAATCTTCTGTG	(Shih *et al.*, 2014)
AtCTS	AT4G39850	GAGATTAGGCATGGCACGTT	GTCGCATTTGTGCATTCATC	(Theodoulou *et al.*, 2005)
AtVSP1	AT5G24780	GCATCTCATACTCAAGCCAAACG	TCCTCAACCAAATCAGCCCA	
AtVSP2	AT5G24770	GTTAGGGACCGGAGCATCAA	TCAATCCCGAGCTCTATGATGTT	(Berger *et al.*, 2002)
VvAct		GCCATTCAGGCTGTTCTTTC	ACTGAGGAGCTGCTCTTTGC	
VvMYB60	GSVIVP01008005001	TTGAGTACGAAAACCTGAATGAT	TTGAGTACGAAAACCTGAATGAT	(Galbiati *et al.*, 2011)
VvSLAC		GGATGGCAGGAAGCTGCTAA	GGATGGCAGGAAGCTGCTAA	
VvSIRK		AGTCCCCGTTACAGGGCTTGGG	AGTCCCCGTTACAGGGCTTGGG	(Pratelli *et al.*, 2002)
VvG3PDH		TTCTCGTTGAGGGCTATTCCA	TTCTCGTTGAGGGCTATTCCA	(Galbiati *et al.*, 2011)
VvHOS10.1	GSVIVT00024036001	CCTCAAGCGGGGAAACTTCA	CCTCAAGCGGGGAAACTTCA	

VvHOS10.2	GSVIVT00008402001	GGTCCCTGATAGCGGGTAGA	GGTCCCTGATAGCGGGTAGA	
VvHOS10.3	GSVIVT00012777001	CGCCGCTTCGATTTCCAAAG	CGCCGCTTCGATTTCCAAAG	
VvERA1		GCGGCGAATGAAAGACCCTA	GCGGCGAATGAAAGACCCTA	
VvERF5	GSVIVT00013 93500	AGAGGCACTACAGAGGCGTA	AGAGGCACTACAGAGGCGTA	
VvNCED1	AY337613	GCAGAGGACGAGAGTGTAAAGGA	GCAGAGGACGAGAGTGTAAAGGA	(Lund *et al.*, 2008)
VvNCED2	AY337614	ATGCTCAAACCGCCTCTGAT	ATGCTCAAACCGCCTCTGAT	
VvABF2		CACAGGATTGATGGGAAACC	CACAGGATTGATGGGAAACC	
VvABF3	GSVIVT00031730001	CAACAACAGCATCAGCCACT	CAACAACAGCATCAGCCACT	
VvSOR	CAO44479	CCACACTTACCGCATGGTCT	CCACACTTACCGCATGGTCT	
VvSnRK2.2	GSVIVT01009074001	CCTGCAAAGAGGATCACCAT	CCTGCAAAGAGGATCACCAT	(Boneh *et al.*, 2012)
VvSnRK2.4	GSVIVT01022427001	AGCTTCTTTCCCGCATTTTT	AGCTTCTTTCCCGCATTTTT	(Boneh *et al.*, 2012)
VvSnRK2.7	GSVIVT01031806001	ACTACCGGTCGGTGACTACG	TCCTCTGTGTTCCCTTCTGG	(Boneh *et al.*, 2012)
VvSnRK2.6	GSVIVT01031806001	CACCAACCCACCTTGCTATT	CACCAACCCACCTTGCTATT	(Boneh *et al.*, 2012)
VvPP2C2	GSVIVT01035420001	GGCATCGAGTTTTTGGTGTT	GGCATCGAGTTTTTGGTGTT	(Boneh *et al.*, 2011)
VvPP2C4	GSVIVT01015308001	TGGGCTTTGGGATGTTATGT	TGGGCTTTGGGATGTTATGT	(Boneh *et al.*, 2011)
VvPP2C8	GSVIVT01016485001	AGTGTTTGATCCTGGCAAGC	AGTGTTTGATCCTGGCAAGC	(Boneh *et al.*, 2011)
VvPP2C9	GSVIVT01024875001	TTAAAGCCCTTCGTGAGCTG	TTAAAGCCCTTCGTGAGCTG	(Boneh *et al.*, 2011)
VvPP2C24	GSVIVT00018464001	CACAGGATTGATGGGAAACC	CACAGGATTGATGGGAAACC	(Boneh *et al.*, 2011)
VvRbohF	GWSUNIT03506984001	CGCATCGCCGAGGAAGAAGT	CGCATCGCCGAGGAAGAAGT	(Boneh *et al.*, 2011)

VvRCAR1	GSVIVT01027078001	TGATGGGAGACCAGGGACAC	TGATGGGAGACCAGGGACAC	(Boneh *et al.*, 2011)
VvRCAR2	GSVIVT01028704001	GGATGTGAAAGTGGGAATGG	GGATGTGAAAGTGGGAATGG	(Boneh *et al.*, 2011)
VvRCAR3	GSVIVT01019517001	GGCAAAGCATTTGAGGAAC	GGCAAAGCATTTGAGGAAC	(Boneh *et al.*, 2011)
VvRCAR4	GSVIVT00035869001	CTGTAATTGGAGGGGACCAC	CTGTAATTGGAGGGGACCAC	(Boneh *et al.*, 2011)
VvRCAR5	GSVIVT00037390001	GTTTTTGTCGACACCATCGTT	GTTTTTGTCGACACCATCGTT	(Boneh *et al.*, 2011)
VvRCAR6	GSVIVT01032747001	TCACCACTCTACACCCTTCC	TCACCACTCTACACCCTTCC	(Boneh *et al.*, 2011)
VvRCAR7	GSVIVT01013161001	GGGAAGGATTACACCATCGTT	GGGAAGGATTACACCATCGTT	(Boneh *et al.*, 2011)
VvPR1	GSVIVT00038575001	GGAGTCCATTAGCACTCCTTTG	GGAGTCCATTAGCACTCCTTTG	(Le Henanff *et al.*, 2009)

Results

Part I: Optimization of technical approaches employed in the study

1.1 Establishing a confocal approach for the in *vivo* analysis of stomatal response

We have established an efficient and reliable protocol for the *in vivo* analysis of stomatal responses to both abiotic and biotic stresses. As opposite to the widely used epidermal peel approach our method allowed for the use of intact leaves, thus providing a more physiological context for the analysis of stomatal activity. In addition, a large number of stomata could be visualized, allowing for reliable statistical analyses and, importantly, changes in the aperture of single stomata in response to different stimuli could be monitored over time.

Our approach is based on confocal microscopy, and it takes advantage of two stomata-specific GFP marker lines developed in our laboratory. The first marker line carries the reporter GFP under the control of the Arabidopsis *AtMYB60* promoter (AT1G08810) that showed highly specific guard cell activity and it is rapidly down regulated by ABA and dehydration stress ($AtMYB60_{pro}$*:GFP* line) (Cominelli *et al.*, 2005; Rusconi *et al.*, 2013). The second marker line carries the reporter GFP under the control of the promoter of the cytochrome P450, *AtCYP86A2* (AT4G00360) gene, which showed constitutive expression in guard cells ($AtCYP86A2_{pro}$*:GFP* line) (Galbiati *et al.*, 2008). Stomatal measurements were performed on whole leaves plunged in MES/KOH buffer, to which ABA, or pathogenic elicitors were added (Figure 1).

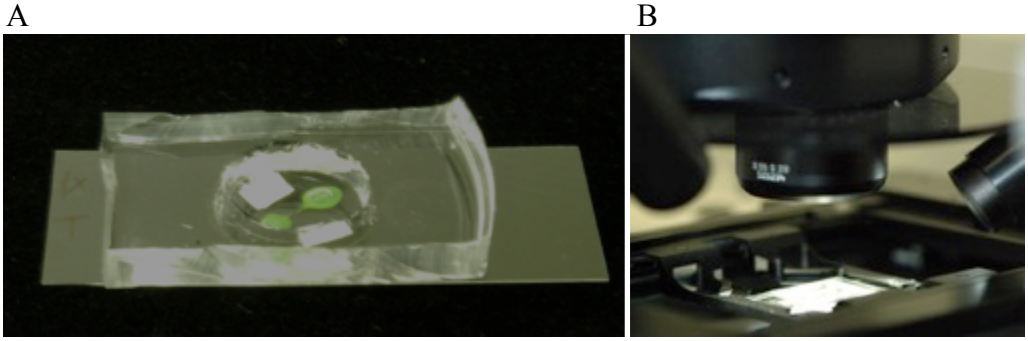


Figure 1 Confocal analysis of Arabidopsis GC in intact leaves. (A) Leaf samples are placed in a chamber made by solid polydimethylsiloxane (PDMS) in MES_KOH/KCl buffer. (B) Specimen under confocal microscope illuminated by two cool white LEDs to stimulate stomatal opening.

Coordinates (x,y,z) for approximately 100 individual stomata were manually set at the beginning of each experiment (Figure 2). A series of sixteen pictures was automatically recorded for each and every stomata, over a period of four hours. Measurements of the aperture of individual stomata from each time-point were digitally recorded.

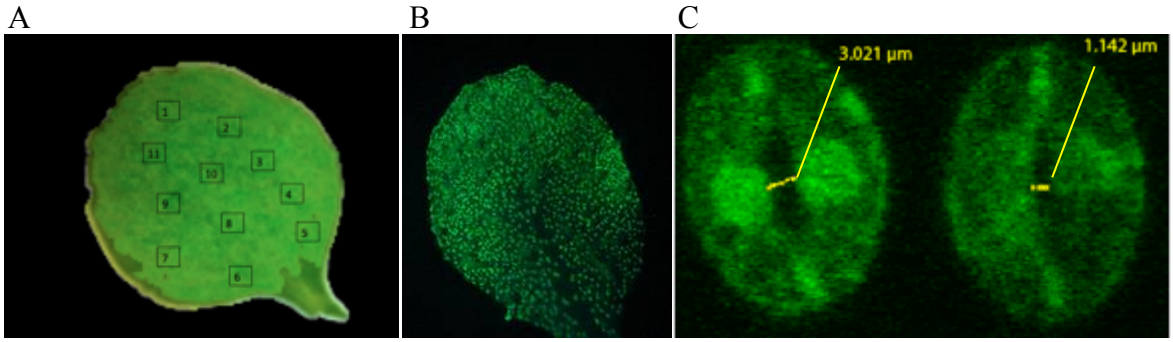


Figure 2 Measurement of stomata aperture. (A) Selection of the areas of interest on the Arabidopsis leaf. During the experiment all stomata present in selected areas are measured. (B) Image of a whole leaf expressing the *AtMYB60*$_{pro}$*:GFP* construct. (C) Measurement of stomatal opening in a WT leaf (left panel) and in a *atmyb60-1* leaf (right panel).

To test the reliability and the robustness of this approach we evaluated the light-induced stomatal opening of plants previously kept in the dark, both in the WT line and *atmyb60-1* mutant, in which light-induced stomatal opening is impaired (Cominelli *et al.*, 2005) (Figure 3A). Measures were taken every minute for the first fifteen minutes of the light treatment and then every ten minutes for a period of three hours. As expected, we detected a continuous and steady opening of the stomata rhyme, both in WT and mutant plants. Consistently with previous reports, the mutant showed a significant reduction in stomatal opening compared with the WT in all time points, thus demonstrating the sensitiveness of this technique (Figure 3A).

To further test the ability of this experimental setting to monitor stomatal responses to chemical stimuli, we first measured the response of stomata to the addiction of ABA. As expected, we observed a very rapid closure of the stomatal pores within 15 minutes after the addition of ABA 100mM (Figure 3B).

Next, we assayed the effect of the pathogen elicitors flg22 (a biologically active peptide derived from flagellin) and lipopoly-saccharide (LPS), known to induce stomatal closure (Melotto *et al.*, 2006). As shown in Figure 3 C and D, flg22 and LPS triggered stomatal closure in both WT and *atmyb60-1* mutant, further confirming the sensitivity of the system and proving evidence that AtMYB60 is not involved in flg22-mediated stomatal closure.

As a whole these results demonstrate that our confocal-based approach is a reliable technique for the *in vivo* analysis of stomatal movement allowing for functional studies in intact leaves.

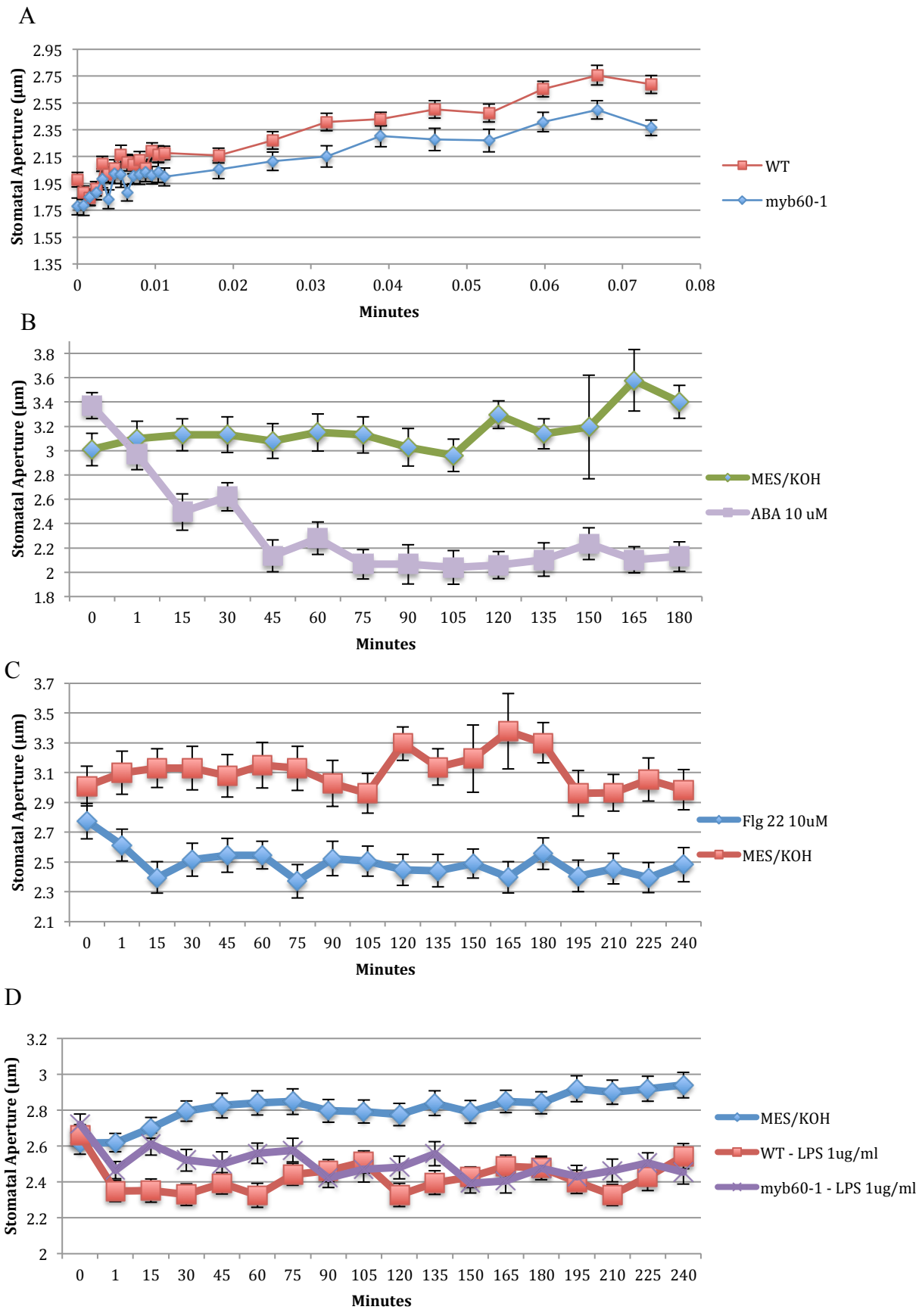


Figure 3 (A) Time course of stomatal responses of WT and *atmyb60-1* leaves to white light. (B) Timecourse of ABA-induced stomatal closure in a WT leaf. ABA was added at time = 0. (C) Timecourse of flg22-induced stomatal closure in a WT leaf. (D) Timecourse of LPS-induced stomatal closure in a WT leaf. Elicitors were added at time = 0.

1.2 Laser-microdissection of Arabidopsis and grape tissues

LMD is a specific form of laser-assisted micro-dissection that uses a UV cutting laser to isolate tissues or individual cells of interest from complex organs (Nelson *et al.*, 2006). Isolated tissues or cells are collected by gravity in the cap of microcentrifuge tubes, located below the sample (Figure 4). RNA extracted from LMD-purified GC was compared with RNA extracted from the other leaf tissues, mainly mesophyll cells. To test quality and purity of RNAs, qPCR of GC marker genes (*AtMYB60* and *Arabidopsis phosphates1* -*AtPHO1*) (Zimmerli *et al.*, 2012) was performed (Figure 5). As expected, both markers resulted more expressed in guard cells compared with the mesophyll.

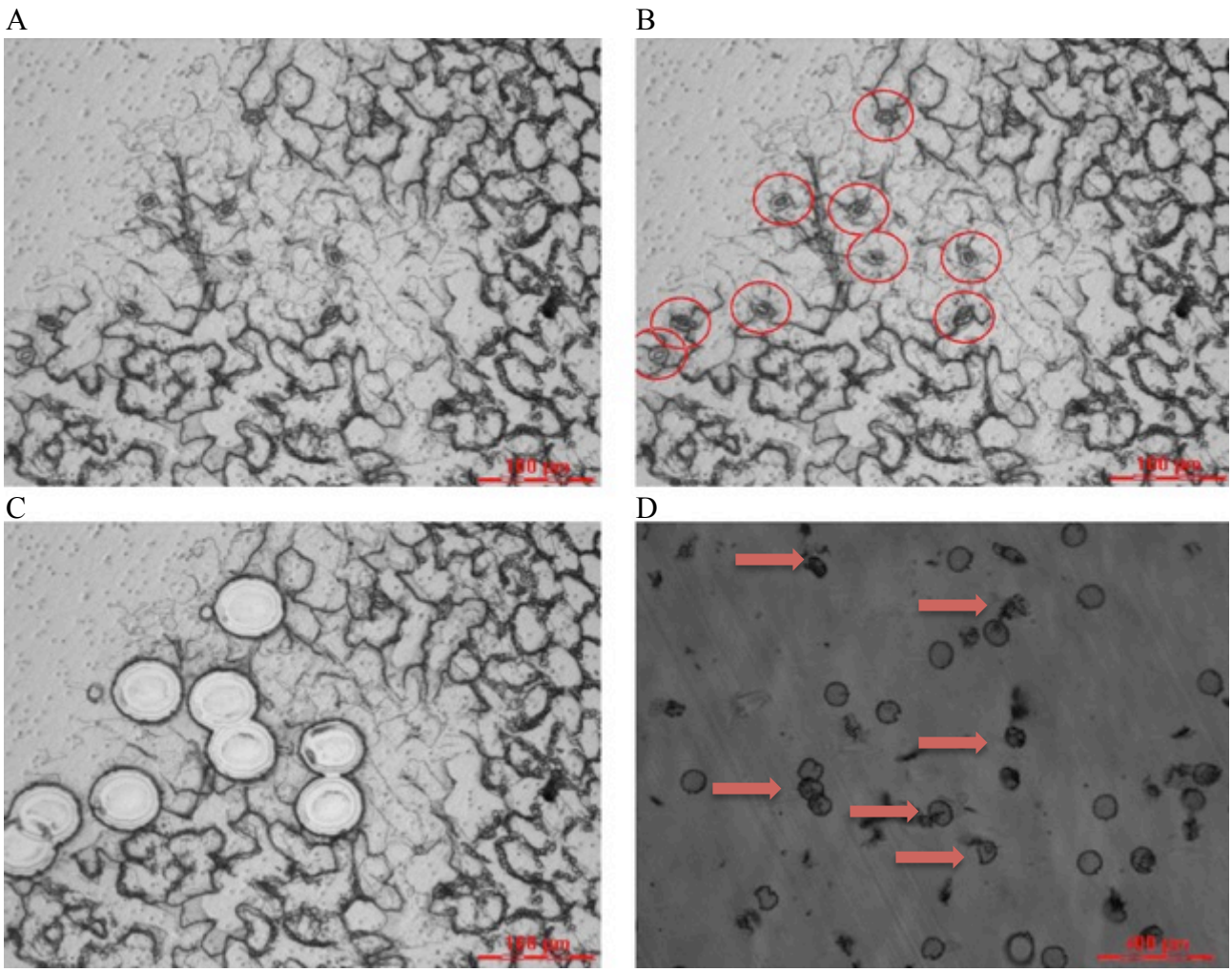


Figure 4 Laser micro-dissection of Arabidopsis leaves. (A) Light microscopy showing the anatomy of the Arabidopsis leaf after fixing and sectioning prior to LMD. (B) Guard cells selected before dissection (red circles). (C) Epidermal section after LMD of GC. (D) Stomata collected after the dissection (red arrows).

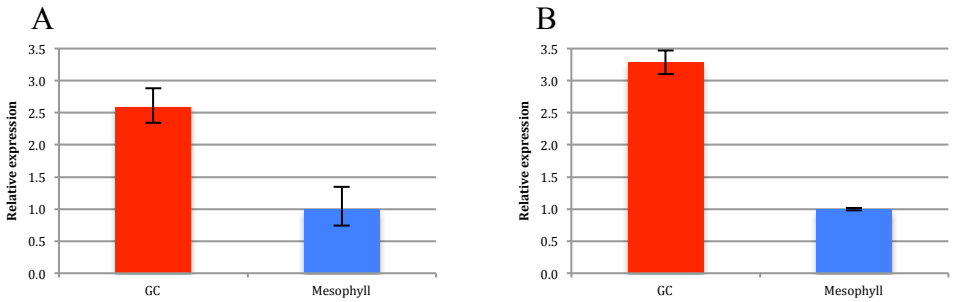


Figure 5 Expression profile of guard cells-specific genes in guard cells and mesophyll cells obtained by laser-microdissection. (A) *AtMYB60*, (B) *AtPHO1*.

As laser microdissection proved to be a very powerful technique for guard cells purification in Arabidopsis, we set for adopting the same strategy as a means to isolate stomata from grapevine leaves. The protocols optimised for the Arabidopsis samples did not yield positive results when applied to grape tissues. Differences in leaf morphology, thickness and accumulation of secondary metabolites between Arabidopsis and grape leaves proved to be crucial issues in obtaining pure cell preparations in Vitis. To overcome some of these obstacles, the main veins of the leaf were manually removed, to prevent shrinking of the tissues during the fixation step and the slide thickness was reduced from 10 to 6 µm to increase the cutting efficiency of the laser beam. Most importantly, different extraction kits had to be tested in order to identify the one capable of yielding good quality RNA. The routinely used kit in Arabidopsis (Applied Biosystems® Arcturus® PicoPure® RNA Isolation Kit) was not suitable for RNA extraction from grapevine guard cells. We tested several commercial kit and we obtained positive results with the PureLink® RNA Mini Kit and RNAqueous® Kit**.**

Despite these precautions a small number of GCs was isolated in each LMD-experiment (on average 1000 stomata for each RNA extraction), thus we experienced very poor yield of RNA. The limited amount of RNA obtained from each microdissection experiment allowed for the production of small amount of cDNAs, suitable for target qPCR analyses, but largely insufficient for genome wide studies (RNAseq or gene-chip microarray).

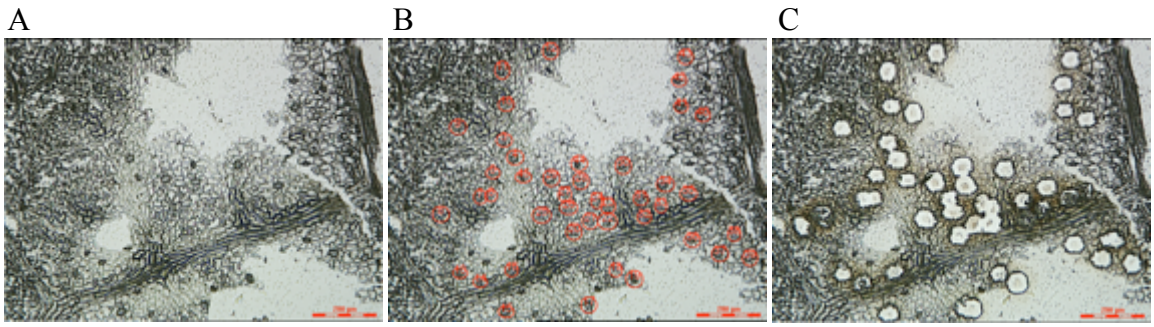


Figure 6 Laser micro-dissection of grape leaves. (B) Selection of stomata on the histological section. (C) Leaf section following dissection of the selected stomata.

The quality of the cDNA used in the qPCR experiments was first assessed by RT-PCR performed with primers corresponding to the *Vitis vinifera Actin1* gene (*VvACT1*) (Figure 16 A). The purity of the GC preparations was tested monitoring the expression of the GC-specific gene *VvSIRK* (Figure 7 B). *VvSIRK* belongs to the Shaker-like family of plant K^+ channels and it is specifically expressed in guard cells (Pratelli *et al.*, 2002).

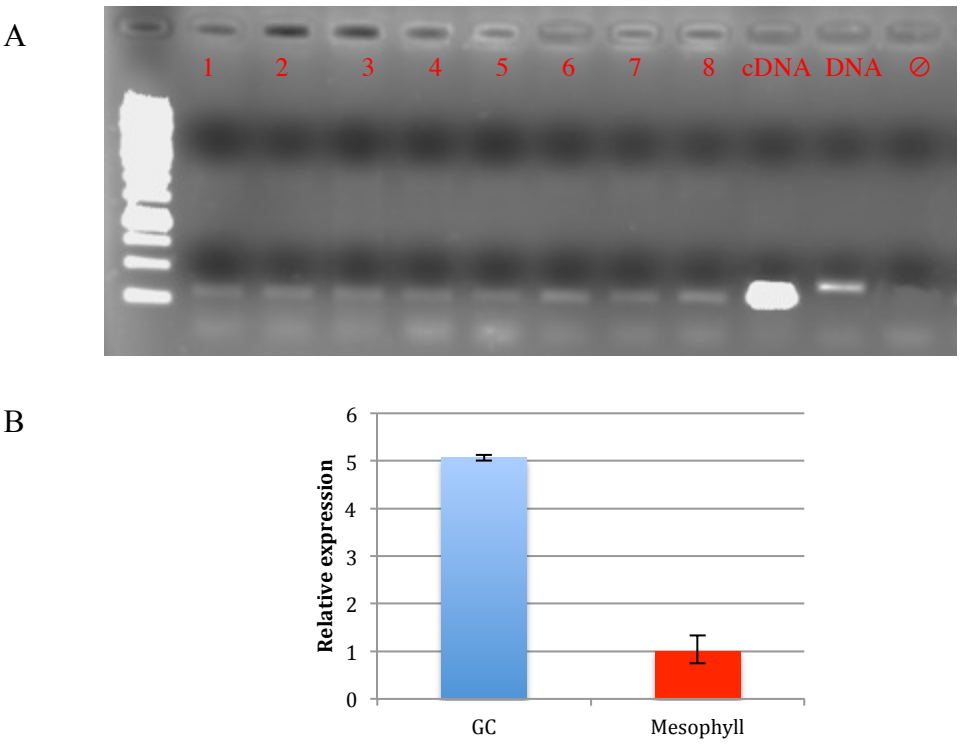


Figure 7 Analysis of LMD sample in grapevine. (A) RT-PCR analysis of the *VvACT* gene to assess RNA quality. (B) qPCR analysis of the GC-specific gene *VvSIRK*, to assess the purity of the GC preparations.

1.3 Purification of guard cells by mechanical disruption of leaf tissues

A rapid method for the isolation of epidermal pieces enriched in GC has been recently described for Arabidopsis plant (Bauer *et al.*, 2013). This method employs the use of a blender for the mechanical disruption of the leaf tissues and the recovery of epidermal pieces trough a microsieve. We adapted the procedure to grape leaves. In brief, the main vasculatures were manually removed from the leaves and samples were subjected to three rounds of blending in ice. Blended tissues were strained through a 250 µm microsieve and visually inspected under an optical microscope to estimate the relative number of intact stomata (Figure 8).

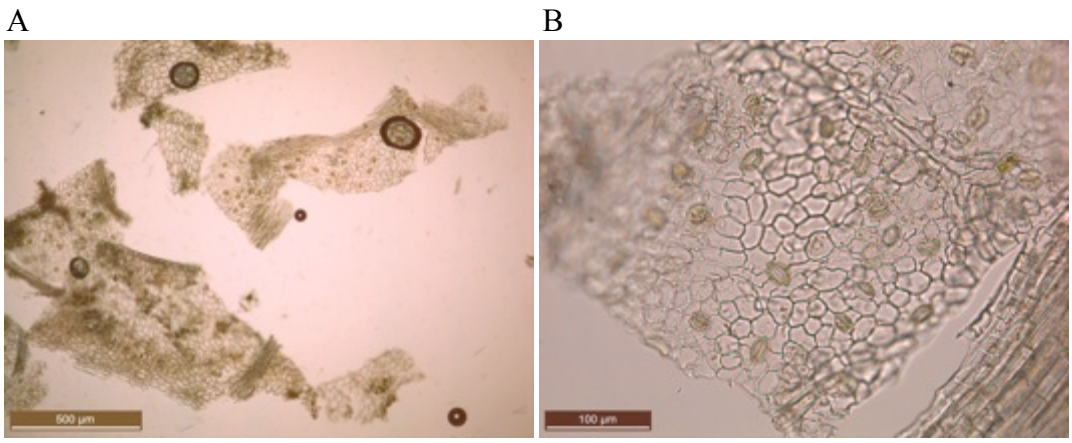


Figure 8 Epidermal pieces obtained by mechanical destruction of leaf tissues obtained with the blender method.

We also produced blended leaves (without separation of epidermal pieces) to be used as controls for the expression studies. Importantly, purified epidermal fragments and control tissues were subjected to the same mechanical disruption, thus avoiding a possible bias introduced by the mechanical stress. To test the actual enrichment of our preparation we investigated the expression level of *VvSIRK* that resulted more expressed in the GC samples compared with the total blended leaves (Figure 9).

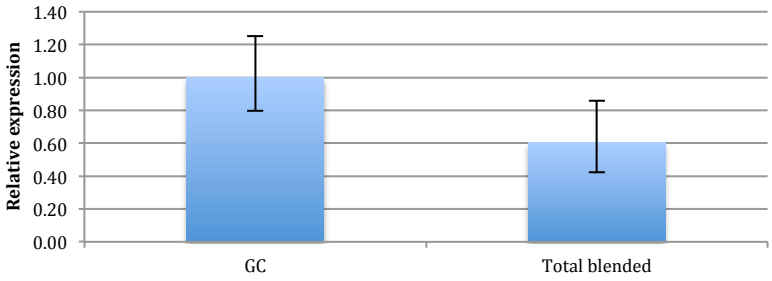


Figure 9 Gene expression of *VvSIRK* in epidermal fragments enriched in intact guard cells (GC) and total leaf blended.

Part II: Functional characterization of the guard cell-specific transcription factor *AtMYB60*

2.1 *atmyb60-1* shows alteration in transcriptional gene regulation

Cominelli and colleagues showed that, in the *atmyb60-1* mutant, the expression of a very limited number of genes, involved in the plant response to water stress or to pathogen attack, was altered (Cominelli *et al.*, 2005). In our lab, in order to improve these results the sequencing of RNAs derived from purified stomata was employed to uncover the complexity of the transcriptional changes triggered in the guard cell by the loss of the *AtMYB60* function. Using 1.5 fold change and corrected 0.05 p-value thresholds 615 and 378 genes were significantly down- or up-regulated, respectively. Among these, 51 genes were previously reported in a guard cell protoplast protein database (Leonhardt *et al.*, 2004). The results from the RNAseq experiment were analysed using PageMan (Usadel *et al.*, 2006), to look for functional categories over- or under-represented in the profiling data (Figure 10).

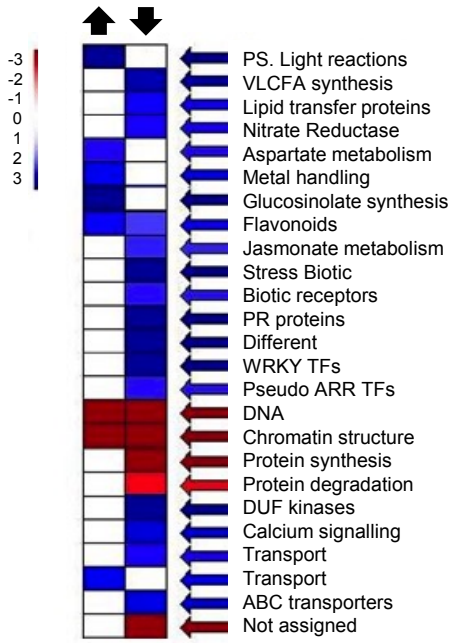


Figure 10 Functional categories over- and under-represented in the transcriptome of *atmyb60-1* GC, compared to WT GC. Differentially expressed transcripts were assigned to functional categories using MapMan and then subjected to overrepresentation analysis using PageMan. Blue indicates over- whilst red under-represented categories.

2.2 The *AtMYB60* loss of function mutant accumulates more oxylipins

Among down-regulated genes in *atmyb60-1*, predominated those involved in biotic stress responses such as the *Pathoghenesis Related Proteins 1*, *3* and *5* (*AtPR1, AtPR3, AtPR5*), the *Plant defensins 1.2* and *1.3* (*AtPDF1.2, AtPDF1.3*), and 13 *WRKY*-type transcription factors (e.g. *AtWRKY33* and *40*). Also enriched were transcripts assigned to fatty acid (FA) synthesis and more specifically to production of very long chained fatty acids (VLCFA) (Figure 10).

To investigate the role of *AtMYB60* in the regulation of FA biosynthesis and their possible involvement in mediating GC activity, we looked for biochemical changes in FA metabolism between WT e *atmyb60-1* plants. As *AtMYB60* is specifically expressed in GC, we profiled the FA composition of both whole leaves and purified epidermal fragments enriched of intact guard cells. GC-enriched epidermal fragments were obtained by mechanical disruption of leaf tissues as described in (Bauer *et al.*, 2013). In order to evaluate the level of purity of the epidermal preparations we tested the expression of known guard cell- and phloem-specific genes by qPCR. The up-regulation of *AtMYB60* and the down-regulation of the vascular-specific *Arabidopsis Interfascicular Fiber1* (*AtIFL1*) genes (Zhong and Ye, 1999), indicated the effective enrichment in GC of our preparations (Figure 11).

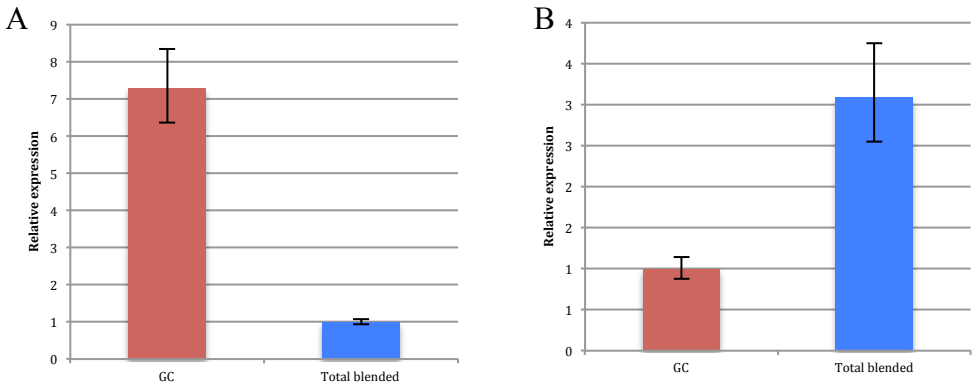


Figure 11 Relative expression of (A) *AtMYB60* and (B) *AtIFL1* in guard cell-enriched samples (GC) compared with blended leaves without GC purification (Total blended).

Metabolite profile of all fatty acid was performed in collaboration with Patrick Galivasco (Max Planck Institute) through the gas chromatography–mass spectrometry (GC-MS) on whole leaves and GCs sample in *atmyb60-1* and WT. In total 193 compounds related to fatty acids were detected in our samples, belonging to nine different chemical families (Table 2).

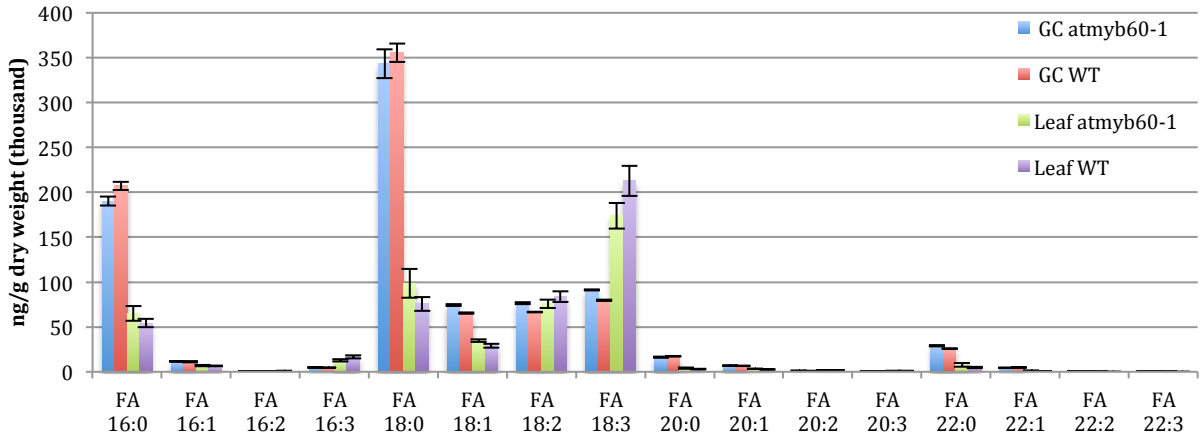
Table 2 List of all fatty acid families detected in whole leaves and GCs

Family	# of compounds
Free Fatty Acid	19
Galactoglycerolipid	22
lyso Phospholipid	13
Oxylipin	5
Phospholipid	40
Pigment	3
Shingolipid	38
Sulfoquinovosylglycerolipid	9
Triacylglyceride	43

Among the 48 compounds detected in the triacylglyceride family a little number of them was accumulated more in GCs than in the whole leaf and four of this (triacylglyceride 48:0, 50:0, 50:1 and 54:6) were accumulated at higher level in GCs of *atmyb60.1* than in GC of WT (Figure 12 H and I). Some short chain fatty acids belonging to the free fatty acid family were accumulated more in the GCs than in the whole leaf (FA 16:0, FA 18:0, FA 20:0, FA 22:0 and the lyso phospholipids 16:0, 18:1_1 and 18:2_2) (Figure 12). However, they showed comparable levels in GCs of the mutant line and of the WT.

Two saturated VLCFAs (C24:0 and C26:0) and two unsaturated VLCFAs (C24:1 and C26:1) were detected (Figure 13). Saturated VLCFAs disclosed an increased accumulation in GCs compared with whole leaves, but did not show significant differences between WT and *atmyb60-1* (Figure 13).

A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300
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200
150
100
50
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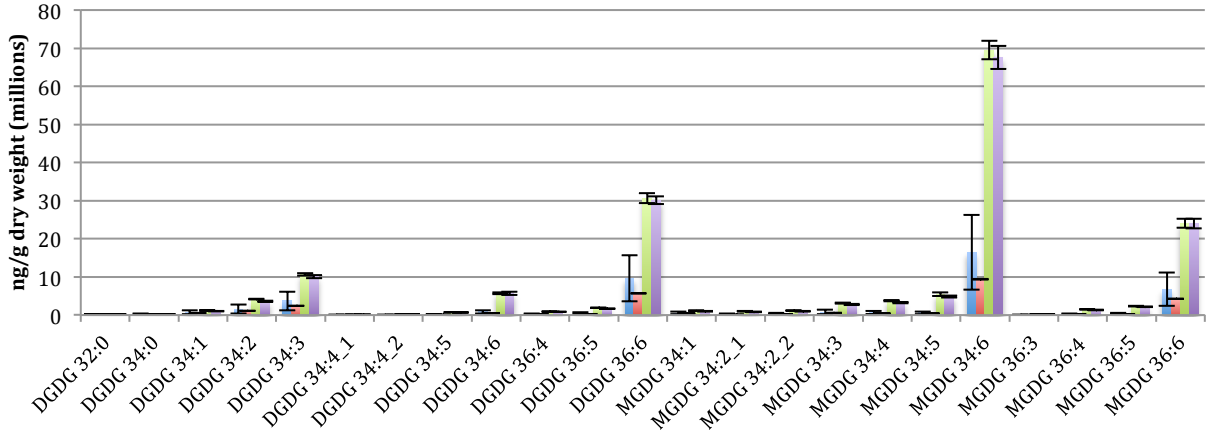
GC WT
Leaf atmyb60-1
Leaf WT
FA 16:0
FA 16:1
FA 16:2
FA 16:3
FA 18:0
FA 18:1
FA 18:2
FA 18:3
FA 20:0
FA 20:1
FA 20:2
FA 20:3
FA 22:0
FA 22:1
FA 22:2
FA 22:3

B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DGDG 34:0
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DGDG 34:2
DGDG 34:3
DGDG 34:4_1
DGDG 34:4_2
DGDG 34:5
DGDG 34:6
DGDG 36:4
DGDG 36:5
DGDG 36:6
MGDG 34:1
MGDG 34:2_1
MGDG 34:2_2
MGDG 34:3
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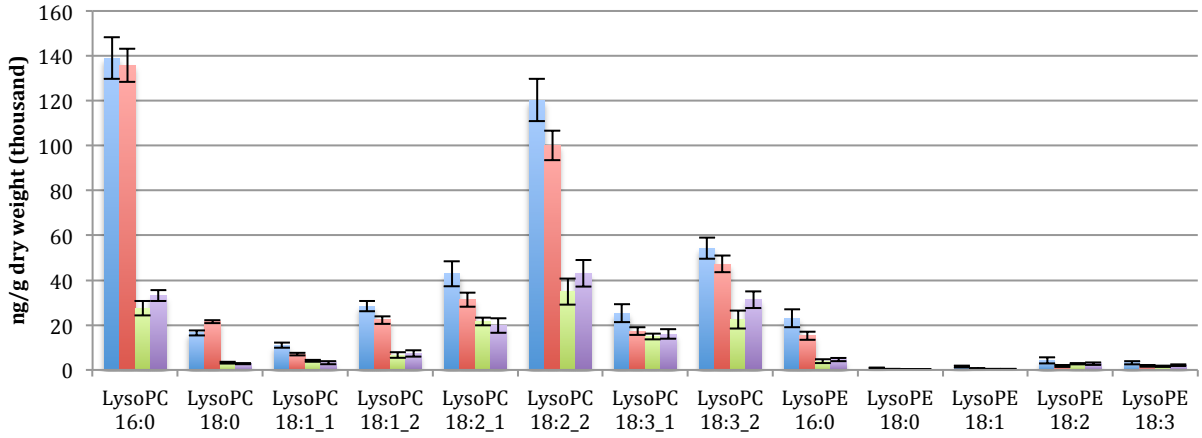
MGDG 36:4
MGDG 36:5
MGDG 36:6

C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120
100
80
60
40
20
0
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LysoPC 18:0
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LysoPC 18:2_1
LysoPC 18:2_2
LysoPC 18:3_1
LysoPC 18:3_2
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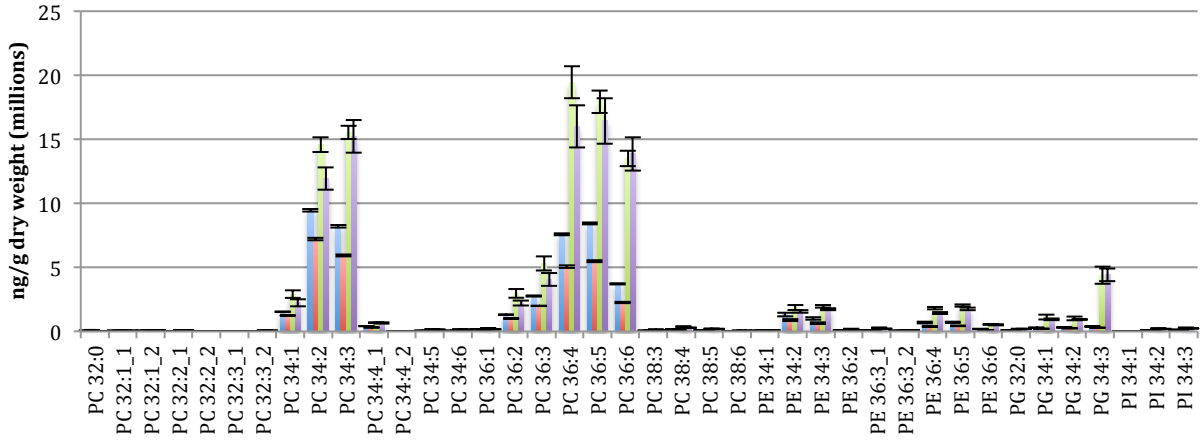
LysoPE 18:2
LysoPE 18:3

D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20
15
10
5
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PC 32:1_1
PC 32:1_2
PC 32:2_1
PC 32:2_2
PC 32:3_1
PC 32:3_2
PC 34:1
PC 34:2
PC 34:3
PC 34:4_1
PC 34:4_2
PC 34:5
PC 34:6
PC 36:1
PC 36:2
PC 36:3
PC 36:4
PC 36:5
PC 36:6
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PE 34:1
PE 34:2
PE 34:3
PE 36:2
PE 36:3_1
PE 36:3_2
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PG 34:3
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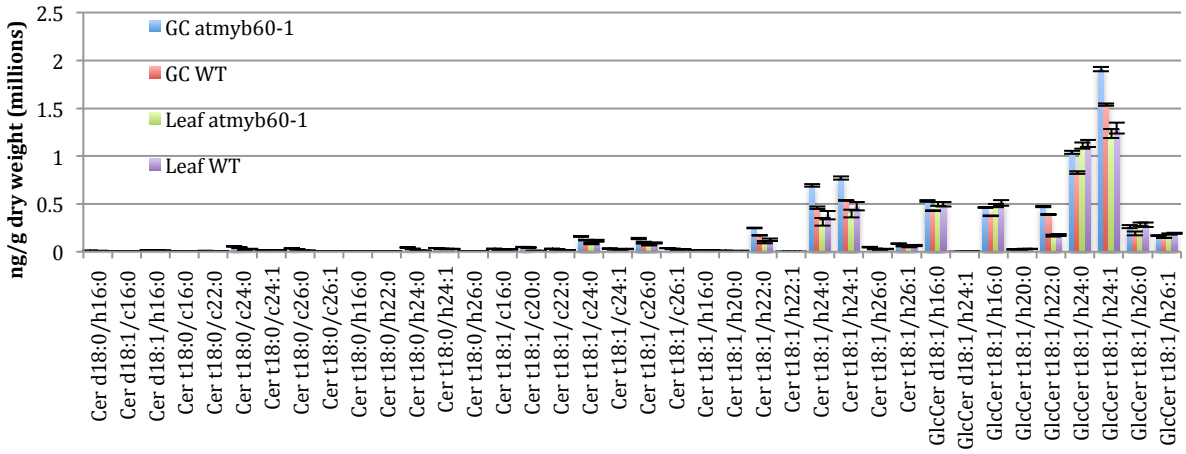
PI 34:3

E


GC atmyb60-1
GC WT
Leaf atmyb60-1
Leaf WT
ng/g dry weight (millions)
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1.5
1
0.5
0
Cer d18:0/h16:0
Cer d18:1/c16:0
Cer d18:1/h16:0
Cer t18:0/c16:0
Cer t18:0/c22:0
Cer t18:0/c24:0
Cer t18:0/c24:1
Cer t18:0/c26:0
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GlcCer t18:1/h22:0
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GlcCer t18:1/h26:1

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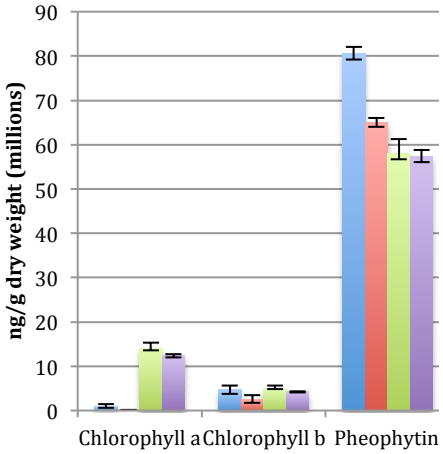
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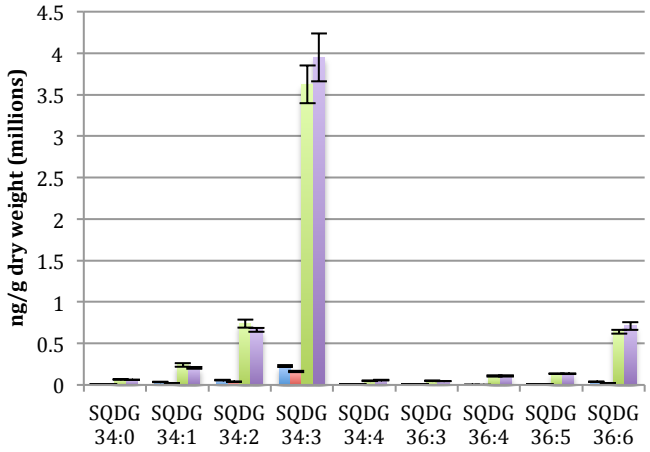
70
60
50
40
30
20
10
0
Chlorophyll a
Chlorophyll b
Pheophytin

G


ng/g dry weight (millions)
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3.5
3
2.5
2
1.5
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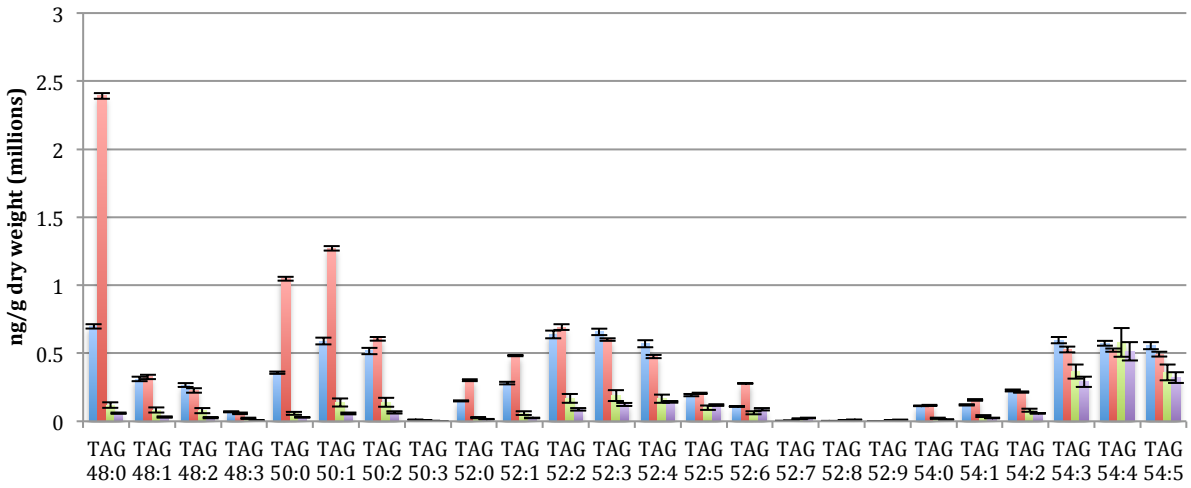
SQDG 34:4
SQDG 36:3
SQDG 36:4
SQDG 36:5
SQDG 36:6

H


ng/g dry weight (millions)
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1.5
1
0.5
0
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TAG 48:1
TAG 48:2
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TAG 50:1
TAG 50:2
TAG 50:3
TAG 52:0
TAG 52:1
TAG 52:2
TAG 52:3
TAG 52:4
TAG 52:5
TAG 52:6
TAG 52:7
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TAG 54:1
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TAG 54:5

I

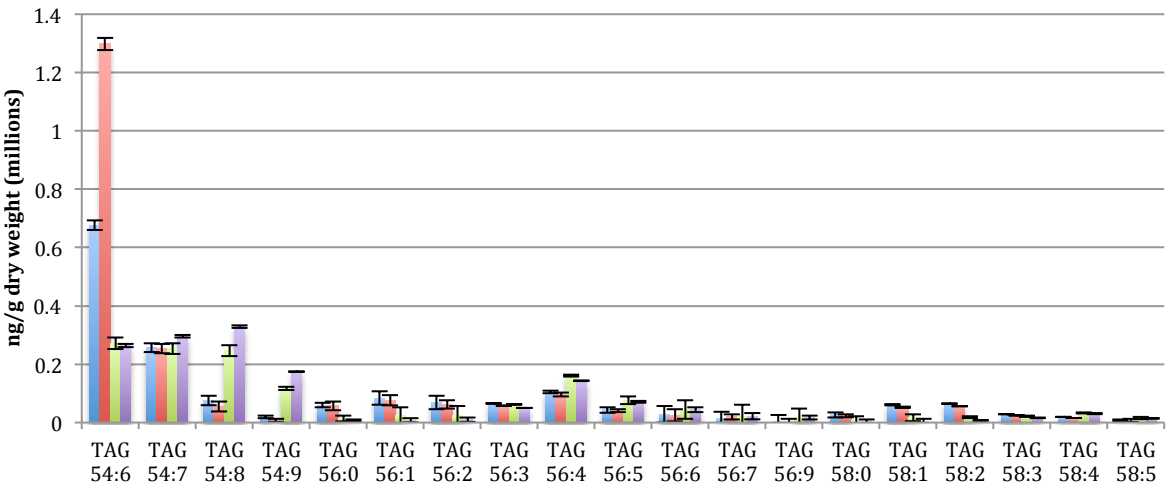
ng/g dry weight (millions)
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Figure 12 GC-MS analysis of lipids and pigments in *atmyb60-1* GC (blue bar), WT GC (red bar), *atmyb60-1* whole leaf (green bar) and WT whole leaf (violet bar). (A) Free fatty acid (B) Galactoglycerolipid (C) Lyso-phospholipid (D) Phospholipid (E) Shingolipid (F) Pigment (G) Sulfoquinovosylglycerolipid (H, I) Triacylglyceride.

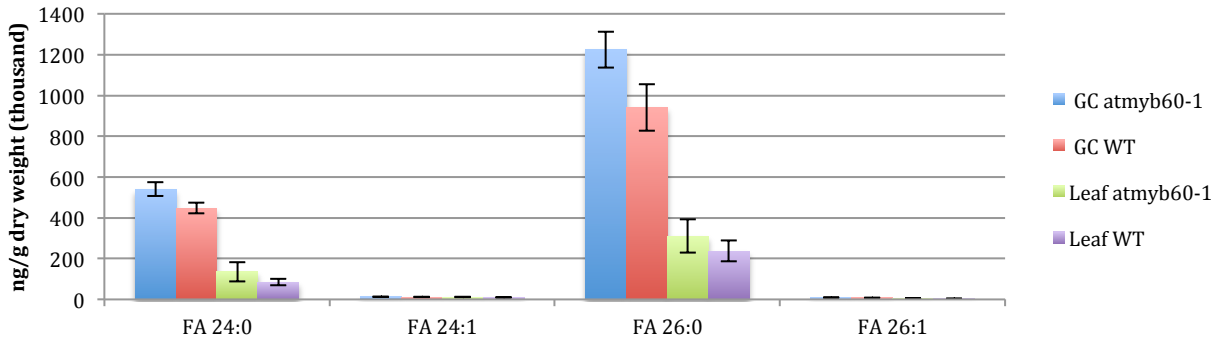

Figure 13 VLCFA quantification in whole leaves and GCs of WT and *atmyb60-1* plants.

Interestingly, the accumulation of some compounds belonging to the oxylipins family disclosed significant differences between WT and mutant plants. The oxylipin family consists of 12-oxo-phytodienoic acid (OPDA), dinor-phytodienoic acid (dn-OPDA), and derivatives of mono- and digalactosyl diacylglycerol (MGDG and DGDG), containing one or two chains of OPDA and/or dn-OPDA, respectively (Kourtchenko *et al.*, 2007). DGDG, MGDG and OPDA accumulated preferentially in guard cells compared to the whole leaves. Most importantly, levels of these oxylipins were enhanced in GCs of the *atmyb60-1* plants compared with GCs of the WT (Figure 14).

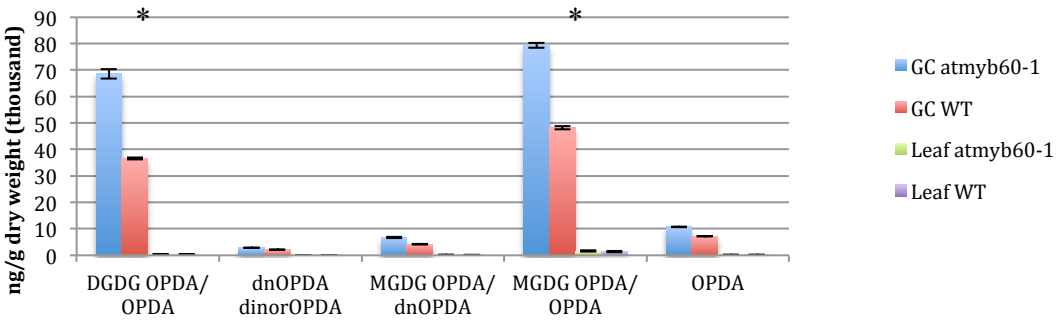

Figure 14 Quantification of oxylipin compounds in whole leaves and GCs of WT and *atmyb60-1* plants. Asterisks indicate significant differences (t-Test, p <0.01).

Interestingly, a recent work showed that oxylipins promotes stomata closure in response to drought, (Savchenko *et al.*, 2014). Oxylipins are synthesize from α-linolenic acid in the chloroplast and then translocated in the peroxisome, via the COMATOSE (AtCTS) transporter, to produce, after a few steps, jasmonic acid (JA) (Feussner and Wasternack, 2002). Similarly to oxylipins, JA accumulates during drought stress and in GC it triggers stomatal closure through a signalling pathway which overlaps with that of ABA (Creelman and Mullet, 1995; Acharya and Assmann, 2009).

The more direct and obvious link between *AtMYB60* activity and the observed over-accumulation of oxylipins in the *atmyb60-1* mutant is the transcriptional control of genes involved in the oxylipin/jasmonate biosynthetic pathway exerted by the AtMYB60 transcription factor.

To test this hypothesis we performed a comprehensive qPCR analysis of the genes directly involved in the oxylipin biosynthetic pathway and in its oxidation to produce JA. To increase the spatial resolution of our analysis to the only leaf cell type in which *AtMYB60* is expressed we adopted a laser micro-dissection (LMD) approach to isolate GCs.

As shown in Figure 15, the octadecanoid pathway in *Arabidopsis thaliana* that gives rise to jasmonic acid, initiates with the conversion of oleic acid catalysed by FATTY ACID DESATURASE-3 (AtFAD3), FATTY ACID DESATURASE-7 (AtFAD7) and FATTY ACID DESATURASE-8 (AtFAD8) which convert linoleic acid (18:2) to linolenic acid (18:3) by inserting a double bond at the v-3 position (Zhang *et al.*, 2012). Linolenic acid is released from cellular lipids in chloroplast by defective in ANTHER DEHISCENTE1 (AtDAD1) (Dave and Graham, 2012). qPCR analysis of whole leaves showed higher expression of *AtDAD1* in *atmyb60-1*compared to the WT (Figure 16D). Yet, analysis of purified GC did not confirm the different regulation of *AtDAD1* expression in mutant and WT plant (Figure 17 D).

The second step of the pathway is the oxidation of octadecatrienoic acid (18:3n-3) by LIPOXYGENASE (AtLOX) to form 13-hydroperoxylinolenic acid. This is then acted on by ALLENE OXIDE SYNTHASE (AtAOS) and ALLENE OXIDE CYCLASE (AtAOC) to give *cis*-(+)-12- oxo-phytodienoic acid (*cis*-OPDA). *cis*-OPDA then travels via the cytosol to the peroxisome with uptake into this organelle being mediated, at least in part, by the ATP binding cassette (ABC) transporter protein, COMATOSE (AtCTS) (Theodoulou *et al.*, 2005). Once in the peroxisome, *cis*-OPDA is reduced (Schaller and Stintzi, 2009) and activated to the CoA ester (Schneider *et al.*, 2005) prior to undergoing three rounds of β-oxidation to form JA (Schilmiller and Howe, 2005).

AtLOX is encoded by six genes belonging to the same gene family. We found that *AtLOX1, AtLOX4* and *AtLOX6* were more expressed in GCs than in mesophyll cells (Figure 17 E, H, J), whereas *AtLOX2*, *AtLOX5* (Figure 17 F, I)*, AtAOS, AtAOC2, AtAOC3* (Figure 17 *K,* L, M)*,* resulted

more expressed in mesophyll cells than in guard cells. Finally, *AtOPR2* (Figure 17 O) was more expressed in GCs, whereas *AtOPR3, AtPKT1* and *AtJMT* were more expressed in mesophyll (Figure 17 P and V). Likewise, in the *AtOPR* family *AtOPR*2 was more expressed in GCs (Figure 17 O). These observations suggest the presence of a guard cell-specific pathway for oxylipins biosynthesis and provide a molecular basis for the over-accumulation of oxylipins that we detected in GC compared with whole leaves.

Despite *AtLOX4, AtLOX6* and *AtOPR*2 being more expressed in GCs we did not find any significant differences between WT and *atmyb60-1* (Figure 17 F, I, J and O).

Besides the analysis of genes encoding biosynthetic enzymes, we analysed the expression of *AtCTS1* that is involved in the transport of OPDA from the chloroplast to the peroxisome (Theodoulou *et al.*, 2005). We did not detected significant differences in the levels of *AtCTS1* expression between WT and *atmyb60-1* plants neither in intact leaves, nor in purified GCs (Figure 18 A and B).

Jasmonic acid is the final product of the oxylipin pathway, produced via the b-oxidation of OPC-8:0 (3-oxo-2-[2'-pentenyl]- cyclopentane-1-octanoic acid) (Howe and Schilmiller, 2002).

As our metabolic profiling did not include JA, we assessed possible differences in JA accumulation in leaves and GC of mutant and WT plant indirectly. To this end, we examined the expression of two well-known JA-responsive genes, namely *Vegetative storage protein1 (AtVSP1)* and *Vegetative storage protein2 (AtVSP2)*, whose expression is directly correlated with JA concentration in plant tissues (Utsugi *et al.*, 1998). Interestingly, both *AtVSP1* and *AtVSP2* disclosed an increase in their expression in GC of the mutant line, suggesting a possible enhanced accumulation of jasmonic acid in *atmyb60-1* stomata (Figure 18 C-F).

As a whole these data provide evidence for a GC-specific oxylipin metabolism, likely involved in the control of stomatal activity. Yet, they do not rule out the direct transcriptional control of *AtMYB60* on the expression of the enzyme involved in this pathway.

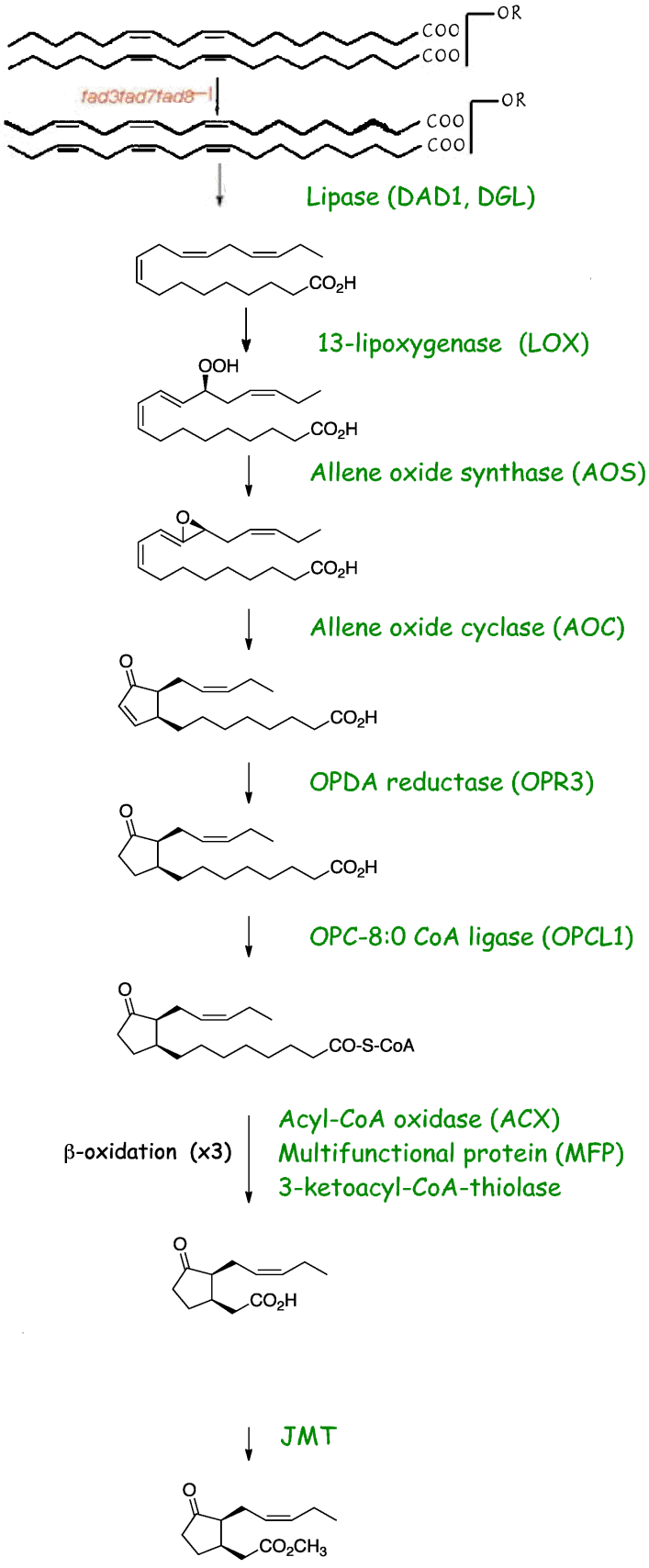

Figure 15 Biosynthetic pathway of oxylipins and jasmonic acid (Modified from http://hormones.psc.riken.jp/img/pathway_ja.png).

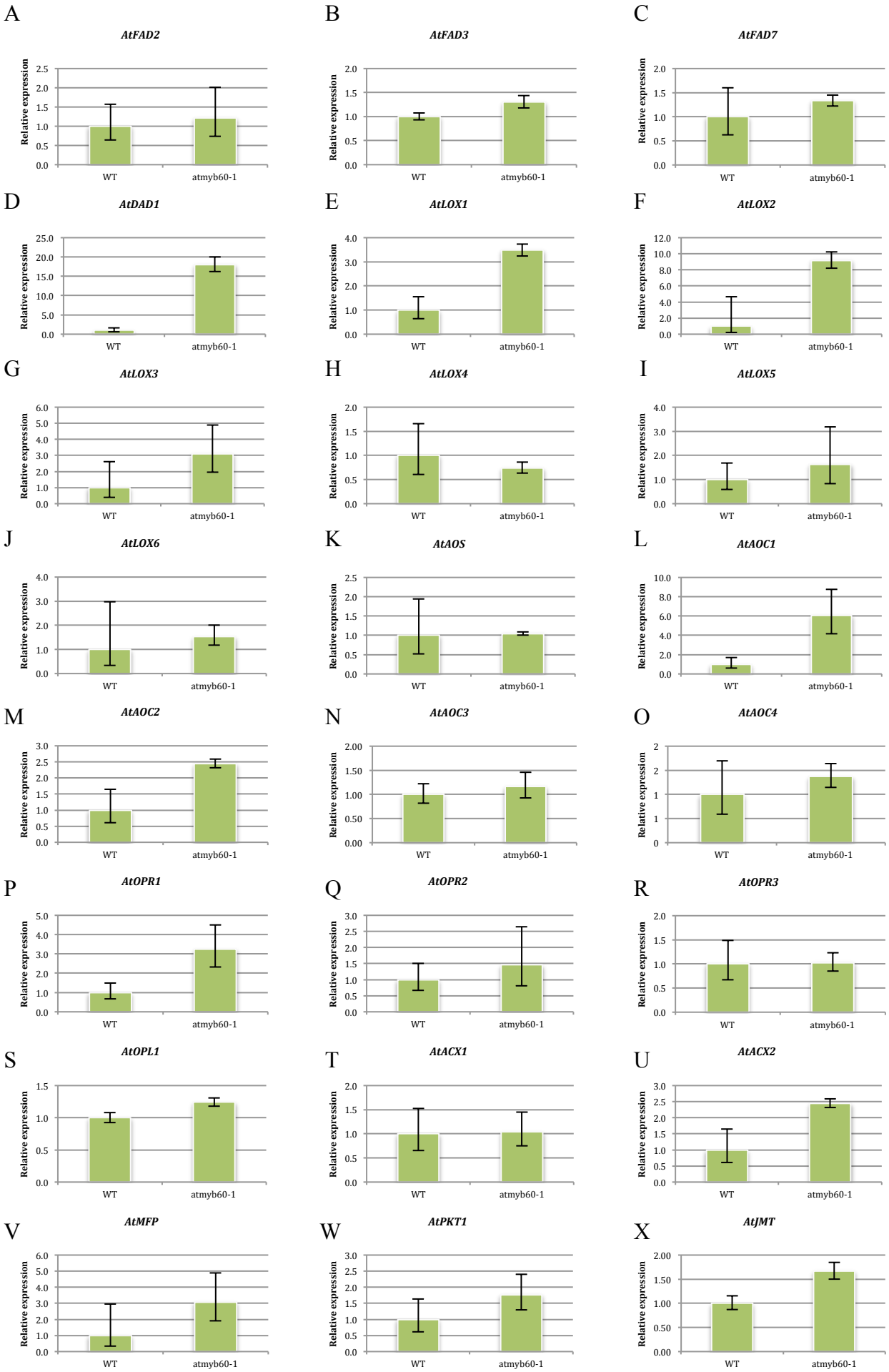

Figure 16 Gene expression analysis of oxylipins and jasmonate biosynthetic pathway performed on whole leaf of WT and *atmyb60-1*(A) *AtFAD2* (B) *AtFAD3* (C) *AtFAD7* (D) *AtDAD1* (E) *AtLOX*1 (F) *AtLOX2* (G) *AtLOX3* (H) *AtLOX4* (I) *AtLOX5 (J) AtLOX6* (K) *AtAOS* (L) *AtAOC1* (M) *AtAOC2* (N) *AtAOC3* (O) *AtAOC4* (P) *AtOPR1* (Q) *AtOPR2* (R) *AtOPR3* (S) *AtOPL1* (T) *AtACX1* (U) *AtACX2* (V) *AtMFP* (W) *AtPKT1* (X) *AtJMT.*

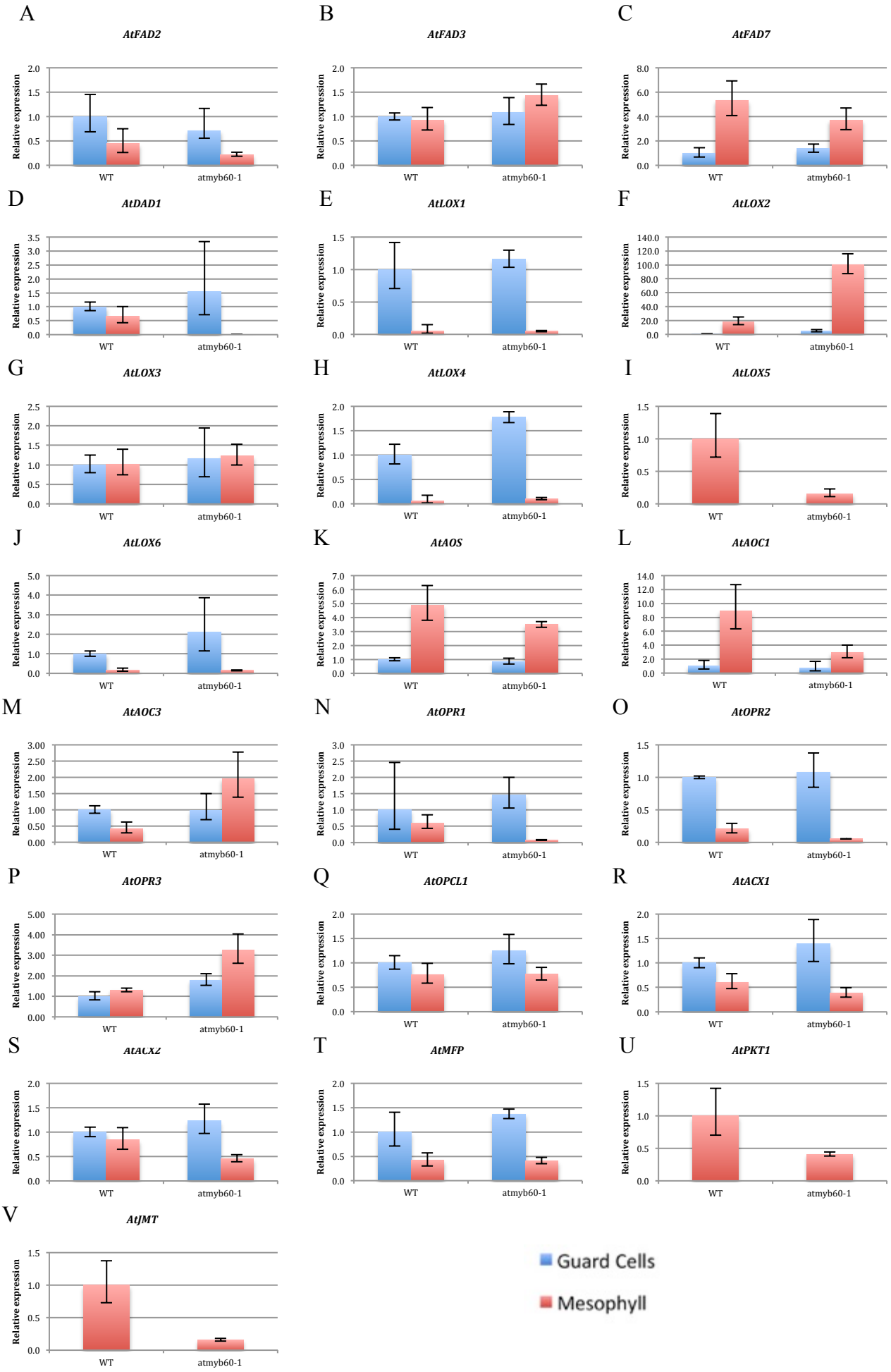

Figure 17 Gene expression analysis of oxylipins and jasmonate biosynthetic pathway performed on LMD samples of WT and *atmyb60-1* (A) *AtFAD2* (B) *AtFAD3* (C) *AtFAD7* (D) *AtDAD1* (E) *AtLOX*1 (F) *AtLOX2* (G) *AtLOX3* (H) *AtLOX4* (I) *AtLOX5 (J) AtLOX6* (K) *AtAOS* (L) *AtAOC1* (M) *AtAOC3* (N) *AtOPR1* (O) *AtOPR2* (P) *AtOPR3* (Q) *AtOPL1* (R) *AtACX1* (S) *AtACX2* (T) *AtMFP* (U) *AtPKT1* (V) *AtJMT.*

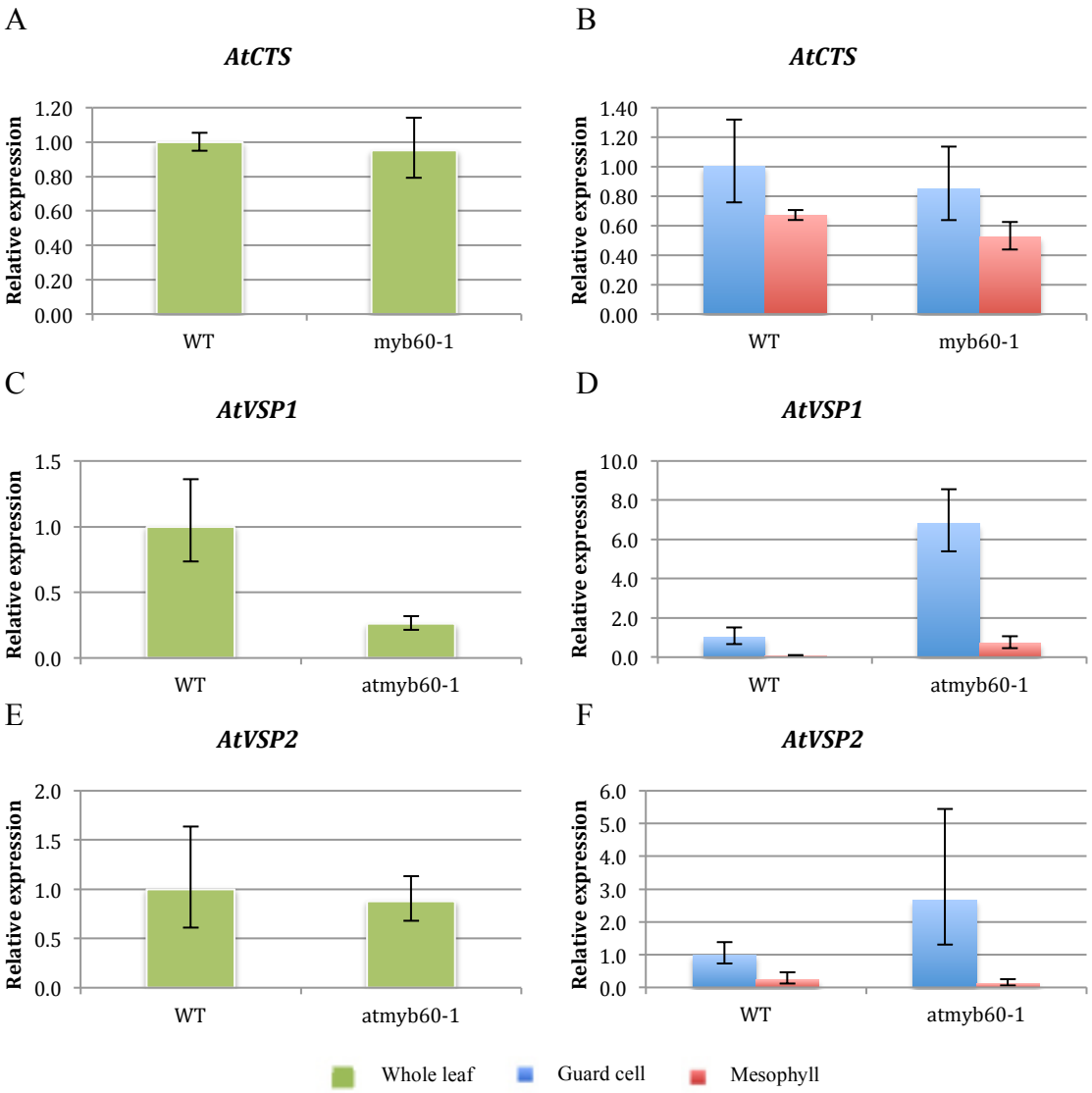

Figure 18 Gene expression analysis of JA-responsive genes in whole leaf (green bar), GC (blue bar) and mesophyll cells (red bar) from WT and *atmyb60-1* plants. (A, B) *AtCTS*, (C, D) *AtVSP1*, (E, F) *AtVSP2*.

2.3 *AtMYB60* mediates SA-induced stomata closure

RNA-seq data indicated that the loss of the *AtMYB60* function resulted in impaired expression of the defence-related and salicylic acid (SA)-induced genes *AtPR1*, *AtPR3* and *AtPR5*. This observation prompted us to investigate the possible link between SA and *AtMYB60*.

SA accumulation is induced by pathogen/microbe-associated molecular patterns (PAMP/MAMP) that are required for bacterium-induced stomatal closure (Melotto *et al.*, 2006). SA triggers intracellular ROS accumulation and K^+ in channel inactivation, which ultimately results in stomatal closure in Arabidopsis (Khokon *et al.*, 2011), in *Commelina communis* and *Vicia faba* (Mori *et al.*, 2001). *AtPR1* expression is induced in response to a variety of pathogens and it is a useful and well established molecular marker for the systemic acquired resistance (SAR) response (Durrant and Dong, 2004). Also, *AtPR1* expression requires the SA-binding protein ARABIDOPSIS NON EXPRESSER OF PR1 (AtNPR1), a central activator of SA-dependent defence genes (Wu *et al.*, 2012). *AtNPR1* loss of function mutants are impaired in SA signalling and *AtPR1* is no longer activated after pathogen attack in *atnpr1-1* plants (Dong, 2004). The requirement of *AtNPR1* for the SA-induced stomatal closure is still unclear.

To investigate the possible involvement of *AtNPR1* in the regulation of stomatal activity we first investigated light-induced stomata opening in WT, *atmyb60-1, atnpr1-1* and *atmyb60/atnpr1-1* plants previously kept in the dark. Confocal microscope analysis confirmed that light-induced stomata opening was impaired in *atmyb60-1* (Cominelli *et al.*, 2005). Stomata aperture of *atnpr1-1* plant did not show any differences from the WT, confirming results reported in (Zeng and He, 2010). Interestingly, the *atmyb60-1/atnpr1-1* double mutant showed a partial rescue of the *atmyb60-1* phenotype, as stomata were more open in *atmyb60-1/atnpr1-1* leaves, compared with stomata from *atmyb60-1* leaves ($p<0.01$) (Figure 19 A).

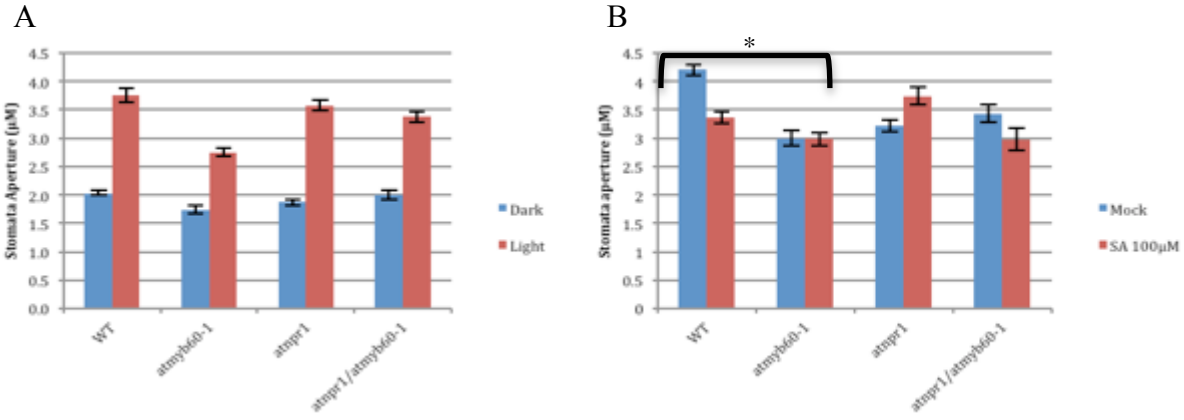

Figure 19 Analysis of stomatal activity in WT, *atmyb60-1*, *atnpr1* and *atmyb60-1/atnpr1*. (A) Light-induced stomata opening (B) SA-induced stomata closure. Asterisk indicate significant differences (t-Test, p <0.01)

As SA induces stomatal closure, we next tested the requirement of *AtMYB60* for stomatal closure in response to salicylic acid treatment. Application of 1 mM SA reduced stomatal aperture by 20% in the WT but, as expected, not in *atnpr1-1*. Interestingly, we found that *atmyb60-1* plants were defective in the stomatal closure response to SA, indeed stomata aperture was not significantly different from the control (Figure 19).

To get more insight into the role of *AtMYB60* in SA-mediated responses, we analysed the transcriptional changes in *AtMYB60, AtNPR1, AtPR1, AtPR2, AtPR3* and *AtPR5*, occurring in WT, *atmyb60-1, atnpr1-1 and atmyb60/atnpr1-1* plants after treatment with 1 mM SA.

As expression of the routinely used housekeeping gene *Arabidopsis Actin 2* (*AtACT2*) has been reported to be affected by SA (Matoušková *et al.*, 2014), the *Arabidopsis eukaryotic translation initiation factor 4A1 (AteIF4)* was also used as a reference to normalise gene expression (Seo and Park, 2010). We analysed *AtPR1* and *AtMYB60* expression normalized against *AtACT* and *AteIF4* and we obtained very similar results (Figure 20). As expected, *AtPR1* expression was strongly induced by SA (Figure 20 A, B). We found that *AtMYB60* was down-regulated after SA treatment, an observation which correlates with the closure of stomata induced by SA. Interestingly, in *AtMYB60* loss of function plants *AtPR1* was more expressed than in WT plants (Figure 20 A, B).

As expected, expression of *AtMYB60* and *AtPR1* was barely detectable in the *atmyb60-1* and *atnpr1-1* single mutants, respectively and in the double *atmyb60/atnpr1-1* mutant. (Figure 20 C, D).

As opposite to *AtMYB60*, *AtNPR1* expression was not significantly regulated by the SA treatment (Figure 20 E).

In order to corroborate the results obtained from gene expression analysis performed on whole leaves, we employed the analysis of purified GC and mesophyll cells, obtained by LMD. As anticipated, *AtMYB60* expression was confined to GC in all the genotypes analysed (Figure 21 A). In GC purified from WT leaves *AtMYB60* expression did not show significant difference compared to whole leaves in response to SA (Figure 21 A). As opposite to what we observed in intact leaves, the basal level of *AtMYB60* expression in *atnpr1-1* GC was significantly enhanced compared to GCs of the WT (Figure 21 A). Following 4h and 8h of treatment with SA, *AtMYB60* expression was down-regulated to the same extent in *atnpr1-1* and WT GC (Figure 21 A). Nevertheless, 24 h after the beginning of the treatment *AtMYB60* expression in GCs was restored to its initial level in *atnpr1-1* but not in the WT (Figure 21 A). These results suggest that *AtNPR1* could act as a repressor of *AtMYB60* expression in GCs.

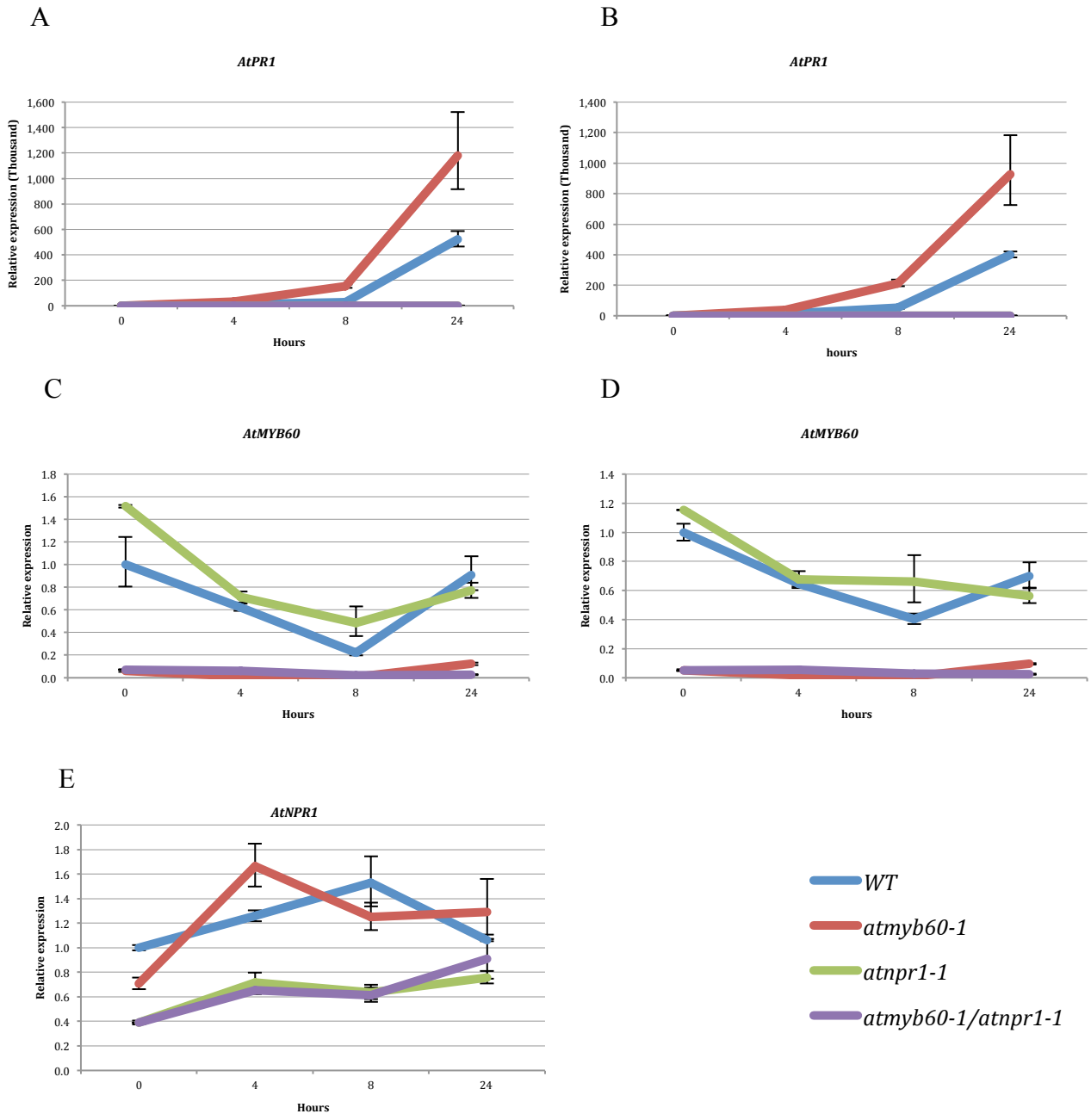

Figure 20 Gene expression in whole leaf after SA treatment. Expression level of *AtPR1* relative to (A) *AtACT* and (B) *AteIF4a*. Expression level of *AtMYB60* relative to (C) *AtACT* and (D) *AteIF4a* and (E) expression level of *AtNPR1* relative to *AtACT*.

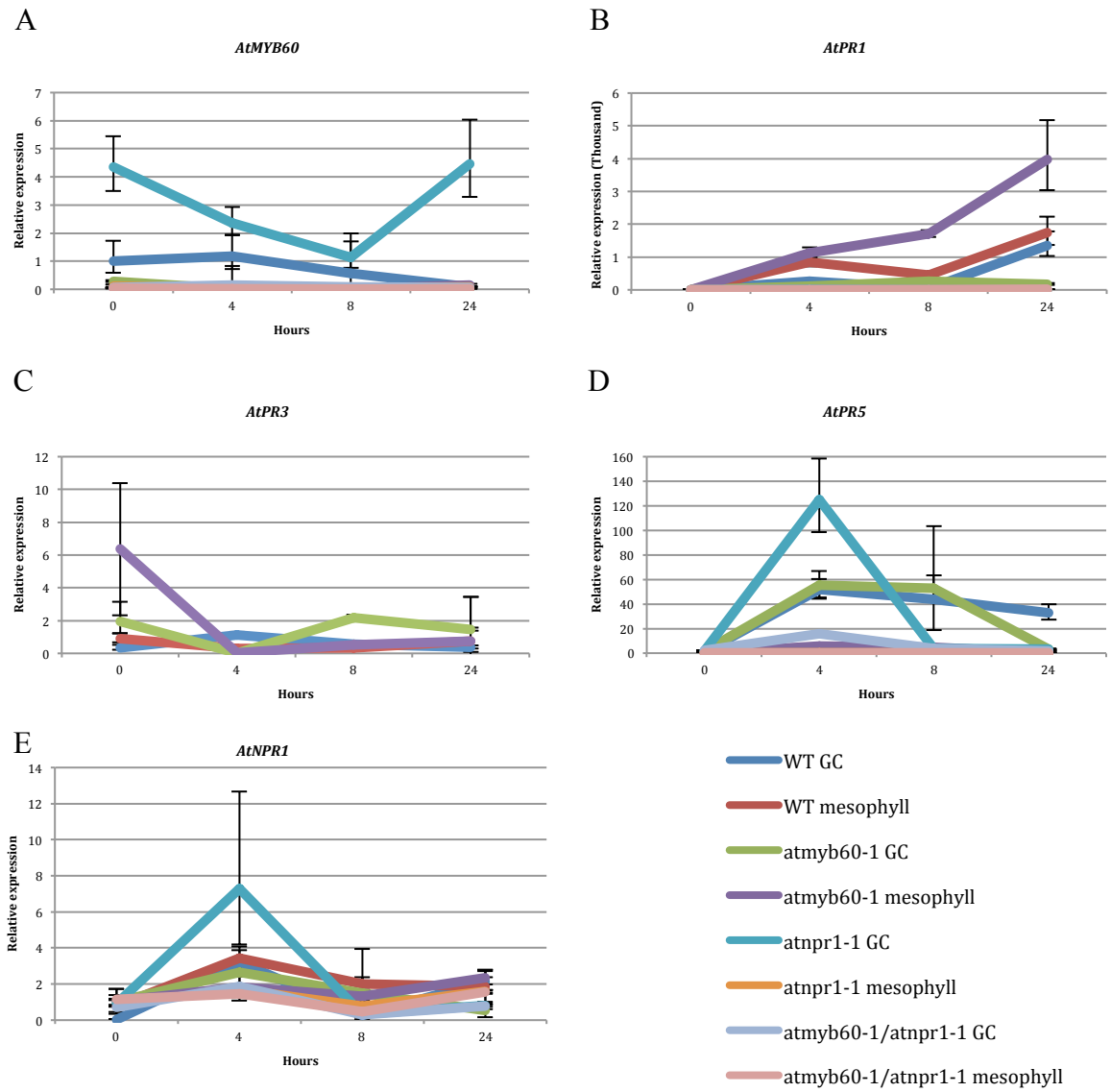

Figure 21 Gene expression in LM-purified GC and mesophyll cells following treatment with SA. (A) *AtMYB60*, (B) *AtPR1*, (C) *AtPR3*, (D) AtPR5, (E) *AtNPR1* in WT, *atmyb60-1*, *atnpr1-1* and *atmyb60-1/atnpr1-1* guard cells and mesophyll cells.

AtPR1 in the WT was induced by SA both in guard cells and mesophyll cells (Figure 21 B). Conversely, in the *atmyb60-1* mutant *AtPR1* was activated only in mesophyll cells. Unexpectedly, in this tissue *AtPR1* expression showed a SA-induced activation significantly greater in the mutant compared with the WT. In GCs of *atmyb60-1* plants *AtPR1* lacked the canonical strong activation usually observed 24 hours after the beginning of the treatment (Figure 21 B). This result suggests a possible role for *AtMYB60* as a positive regulator of *AtPR1* expression in GCs. Consistent with previous reported, *AtPR1* in *atnpr1-1* was not expressed neither in GCs nor in mesophyll cells (Figure 21 B).

From RNA-seq data *AtPR3* and *AtPR5* expression resulted impaired in *atmyb60-1*. qPCR analysis revealed that *AtPR3* is not expressed in GC and that its expression in the mesophyll is not affected

by SA or by the *atmyb60-1* or *atnpr1-1* mutations (Figure 21C). Conversely, *AtPR5* resulted preferentially expressed in GCs (Figure 21 D). Following treatment with SA, *AtPR5* expression was up-regulated both in WT, *atmyb60-1* and in *atnpr1-1* plants, although with different intensity (Figure 21 D). While in *atmyb60/atnpr1-1* the expression of *AtPR5* did not change (Figure 21 D).
AtNPR1 did not show any variation at the transcriptional level, in response to SA, neither in GCs neither in mesophyll cells, in agreement with what was seen on the whole leaf (Figure 21 E).
In sum, our results suggest that *AtMYB60* is essential in guard cells to modulate *AtPR1* expression in response to SA, thus indicating *AtMYB60* as a likely transcriptional regulator of SA-induced stomatal closure.

Part III: Analysis of the transcriptional regulation of stomatal activity in *Vitis vinifera*

3.1 Cellular specificity of *VvMYB60* expression in the grape leaf

The grape *VvMYB60* gene, encoding a R2R3-type MYB transcription factor, has been shown to represent a true functional orthologue of the Arabidopsis *AtMYB60* gene (Galbiati *et al.*, 2011). Functional studies and promoter:reporter analyses on *VvMYB60* were mainly performed in Arabidopsis, so that little information is available on *VvMYB60* in *Vitis*. In order to gain more insights into the function role of *VvMYB60* in grapevine we first investigated the cellular specificity of its expression in the grape leaf. *AtMYB60* mediates stomatal activity in Arabidopsis (Cominelli *et al.*, 2005). In particular, up-regulation of *AtMYB60* expression following exposure to light has been shown to promote stomatal opening, whereas down-regulation of *AtMYB60* in response to exogenous application of ABA or exposure to drought stress, results in stomatal closure (Cominelli *et al.*, 2005).

It was shown that activity of the grape *VvMYB60* promoter is localized to guard cells of Arabidopsis stable lines carrying a *VvMYB0pro:GUS* construct (Galbiati *et al.*, 2011). In order to confirm the guard cell specificity of *VvMYB60* in grapevine the analysis of its transcript was performed on both total blended and on epidermal pieces enriched in intact guard cells (Figure 22 A).

To further investigate the cellular specificity of *VvMYB60* expression in the grape leaf, we employed the use of LMD-purified GC and mesophyll cell (Figure 22 B).

These results clearly demonstrate that, similarly to its Arabidopsis ortholog, *VvMYB60* is specifically expressed in GC in the grape leaf.

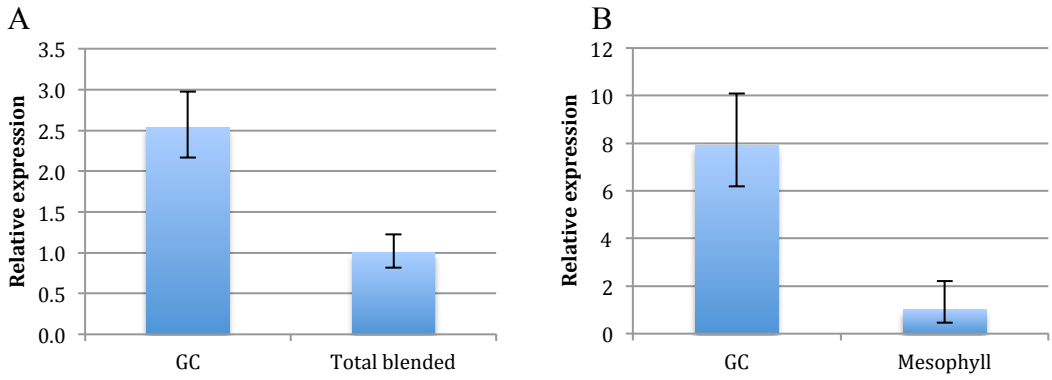

Figure 22 Analysis of *VvMYB60* expression in grape GC. (A) *VvMYB60* expression in GC-enriched epidermal fragments obtained by leaf blending and total blended leaves. (B) *VvMYB60* expression in LMD-purified GCs and mesophyll cells.

3.2 Correlative analysis of *VvMYB60* expression and stomatal activity in the grape leaf

In Arabidopsis *AtMYB60* has been identified as a key transcriptional regulator of stomatal activity (Cominelli *et al.*, 2005). In particular, up-regulation of *AtMYB60* expression following exposure to light has been shown to promote stomatal opening, whereas down-regulation of *AtMYB60* in response to exogenous application of ABA or exposure to drought stress, results in stomatal closure (Cominelli *et al.*, 2005). Stomatal opening is impaired in the Arabidopsis *atmyb60-1* knock-out mutant.

The introduction of the *VvMYB60* genomic sequence in the *atmyb60-1* background fully rescued the mutant phenotype, thus indicating the functional conservation of the grape and Arabidopsis MYB60 proteins (Galbiati *et al.*, 2011).

As a first approach to confirm the role of *VvMYB60* in mediating stomatal activity in grape, we investigated weather its expression was positively correlated with stomatal conductance. To this end we analysed *VvMYB60* expression in different genotypes and in different rootstock-scion combinations grown under both normal and drought stress conditions and we monitored changes in the stomatal conductance of the leaf.

First, we analysed the commercial rootstocks, Paulsen 1103 (1103P) and Koiber 5BB (K5BB), as they display opposite responses to drought stress. 1103P was selected in Southern Italy for its strong drought tolerance and its ability to grow well on lime-based soils, while K5BB is generally considered relatively susceptible to water stress (www.viten.net). The study was carried out under semi-controlled environmental conditions on un-grafted plants grown in pots. Several biometrical parameters addressing the plant water status and leaf gas exchange were measured. These included internode elongation, leaf area, leaf water potential (Ψleaf), and stomatal conductance (g_s). Control plants were grown under normal water conditions (NW) representing 90% of the field capacity (FC), whereas treated plants were grown under low water conditions (LW), corresponding to 30% of FC. The sampling of leaves for expression studies was performed the ninth day from the beginning of the stress treatment, corresponding to the third day at 30% of FC. At the same time leaves were sampled from control plants constantly maintained at 90% FC. At the end of the stress treatment, plants were gradually rehydrated up to 90% of FC.

Among the various stomatal characters, stomatal density (SD) is an important eco-physiological parameter that affects gas exchange and photosynthesis, together to stomata main aperture (Pompelli *et al.*, 2010). SD is determined by stomatal initiation during ontogenesis and by epidermal cell expansion at a later stage (Salisbury, 1928). The stomatal density has been found to increase in plants with decreased ABA concentrations, which also have increased transpiration

(Franks and Farquhar, 2001; Lake and Woodward, 2008). The stomatal index, the number of stomata relative to the number of epidermal cells, was also found to increase with soil moisture (Arve *et al.*, 2011). In *Vicia faba* drought and salinity stress has been found to increase the stomatal density and stomatal index, facilitating water uptake under water stressed conditions (GAN *et al.*, 2010; Yoo *et al.*, 2010).

First, we evaluated stomata distribution: Paulsen leaves displayed a stomatal density of 71.54 stomata per mm^2 (± 5.30) while Kober leaves disclosed a significantly increased density of 143.83 per mm^2 (± 6.24) (Figure 23 A). Interestingly, despite having a reduced stomatal density, Paulsen plants, grown under standard conditions showed a 6.5 fold ($p<0.1$) increase in stomatal conductance compared with Kober (Figure 23 B). Nevertheless, under drought stress Paulsen drastically reduced g_s, reaching a value equal to that of Kober. Conversely, Kober showed a moderate difference in g_s between control and drought stressed plants (Figure 23 B). These results confirmed the anisohydric behaviour displayed by 1103P and the isohydric behaviour of K5BB.

The expression of *VvMYB60* in leaves of plants kept under NW conditions resulted reduced in K5BB compared with 1103P (Figure 23 C). Consistent with the reduced expression of *VvMYB60*, K5BB leaves showed a constitutive reduction of stomatal opening. Under LW conditions K5BB leaves did not show changes in the expression level of *VvMYB60* (Figure 23 D). Accordingly, stomatal conductance did not show variations in response to the stress treatment (Figure 23 A). On the contrary, 1103P displayed a drastic reduction in stomatal conductance under LW (Figure 23 A), which correlated with the strong down-regulation of *VvMYB60* (Figure 23 D). Interestingly, analysis of the GC-specific gene *VvSIRK* resulted in an expression profile very similar to *VvMYB60* in both genotypes (Figure 23 E, F).

All together these results suggest that there is a positive correlation between stomata conductance and the level of expression of *VvMYB60* in the rootstocks 1103P e K5BB. Moreover, these results suggest that 1103P and K5BB adopted two different mechanisms of drought response. K5BB showed an escape behaviour instead 1103P adopted an avoidance behaviour, in agreement with the isohydric and anisohydric behaviour described for these two genotypes.

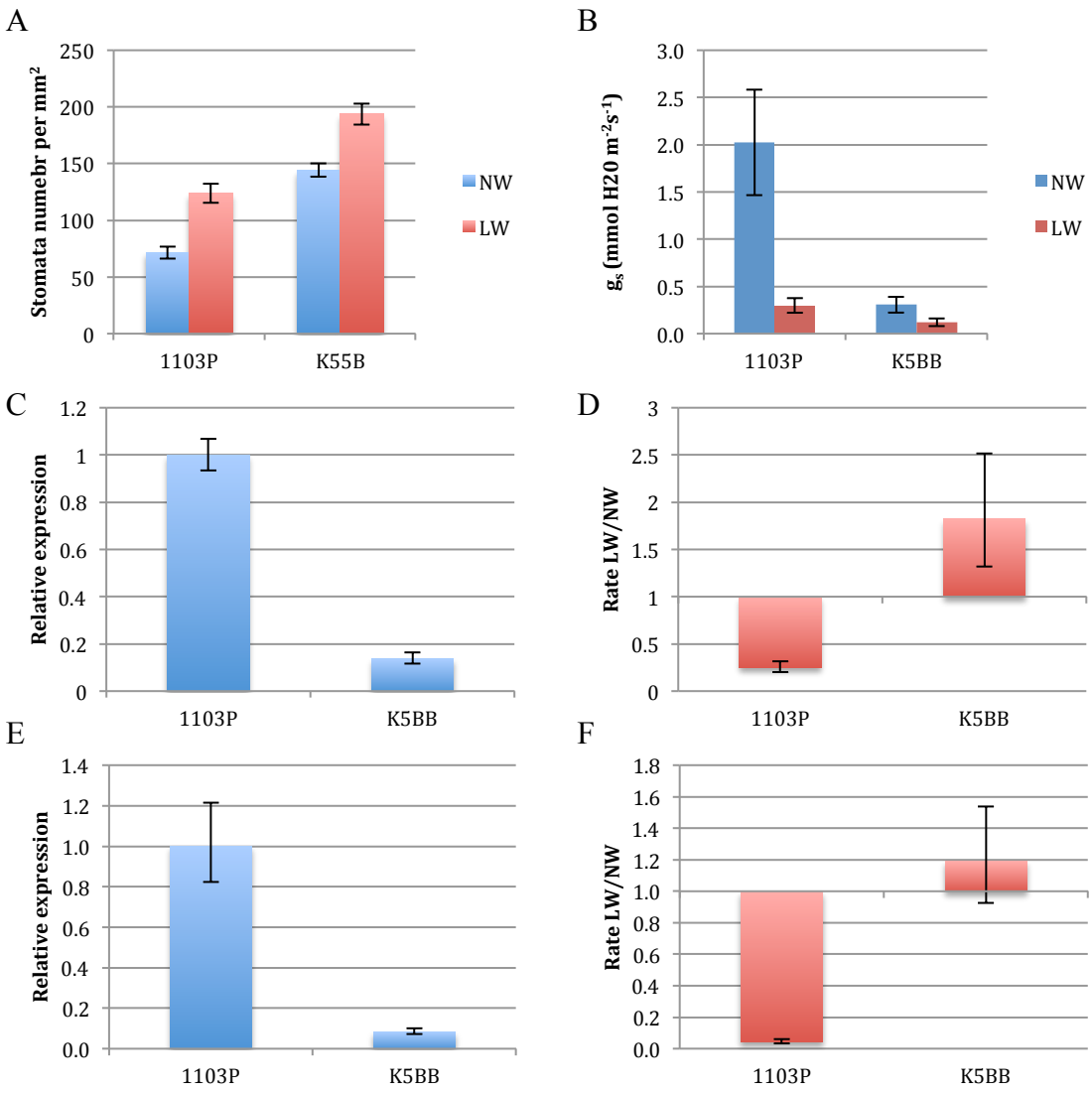

Figure 23 Comparison of GC responses under drought stress in 1103P and K5BB. (A) Analysis of stomatal density. (B) Analysis of stomatal conductance index. (C) Relative expression of *VvMYB60* in whole leaves from 1103P and K5BB grown under normal watering (NW) conditions. (D) Ratio of *VvMYB60* expression in whole leaves from 1103P and K5BB grown under normal water (NW) or low water (LW) conditions. (E) Relative expression of *VvSIRK* in whole leaves from 1103P and K5BB grown under NW conditions. (F) Ratio of *VvSIRK* expression in whole leaves from 1103P and K5BB grown under NW or LW conditions.

Next, we extended our analysis to other well characterised rootstock genotypes, M4 and 101.14. In a previous screening, among the novel candidate genotypes established by the DiSAA research group, the Milano4 (M4) [(*V. vinifera* × *V. berlandieri*) × *V. berlandieri* cv. Resseguier n. 1] rootstock was selected for its relatively high tolerance to water deficiency and high soil salt concentration (Porro *et al.*, 2013). The stomata activity of M4 was compared against the commercial rootstock 101.14, considered susceptible to drought (Alsina *et al.*, 2011). Importantly, the different adaptation of these two genotypes to water stress has been characterised in details within the SERRES project (Meggio *et al.*, 2014). M4 and 101.14 were grown under the same experimental conditions described for 1103P e K5BB. Stomata density between M4 and 101.14 was

comparable (Figure 24 A). Under NW conditions, M4 and 101.14 also disclosed comparable values of g_s (Figure 24 B). Exposure to water stress induced a nearly complete stomatal closure in 101.14 leaves, whereas in M4 only resulted in a partial reduction of g_s that reached a value of about 30% of the control plant (Figure 24 B). Under NW both genotypes showed enhanced expression of *VvSIRK* (Figure 24 C), while interestingly, under LW conditions 101.14 leaves showed a significantly enhanced down-regulation of both *VvMYB60* and *VvSIRK* (Figure 24 D).

These results, confirm the positive correlation between stomatal conductance and *VvMYB60* (and *VvSIRK*) expression previously observed in 1103P e K5BB and indicate a more drought-avoiding behaviour for M4 compared with that of 101.14, which appears to maintain a partial stomatal aperture as recently found also by Meggio and colleagues (Meggio *et al.*, 2014).

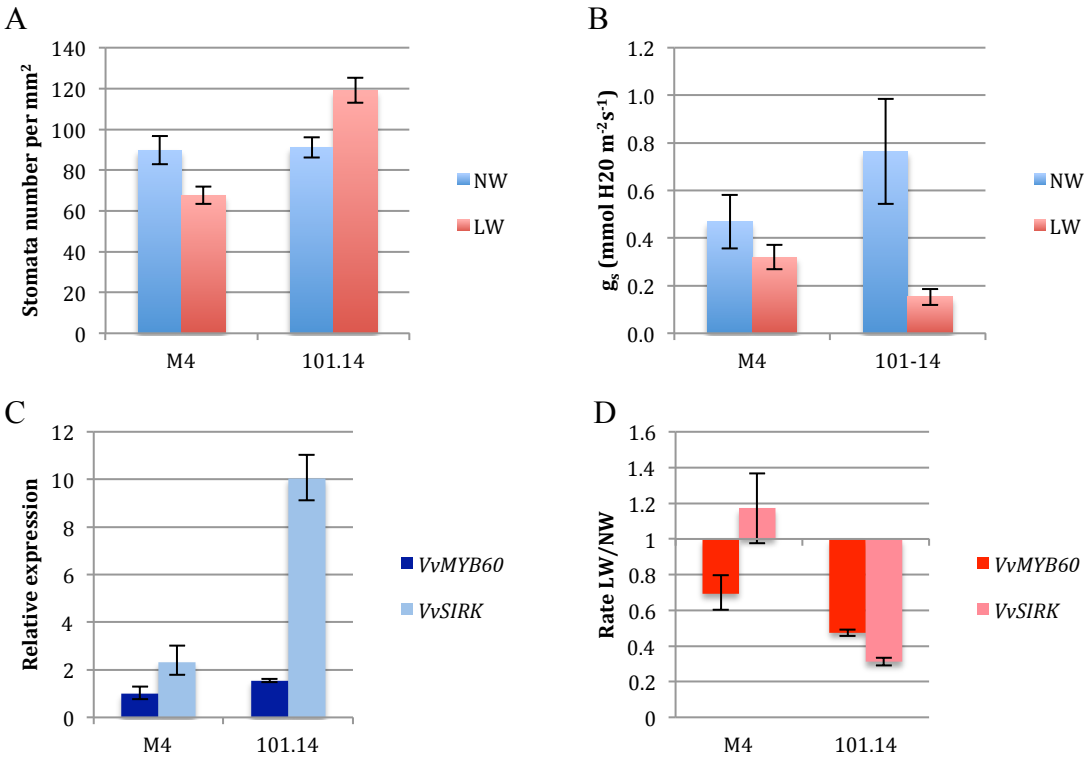

Figure 24 Comparison of GC responses under drought stress in in M4 and 101.14. (A) Analysis of stomatal density. (B) Analysis of stomatal conductance index. (C) Relative expression of *VvMYB60* and *VvSIRK* in whole leaves from M4 and 101.14 grown under normal watering (NW) conditions. (D) Ratio of *VvMYB60* and *VvSIRK* expression in whole leaves from M4 and 101.14 grown under NW or LW conditions.

3.3 Correlative analysis of *VvMYB60* expression and stomatal activity in rootstocks of the M- and SM-series

To further investigate the functional relationship between *VvMYB60* expression and stomatal activity, we extended our analysis to additional rootstocks available in the SERRES project. In particular, we focused our attention on the extended Milano (M) series (Porro *et al.*, 2013) and the San Michele (SM) series (Table 3).

Table 3 Grape rootstock genotypes used in this work

Rootstock	Constitutor	Parental genotypes
M1	UNIMI	106/8 [*V.rip*. x (*V. cord*. X *V. rup*.)] x Resseguier n°1 (*V. berl*.)
M2	UNIMI	Teleki 8B (*V.berl*. x *V.rip*.) x 333 E.M. (*V.vin*. x *V.berl*.)
M3	UNIMI	R 27 (*V.berl*. x *V.rip*.) x Teleki 5C (*V.berl*. x *V.rip*.)
M4	UNIMI	41 B (*V.vin*. x *V.berl*.) x Resseguier n°1 (*V.berl*.)
101.14	Commercial	*Vitis riparia* x *Vitis rupestris*
K5BB	Commercial	*Vitis berlandieri* x *Vitis riparia*
SO4	Commercial	*Vitis riparia* x *Vitis berlandieri*
1103P	Commercial	*Vitis berlandieri* x *Vitis rupestris*
SM24	San Michele	NA
SM79	San Michele	NA
SM82	San Michele	NA
SM93	San Michele	NA

NA: not available

All the genotypes were analysed under the same experimental setting described in the previous paragraph. As a preliminary characterization of the new genotypes we determined the relative stomatal density in leaves of plant grown under NW or LW conditions. To facilitate the comparison with the previously analysed rootstocks, M4, 101.14, 1103P and K5BB were included in the study.

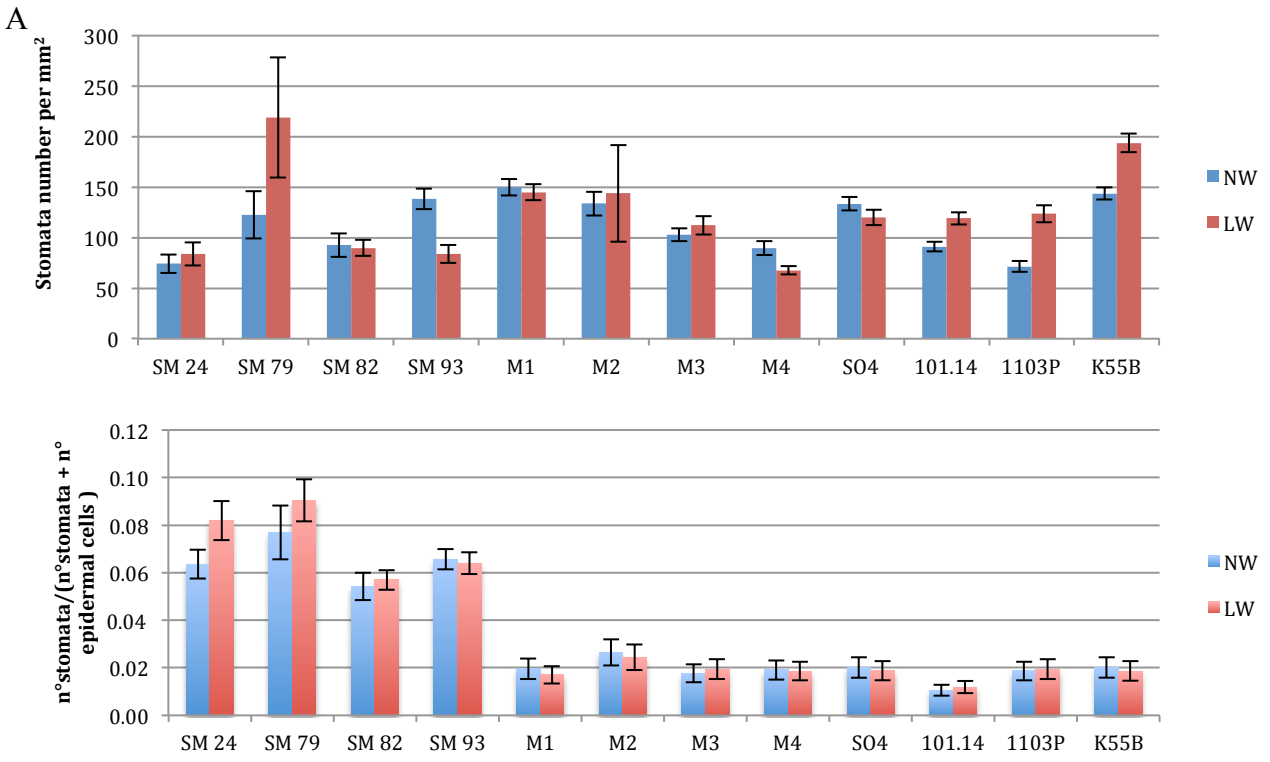

Figure 25 Analysis of (A) stomatal density and (B) stomata index in leaves of plants grown under normal water (NW) or low water (LW) conditions for each genotype.

As shown in Figure 25 all the genotypes analysed did not show significant differences in stomatal density and index between treated and control plants. Interestingly, even under NW conditions there was a large difference in both stomatal index and stomatal density among different genotypes, with M1 and K5BB showing the highest value of stomatal density and SM24 and 1130P disclosing the lowest stomatal density (Figure 25 A). This high variability likely reflects the ample genetic variability embedded in the collection of rootstocks.

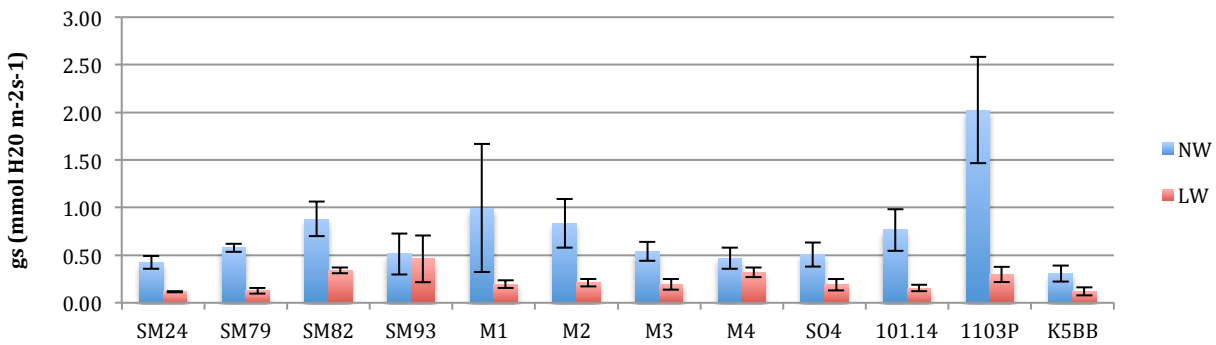

Figure 26 Analysis of stomatal conductance in leaves of plants grown under normal water (NW) or low water (LW) conditions for each genotype.

Under drought stress conditions, we observed a concurrent decrease of stomatal conductance in SM24, SM79 and SM82 but not in SM93 (*t-test*), where g_s did not change (Figure 26). Besides M4, M series included M1, M2 and M3; these genotypes showed a very similar behaviour, decreasing drastically stomatal conductance under drought stress. SO4 that is considered moderately tolerant to drought (www.viten.net) showed a stomatal conductance very similar to other M genotypes, both in NW and in LW.

Comparative analysis of *VvMYB60* and *VvSIRK* disclosed a general variation in NW among the genotypes; moreover *VvSIRK* was always relatively more expressed than *VvMYB60* (Figure 27 A). In agreement with previous data both *VvMYB60* and *VvSIRK* were generally down-regulated under drought stress, with the exception of SO4, in which *VvMYB60* was not down-regulated and of M1, in which neither *VvMYB60* nor *VvSIRK* were down-regulated (Figure 27 B).

In sum, our results showed that *VvMYB60* in different genotypes was down-regulated under drought stress, in agreement with its role of positive regulator of stomatal opening.

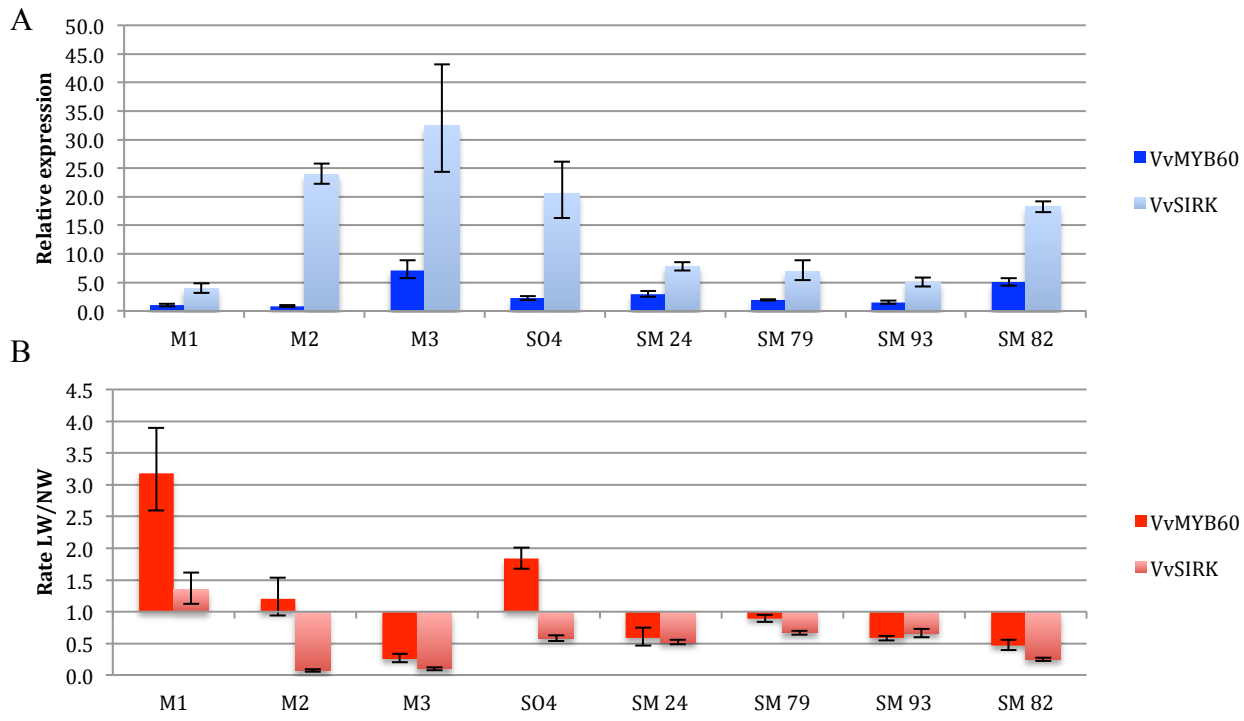

Figure 27 Analysis of *VvMYB60* and *VvSIRK* expression in whole leaves from M or SM rootstocks. (A) *VvMYB60* and *VvSIRK* expression in leaves from plants grown normal water conditions. (B) Ratio of *VvMYB60* and *VvSIRK* expression in whole leaves from M and SM plants grown under normal water and low water conditions.

3.4 Analysis of the transcriptional regulation of ABA synthesis, perception and signalling

In grapevine, stomatal conductance is negatively correlated with ABA synthesis in the root, its concentrations in the xylem sap and its relative abundance in leaf tissues (SOAR *et al.*, 2006). The phytohormone ABA is a key endogenous messenger in the plant response to stress (Zhu, 2002).
In addition to triggering stomatal closure in the leaf, ABA regulates the expression of specific stress-related genes which mediate several molecular mechanisms involved in abiotic stress tolerance (Wang *et al.*, 2003). We analysed the expression of genes involved in ABA biosynthesis and signal transduction and of ABA-regulated target genes in the different rootstock genotypes to assess the relationship among GC-activity, adaptation to stress and ABA regulation.

3.4.1 ABA-related genes analysed in the study

ABA is derived from C40-cis-epoxycarotenoids (90-cis-neoxanthin, 90-cis-violaxanhin), which are cleaved by a 9-cis-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin, the direct C15 precursor of ABA (Liotenberg *et al.*, 1999). NCED is the key enzyme in the ABA biosynthetic pathway in plants and it is considered to be the rate-limiting factor in ABA accumulation. Ectopic expression of the *AtNCED1* gene in Arabidopsis, has been shown to enhance water-stress tolerance (Wan and Li, 2006). Since its first isolation from the maize *vp14* mutant (Tan *et al.*, 1997), the *NCED* gene has been cloned and characterized in various plant species including grapevine (M., Zhang *et al.*, 2009; Koyama *et al.*, 2010). Importantly, expression of the grape *VvNCED1* has been directly correlated with ABA accumulation in the plant tissues in response to stress (SPEIRS *et al.*, 2013). We thus analysed the expression of *VvNCED1* and *VvNCED2* as markers for ABA biosynthesis (Boneh *et al.*, 2011).
The core of the ABA signalling pathway is composed by ABA receptors (REGULATORY COMPONENTS OF ABA RECEPTOR- RCAR), 2C-type PROTEIN PHOSPHATASES (PP2Cs) and SNF1-related protein kinase (SnRKs) acting in a hierarchical order (Raghavendra *et al.*, 2010).
Previous analysis of *RCAR*-coding genes from grape, revealed that in general they are down-regulated under stress (Boneh *et al.*, 2011). The RCAR 1-7 were include in our analysis.
VvPP2Cs are generally up-regulated in drought stress, in agreement with its role in feedback regulation of ABA (Boneh *et al.*, 2011). *VvPP2C1-9* and *24* were analysed.

VvSnRKs were characterized by (Boneh *et al.*, 2012) and in general they are down-regulated under drought stress (Boneh *et al.*, 2012) although they are strong regulated at the post-transcriptional level.

Additional components of ABA signalling analysed in our study include, the grape homolog of the plasma membrane *NADPH oxidase Arabidopsis thaliana Respiratory burst oxidase homolog F* (*AtRbohF*) and the homolog of the *Arabidopsis Enhanced response to ABA* (*VvERA1*) gene.

The RBOHF protein is a direct target of the GC-specific SnRK open stomata 1 (OST1) and mediates the production of Reactive Oxygen Species (ROS) in guard cells, which in turn leads to stomatal closure (Hauser *et al.*, 2011; Kwak *et al.*, 2006).

VvRBOHF was identify by (Boneh *et al.*, 2012) while we identified the grape GWSUNIT03506984001 gene as the closest homolog to the *AtERA1,* based on protein sequence conservation.

AtERA1 encodes a FARNESYL TRANSFERASE which has been shown to function in early guard cell signalling, where it act as negative regulator of guard cell ABA-induced stomatal closure (Schroeder *et al.*, 2001). The *atera1-1* mutant shows ABA hypersensitive stomatal closing and ABA hypersensitive activation of S-type anion currents (Schroeder *et al.*, 2001). Furthermore, *atera1* plants show reduced water loss during drought (Schroeder *et al.*, 2001).

Marguerit and colleagues performed a QTL analysis of transpiration efficiency on grapevine (Marguerit *et al.*, 2012). Besides genes that we have already analysed, Marguerit and co-worker identified some genes involved in ABA response, including *VvABFs*, *VvERF5* and *VvHOS10* (Marguerit *et al.*, 2012).

Downstream targets of ABA signalling include several transcription factors, whose expression is activated in response to stress in a ABA-dependent manner (Boneh *et al.*, 2011). Among them we analysed the expression of the ABRE-BINDING FACTOR2 and 3 (ABFs), which regulate ABRE-dependent ABA signalling involved in drought stress tolerance.

ABA-regulated transcription factors include members of the APETALA2/ETHYLEN BINDING FACTOR (AP2/ERF) superfamily, one of the largest groups of transcription factors in plants (Wessler, 2005). AP2/ERF transcription factors regulate a number of biological processes including development, reproduction, responses to hormones, adaptation to biotic and abiotic stresses (Licausi *et al.*, 2010). We focused our attention on the Vitis *Ethylene response factor 5* (*VvERF5*) that was the only genes belonging to the *ERF* family identify in QTLs (Marguerit *et al.*, 2012).

R2R3-type MYB transcription factors are also found among ABA targets. This gene family include the ABA-responsive *High expression of osmotically responsive* (*HOS*) genes. Interestingly, in Arabidopsis, *AtHOS10* have been shown to activate expression of ABA-biosynthetic genes during

stress (Xiong and Zhu, 2003). Through QTL analysis Marguerit identified three genes belong to *VvHOS10* family: *VvHOS10.1, VvHOS10.2* and *VvHOS10.3* (Marguerit *et al.*, 2012) that were analysed in this work.

Finally, GC-related genes, regulated at the transcriptional level by ABA, were included in the analysis. These comprised the plasma membrane proteins Slow anion channel associated-1 (*VvSLAC1*), that is a component of the slow (S-type) anion efflux channel function that controls stomatal pore closure, the inwardly rectifying K^+ channel *VvSIRK*, *Vitis vinifera shaker-like potassium channel* (*VvSOR*) and *VvMYB60.*

3.4.2 Expression profiles of ABA-related genes: an overview

Gene expression was analysed by qPCR. Results obtained from the analysis of ABA-related gene expression in the 12 genotypes included in the study are summarised in Figure 28. Relative expression was calculated as ΔΔCt of the gene of interest (GOI) in plant grown in the low watering conditions compared to ΔΔCt of the same GOI of plant grown in control conditions. Thus, changes in the level of gene expression are independent from the expression of the gene at the steady state; rather they reflect variations induced by drought stress.

Colour code gives a qualitative overview of the changes that occurs in the collection in response to drought stress. Green colour indicates down-regulation following exposure to stress, whereas red colour indicate up-regulation. The intensity of the colour scale relates to the fold changes observed in the expression of a given gene (Figure 28).

As a whole gene expression data indicate a great variability in the transcriptional response to drought disclosed by the different genotypes for the genes considered in the study.

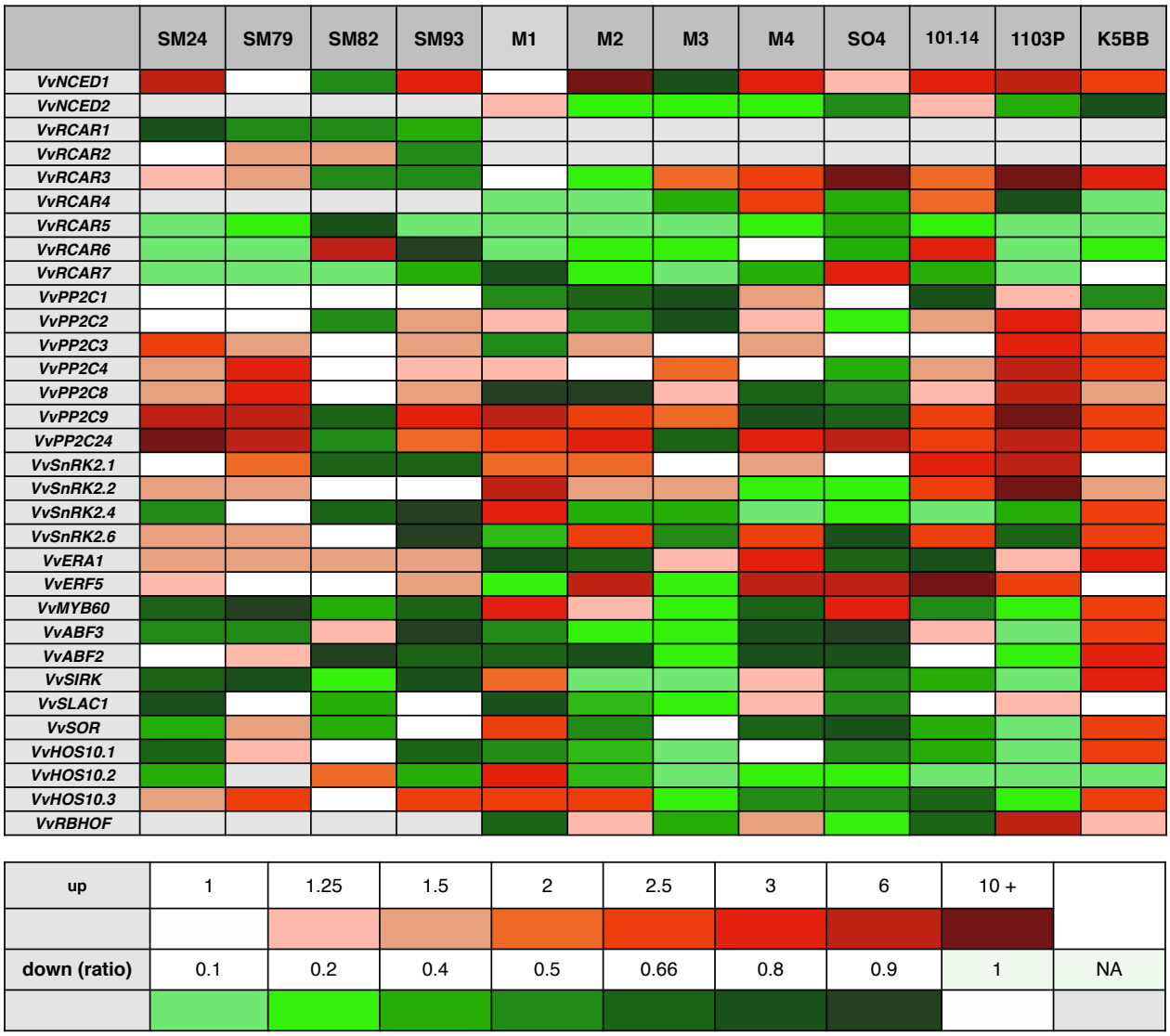

Figure 28 Summary of qPCR analysis of changes gene expression in response to water stress in whole leaves from the 12 genotypes analysed in this study. Changes in gene expression were calculated as the ratio of relative gene expression under drought stress and the relative gene expression under control conditions. The green to red colour scale represents low to high levels of change in gene expression. Light green indicates strong down-regulation, whereas dark red means strong up-regulation. White indicates an equal level of expression under both control and stress conditions. Grey boxes represent not available data (NA).

3.4.3 Comparative analysis of ABA-related genes under drought stress

The ABA-biosynthetic gene *VvNCED1* has been shown to be transcriptionally activated in response to drought stress (Tan *et al.*, 1997; Boneh *et al.*, 2011). Interestingly, we did not detect such an up-regulation in all the rootstocks. In particular, M1, M3, SO4 and SM79 did not show substantial differences in *VvNCED1* expression in response to drought. M2, SM24 and SM93 disclosed the highest level of induction (Figure 29 A) while, we observed an unexpected down-regulation of *VvNCED1* expression in SM82 (Figure 29 A). In contrast, *VvNCED2* expression was largely unaffected or moderately down-regulated under stress conditions. This result is in agreement with previous reports, which indicate that *VvNCED2* expression is modulated by ABA preferentially in seeds (Soar *et al.*, 2006) (Figure 29 B).

In grapevine it has been shown that *VvRCAR* genes were differently regulated after drought and ABA treatment (Boneh *et al.*, 2011). In our experiment *VvRCARs* were generally down-regulated in agreement with (Raghavendra *et al.*, 2010). *VvRCAR7*, putative ortholog of *AtPYL1* showed the strongest and more widespread down-regulation (Figure 29 H).

We found that in leaves of plant grown under NW conditions, *VvRCAR1* and *VvRCAR2* were the most expressed receptors (data not shown). This observation is in contrast with previous reports which indicate *VvRCAR6* as the most express ABA-receptor in leaves (Boneh *et al.*, 2011).

In general, transcript levels of the ABA phosphatases (PP2C) is increased under drought stress (Raghavendra *et al.*, 2010). As show in Figure 30, PP2C genes displayed a general up-regulation under drought conditions. However, not all the phosphatase showed the same behaviour, *VvPP2C1* was down-regulated in M1, M3 and M4 or its expression was unchanged in SM group. Remarkably, SM82 did not disclose any up-regulation of PP2C expression (Figure 30 G). In this rootstock, *VvPP2C24* ortholog of the ABA-induced *AtABI1*, was down-regulated under stress conditions. This behaviour correlated with the non-activation of *VvNCED1* observed for this genotype. Similarly, SO4 showed down-regulation of PP2C genes, with the exception of *VvPP2C24* which resulted up-regulated (Figure 30).

The genes *VvPP2C9* and *VvPP2C24* disclosed a strong up-regulation in response to drought in SM24, SM79 and SM93 (Figure 30 F, G). Interestingly, the same genes resulted in a moderate down-regulation in SM82. This result correlates with the lack of *VvNCED1* activation in SM82 plants exposed to water stress (Figure 29 A).

As described in literature, the SnRK protein kinases are generally regulated at the post transcriptional level (Kobayashi *et al.*, 2005). Consistently, we observed small variations in the expression level of the different SnRK-genes in our experiment (Figure 31). In particular, SM93 did

not show up-regulation of any kinases while M2 disclosed an activation of *SnRK2* and *SnRK4* expression following the stress treatment (Figure 31 B and C).

VvERA1 did not show evident changes in the level of expression in most genotypes (Figure 31 E).

The transcription factor *VvERF5* was strongly up-regulated in SO4 and in M2, according to the expression of other genes in M2, instead it was strongly down-regulated in M1 and M3 (Figure 31 F). *VvRBOHF,* expression was moderately up-regulated under drought stress in most genotypes (Figure 31 G).

Analysis of the *VvHOS10* genes revealed the strong down-regulation of *VvHOS10.1* and *VvHOS10.2* under drought stress, in most genotypes (Figure 31H). This observation is in contrast with data from Zhu and colleagues, who reported the up-regulation of these genes following exposure to stress (Zhu *et al.*, 2005). Conversely, *VvHOS10.3* did not show significant variations in gene expression in nearly all the genotypes.

Finally, the analysis of GC-related genes revealed a similar behaviour for *VvSIRK* and *VvSOR*. Both genes showed a substantial down-regulation in most rootstocks, with the exception of M1, SM79 and SM93 (Figure 32 A, B). Interestingly, compared to the other SM rootstocks, SM82, under NW conditions displayed higher expression *VvSLAC* and *VvSOR* (together with the previous described *VvMYB60* and *VvSIRK*, data not shown). This observation is in agreement with the higher stomatal conductance previously observed in SM82 in comparison with the other genotypes. Nevertheless, under drought stress SM82 leaves showed a significant (and sometime enhanced) down-regulation of GC-related genes (Figure 32) This result is in apparent contrast with the observation that *VvNCED1* and *VvPP2C* genes were not activated in SM82 in response to drought and likely suggests a possible ABA-independent down-regulation of *VvSIRK, VvSLAC* and *VvSOR* in this genotype (Figure 32).

The comparison of M4 with 101.14, 1103P with K5BB resulted useful in order to get more insight into guard cell genes regulation in response to drought.

VvNCED1 was equally up-regulated in M4 and 101.14 under drought stress (Figure 33 A). Instead *VvNCED1* resulted more up-regulated in K5BB compared to 1103P (Figure 33 A). Many genes of the core of ABA signalling pathway resulted more up-regulated in 101.14 and 1103P compared to M4 and K5BB.

Among these *VvRCAR6* (Figure 33 B), the phosphatases *VvPP2C4, VvPP2C9* and *VvPP2C24* (Figure 33 C), and kinases, like *VvSnRK2.1, VvSnRK2.2* and *VvSnRK2.6* (Figure 33 D) showed the greatest increase.

Moreover *VvERF5* resulted strongly activated in 101.14 and not in M4 (Figure 33 D). The function of *VvERF5* has not been established, however it was also strongly up-regulated in young berries

before veraison (Sweetman *et al.*, 2012), a process in which ABA is involved. This strong activation of the ABA pathway in 101.14 and K5BB compared to M4 and 1103P also affected the synthesis of genes involved in the stomatal activity. As descripted before *VvMYB60* and *VvSIRK* down-regulation was more marked in 101.14 and 1103P than M4 and K5BB (Figure 33 D). At the contrary *VvSOR* and *VvSLAC* did not show any significant differences.

In sum our results indicate that there is a strong correlation between stomata conductance and the level of expression of involved in drought stress like *VvNCED1* and guard cell related genes. Moreover we found a strong link between the activation of genes involved in ABA pathway and the regulation of genes involved in stomata activity.

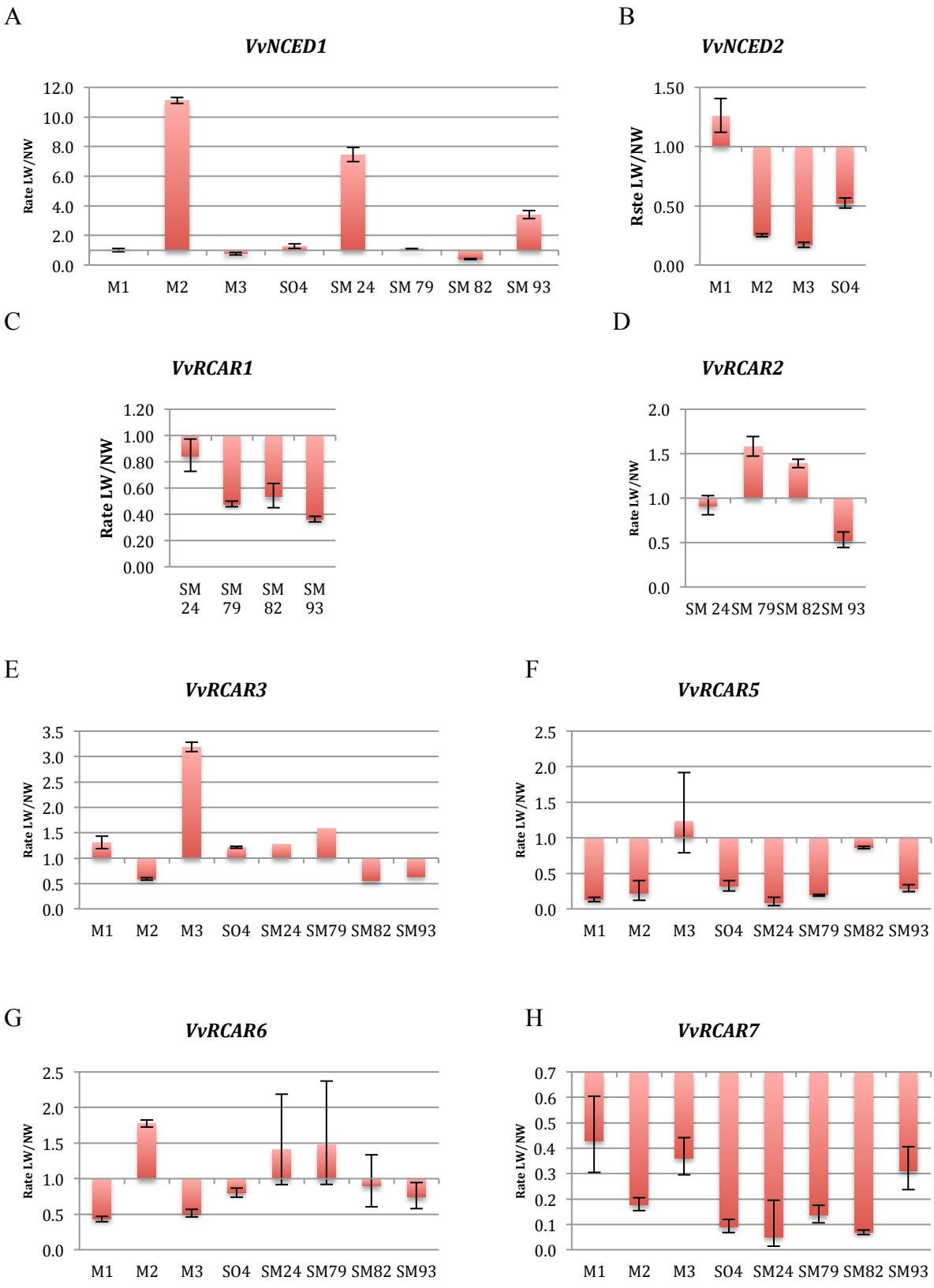

Figure 29 Gene expression analysis of ABA-biosynthetic (*VvNCED*) and ABA-receptor (*VvRCAR*) genes in whole leaves from M and SM plants. Data correspond to the ratio of relative expression under NW and LW. (A) *VvNCED1* (B) *VvNCED2* (C) *VvRCAR1* (D) *VvRCAR2* (E) *VvRCAR3* (F) *VvRCAR5* (G) *VvRCAR6* (H) *VvRCAR7*.

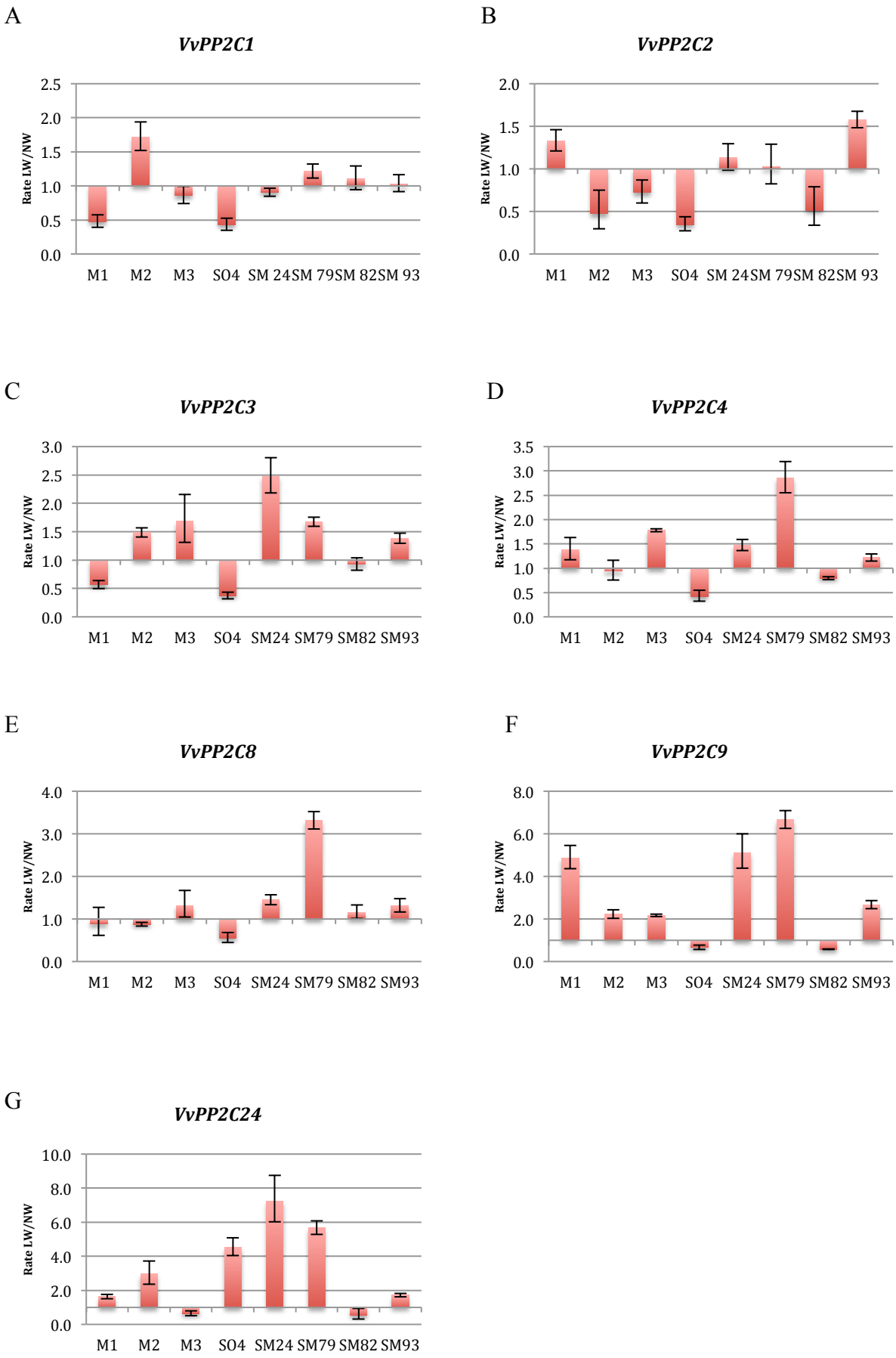

Figure 30 Gene expression analysis of PP2C-genes in whole leaves from M and SM plants. Data correspond to the ratio of relative expression under NW and LW. (A) *VvPP2C1* (B) *VvPP2C2* (C) *VvPP2C3* (D) *VvPP2C4* (E) *VvPP2C8* (F) *VvPP2C9* (G) *VvPP2C24.*

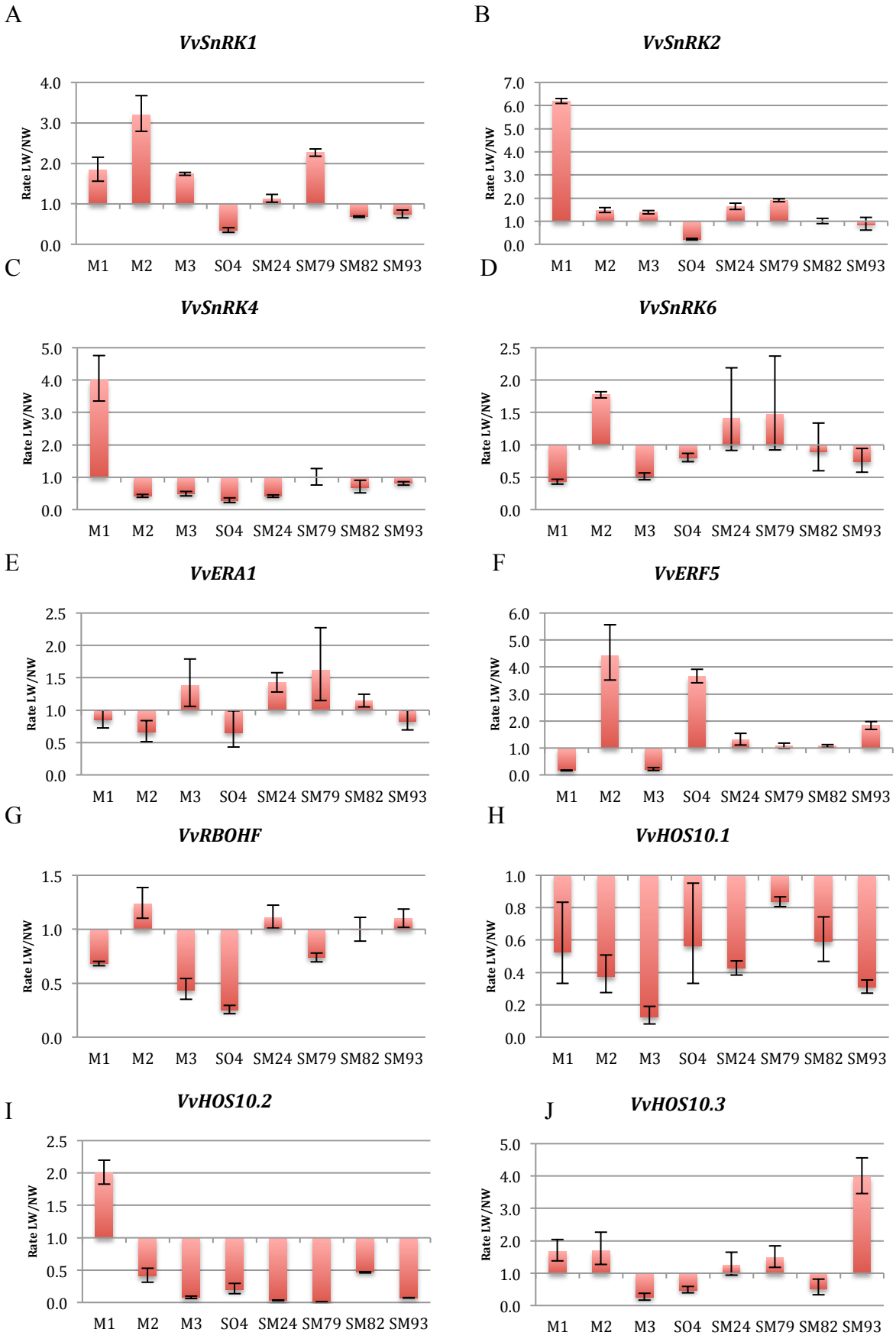

Figure 31 Gene expression analysis of SnRK-genes and ABA-responsive genes in whole leaves from M and SM plants. Data correspond to the ratio of relative expression under NW and LW. (A) *VvSnRK1* (B) *VvSnRK2* (C) *VvSnRK4* (D) *VvSnRK6* (E) *VvERA1* (F) *VvERF5* (G) *VvRBOHF* (H) *VvHOS10.1* (I) *VvHOS10.2* (J) *VvHOS10.3.*

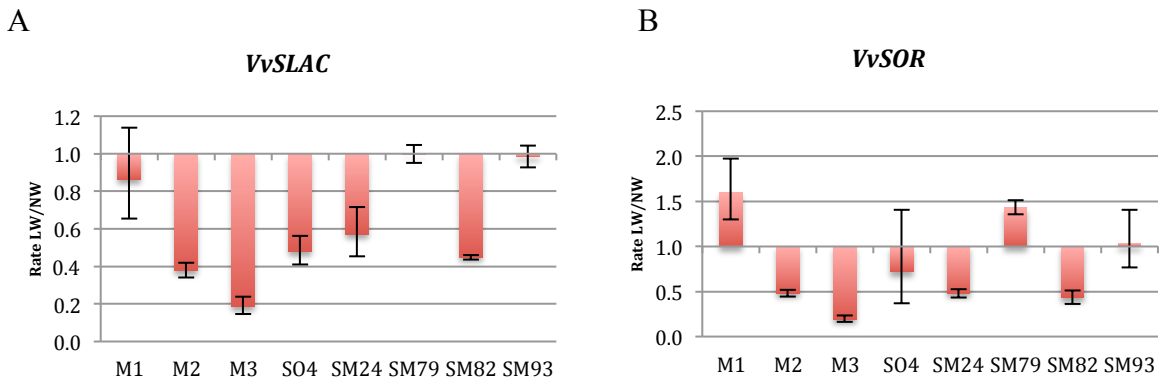

Figure 32 Gene expression analysis of GC-related genes in whole leaves from M and SM plants. Data correspond to the ratio of relative expression under NW and LW. (A) *VvSLAC* (B) *VvSOR*.

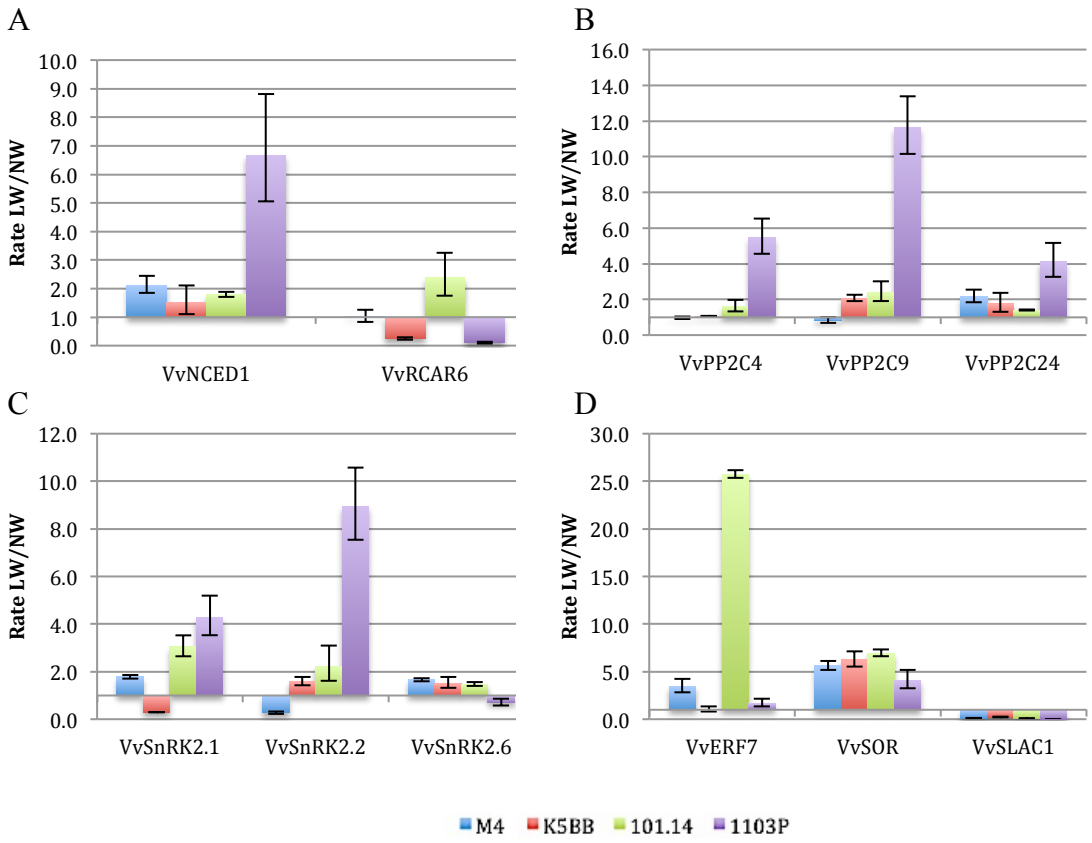

Figure 33 Gene expression analysis of ABA-related genes in whole leaves from M4 and commercial rootstocks (K5BB, 101.14, 1103P). Data correspond to the ratio of relative expression under NW and LW of M4 (blue bars), K5BB (red bars), 101.14 (green bars) and 1103P (violet bars.

3.5 Analysis of the contribution of the rootstock on the regulation of gene expression in the scion.

There is strong evidence that the physiological status of the root plays an important role in modulating physiological responses in the shoot, including stomatal closure (Milligan and Dale, 1988; Iacono *et al.*, 1998).

ABA is considered to be the chemical most likely involved in root-to-shoot signalling (FORT *et al.*, 1997). Information on the effects of rootstocks on growth, drought tolerance and water relations in grafted grapevine are largely incomplete and no information are available on the contribution of the rootstock to the regulation of gene expression in the scion.

In order to investigate the influence of the rootstock on the regulation of gene expression occurring in guard cells distributed on leaves of the scion, we compared gene expression in grafted and un-grafted plants combination. More into details, we surveyed expression of stress- and ABA-related genes in leaves derived from different rootstock-scion combinations.

At first we compared gene expression in leaves of M4 and 101.14 ungrafted, with that of leaves from M4 grafted on M4 (M4/M4) and 101.14 grafted on 101.14 (101.14/101.14).

Interestingly, under normal watering conditions, the grafted plant showed higher expression of *VvNCED1* and *VvABI1* compared to non-grafted plants (Figure 34 A and B). This suggests that the steady state of ABA synthesis and perception in grafted plant was higher compared with ungrafted plant. This did not affect the expression of guard cell related genes and even the expression of *VvMYB60* in M4/M4 was higher than its the expression in M4. However, this could indicate a perturbation in gene regulation due to the graft. Moreover *VvHOS10.1* in M4 and *VvHOS10.1* and *VvHOS10.2* in 101.14, usually up-regulated in different kind of stress, resulted more expressed in grafted plants (Figure 34 A and B). This preliminary result could suggest that rootstocks affected, in some degree, gene regulation even if rootstocks and as scion belong to the same genotype.

Conversely, under drought stress condition, the differences in ABA pathway between grafted and ungrafted were reversed. Comparing gene expression in LW with those in NW we found that the transcript of ABA gene were higher in un-grafted plants compared with grafted plants. The most significant differences disclosed by both M4 and 101.14 ungrafted compared to grafted plants were the higher expression *of VvNCED1* and *VvABI1.1* and the strong down-regulation of *VvERA1* (Figure 35 A and B). In M4/M4 and in 101.14/101.14 in LW the expression of ABA related genes, already high in NW, did not increase more. *VvMYB60* regulation in LW proved differences between the two genotypes. It resulted more down-regulated in ungrafted plant comparing *101.14* and 101.14/101.14 but not in M4 and M4/M4 (Figure 35 A and B). Instead *VvSIRK* and *VvSLAC*

resulted more down-regulated in M4 and 101.14 compared to M4/M4 and 101.14/101.14 (Figure 35 A and B).

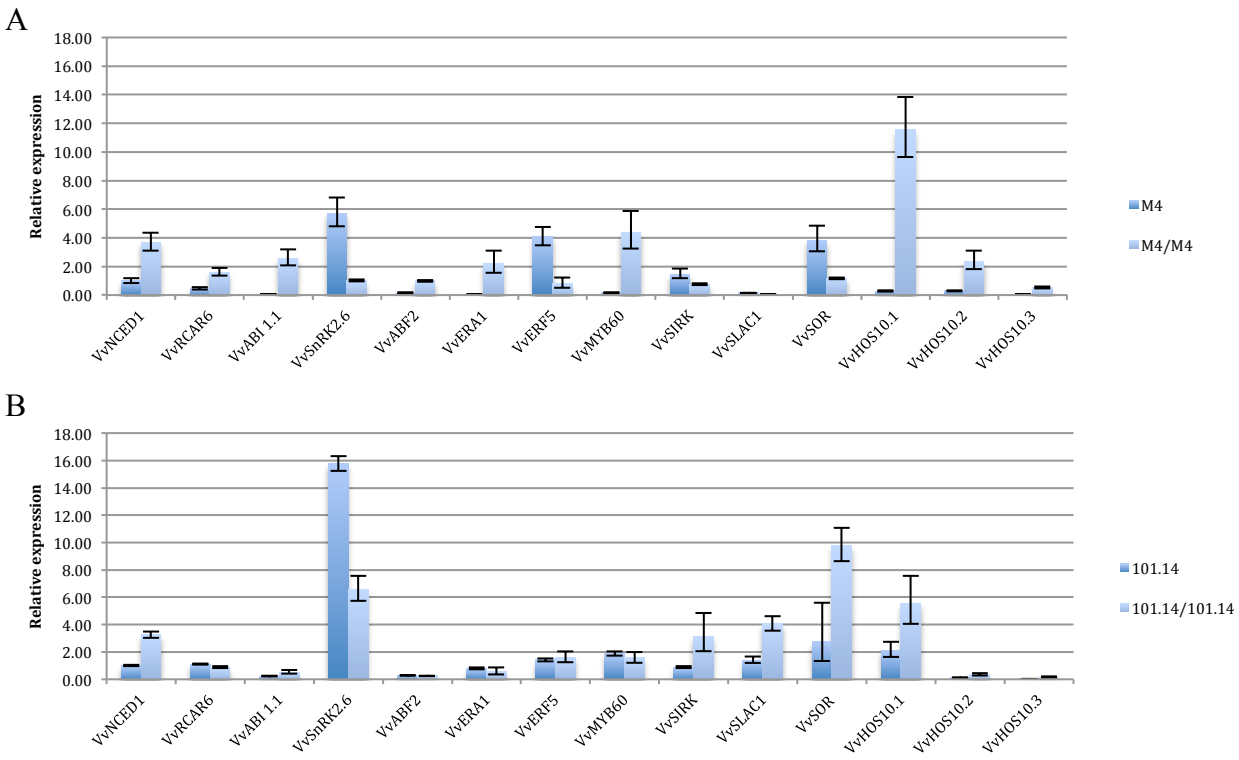

Figure 34 Comparative analysis of ABA- and GC-related gene expression in ungrafted and grafted M4 (A) or 101.14 (B) plants grown under normal water conditions.

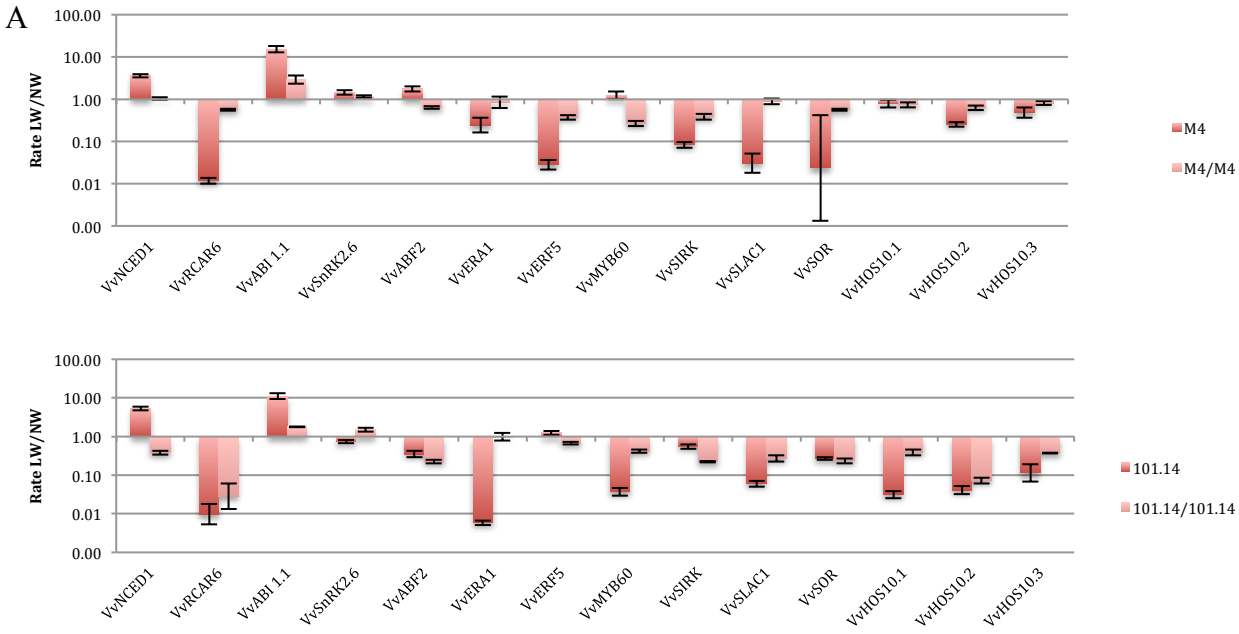

Figure 35 Changes in gene expression in response to water stress in whole leaves from ungrafted and grafted M4 (A) or 101.14 (B) plants. Data correspond to the ratio of relative expression under NW and LW.

3.6 Analysis of rootstock influence on Cabernet Sauvignon under drought stress

In order to improve our knowledge about plant with agronomic interest, we focused on CS grafted on CS and on the two reference rootstock M4 and 101.14 and, as control, the two rootstocks grafted on themselves.

This experiment was performed for three years in two different environmental conditions (two different greenhouse); also in the second and in the third year we adopted a guard cell enrichment approach, blender mediated, in order to better understand the stomata role in drought stress response.

In the first year experimental design and conditions were the same as previous experiment. We compared CS/CS, CS/M4 and CS/101.14. As shown in Figure 36 A and B, under both normal water and low water conditions the analysis of the transcript of whole leaf did not revealed any significant difference among the three genotypes analysed.

However physiological data indicated that CS/101.14 close stomata earlier and stronger under drought stress than CS/M4 (data not shown). Whole leaf RNA extraction could have affected analyses of GC related genes and this could explain because the differences in stomatal aperture under drought stress were not correlated with the regulation of the genes in GC.

For this reason we adopted a GC enrichment approach, blender mediated in order to improve the accuracy of guard cells related genes. We compared CS grafted on M4 and 101.14 and as control M4/M4 and 101.14/101.14. Moreover, in order to appreciate differential in gene regulation that occurred in GC at the early stage of drought response the leaves sampling was performing at three-time point. We analysed plants at 90% of FC, at 60% and finally at 30%.

VvNCED1 was more expressed in 101.14/101.14 than CS/M4, CS/101.14 and M4/M4 already at 60% of FC but much more at 30% of FC (Figure 37 A).

Interestingly, in plants with as rootstocks 101.14 (101.14/101.14 and CS/101.14) *VvABI1* was more expressed than in CS/M4 and M4/M4 (Figure 37 B). Yet, this hyper-activation of ABA pathway induced an alteration in guard cell related genes regulations. Indeed *VvMYB60* was more down-regulated in the leaf of CS and 101.14 having 101.14 as rootstocks under drought stress compared to CS grafted on M4 and M4 grafted on itself (Figure 37 C).

As seen before the expression of *VvNCED1* in the leaf of 101.14 non-grafted plants, under drought stress, was more induced that in the other genotype, now we found that the stronger activation of ABA pathway in the rootstocks can influence the synthesis of some genes in the scion.

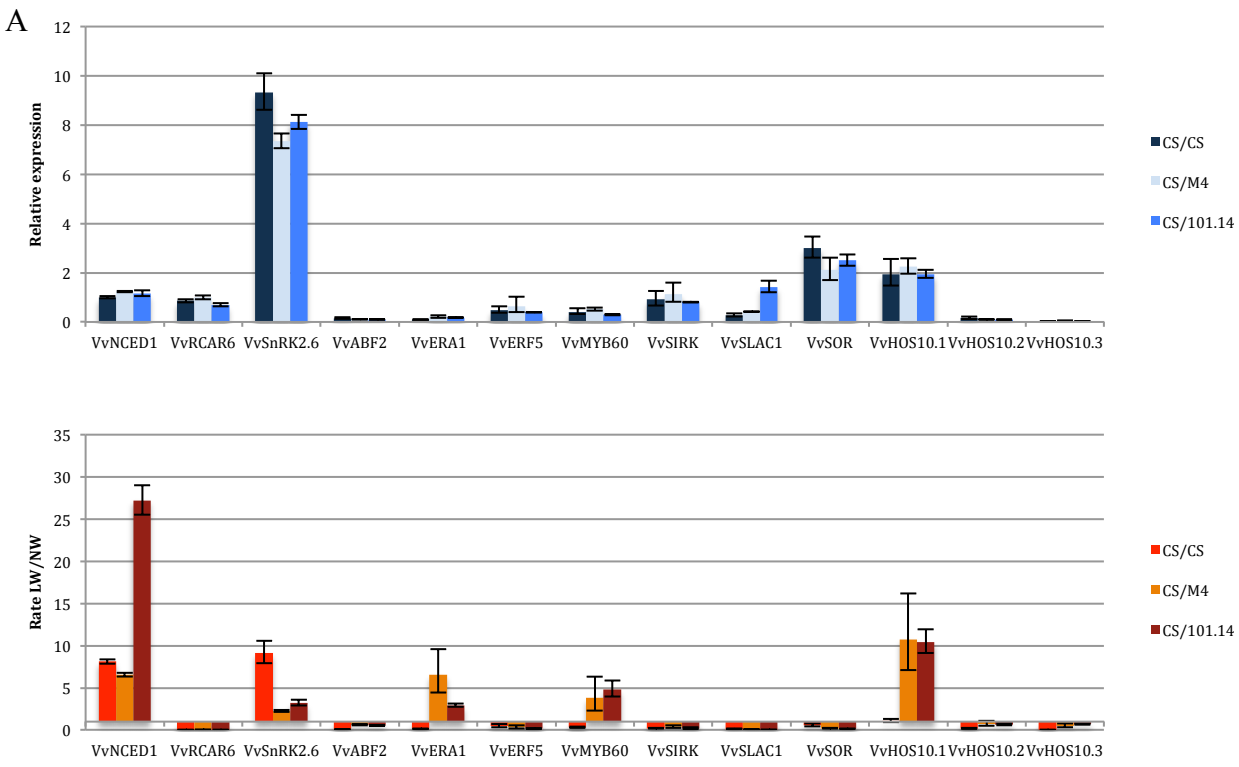

Figure 36 Comparative analysis of ABA- and GC-related gene expression in whole leaves of CS grafted on itself (CS/CS), on M4 (CS/M4) or on 101.14 (CS/101.14). (A) Gene expression in leaves from plants grown under normal water conditions. (B) Changes in gene expression in response to water stress. Data correspond to the ratio of relative expression under NW and LW.

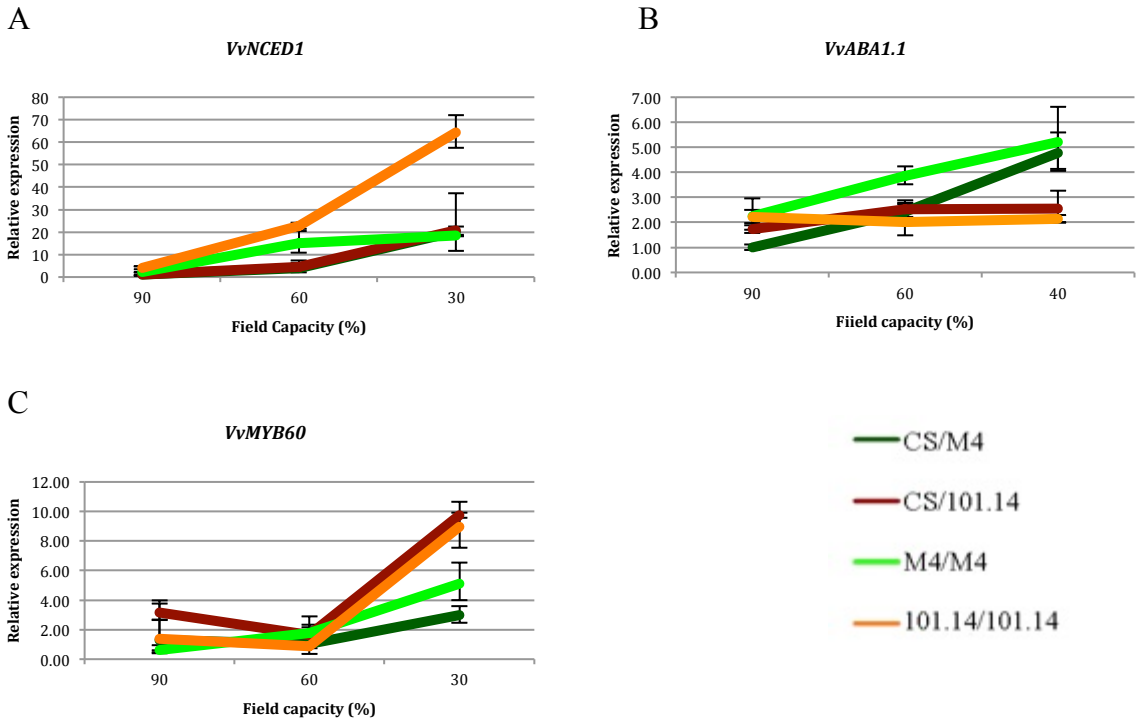

Figure 37 Comparative analysis of ABA-related and GC-related gene expression in GC-enriched epidermal fragments from CS grafted on M4 (CS/M4) or on 101.14 (CS/101.14), M4 grafted on itself and 101.14 grafted on itself, in response to water stress. (A) *VvNCED1*, (B) *VvABA1.1* and (C) *VvMYB60*. Leaf samples were collected from plants maintained at 90%, 60% and 30% of field capacity.

3.7 *VvMYB60* is down-regulated by salicylic acid

In this work we showed that *AtMYB60* was down-regulated by salicylic acid. We have also shown how *VvMYB60* regulation could be influenced by the rootstocks. In order to understand if *VvMYB60* was involved in salicylic acid response as well and whether this response could be influenced by the rootstocks, different grafted combination were treated with 100µM of SA. Leaves were collected from CS/M4, CS/101.14, M4/M4 and 101.14/101.14 plants, 6 and 24 hours after treatment and compared with the untreated control.

Analysis of gene expression was performed on both whole leaf and epidermal pieces enriched in guard cells.

We monitored the expression of a grapevine *VvPR1* gene, whose sequence is the most closely related to *AtPR1* (Le Henanff *et al.*, 2009), but however no information were available of its expression after SA treatment. As shown in Figure 38 A, *VvPR1* in whole leaf was weakly stimulated by SA after 6 hours and then its expression decreased. At the contrary in the GCs sample, *VvPR1* expression was induced only in CS leaves, independently by the rootstocks. In M4/M4 and 101.14/101.14 *VvPR1* was slightly down-regulated (Figure 38 B).

As shown in (Figure 38 C and D), *VvMYB60* was down-regulated after SA treatment in all genotypes after 6 hours and later the change in expression level was very little. This down-regulation took place both in the analysis of whole leaf and GCs.

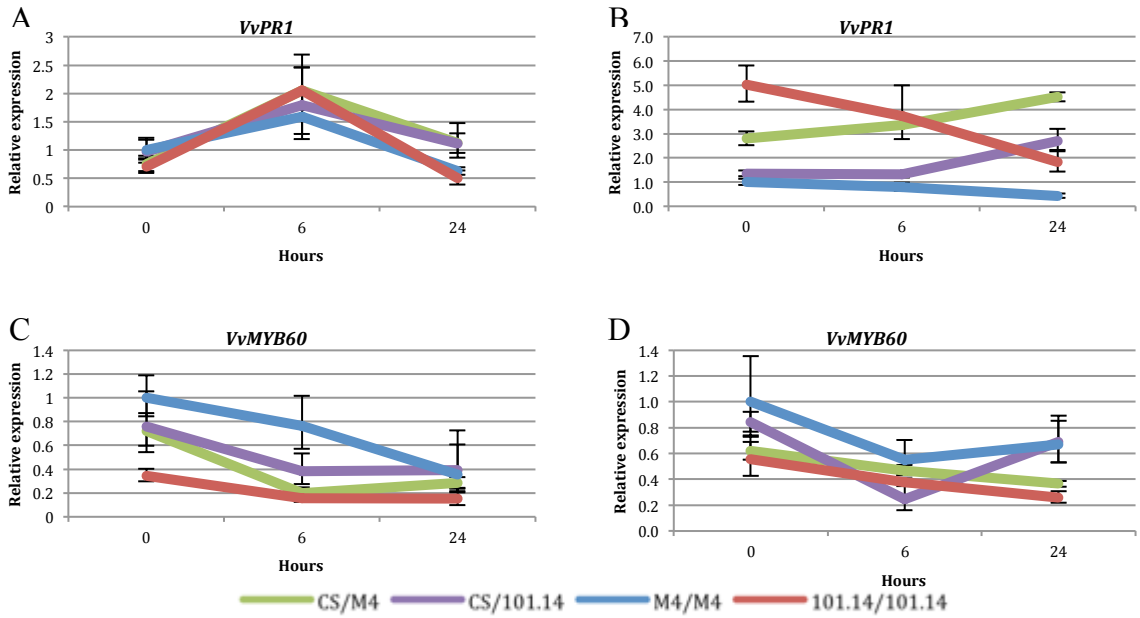

Figure 38 Analysis of *VvPR1* and *VvMYB60* expression in response to SA (100µM) in whole leaves and GC-enriched epidermal fragments from M4/M4, 101.14/101.14, CS/M4 and CS/101.14 graft combinations. (A) *VvPR1* expression in whole leaves. (B) *VvPR1* expression in GC-enriched epidermal pieces. (C) *VvMYB60* expression in whole leaves. (D) *VvMYB60* expression in GC-enriched epidermal pieces. M4/M4 blue line, 101.14/101.14 red line, CS/M4 green line and CS/101.14 violet line.

Discussion

This work aims at investigating the molecular basis of stomatal responses, through the analysis of the transcriptional changes occurring in guard cells (GC) in response to biotic and abiotic stress. First we focused on the plant model system Arabidopsis in order to get more insight into the transcriptional mechanisms that regulate stomatal activity. Next, we applied this knowledge to grapevine.

Stomata are microscopic pores distributed on the surface of leaves and stems, surrounded by two highly specialized guard cells. The opening and closure of the pore, in response to internal signals and environmental cues allows the plant to cope with the conflicting needs of ensuring adequate uptake of CO_2 for photosynthesis and preventing water loss by transpiration (Schroeder *et al.*, 2001). Nearly 90% of water loss from the plant tissues occurs through stomata (Sang *et al.*, 2001). Under optimal growth conditions, transpirational water loss is the main driver for water flux and its circulation throughout the plant. Under drought conditions, the rapid closure of the stomatal pores represents the first adaptive response to enhance plant survival (Schroeder *et al.*, 2001).

In grapevine, stomatal conductance is a key trait as it directly determines the isohydric/anisohydric behaviour displayed by different genotypes. These differences are due to stomatal control over evaporative demand rather than stomatal density in vegetative tissues (Rogiers *et al.*, 2009).

Stomata also represent the primary port of entry for bacterial and fungal pathogens, and are known to play an active role in the plant innate immune-system (Melotto *et al.*, 2006). Moreover stomata have also been found to respond to various microbe-derived compounds (Melotto *et al.*, 2006). This phenomenon has been defined as stomatal immunity (Melotto *et al.*, 2006) as it requires well-known molecular components of the plant innate immune system (Zeng and He, 2010). In grapevine, the penetration and sporulation of *Plasmopara viticola* occurs via stomata, suggesting functional relationships between guard cells and the pathogen (Allègre *et al.*, 2007). The analysis of the relationship between stomatal regulation and bacterial activity is a key issue in understanding the crosstalk between biotic and abiotic stress-signalling, and is instrumental in developing new plant varieties with enhanced stress tolerance (Lee and Luan, 2012).

The regulation of stomatal activity involves the activation of the canonical ABA-signalling components as well as changes in gene expression patterns and in RNA processing (Cominelli *et al.*, 2010). The Arabidopsis R2R3-type MYB transcription factor *AtMYB60* has been identified as a transcriptional integrator of abiotic and biotic stress responses in GC (Cominelli *et al.*, 2005).

MYB factors represent a family of proteins that include the conserved MYB DNA-binding domain. In contrast to animals, plants contain a MYB-protein subfamily that is characterised by the R2R3-type MYB domain (Stracke *et al.*, 2001). R2R3-type *MYB* genes control many aspects of plant secondary metabolism, as well as the identity and fate of plant cells (Stracke *et al.*, 2001). R2R3 *MYB* genes are well characterized in grape for their role in phenylpropanoid pathway, including anthocyanins, flavonols, and flavan-3-ols (Deluc *et al.*, 2006). Interestingly, many of the white grape cultivars present in the world today arose from multiallelic mutations of the *VvMYBA1* and *VvMYBA2* genes which control the last biosynthetic step of anthocyanin synthesis (Kobayashi *et al.*, 2004).

In Arabidopsis, *AtMYB60* is specifically expressed in guard cells and its expression is negatively modulated during drought. A null mutation in *AtMYB60* results in the constitutive reduction of stomatal opening and in decreased wilting under water stress conditions, whereas an accumulation of *AtMYB60* induces stomata opening (Cominelli *et al.*, 2005). Interestingly, the loss of the *AtMYB60* function in the null mutant *atmyb60-1* results in impaired expression of both pathogen- and stress-related genes (Cominelli *et al.*, 2005).

The grape *VvMYB60* was identified as the true ortholog of *AtMYB60* (Galbiati *et al.*, 2011). This finding is supported by the fact that the aminoacidic sequence of the VvMYB60 and AtMYB60 proteins is highly conserved (Figure 39) (Galbiati *et al.*, 2011), the *VvMYB60* and *AtMYB60* genes show very similar expression profiles (Galbiati *et al.*, 2011), their promoters drive expression of reporter genes exclusively in guard cells and, finally, the expression of *VvMYB60* in the *atmyb60-1* mutant background completely rescues the loss of the *AtMYB60* function (Galbiati *et al.*, 2011).

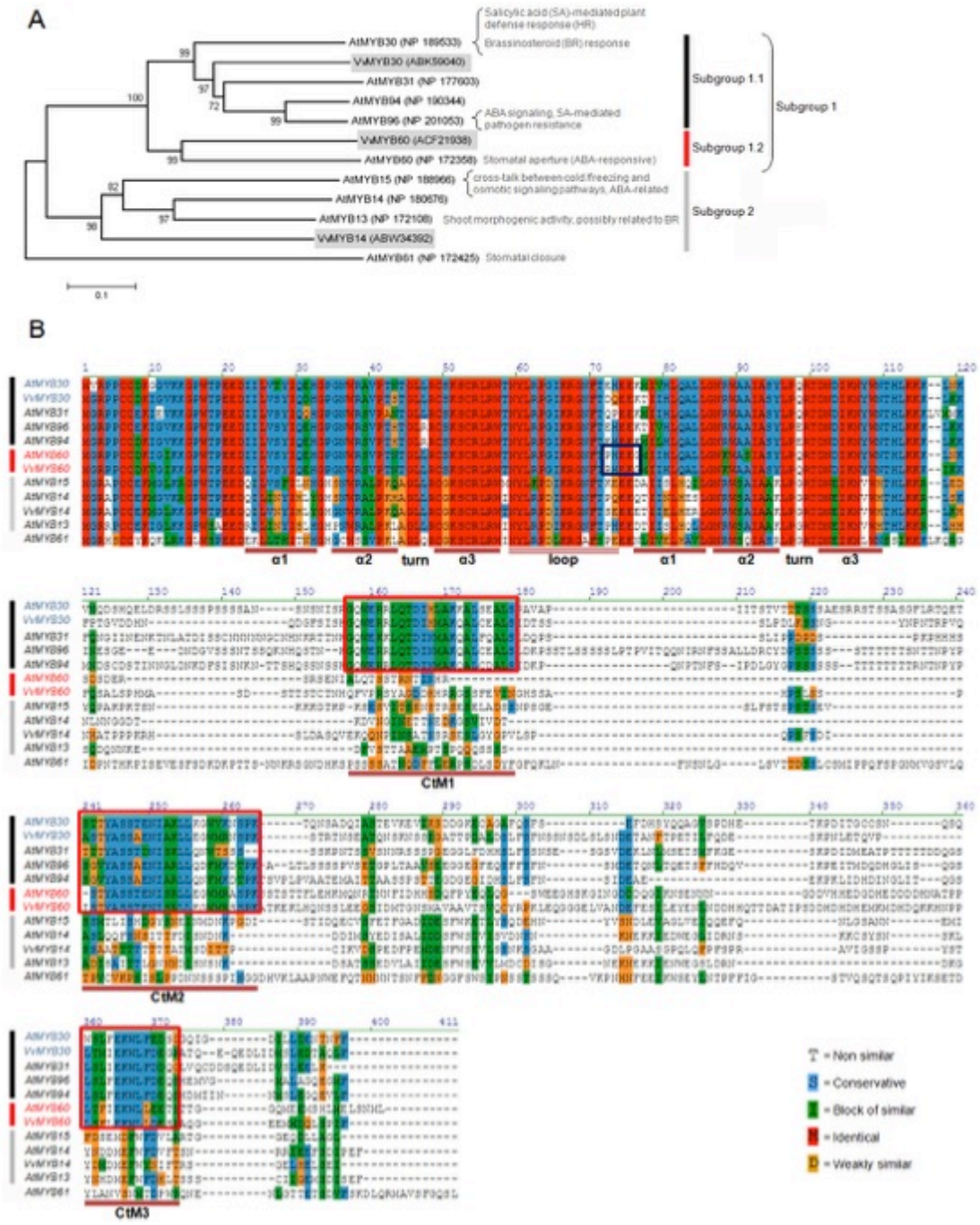

Figure 39 Analysis of grape and Arabidopsis MYB homologues as reported in (Galbiati et al., 2011). (A), Phylogenetic relationships between Arabidopsis and grape subgroups 1 and 2 of R2R3 MYB factors, as described by (Kranz et al., 1998). (B) Alignment of deduced amino acid sequences of subgroup 1 and 2 R2R3 MYB homologues from Arabidopsis and grape. The R2 and R3 repeats lie between the three alpha helices of each repeat. Boxes represent the C- terminal motifs CtM1, CtM2 and CtM3 (red boxes) conserved in members of subgroup 1 and the PHEEG signature (blue box), distinctive of AtMYB60 and VvMYB60 (subgroup 1.2). Amino acid residues are shaded in different colours, as indicated in the legend. Dots represent gaps introduced to improve the alignment (Galbiati *et al.*, 2011).

Part I: Technical approaches to study stomatal activity

In this thesis we refined some techniques to improve the precision to study stomatal regulation. We adopted a confocal microscopy-based approach to analyse stomata movement in more physiological conditions and the laser microdissection technique to better assess the regulation of gene expression in guard cells.

1.1 A confocal approach for assessing stomatal response to different stimuli

The routinely technique to observer stomata movement at the microscope, namely epidermal peel, foresees the disconnection of epidermal layer from the underlying mesophyll. Moreover the sample preparation requires removing the epidermal layer from the physiological buffer during the microscope analysis.

A confocal approach, adapted from (Chitrakar and Melotto, 2010), allows to analyse stomatal activity in more physiological condition than the routinely used epidermal peel. Confocal analysis reduces leaf stress during the incubation time and during the image acquisition as well. Indeed, this technique does not disconnect epidermal layer allowing a stomatal assessing in continuously cross-talk with the rest of the leaf. This method allows for the observation of the same live leaf sample over extended periods of time. The sample is always embedded in the physiological buffer during the image acquisition.

Moreover some stomata responses take place in a very short period of time and the strength and the timing could vary among different genotypes. Non continuous sampling mode performed by epidermal peels reduces the assessing of rapid stomata movement. On the contrary, confocal-based measurements allow a real time analysis of stomata activity, within few seconds after the perturbation (chemicals, light, hormones etc.).

The analysis performed on the whole leaf of WT and *atmyb60-1* following exposure to dark or light, chemicals (ABA), or elicitors (flg22 and LPS) confirmed the sensibility of this method and its potential in studying GC responses to different kind of stress.

1.2 Laser Microdissection-based purification of guard cells

The guard cell is autonomous, making it a useful model to understand cell type responses to stresses (Obulareddy *et al.*, 2013). Plant adaptation to stress requires the fine-tuning of their plasticity to the environment, which is achieved by the integration and coordination of several cell-specific responses (Pandey and Somssich, 2009). Global analysis of gene expression in whole plants or heterogeneous organs has greatly limited our understanding of how multi-cellular organisms cope with environmental changes at the cellular level, and prevented the identification of key genes involved in the regulation of subtle but significant cell-specific responses. Increasing repositories of publicly available data have opened new avenue to genome-wide analysis of plant stress responses, yet, most studies focused on the analysis of transcriptional changes associated with immediate responses or resistance to individual stresses, traits of little relevance to water use efficiency and yield protection in crops (Bohnert and Jensen, 1996). In addition, the majority of available datasets results from gene profiling of whole plants or plant organs, in which the contributions of multiple cell types are homogenised. Evidence indicates that most of the plant responses to the environment occur in a cell type-dependent manner (Rogers *et al.*, 2012). "Bulk" analyses of heterogeneous tissues often prevent the identification of key genes involved in the regulation of subtle but significant cell-specific responses (Nelson *et al.*, 2006). In this perspective, methods to address gene expression or biochemical properties in single cells are instrumental in understanding plant responses to stress.

The analysis of GC-specific features, including gene expression, traditionally employed the use of purified guard cell protoplasts (GCPs). Procedures to isolate GCPs for Western blotting, reverse transcription polymerase chain reaction (RT-PCR), microarray analysis, and electrophysiological studies have been previously reported (Leonhardt *et al.*, 2004). With the advent of novel high-throughput methods such as direct RNA sequencing (RNA-seq), the quantity, quality, and differential decay of RNA molecules, as well as the preservation of whole cell transcriptomes during protoplasting have become critical issues for the success of gene expression studies (Obulareddy *et al.*, 2013). GCPs preparation requires transcription inhibitors during the complete digestion of the cell wall to avoid induction of stress-related genes (Leonhardt *et al.*, 2004). However, the long procedure (>5 h) to release guard-cell protoplasts may lead to RNA decay (Obulareddy *et al.*, 2013).

Alternative methods, based on the mechanical destruction of the leaf, to produce epidermal pieces enriched in intact guard cells have been described (Bauer *et al.*, 2013; Geiger *et al.*, 2009). This approach is suitable for both molecular analysis and for microscope analysis. An ice-cool setting

condition reduces stress induced by blending of leaf tissues. This approach has been recently employed to produce a genome-wide analysis of transcriptional responses in Arabidopsis GCs (Bauer *et al.*, 2013).

Finally, laser microdissection (LMD) has also been exploited for the purification of GCs. LMD is a technique by which individual cells can be harvested from tissue sections while they are viewed under the microscope, by cutting selected cells with a laser beam (Nelson *et al.*, 2006).

The advantage that this technology offers, with respect to other techniques, is that LMD can be usually applied to all cells that can be identified by conventional microscopy without the obligate use of specific cell markers or genetic lines (Balestrini *et al.*, 2009). LMD returns a sample reasonably composed by only cells picked, greatly reducing the stress added. Its relative disadvantage is that the number of cells that can be recovered is limited by the abundance and recognition of cellular targets in histological sections. On the other hand protoplast isolation *per se* is a stress-inducing procedure, particularly during the enzymatic isolation, with accumulation of peroxides and degradation products that induce cell lysis (Davey *et al.*, 2005). In comparison, LMD produces a lower yield but purest sample of intact guard cells avoiding the activation of stress-induced responses.

In this thesis blended-derived epidermal pieces, and LMD-based cell purification were used in Arabidopsis and in grapevine. Blender-purification of GC from grape tissues has not been reported in the literature. Starting from the protocol developed in Arabidopsis, we set the experimental conditions for grape tissues. Compared to LMD, this method allows obtaining intact and alive GCs in a short period of time and in relatively large amounts. Analysis of the expression of GC-specific marker genes, *AtMYB60* and *VvSIRK* for Arabidopsis and grapevine respectively, confirmed the effective enrichment in GCs of our preparations (Figure 9 and Figure 22). LMD in grapevine has successfully been used to study gene expression in different plant tissues, such as vessel-associated cells (Chitarra *et al.*, 2014), leaf phloem infected (Santi *et al.*, 2012) and different parts of the root tissue (Gambetta *et al.*, 2013). We adapted the described sample preparation and microdissection conditions to isolate GC from grape leaves. Again, expression of marker genes revealed a high degree of GC purification, yet we experienced little yield of total RNA from the microdissected samples that prevented the possibility to perform gene profiling studies on a whole genome scale.

Part II: Arabidopsis *AtMYB60* is involved in the regulation of GC activity in response to both abiotic and biotic response

2.1 The role of *AtMYB60* in oxylipin-mediated guard cell regulation

RNA profiling of guard cells obtained from WT and mutant plants, reveals few hundred up and down regulated genes. Functional analysis of the differentially expressed transcripts revealed significant down-regulation of genes classically associated with biotic and abiotic stresses, such as pathogenesis related proteins (PR). The loss of the *AtMYB60* function also results in lower expression of genes involved in VLCFA and cutin synthesis (Figure 10).

Interestingly, *AtMYB60* paralogs in Arabidopsis *AtMYB30*, *AtMYB94* and *AtMYB96* (Figure 40), are also linked to VLCFA and cutin metabolism (Raffaele *et al.*, 2008; Seo and Park, 2010; Lee *et al.*, 2014). Moreover, *AtMYB60, AtMYB30, AtMYB94* and *AtMYB96* are involved in drought response and ABA pathway. Most notably, *AtMYB96* is induced by drought and ABA and it controls VLCFA synthesis and wax deposition (Seo *et al.*, 2011).

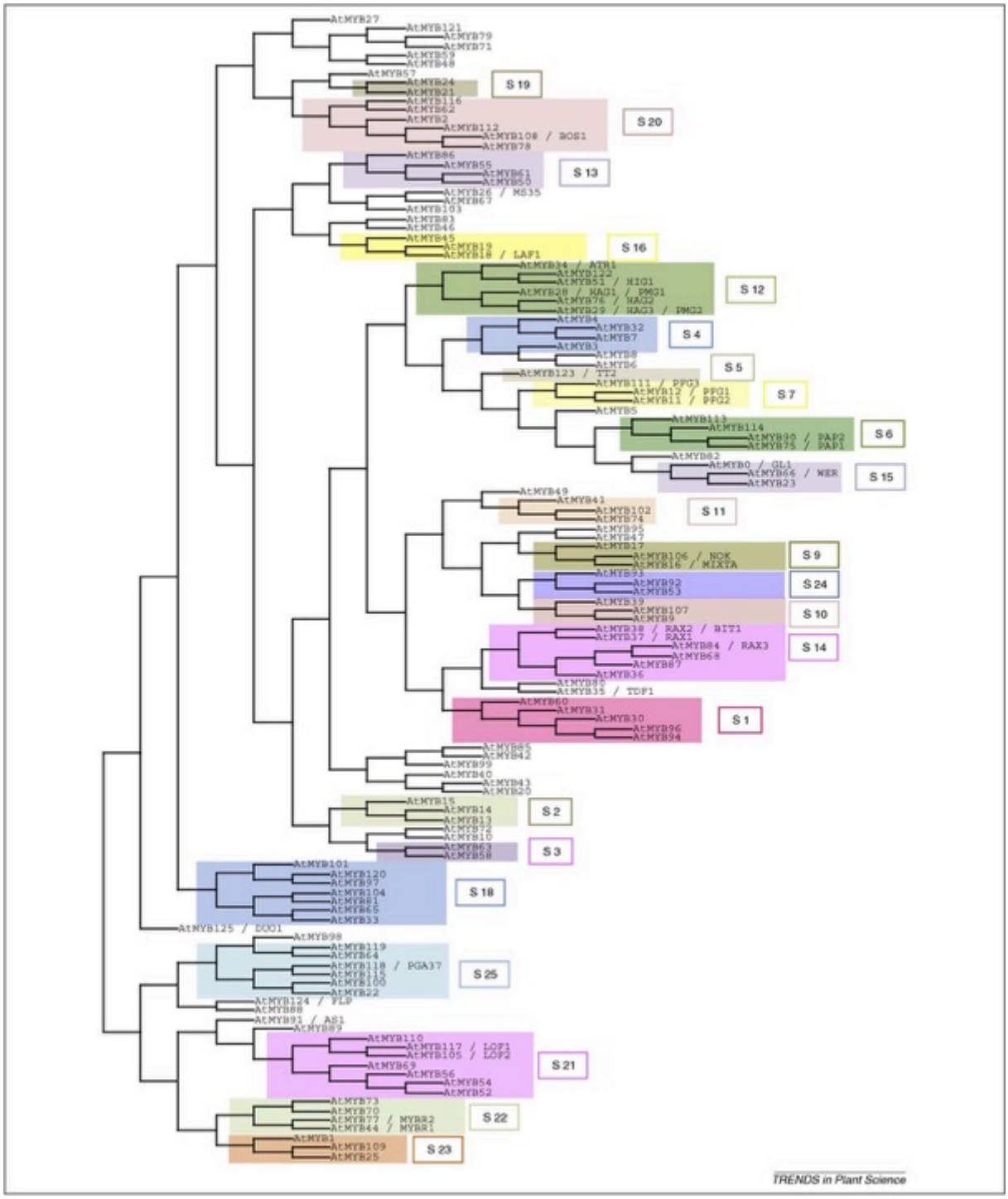

Figure 40 Schematic representation of the relationships between the different R2R3-MYB subgroups, as reported in Dubos *et al.*, 2010.

Although the over-expression of *AtMYB30* increases wax synthesis, his inactivation does not induce significant effect on wax accumulation, suggesting that the lack of *AtMYB30* activation can be overridden by stronger wax pathway activators in *AtMYB30* loss of function plants (Raffaele *et al.*, 2008). From our RNAseq data, *atmyb60-1* results impaired in genes involved in VLCFA synthesis, nevertheless the metabolic analysis did not show any differences in VLCFA accumulation between WT and mutant leaves (Figure 13).

Furthermore metabolomic analysis showed a significant accumulation in oxylipins in the mutant line compared to WT (Figure 14). Oxylipins are regulators of stomatal closure in response to drought (Savchenko *et al.*, 2014). Moreover, oxylipins resulted more accumulated in the GCs compared to the whole leaf and, most importantly, GCs of *atmyb60-1* accumulated much more oxylipins than GCs of WT (Figure 14). This cell specific storage is also supported by molecular data: genes coding for some metabolic enzymes involved in oxylipins synthesis (*AtLOX1*, *AtLOX4, AtLOX6* and *AtOPR2)* are more expresses in GCs compared to the mesophyll (Figure 17). This result supports the presence of a stomata-specific pathway for oxylipins biosynthesis. Thus, an intriguing hypothesis is that drought induces down-regulation of *AtMYB60*, which in turn results in accumulation of oxylipins in GCs.

Oxylipins comprise a vast array of bioactive metabolites that are generated from membrane lipids as a result of lipid peroxidation (Dave and Graham, 2012). Among them, jasmonates are also found, a family of signalling molecules that act as phytohormones in the regulation of developmental processes and stress responses in plants (Ballaré, 2011). 12-OPDA is a precursor of jasmonic acid (Mosblech *et al.*, 2009). Moreover Savchenko and co-workers have identified drought as a stress signal that uncouples the conversion of 12-OPDA, one of the five oxylipins analysed, from JA (Savchenko *et al.*, 2014). Both oxylipins and jasmonate are involved in the regulation of stomatal activity. JA is accumulated during drought and wounding and it has been shown to act as positive regulator in stomatal closure (Munemasa *et al.*, 2007b).

Notably, only drought (and not wounding) induces the production of 12-OPDA in Landsberg *erecta* (L*er*) and Wassilewskaija (Ws) ecotypes, whereas, in Columbia (Col-0), 12-OPDA are equally induced by drought and wounding. This is potentially due to a higher available pool of substrates in Col-0, which carries a dysfunctional *Arabidopsis Hydroperoxide lyase* (*AtHPL)* gene (Savchenko *et al.*, 2014). AtHPL catalyses the oxidative cleavage of the hydrocarbon backbone of fatty acid hydroperoxides (Feussner and Wasternack, 2002). This process, which removes substrate for the synthesis of OPDA, is inhibited in the ecotype Col-0, the ecotype used in this thesis. JA levels in all the three ecotypes are enhanced solely in response to wounding (Savchenko *et al.*, 2014). Thus in Col-0 wounding induces both OPDA and JA.

As AtMYB60 is a guard cell specific transcription factor our hypothesis is that it controls stomata activity by tuning the expression of one o more guard cell genes. Transcript analysis showed alteration of some stress related genes in mutant plant compared to WT. Thus the most direct and obviously link with oxylipins/jasmonate pathway should be the control of transcriptional regulation of genes involved in this synthesis. Analysis of the transcript of metabolic enzyme involved in

oxylipins and jasmonate biosynthesis did not disclose significant differences between WT and *atmyb60-1* plants.

Furthermore, the analysis of *AtVSP1*, a well-known marker gene for jasmonate accumulation (Feys *et al.*, 1994) suggested that, likely, JA is more accumulated in GCs of *atmyb60-1* than in the GCs of WT. However, our data show that, at the transcriptional level, the metabolic enzymes involved in the biosynthesis of oxylipins and jasmonic acid are equally expressed in the GCs of WT and in the GCs of mutant plants (Figure 17).

In *atmyb60-1*, 12-OPDA and JA are hyper-accumulated like in Col-0 (the ecotype mutant for *AtHPL*) after wounding. This adds another link among 12-OPDA, JA and *AtMYB60*. It is tempting to speculate that the *atmyb60-1* null mutant is additive to the *AtHPL* mutation and this results in an increased production of oxylipins and jasmonate under normal grown conditions.

One reason for OPDA accumulation could be related to its transport within the cell (Theodoulou *et al.*, 2005). 12-OPDA is produced in the chloroplast then it is translocated to the peroxisome, via a process mediated by the transporter Arabidopsis comatose (AtCTS) (Hooks *et al.*, 2007). However, the regulation at the transcriptional level of *AtCTS* is not impaired in the *atmyb60-1* mutant plants (Figure 18 A, B).

Among the five oxylipins detected in leaves and in GCs only 12-OPDA are known to be involved in stomatal activity (Howe and Schilmiller, 2002). Interestingly, DGDG OPDA and MGDG OPDA are also more accumulated in GCs of *atmyb60-1* than in the GCs of WT (Figure 14). Moreover, it has been reported that the levels of oxylipin containing galactolipids (like DGDG OPDA and MGDG OPDA) drastically rise in response to mechanical wounding (Buseman *et al.*, 2006). The role of oxylipin-containing lipids in the plant has not been elucidated yet, but it is well established that free JA and its precursor OPDA are signalling molecules occurring in most, if not all, plant species (Stelmach *et al.*, 1998), fulfilling several important roles in defence and developmental processes (Taki *et al.*, 2005). Genes involved in this branch of oxylipins synthesis have not been analysed in this work. Clearly, they will be the focus of further analysis.

In sum, analysis of the transcriptional regulation of the genes involved in oxylipins synthesis did not provide hints on the molecular mechanisms by which the *atmyb60-1* mutant accumulates increased levels of such molecules compared to the WT. More work will be needed to uncover the bases for oxylipins over-accumulation in GC of the mutant. Nevertheless, metabolic data clearly indicate an important mechanism underlying the constitutive reduction of the opening of the stomatal pore in the *atmyb60-1* background.

Finally, an unexpected result emerged from the analysis of gene expression in GCs and mesophyll cells. Genes as *AtLOX2, AtLOX5* (Figure 17 F, I)*, AtAOC, AtAOC3* (Figure 17 L, M)*, AtOPR3* and

AtJMT (Figure 17 P, V) were up-regulated in the mesophyll of the mutant line compared to the WT. This suggests that disruption of a guard cell specific transcription factor, as *AtMYB60*, can have detectable consequences on gene expression in the mesophyll. This effect can be indirect and involve signals moving from GC to mesophyll and *vice versa*. However, the bases for this type of regulation remain largely unknown.

2.2 The role of *AtMYB60* in SA-mediated guard cell regulation

In addition to its function in oxylipins and jasmonate metabolism *AtMYB60* might play another role in plant defence. From RNA-seq data the expression of genes involved in salicylic acid response, like *AtPR1*, was impaired in the *atmyb60-1* mutant. Recognition of microbe-associated molecular patterns (MAMPs) rapidly induces stomatal closure to prevent pathogen entry. This response requires components of the SA and ABA signalling pathways (Melotto *et al.*, 2006).

Salicylic acid is associated with stomatal closure (Zeng and He, 2010). Consistent with its role of positive regulator of stomatal opening, *AtMYB60* expression was strongly down-regulated following exogenous applications of SA (Figure 21 A). Most importantly, *atmyb60-1* plants disclosed an impaired response to SA, similar to the one exhibited by the *atnpr1-1* mutant (Figure 19 B). In contrast, exogenous ABA was still able to induce stomatal closure in *npr1-1* as well as in *atymb60-1* plants (Cominelli *et al.*, 2005; Zeng and He, 2010). This observation suggests a possible involvement of *AtMYB60* in mediating SA-mediated stomatal regulation.

Gene expression analysis established a further link between *AtNPR1* and *AtMYB60*. The analysis of *AtMYB60* expression performed on whole leaves from WT and *atnpr1-1* plants did not reveal significant differences between the two genotypes (Figure 20). Nevertheless, qPCR analysis of LMD-purified GCs and mesophyll cells uncovered a possible stomata-specific control exerted by *AtNPR1* on *AtMYB60* expression. More into details, in the absence of SA, expression of *AtMYB60* was significantly up-regulated in guard cells of *atnpr1-1* plants, suggesting that *AtNPR1* may act as a transcriptional repressor of *AtMYB60* (Figure 21 A). Following SA treatment we observed a significant decrease in *AtMYB60* expression both in WT and *atnpr1-1* leaves. This implies that *AtNPR1* is not required for the SA-induced *AtMYB60* transcriptional inactivation. In the context of disease defence responses, the existence of a SA-dependent but *AtNPR1*-independent pathway that leads to pathogen resistance has been well documented (Dong, 2001). According to this observation, it is tempting to speculate that *AtMYB60* belongs to the *AtNPR1*-independent pathway. It is important to note that, 24 hours after the beginning of the treatment *AtMYB60* resulted strongly

down-regulated in GCs of the WT, whereas in *atnpr1-1* its expression was restored to the same level observed in untreated plants. Evidence indicates that activation by SA of early and late-responsive genes occur by different mechanisms (Uquillas *et al.*, 2004). In particular, activation of immediate early genes by SA proceeds through an NPR1-independent pathway. We can thus speculate that the SA-induced *AtMYB60* down-regulation does not require *AtNPR1* in the early phase, while *AtNPR1* controls the inactivation of *AtMYB60* expression at a later stage.

Further support to the involvement of *AtMYB60* in mediating SA responses in GC comes from the analysis of *AtPR1* expression, a late SA-responsive gene. In whole leaves from the *atmyb60-1* mutant, up-regulation of *AtPR1* expression following SA treatment was slightly enhanced compared with leaves from the WT (Figure 20 A and B). Profiling of LM-purified GCs and mesophyll cells revealed interesting and in part contrasting features of *AtPR1* expression in the leaf. In the WT, *AtPR1* was activated to the same extent in GC and mesophyll cells 24 hours after the beginning of the SA treatment (Figure 21 B). Conversely, *AtPR1* expression was not activated by SA in GC from the *atmyb60-1* mutant, whereas its activation in the mesophyll was significantly enhanced compared with the WT (Figure 21 B). Two main conclusions can be drawn by these contrasting results. First, a functional *AtMYB60* gene is required for proper activation of *AtPR1* expression in GC, thus reinforcing the notion that *AtMYB60* mediates the transcriptional response to SA in GC. Second, despite the stomata-specific expression of *AtMYB60*, the lack of its function affects responses taking place in the mesophyll. Even if the molecular bases of such response is unclear, the hyper activation of *AtPR1* in the mesophyll of the mutant explains the differences observed for the *AtPR1* expression in whole leaves from *atmyb60-1* and WT plants (Figure 16). Clearly, more work will be needed to investigate the influence of the *atmyb60-1* mutation on the regulation of gene expression in the mesophyll. Although *AtMYB60* is specifically expressed in GCs, its mutation triggers alteration in the regulation of genes expressed in mesophyll cells. Likewise the up-regulation of *AtPR1* after SA treatment in *atmyb60-1* plant some genes in oxylipins biosynthesis are up-regulated in mesophyll compared to WT plants.

Evidence indicates a high degree of cross-talk among stomatal pathways, which are induced by water stress, pathogenic elicitors, and related hormonal responses. Three-sided antagonistic interactions among SA-, JA-, and ABA-mediated signalling appear to participate in the regulation of responses to both biotic and abiotic stresses. SA and JA act synergistically when applied to the plant in low concentrations, whereas a high concentration of one hormone antagonizes the other (Mur *et al.*, 2006). SA- and JA-dependent signalling pathways, which are activated in response to biotrophic and necrotrophic pathogens herbivorous insects, respectively (Spoel and Dong, 2008), can be suppressed by ABA-mediated signalling (Yasuda *et al.*, 2008). In particular, ABA inhibits

the accumulation of SA and the expression of genes involved in basal resistance (Spoel and Dong, 2008).

In guard cells activation of ABA, SA or JA signalling results in stomata closure. SA accumulation in leaf tissues triggers intracellular ROS accumulation and K^+ in channel inactivation, which ultimately results in stomatal closure in response to bacterial infections (Khokon *et al.*, 2011). At the early stage during drought stress, endogenous JA in combination with high ABA level stimulate the preparatory response needed for drought acclimation, including stomatal closure (Harb *et al.*, 2010).

Based on our results, it is intriguing to speculate that *AtMYB60* could integrate multiple hormonal signal-transduction processes (e.g. SA, JA and ABA) by modulating the expression of genes involved in specific guard cell responses.

Part III: Grapevine under drought stress: transcriptional regulation of stomatal activity

3.1 *VvMYB60* is involved in the regulation of stomata activity in response to water stress

A reporter gene approach in a heterologous as Arabidopsis clearly indicated that the activity of the *VvMYB60* promoter is restricted to guard cells and is negatively modulated by ABA. This result is consistent with the observation that the endogenous *VvMYB60* gene is expressed in grape leaves and berry skin, which both contain stomata, and with the lack of expression in roots (Galbiati *et al.*, 2011). Interestingly, in berry skin *VvMYB60* expression was higher before veraison, when the grape berry is photosynthetically active and stomata are functional, and was reduced after veraison, when stomata evolve into non-functional lenticels. At this stage, the onset of ripening and the accumulation of sugars are correlated to increasing levels of ABA in the berry (Gambetta *et al.*, 2010) suggesting a possible negative effect of the hormone on the expression of *VvMYB60* in grape tissues. Indeed, treatment of leaves with exogenous ABA resulted in the rapid down-regulation of *VvMYB60* expression (Galbiati *et al.*, 2011).

Analysis of promoter:reporter transcriptional fusions in Arabidopsis have been shown to provide efficient and reliable tools to investigate the cellular specificity of grape genes (Pratelli *et al.*, 2002).

It is thus reasonable to conclude that the stomata-specific activity of the *VvMYB60* promoter in Arabidopsis mirrors the expression of the endogenous gene in grape guard cells.

We employed two complementary approaches to confirm this hypothesis directly in grape tissues. The analysis of *VvMYB60* expression in whole grape leaves and in GC-enriched epidermal fragments revealed a preferential expression of the gene in the GC-fraction (Figure 22 A). Additionally, the analysis of pure preparations of GCs and mesophyll cells, obtained by laser microdissection of grape leaves, confirmed the up-regulation of *VvMYB60* in stomata (Figure 22 B). Gene expression in guard cells relies on transcriptional mechanisms, employing *cis*-acting elements and their cognate transcription factors. In guard cells expression is mediated by the DOF recognition DNA motif [A/T]AAAG (Plesch *et al.*, 2001). Interestingly, a cluster of [A/T]AAAG DOF target sites was identified in close proximity to the *VvMYB60* translational start codon (Galbiati *et al.*, 2011). Such a cluster has been described as a guard cell-specific *cis*-regulatory element in different plant species. The occurrence of [A/T]AAAG motifs in the *VvMYB60* grape promoters indicates the conservation of the *cis*- and, possibly, *trans*- mechanisms that direct expression in guard cells in distantly related plant species as Arabidopsis and grape. Interestingly, an [A/T]AAAG is also present in *VvSIRK* supporting the guard cell-specificity of this cluster (Galbiati *et al.*, 2011) (Figure 41).

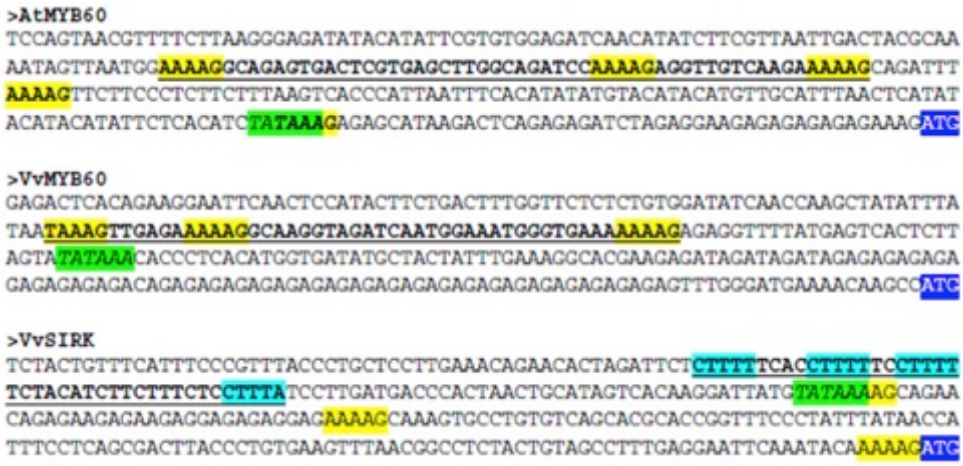

Figure 41 Occurrence of [A/T]AAAG motifs in the 300 bp regulatory region located upstream of the translational start codon of the *AtMYB60*, *VvMYB60* and *VvSIRK* genes. [A/T]AAAG nucleotides on the + strand are highlighted in yellow, whereas [A/T]AAAG nucleotides on the - strand are highlighted in pale blue. The predicted TATA box is in italic and highlighted in green, the ATG codon is highlighted in dark blue. Sequences encompassing clusters of [A/T]AAAG motifs (see text for definition) are in bold and underlined. Modified from Galbiati *et al.*, 2011.

Expression analyses demonstrated the strong conservation of the cellular specificity of the Arabidopsis and grape *MYB60* genes. Protein alignment revealed a surprising degree of homology between AtMYB60 and VvMYB60. The ability of *VvMYB60* to fully complement the stomatal

defects exhibited by the *atmyb60-1* mutant indicated the conservation of gene function between the Arabidopsis and grape genes and identified *VvMYB60* as a true ortholog of *AtMYB60* (Galbiati *et al.*, 2011).

Our analysis of *VvMYB60* expression in different rootstocks highlighted a positive correlation between the level of gene expression and the regulation of stomatal conductance in response to water stress. The enhanced g_s observed in 1103P compared with K5BB in plants grown under control conditions was associated with increased expression of *VvMYB60* in 1103P. The comparison of M4 and 101.14, the major reference genotypes analysed in the SERRES project, revealed that M4 was able to maintain higher values of g_s compared with 101.14 under both water stress and high NaCl treatment (Meggio *et al.*, 2014). Interestingly, this response correlated with a reduced down-regulation of *VvMYB60* expression in M4 leaves compared to 101.14 (Figure 24 D). This result is consistent with recent findings which indicate that the enhanced capacity of M4 to respond to water stress primarily dependents upon root responses, in which the accumulation of osmolytes plays a central role in the rate of water uptake under stress conditions (Meggio *et al.*, 2014).

As a whole our data suggest a positive correlation between the level of *VvMYB60* expression and stomatal activity, even though differences in gene expression do not necessarily directly relate to augmented drought avoidance responses.

3.2 ABA-related genes are differently regulated in different rootstock genotypes in response to water stress

Guard cell signalling integrates water status, hormonal stimuli, light, CO_2 levels and other environmental conditions to regulate stomatal apertures (Schroeder, Kwak, *et al.*, 2001). Drought induced stomatal closure is the results of several biochemical process largely related to the ABA pathway (Jin *et al.*, 2013). In guard cells, ABA triggers a signalling cascade that reduces cellular turgor by causing the efflux of K^+ and Cl^- and the removal of organic osmolytes (Schroeder *et al.*, 2001). The many biochemical, cellular and structural studies carried out in Arabidopsis, have shown that the ABA-induced responses rely on de novo ABA biosynthesis, ABA translocation form the site of production to the site of action, ABA perception and transcriptional regulation of downstream target genes (Shinozaki and Yamaguchi-Shinozaki, 2006).

Previous studies revealed a great variability in the expression of genes related to ABA synthesis, perception and signalling in response to stress across grape genotypes (Chaves *et al.*, 2010). In this

work, we had the opportunity to survey the expression of ABA-related genes in a wide array of grape genotypes, inclusive of commercial rootstocks and novel rootstocks developed at the University of Milano (M series) or at the Fondazione Muck in San Michele (TN) (SM series).

Analysis of the ABA synthesis marker gene *VvNCED1*, showed a general up-regulation in response to water stress. Yet, we also detected genotypes in which expression of *VvNCED1* was not activated under drought (SM79, M1 and SO4) (Figure 29 A) and genotypes which showed down-regulation of *VvNCED1* expression (SM82 and M3) (Figure 29 A). Despite the lack of *VvNCED1* transcriptional up-regulation, SM79, M1 and SO4 revealed activation of ABA signalling, as highlighted by the general up-regulation of PP2C genes (Figure 30) and the down-regulation of the guard cell-related genes *VvMYB60* and *VvSIRK* (Figure 32).

Consistent with the observed down-regulation in SM82 and in M3, we did not detect activation of PP2Cs, following exposure to stress. However, in these genotypes all GCs related genes analysed (*VvMYB60, VvSIRK, VvSLAC* and *VvSOR*) showed a strong down-regulation. These results suggest the prevalence of an ABA-independent regulation of such genes, which might regulate stomatal activity in response to stress. Interestingly, it has been shown that the down-regulation of the *AtMYB60* promoter activity can occur in an ABA-independent manner (Rusconi *et al.*, 2013). In the light of the strong conservation of the *AtMYB60* and *VvMYB60* promoters it is tempting to speculate that expression of *VvMYB60* in grape can be modulated by both ABA-dependent and ABA–independent pathways.

Extensive analysis of ABA receptor genes in grape revealed an high degree of variability among *VvRCARs* expression both after ABA treatment and drought stress (Boneh *et al.*, 2011). Our data indicated a general down-regulation of *VvRCARs* in most genotypes analysed, with the exception of *VvRCAR3*, whose expression resulted mostly up-regulated (Figure 29).

In accord to previous report (Boneh *et al.*, 2012), most PP2C genes were generally up-regulated under drought stress. In particular, expression of *VvPP2C24*, identified as the putative ortholog of *AtABI1* (Marguerit *et al.*, 2012), was strongly up-regulated in almost all genotypes. Interestingly, *VvPP2C24* regulation was tightly correlated with *VvNCED1* expression. Indeed, only SM82 and M3, in which *VvNCED1* was not activated during stress, showed a down-regulation of *VvPP2C24*. In addition to *VvPP2C24*, SM82 failed to activate the remaining PP2C genes, thus confirming the lack of activation of ABA signalling pathway (Figure 30).

Boneh and co-worker showed that *SnRK* genes are generally down-regulated under drought stress (Boneh *et al.*, 2012). Among the genotypes employed in our study, the transcriptional regulation of *SnRK* genes showed high variability, without a clear correlation with ABA synthetic genes (Figure 31).

The focus of this work being the regulation of stomatal activity under stress, we analysed the expression of several ABA-regulated guard cell related genes. These included *VvERA1*, the grape homologue of *AtERA1*. Disruption of the *Arabidopsis* farnesyltransferase gene *AtERA1* or application of farnesyltransferase inhibitors results in ABA hypersensitivity of guard cell anion-channel activation and of stomatal closing (Goritschnig *et al.*, 2008). The *AtERA1* mutation decreases the transpiration rate of leaves and consequently slows desiccation during drought. In our experiment the transcript level of *VvERA1* show little variations under drought stress. In particular, *VvERA1* resulted more up-regulated in K5BB and in M4 compared to 1103P and 101.14. This could contribute to the enhanced stomatal responses disclosed by 1103P and 101.14.

Among other ABA-regulated genes, *VvERF5* was largely up-regulated in our collection as expected, based on previous reports (Sweetman *et al.*, 2012). Interestingly, *VvERF5* showed the same expression level in K5BB grown under standard or stress conditions. Conversely, in 1103P its expression was up-regulated under drought stress (Figure 33 D), suggesting a possible involvement of *VvERF5* in the increased drought tolerant phenotype exhibited by 1103P.

Genes belonging to the *ABF* and *HOS* families were first characterized in Arabidopsis as ABA-inducible genes (Yoshida *et al.*, 2010; Zhu *et al.*, 2005). Unexpectedly, under drought stress, *VvABF2,3* and *VvHOS10.1-3* were largely down-regulated in almost all the rootstock genotypes analysed (Figure 31), suggesting a possible different mode of regulation for these genes in Arabidopsis and grape. This result emphasises the importance of reassessing experimental data obtained in model systems directly in the plant of interest. Clearly further studies will be necessary to investigate the different transcriptional response of *ABF* and *HOS* genes in Arabidopsis and grape.

Purely based on the results of our gene expression analysis it is not easy to cluster the rootstock genotypes, according to homogenous responses to water stress. This is mainly due to the high variability in the transcriptional responses found in the rootstock collection. Nevertheless, our results suggest that the genotypes analysed can be grouped into three major classes, based on the different transcriptional regulation of ABA-related genes.

The first class includes SM24, SM93, M2, M4, 101.14, 1103P and K5BB. In these genotypes, under drought stress, ABA-biosynthetic genes are activated and, consistently, genes involved in ABA perception and signal transduction are also activated. In turn, this resulted in the activation of down-stream GCs related genes.

The second class only includes SM79 and M1. In these two genotypes, even if ABA-biosynthetic genes were non up-regulated following exposure to stress, we observed a significant activation of ABA-signalling components and of, GC-related genes.

Finally, the third category comprises SM82, M3 and SO4. In these rootstocks, as observed in the other genotypes, GC-related genes are down-regulated under stress. Yet, this is not preceded by the transcriptional activation of ABA-biosynthetic and ABA-signalling genes. Thus we can suppose that in SM82 and M3 GCs related genes are regulated in ABA independent way.

In order to strengthen our comprehension of the molecular bases that characterize drought stress response in grapevine we attempted to correlate stomata activity, as judged by measurements of stomatal conductance, with the regulation of guard cell-related genes, but also with the regulation of the genes involved in ABA pathway. Once again, the comparison of the two reference rootstocks of the SERRES project, M4 and 101.14, proved to be useful for this purpose. Our data, in agreement with the work of (Meggio *et al.*, 2014), suggest that the drought tolerant behaviour displayed by M4 is mainly linked to the root system and is not related to stomatal activity. Indeed, 101.14 showed a significant decrease of g_s under drought stress (Figure 26). This response correlated positively with the perception of ABA. M4 and 101.14 disclosed the same relative expression of *VvNCED1* under drought stress. This suggests that ABA synthesis in response to stress in both genotypes is comparable. The different response between M4 and 101.14 could be due to differences in ABA perception. 101.14 showed a stronger up-regulation of *PP2C* genes compared with M4. In particular, *VvPP2C4* and *VvPP2C9*, the major interacting partners of ABA receptors in the grapevine (Boneh *et al.*, 2011), were up-regulated in 101.14, but not in M4, under drought stress (Figure 33 B). Moreover, the previously described *VvERA1* was down-regulated in 101.14, but up-regulated in M4. Similarly, *VvERF5* up-regulation was enhanced in 101.14 compared with M4. Consistently, the guard cell-specific genes *VvMYB60* and *VvSIRK* were more down-regulated in 101.14 than M4. The different transcriptional regulation of ABA- and GC-related genes displayed by M4 and 101.14 could contribute to the different phenotypic response shown by the two genotypes under water stress.

3.3 Influence of the rootstock on the regulation of gene expression in the scion

In addition to the well-established resistance to phylloxera, grapevine rootstocks can confer resistance to various pathogens and tolerance to abiotic stresses (Corso and Bonghi, 2014). Indeed, the physiological status of the root plays an important role in modulating the behaviour of the shoot, including stomatal activity (Iacono *et al.*, 1998).

Root sensing of water deficit has been widely studied (Schachtman and Goodger, 2008). The transport through the xylem of chemical signals (including ABA) originated in the root, in the early

stages of water-deficit, ultimately reduces leaf transpiration and restrains leaf growth (Goodger *et al.*, 2005). It has been recently shown that, in Arabidopsis, guard cells are capable of autonomous ABA accumulation, as they possess the entire biosynthetic pathway (Bauer *et al.*, 2013). Nevertheless, ABA produced outside leaves up-regulates ABA synthesis in GCs, through a positive feedback (Bauer *et al.*, 2013).

Our analysis of gene expression in grafted and un-grafted grapevines yielded interesting results. In both M4 and 101.14 we observed a constitutive up-regulation of ABA biosynthetic (e.g. *VvNCED1*) and signalling genes (e.g. *VvPP2C24*) in leaves from grafted plants (M4/M4; 101.14/101.14) compared with ungrafted plants (Figure 35). This suggests that the graft itself could be perceived by the scion as a stress condition, which in turn activates ABA-mediated responses. Even if further studies will be needed to elucidate this behaviour, such an up-regulation of the ABA pathway does not translate into a constitutive enhanced expression of ABA-dependent GC-related genes, including *VvMYB60*. Is important to emphasize that in this experiment expression analyses were performed on RNAs derived from whole leaves. It is thus possible that changes in the level of expression of GC-specific genes were largely underestimated.

To gain more insight into the possible influence of the rootstock in the control of gene expression in stomata, we analysed different graft combination involving Cabernet Sauvignon (CS), M4 and 101.14. In this experimental setting, gene expression was assessed in both whole leaves and in the GC-enriched fraction.

Comparative analysis of CS/M4 and CS/101.14 graft combinations revealed interesting features on the possible effects of the rootstocks on gene expression in the scion. Expression of *VvNCED1* in GCs of the scion was essentially independent from the genotype of the rootstock. Despite the enhanced up-regulation of *VvNCED1* observed in GCs from 101.14/101.14 compared with GC from M4/M4, the GC-enriched fraction derived from CS leaves showed the same level of expression regardless the genotype of the rootstock (101.14 or M4). This observation is consistent with the existence of a cell-autonomous ABA biosynthetic pathway in GCs, as suggested by (Bauer *et al.*, 2013). Conversely, expression of downstream genes involved in ABA signalling appeared to be affected by the genotype of the rootstock. Expression of *VvABI1*, a key modulator of ABA signalling in GCs, was up-regulated to same extent in GCs from CS/M4 and M4/M4 graft combinations compared with CS/101.14 and 101.14/101.14 (Figure 37). Interestingly, we observed a similar rootstock-dependent response for the regulation of *VvMYB60* expression.

3.4 A possible role for *VvMYB60* in salicylic acid response

In order to further investigate the possible role of *VvMYB60* we have begun to analyse if, like its ortholog in Arabidopsis, the grape gene is involved in salicylic acid response as well.

Preliminary results suggest a very similar behaviour of *VvMYB60* compared to *AtMYB60*. SA treatment induced the down-regulation of *VvMYB60* expression, both after 6h and after 24h from the beginning of the treatment (Figure 38 C and D).

Moreover, *VvPR1* disclosed a different expression between whole leaf and stomata, similarly to what already described in Arabidopsis. The analysis of *VvPR1* in whole leaf showed an up-regulation in after 6h from the start of the treatment and it returned to the basal level after 24h (Figure 38 A and B). Completely different is the analysis in the GCs enrichment sample. *VvPR1* was up-regulated mainly after 24h and only in CS leaf (CS/M4 and CS/101.14) and not in M4/M4 and 101.14/101.14.

These are preliminary results but could suggest a genotype-dependent response to SA in the scion, which occurs independently form the rootstock. The absence of an effect of the rootstock is, at least in part, expected based on our experimental setting. Indeed, SA was directly applied locally to the leaves, and it is thus unlikely that the roots could contribute to modulate this type of response. It is interesting to note that, local application of SA on M4 and 101.14 did not result in the up-regulation of *VvPR1* expression, as observed on CS leaves. Cleary, additional experiments, performed under more accurate conditions (e.g. infection with pathogens), will be necessary to understand the lack of *VvPR1* activation in these two genotypes.

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