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CYCLE: XXVI

A TRANSCRIPTOMIC APPROACH TO DISSECT THE EFFECT OF GRAPEVINE ROOTSTOCKS ON PLANT TOLERANCE TO ABIOTIC STRESSES AND BERRY RIPENING

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

January 31st, 2014 Massimiliano Corso

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In memory of Angelo Ramina
(1944-2013)

Science is not illusion.
But it would be an illusion
to suppose that we could get
anywhere else
what it cannot give us.

Sigmund Freud, 1927

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RIASSUNTO

La vite (genere *Vitis*) rappresenta una delle principali specie coltivate su scala mondiale , con una produzione che si avvicina ai 70 milioni di tonnellate e una superficie coltivata di oltre 7 milioni di ettari . Tra le 60 specie all'interno del genere *Vitis*, *Vitis vinifera L.* è la più utilizzata per la produzione di vino e distillati. Prima della devastazione della viticoltura europea causata dall'introduzione del parassita fillossera dal Nord America, le varietà di *V. vinifera* usate per la produzione di vino in Europa non erano innestate. Successivamente, l'utilizzo di portinnesti di origine americana ha permesso di fornire una maggiore resistenza al parassita e ad altre malattie che stavano seriamente compromettendo la viticolture Europea. I portinnesti più usati commercialmente derivano da incroci di svariate specie di vite, tra cui *V. berlandieri, V. riparia* e *V. rupestris*, e, oltre a migliorare la resistenza alla fillossera e altri patogeni, conferiscono caratteristiche di tolleranza a stress abiotici (come siccità, elevata salinità e Fe-carenza), regolano la crescita dell'acino, contribuiscono alla maturazione e alla qualità dei frutti, possono alterare alcuni aspetti legati alla qualità in post-raccolta dell'acino.

I risultati presentati in questa tesi di dottorato sono parte integrante di un progetto multi-disciplinare chiamato SERRES (selezione di nuovi portinnesti di vite resistenti a stress abiotici attraverso lo sviluppo e la validazione di marcatori molecolari) e finanziato dalla fondazione Ager. La selezione e la caratterizzazione di portainnesti che conferiscano un maggiore grado di tolleranza agli stress abiotici è essenziale per lo sviluppo di modelli agricoli sostenibili e, allo stesso tempo, per l'induzione di un rapporto equilibrato tra fase vegetativa e produttiva, una progressione diversa della maturazione dell'uva, così come, differenze a livello qualitativo. Migliorare la conoscenza delle basi molecolari, biochimiche e fisiologiche della resistenza allo stress è un requisito fondamentale per la selezione di genotipi in grado di far fronte alle condizioni di stress senza conseguenze negative su crescita vegetativa e produzione di uva ad alta qualità.

Lo stress idrico ha un impatto enorme sulla produzione agricola, infatti, è uno dei principali fattori che limitano la produttività delle piante e causano una grave riduzione della resa. Sulla base dei modelli climatici globali, che prevedono un aumento delle aree aride nel prossimo futuro, la carenza idrica può diventare il principale fattore limitante per la coltivazione. In questo contesto, i portinnesti potrebbero assumere un ruolo importante

nel limitare la perdita di raccolto migliorando l'efficienza dell'uso dell'acqua, il potenziale di sopravvivenza della pianta e la capacità di crescita del frutto in presenza di condizioni avverse come siccità ed elevata salinità del suolo (stress osmotici). Lo stress idrico porta a molti cambiamenti morfologici e fisiologici, tra cui ridotta espansione della parte aerea, limitazione della crescita radicale, diminuzione della traspirazione fogliare e dell'efficienza fotosintetica, accumulo di ioni e osmoliti, attivazione di processi di disintossicazione e parallelamente la regolazione a livello trascrizionale di un elevato numero di geni. In seguito allo stress idrico, si innesca uno stress secondario legato all'accumulo di specie reattive dell'ossigeno (ROS), quali H_2O_2, O^{2-}, -OH, $^1O_2$ e NO. Le ROS sono responsabili della maggior parte dei danni ossidativi nei sistemi biologici e nelle componenti cellulari. Un rigoroso controllo dei livelli delle ROS è obbligatorio per la sopravvivenza delle piante e il cross-talk tra l'accumulo di ROS lo stato redox è parte integrante di un preciso controllo omeostatico che gioca un ruolo fondamentale nella risposta agli stress. Le piante innescano svariati meccanismi di riduzione del livello di ROS (ROS-scavenging) volti all'induzione dell'espressione di geni che codificano per gli enzimi superossido dismutasi (SOD) , catalasi (CAT), ascorbato perossidasi e glutatione perossidasi.

Recentemente è stato condotto uno studio di caratterizzazione a livello biochimico e fisiologico di M4 [(*V. vinifera* x *V. Berlandieri*) x *V. berlandieri* cv Resseguier n.1], un nuovo genotipo di vite candidato ad essere utilizzato come portinnesto. Questo genotipo, studiato dal 1985 dal gruppo di ricerca DiSAA dell'Università degli studi di Milano, è stato selezionato per la sua alta tolleranza allo stress idrico (WS) e salino (SS). Se confrontate con il genotipo commerciale 101.14, le piante di M4 sottoposte a deficit idrico hanno mostrato una maggiore capacità di tolleranza e una più elevata attività fotosintetica anche in condizioni di stress gravi.

Nella prima parte di questa tesi sono stati osservati i risultati ottenuti da un'analisi trascrittomica condotta su larga scala (RNA -Seq), effettuata su foglie e radici dei portinnesti M4 e 101.14 campionati in condizioni di stress idrico progressivo (5 time-points). Le analisi fisiologiche sono state effettuate sulle piante trattate (deficit idrico, WS) e di controllo (irrigate, WW) lungo tutto il campionamento. L'analisi multifattoriale, che è stata condotta sui dati mRNA-Seq, ci ha permesso di valutare il peso di tre diverse

componenti sulla risposta allo stress: genotipo (R : 101.14 e M4), tipo di stress imposto (Trattamento, T : WW e WS) e time-point considerato (P : T1 - T4). Con questa analisi stato inoltre possibile identificare i geni differenzialmente espressi (GDE) legati all'azione specifica o combinata di questi fattori (R:T , R:P , T:P e R:T:P). In WS radice si è sempre osservati un numero maggiore di GDE rispetto alla foglia. Una prima osservazione generale confrontando i risultati delle analisi multifattoriali eseguite su foglie e radici, è che nel tessuto radice il "trattamento" sembra essere la variabile che ha un impatto maggiore sull'espressione genica, mentre nel tessuto fogliare il peso del genotipo (portinnesto) sembra essere il più elevato. Questa osservazione non è sorprendente, considerato che il sistema radicale è il primo organo a percepire lo stress causato dalla carenza idrica e quello principale atto alla risposta. In questo caso è chiaro che il tipo di trattamento imposto rappresenta la variabile principale che influenza l'espressione genica mentre l'effetto del genotipo è meno determinante. Con i dati RNA-seq è stata eseguita una "Differential Cluster Analysis" (DCA), che si basa sul confronto delle correlazioni tra le espressioni dei trascritti di un organismo "reference" e di un "target". Questa analisi ci ha permesso di identificare i pattern di co-espressione genica (T1-T4) conservati e pattern non-conservati tra M4 e 101.14. Per quanto riguarda gli ormoni vegetali, è stata osservata un'induzione dei geni legati ad auxine, jasmonati ed etilene nelle radici di M4 sottoposte a stress, mentre una sovra-regolazione degli stessi trascritti è stata osservata in 101.14. La categoria metabolica più interessante, emersa dall'analisi DCA, è quella legata ai metaboliti secondari. Infatti sono stati individuati diversi GDE legati a questa categoria sia in radice che in foglia di M4, indotti in condizioni di stress, ed è stata evidenziata una forte specificità di espressione tra i due tessuti. Infatti, in condizioni di carenza idrica, radici e foglie del genotipo tollerante M4 mostrano rispettivamente una maggiore induzione dei geni legati agli stilbeni (*i.e.* STS) e ai flavonoidi (*e.g.* CHS, F3H, LDOX, FLS). Il ruolo di questi geni potrebbe essere legato al controllo e al bilanciamento delle specie reattive dell'ossigeno (ROS), in aggiunta ai classici meccanismi di ROS-scaveging (meccanismi antiossidanti primari). In presenza di stress idrico, M4 potrebbe attuare meccanismi differenziali in radice e foglie che portano alla produzione di molecole, come resveratrolo e flavonoidi, correlate ad un sistema antiossidante secondario presente solo

nel portinnesto più tollerante. La maggiore tolleranza allo stress idrico di M4, in confronto a quanto osservato in 101.14, potrebbe essere relativo a questi eventi.

Nella seconda parte di questa tesi, è stato valutato l'effetto dei portinnesti M4 e 1103P su sviluppo, maturazione e qualità delle bacche di Cabernet Sauvignon (CS). Per questo esperimento sono stati campionati da piante di CS/M4 e CS/1103P acini interi a 45, 59 e 65 giorni dopo la piena fioritura (GDF). Successivamente la maggior parte delle bacche di CS/M4 avevano raggiunto l'invaiatura, si è quindi deciso di separare bucce e polpe per i campionamenti successivi, condotti a 72, 86 e 100 GDF. Sulla base dei parametri fisici (volume e colore) e chimici (solidi solubili totali, SSC), i due portinnesti hanno mostrato una diversa influenza sulla cinetica di sviluppo e maturazione delle bacche di CS. Per identificare le stesse fasi di sviluppo dei frutti raccolti da CS/1103P e CS/M4, è stato condotta un'analisi di espressione preliminare, mediante sistema real-time PCR, sui geni coinvolti nella biosintesi di fenoli, zuccheri e acidi organici. Questo approccio ha permesso di identificare la fase verde a 45 DAFB in entrambe le combinazioni d'innesto, mentre l'invaiatura è stata individuata a 72 e 86 DAFB rispettivamente per CS/M4 e CS/1103P. Le analisi mRNA-seq e micro-RNAseq sono state effettuate sulle bacche in fase di pre-invaiatura (45 GDF), invaiatura (72 GDF per CS/M4 e 86 GDF per CS/1103P) e epoca di raccolta tradizionale di CS (100 GDF). Le analisi statistiche sono state condotte sui dati RNA-seq confrontando il rapporto tra i dati di espressione di CS/M4 e CS/1103P ad ogni punto della cinetica e per entrambi i tessuti. Le analisi di "clusterizzazione" e di arricchimento hanno evidenziato la presenta di un elevato numero di GDE legati a metabolismi auxinici. Le auxine hanno un ruolo fondamentale durante lo sviluppo e sulla maturazione della bacca, si è quindi deciso di concentrare la nostra attenzione su questa classe ormonale e di eseguire una caratterizzazione e un'analsi filogenetica delle famiglie geniche ARF e AUX / IAA sul genoma di PN40024. Il ruolo delle auxine in questi processi è stato studiato anche in un altro un altro lavoro presentato in questa tesi, durante il quale è stato dimostrato che un trattamento sugli acini d'uva in fase di pre-invaiatura con l'auxina sintetica NAA causa un ritardo nella maturazione, che si manifesta a livello fisiologico e di espressione genica, parallelamente alle quali è stata osservata l'induzione di un elevato numero di trascritti atti a controllare l'omeostasi delle auxine. Le analisi condotte con il software HORMONOMETER hanno suggerito che il recupero

omeostatico atto a portare i livelli dell'ormone a concentrazioni meno elevate è avvenuto a soli 7 giorni dal trattamento. Questa ipotesi è fortemente supportata dalla sovra-regolazione di geni coinvolti nella coniugazione (GH3 -like) e nell'azione (IAA4 e IAA31 -like) delle auxine. Considerando questi risultati, le differenze osservate tra CS/M4 e CS/1103P durante lo sviluppo e la maturazione della bacca potrebbero essere collegate ad una diversa regolazione dell'auxina. Infatti, i dati di espressione (mRNA-seq, microRNA-seq e qPCR) evidenziato importanti differenze nel metabolismo auxinico tra le due combinazioni d'innesto. I nostri dati suggeriscono un coinvolgimento importante dell'ormone nel controllo dello sviluppo/maturazione della bacca grazie all'espressione di legati, da un lato all'azione delle auxine (ARF e AUX/IAA) e, dall'altro , all'omeostasi di questo ormone attraverso trascritti coinvolti nella coniugazione (GH3) e nel trasporto (PIN e ABCB). In questo contesto , anche i miRNA hanno un ruolo importante, in particolare esercitando un controllo sulla trascrizione dei geni ARF (e.g. miR160 e miR167). In fase di pre-invaiatura, le auxine hanno un'azione positiva sulla trascrizione dei geni che controllano le dimensioni della bacca (e.g. espansine) e di geni legati alla famiglia delle ARF (ad esempio VvARF8A e VvARF1A). Parallelamente all'induzione di geni che appartengono alla famiglia ARF, è stata osservata l'induzione di trascritti che controllano i livelli (*e.g.* VvGH3-1) e l'azione (VvIAA9, VvIAA15A, VvIAA16) dell'ormone, suggerendo un'accurata regolazione dei livelli auxinici in queste fasi importanti dello sviluppo del frutto. Inoltre, il controllo dei livelli di auxina nella bacca d'uva sembra essere legato anche ad altri meccanismi legati all'induzione di geni legati al trasporto ormonale durante le fasi precoci (ABCBs) e tardive (PIN) della maturazione del frutto. Tenendo conto delle differenze osservate tra CS/M4 e CS/1103P nell'espressione di trascritti legati al metabolismo dell'auxina, questo ormone sembra esercitare un'azione negativa su alcuni geni legati alla maturazione della bacca (e.g. flavonoidi), ma la sua induzione nella fase di pre-invaiatura potrebbe essere necessaria per far scattare altri processi metabolici coinvolti nella maturazione dell'acino d'uva.

SUMMARY

Grapevine represents one of the major economic crop species on a worldwide scale, with a world production approaching 70 million of tons and a harvest area of over 7 million hectares. Amongst the 60 species within the *Vitis* genus, *Vitis vinifera* L. is the mostly used for the production of wine and distilled liquors. Before the devastation of European viticulture caused by of the introduction of phylloxera from North America, varieties of *V. vinifera* used commercially for wine production in Europe were traditionally grown on their own roots. Subsequently, the use of rootstocks from the pest's origin was introduced to provide resistance to this and other deleterious diseases and to save the fate of European viticulture. Rootstocks have been bred from a number of Vitis species, especially *V. berlandieri*, *V. riparia*, and *V. rupestris*, and are known, in addition to the enhanced resistance to phylloxera and other pathogens, confer tolerance to abiotic stresses (*e.g.* drought, high salinity and Fe-deficiency), regulate the size of the scion, affected fruit development/ripening, contribute to fruit quality and can alter specific aspects of postharvest fruit quality of a scion.

Results presented in this Ph.D thesis are a part of a larger multi-disciplinary project called SERRES (Selection of new grape rootstocks resistant to abiotic stresses through the development and validation of molecular markers) granted by Ager foundation. Selection of resistant rootstocks is crucial for the development of sustainable agricultural models and, at the same time, for inducing a balanced vegetative/productive ratio, a different ripening progression in grape berries and, as well as, differences in their global quality. Improving the knowledge about the molecular, biochemical and physiological bases of stress resistance is an absolute requirement for the selection of genotypes able to cope with stress conditions without any negative consequences on the vegetative growth and production of high quality grape.

Drought has an enormous impact on crop production, indeed, it is one of the major factors limiting plant productivity and cause a severe yield reduction. Based on the global climate models, which predict an increase in the aridity in the next future, water deficit may became the major limiting factor. 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. Water deficit leads to many

morphological and physiological changes across a range of spatial and temporal scales, including reduced expansion of aerial organs, maintenance of root growth, decrease in transpiration and photosynthesis, accumulation of osmotic compounds and ions, activation of detoxifying processes and, in parallel, the transcriptional regulation of a large number of genes. Oxidative stress is related to the accumulation of reactive oxygen species, such as H_2O_2, O^{2-}, -OH, $^1O_2$, and NO. These ROS are responsible for most of the oxidative damages in biological systems and cellular components. Thus, a strict control of ROS levels, throughout the expression of genes coding for superoxide dismutases (SOD), catalase (CAT), ascorbate peroxidase and glutathione peroxidise ROS scavenging enzymes, is mandatory for plant survival and the cross-talk between ROS accumulation and redox state is integrating part of a fine homeostasis control that plays a pivotal role in the plant response to stresses.

Recently, a biochemical and physiological study of the M4 [(V. vinifera x V. berlandieri) x V. berlandieri x cv Resseguier n.1] novel candidate genotype to be used as rootstock in grapevine was performed. This genotype, established from 1985 by the DiSAA research group operating at the Milan University, was selected for its high tolerance to water deficit (WS) and salt exposure (SS). In comparison with the 101.14 commercial genotype, M4 un-grafted plants subjected to water and salt stress showed a greater capacity to tolerate WS and SS maintaining photosynthetic activity also under severe stress conditions and accumulating, especially at the root level, osmotic compounds and ions.

In the first part of this thesis were reported results obtained from a large scale whole transcriptome analyses (RNA-seq) performed on root (whole apparatus) and leaf tissues of 101.14 (drought susceptible) and M4 plants sampled in progressive drought (five time points). Physiological analyses were performed on treated (water-stress, WS) and control (well-watered, WW) plants over all the sampling. The multifactorial analysis , which was performed on mRNA -seq data concerning to both the analyzed tissues (leaf and root), allowed us to evaluate the relative weight of the genotype (R: 101.14 and M4), of the type of stress imposed (Treatment, T: WW and WS) and of the time point considered (P: T1-T4), and to identify Differentially expressed Genes (DEGs) that are affected in a specific way or the combined action of these factors (R:T, R:P, T:P and R:T:P). In WS root dataset, all considered components (R, T and P) were found to affect the higher number of

genes in comparison to other dataset (WS leaf). A first general observation comparing results of the multifactorial analyses performed on leaves and roots is that in root tissue the "treatment" seems to be the main variable explaining differential gene expression depend on the kind treatment imposed, whereas in leaf tissue the weight of the genotype (rootstock) appear to be the highest. This observation is not surprising, considering that the root system is the first organ perceiving the water deprivation stress and the main one actively responding to it. In this case it's clear the kind of treatment imposed represent the main variable influencing expression whereas the effect of the genotype is less determinant on differential expression of genes. RNA-seq data were used to performed a Differential Cluster Analysis (DCA), which is based upon comparison of correlation between genes expression of a "reference" and a "target" organism and allowed us to identify conserved and diverged co-expression patterns between related organisms. This analysis allowed us to compared the transcriptomic responses of M4 and 101.14 rootstocks. As concerns plant hormones, it was showed an induction of auxin, JAs and GAs related-genes at the beginning of the stress kinetic in M4 stressed roots, whereas a up-regulation of these transcripts in unstressed root was observed in 101.14. The most interesting metabolic category was the "Secondary metabolism" one because several DEGs belonging to these metabolisms were founded in both root and leaf upon WS, but with a strong specificity of DEGs expression among two considered organs. Indeed, upon WS, roots and leaves of the tolerant genotype M4 exhibit an higher induction of stilbenes (*i.e.* STS) and flavonoids (*e.g.* CHS, F3H, LDOX, FLS) biosynthetic genes, respectively. We hypothesized the role of these genes in the control and balance ROS levels, in addition to the others well known ROS scavengers. In presence of water stress, M4 rootstock may acts differential mechanisms in root and leaves which leads to the production of molecules, such as resveratrol and flavonoids and these events may be related to a secondary antioxidant system in this rootstock. The higher resistance of M4 rootstock to water stress, in comparison to what observed in 101.14, should be related to these events.

In the second part, in order to evaluate the effects of the rootstocks on grape berry quality and development/ripening, an RNA-seq experiment on Cabernet Sauvignon (CS) grafted onto M4 and 1103 Paulsen rootstocks was carried out. Whole berries were collected from CS/1103P and CS/M4 bunches at 45, 59, 65 days after full bloom (DAFB), in

correspondence to the end of lag phase. At this moment most of grape berries reached véraison, the other samples (separating skin and pulp) were collected at 72, 86 and 100 DAFB. On the basis of physical (volume and colour) and chemical (Soluble Solids Concentration, SSC) parameters, the two rootstocks seem to induce a different development and ripening pattern on CS berries. To identify the same developmental phases of berries collected from CS/1103P and CS/M4, the expression profile of genes involved in phenols, sugar and organic acids metabolisms were overlapped. This approach allowed to establish that the green phase occurred at 45 DAFB in both combinations, while véraison happened at 72 and 86 DAFB for CS/M4 and CS/1103P, respectively. An mRNA-seq and a microRNA-seq experiments were carried out on CS berries sampled at pre-véraison (45 DAFB), véraison (72 and 86 DAFB for M4 and 1103P, respectively) and traditional CS vintage date (100 DAFB). For the statistical analyses on RNA-seq data a pairwise comparisons between M4 and 1103P genotypes were accomplished at each time point and a large numbers of DEGs related to auxin metabolisms were identified with enrichment and clustering analysis. It is well known the important role of auxins on grape berry development, so, it was decided to focus our attention on this hormone and to performed a characterization of grape ARF and AUX/IAA gene families. Indeed, in another work presented in this thesis, we showed that an NAA treatment just before véraison caused delayed grape berry ripening at the transcriptional and physiological level, along with the recovery of a steady state of its intracellular concentration. Hormone indices analysis carried out with the HORMONOMETER tool suggests that biologically active concentrations of auxins were achieved throughout a homeostatic recovery. This occurred within 7 days after the treatment, during which the physiological response was mainly unspecific and due to a likely pharmacological effect of NAA. This hypothesis is strongly supported by the up-regulation of genes involved in auxin conjugation (*GH3-like*) and action (*IAA4-* and *IAA31-like*). Considering these results, the differences observed among CS/M4 and CS/1103P in grape berry development and ripening should be related to a different regulation of auxin metabolism. Indeed, all transcripts/miRNAs analyses performed (RNA-seq, microRNA-seq and qPCR) highlighted important differences in the auxin metabolism among the two scion/rootstock combination. Our data suggest an important involvement in the control of grape berry development/ripening of genes that

are related, on one hand to auxin action (*ARF* and *AUX/IAA*) and, on the other hand, to homeostasis of this hormone through the expression of genes involved in conjugation (*GH3*) and transport (*PIN* and *ABCB*). In this context, also miRNA have an important role, especially by controlling *ARF*–related genes (*e.g.* miR160 and miR167). In the case of fruit ripening, auxin acted as a positive regulator of genes that control grape berry size (*e.g.* expansin-related genes) before the véraison stage; it was indeed observed the up-regulation at the pre-véraison stage, which was different for CS/M4 and CS/1103P, of transcripts that control auxin-responsive genes (*e.g. VvARF8A* and *VvARF1A*). The induction of genes that belonged to *ARF* family was paralleled by the expression of transcripts that control auxin level(*e.g. VvGH3-1)* and action *(VvIAA9, VvIAA15A, VvIAA16*), suggesting that an accurate regulation of auxin homeostasis in grape berries at these phases. Moreover, control of auxin levels in grape berry seems pass through other mechanisms which involved control of transport-related genes in the early (ABCBs) and late (PINs) phases of berry development. Taking into accounts that at commercial CS harvest, CS/M4 berries berries were showing differences in some processes ripening-related (*e.g*. flavonoids metabolism) and a different regulation of auxin metabolisms, when compared to those of CS/1103P, auxin seems to act as negative regulators on some genes related to grape berry ripening but its induction at the pre-véraison stage could be necessary to triggers other metabolism involved in ripening processes.

Chapter I

GENERAL INTRODUCTION

1. Grapevine rootstocks

Grafting, which involves the combination of two different varieties or species to form a plant with new characteristics, is a technique extensively used in the cultivation of several horticultural species, such as grapevine, apple and peach. Grafting technique involves the areal part, called scion, which is grafted onto the basal portion, called rootstock, of the plant (Arrigo and Arnold, 2007; Lee *et al.*, 2010). So, the new-formed individual is made up by two bionts (scion and rootstock), which are characterized by two different genotypes. Grafting has two parameters of importance: ease of grafting and affinity between scion/rootstock combination (Gregory *et al.*, 2013). To obtain a successful grafting the vascular cambium, responsible for cell division, of both bionts has to be in contact in order to connect xylem and phloem (Marguerit *et al.*, 2012). In viticulture, practice of grafting was already widespread in ancient times but the principal reason for the widespread use of grafting in viticulture was the *Daktulosphaira vitifoliae* (phylloxera) epidemic. Phylloxera, native to North America, was introduced into Europe at the end of the nineteenth century and destroyed around four million of vineyard hectares. There are some evidences that a Bordeaux grower called Leo Laliman was the first to advise grafting European grape vines, *Vitis vinifera*, onto rootstocks from *Vitis* species originate from North America. The higher resistance to this pests observed in the American species is related to their co-evolution with phylloxera, which leads to the development of resistance mechanisms that still are not completely understood. Proper sanitation may reduce the risk of phylloxera infestation, but it is no guarantee against its spread. The potential economic loss from phylloxera infestation is so great that planting on resistant rootstocks is recommended even in regions where phylloxera is not yet present (Arrigo and Arnold, 2007).

In addition to the enhanced resistance to phylloxera, the growth of many plants in cultivated systems is profoundly affected by selection of appropriate rootstocks. Rootstocks have been bred from a number of *Vitis* species, especially *V. berlandieri, V. riparia,* and *V. rupestris,* and are known to confer resistance to various pathogens, tolerance to abiotic stresses (*e.g.* drought, high salinity and Fe^{2+}-deficiency), regulate the size of the scion, affected fruit development/ripening, contribute to fruit quality and further they can alter specific aspects of postharvest fruit quality of a scion (Arrigo and

Arnold, 2007; Lee *et al.*, 2010; Gregory *et al.*, 2013; Marguerit *et al.*, 2012; Fisarakis *et al.*, 2001; Grant and Matthews, 1996; Walker *et al.*, 2002, 2004).

1.1 Influence of rootstocks on grapevine abiotic stresses tolerance

In addition to their ability to helped scion to cope with biotic stresses, rootstocks can confer also tolerance to a large range of abiotic stresses. Among these, drought and high salinity have an enormous impact on crop production, indeed, they were ones of the major factors limiting plant productivity and cause a severe yield reduction (Cramer *et al.*, 2007). So, breeding of crop varieties that use water more efficiently is a key strategy for the improvement of agro systems (Marguerit *et al.*, 2012). Based on the global climate models, which predict an increase in the aridity in the next future (Dai, 2013), water deficit may became the major limiting factor. 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).

Rootstocks exhibit differential degrees of tolerance in response to drought, for example 101-14 and Schwarzmann are considered less tolerant, while Lider 116-60, Ramsey, 1103 Paulsen, 140 Ruggeri, Kober 5BB and Richter 110 confer to scion higher drought tolerance (Flexas *et al.*, 2009).

The ability of these rootstocks to confer high tolerance to water stress depend on several factors. For some perennial crop species, altered scion vigour has been linked to differences in hydraulic parameters of the root system. Gambetta et al. (2012) hypotized a pivotal role of aquaporins proteins in relation to grapevine rootstocks vigour and control of water use during drought. In this study they showed that Vv*PIPs* expression was consistently higher in high-vigour rootstock and demonstrate their role in control of rootstocks vigour. The hydraulic capacity of a root system to deliver water scion is related to the increase in Lpr (per root surface area or per biomass), and/or whole-root-system surface area. Indeed, (Alsina *et al.*, 2011) found that grapevines grafted onto 1103P rootstock (high vigour) exhibited greater whole-root-system hydraulic conductance compared to 101-14 (low vigour) resulting from continued growth at greater depth during the warmer and drier summer months.

Stomata have another important role in regulating water loss during water stress (Marguerit *et al.*, 2012), and stomatal closure is one of the earliest responses to water deficit (Damour *et al.*, 2010). So, grapevine rootstocks which increased the efficiency of stomatal closure control and water use efficiency induced also a major tolerance to water stress.

Salt stress is another environmental perturbation which negatively affects grapevine growth and yield. High salinity cause problems in water uptake and availability of micronutrients, increasing toxic-ion concentration and degradation of soil structure (Ismail *et al.*, 2013). *Vitis vinifera* is moderately sensitive to high salinity in the soil and damages caused by this stress are primary related to the chloride ions. The inhibition of grapevine growth and CO_2 assimilation in relation to high salinity is mainly due to changes in stomatal conductance (similarly to what observed for water stress), electron transport rate, leaf water potential, chlorophyll, fluorescence, osmotic potential, and leaf ion concentrations (Cramer *et al.*, 2007). Together with these physiological problems, salt stress causes, at molecular level, formation of reactive oxygen species (ROS), membrane disorganization, metabolic toxicity and reduced nutrient acquisition, as well as induction of several genes related to plant hormones (*e.g.* abscissic acid and jasmonates) (Cramer *et al.*, 2007; Ismail *et al.*, 2012). Grapevine responses to salinity depends on several factors, such as soil type, rootstock–scion combination, irrigation system and climate. Grapevine are more sensitive to Cl^- toxicity than Na^+ toxicity (Cramer 2007). Rootstocks obtained from wild *Vitis* species differ widely in their ability to exclude Cl^- (in reducing order *V. rupestris, V. cinerea, V. champini* and *V. berlandieri*), and consequently in their capability to higher tolerate salinity; so, response efficiency of the scion in presence of salt soils vary in relation to the comparative exclusion of sodium versus chloride by the genotype of the root system (Fisarakis *et al.*, 2001). Fisarakis *et al.* (2001) showed that there is a great variability in the uptake and accumulation of Na+ and Cl– among rootstocks. In this work they showed that *V. berlandieri* specie had a great ability for Cl– and/or Na+ exclusion, although this ability is reduced in hybrids having *V. vinifera* as parent. This explains the reduced ability for Cl– exclusion of 41B (*V.berlandieri*×*V.vinifera*) compared to other rootstocks. Salinity, as well as water stress, negatively affects grapevine yield. In Walker *et al.* (2002) was shown a strong influence of rootstock on scion production upon salt

stress. In particular they observed that rootstocks imparting most vigour at high salinity (*e.g.* Ramsey, 1103 Paulsen and R2), determined by the weight of one-year-old pruning wood in each year also produced a higher number of bunches per vine at both the medium and high salinity treatments.

Iron chlorosis is further physiopathology that affects grapevine grown on calcareous soil. Iron (Fe) deficiency chlorosis, associated with high levels of soil bicarbonate is one of the main nutritional disorders observed in sensitive grapevine genotypes. Fe deficiency causes a reduction of grapevine longevity and productivity, affected growth and reduced yield (Covarrubias and Rombolà, 2013). Grapevine upon iron chlorosis stress enhance the activity of Fe-reductase enzyme and increase the release of protons and organic compounds in roots. This result in a lower pH and higher solubility of Fe(III) and is known as strategy I (Jiménez *et al.*, 2007). In this context bicarbonate concentration is particularly important, indeed bicarbonate is one of the main factors causing Fe chlorosis in strategy I plants but mechanisms of its involvement in this stress are still not clear (Covarrubias and Rombolà, 2013). Several *V. vinifera* cultivars are subjected to stress induced by calcareous soils, however the use of selected rootstocks can solve this problems. For example, Bavaresco and Lovisolo (2000) showed that different scion/rootstock combinations among three Pinot blanc cultivars and two rootstocks (SO4 and 3309C) lead up to different results in response to iron chlorosis, strongly related to the chlorophyll content and vegetative growth which were correlated with specific conductivity in scion/rootstock surface. In another work, Bavaresco *et al.* (1993) compared the response of 140 Ruggeri and 101.14 rootstocks to iron choloris showing that the iron-efficient rootstock (140 Ruggeri) did not induce chlorosis when growing on the calcareous soil, while the opposite occurred with the iron-inefficient rootstock (101.14).

1.2 Rootstocks widely used in viticulture and characterization of new genotypes with OMICS techniques

Widely used grapevine rootstocks are individuals derived from crosses of two or more species belonging to the genus *Vitis*. In particular, the majority of commercial rootstocks used in viticulture belong to *V. riparia, V. berlandieri and V. ruprestris* hybrids (Arrigo

and Arnold, 2007), leading to a narrow genetic variability. Indeed, 90% of cultivated vines are grafted onto less than ten rootstocks.

This situation may cause several risk, such as the onset of pathogens, nematodes and insects mutations which leads these species to overcome resistance of the root system. An example is the AxR1 Californian rootstock (*V.vinifera x V. rupestris*), which is no longer used for effectiveness loss (Grant and Matthews, 1996).

Currently, *non-vinifera* rootstocks, which exhibit an higher tolerance to phylloxera and nematode infestation, in comparison to *Vitis vinifera*, confer more resistance to the plant to these pests, but they cannot prevent the proliferation of the aphid. A scheme of the widely used rootstocks and their parental is reported in Figure 1.

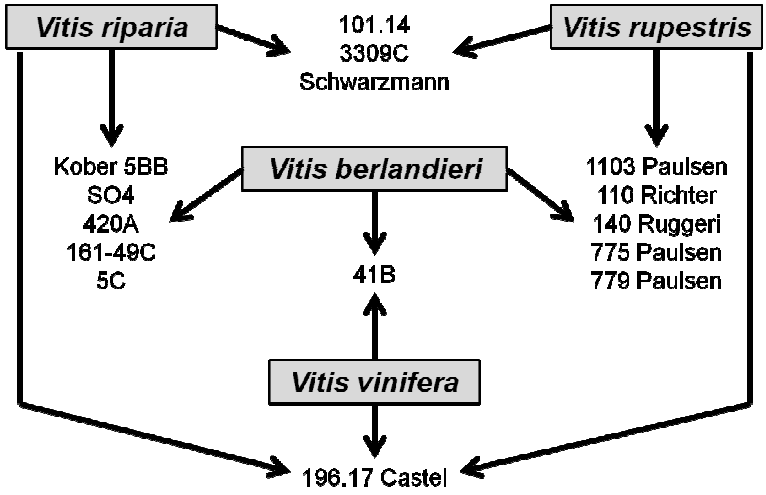

Figure 1. Widely used rootstock in Italian viticulture.

The development of new rootstocks able to confer tolerance to biotic and abiotic stresses, and contribute to grape quality and ripening/development, is an important step for the future of viticulture.

In the lasts years, significant efforts have been done for the selection of the optimal rootstock/scion combinations to satisfy specific grape growing needs (Hamdan and Basheer-Salimia, 2010; Komar *et al.*, 2010; Koundouras *et al.*, 2009). The selection of new rootstocks was initially carried out by phenotypic and genetic techniques. In order to better characterize new rootstocks and give insights into the mechanisms which allow them to have the desired characteristics, we need more accurate information than the

phenotypical one. Actually, the developmente of the "omics" sciences, such as transcriptomic, proteomic and metabolomic approaches became essential to functionally characterize the selected rootstocks and to understand the effect of these rootstocks on the scion (Deluc *et al.*, 2009; Grimplet *et al.*, 2009a; Grimplet *et al.*, 2009b; Rodríguez-Celma *et al.*, 2013; Wang *et al.*, 2009).

A multi-disciplinary approach was carried out with the AGER-SERRES project (http://users.unimi.it/serres/index.html). The aim of the SERRES (Selection of new grape rootstock resistant to abiotic stresses through the development and validation of molecular markers) project was to create the basis for a new sustainable viticulture, which is currently limited by the low availability of rootstocks able to confer more tolerance to adverse growing conditions, such as water scarcity, salinity or excess of limestone. Within this project, the adaptive responses to environmental stresses (*i.e.* drought, high salinity and calcareous soils) of new putative-tolerant rootstocks, selected by the University of Milan and named "M series", and the effect of these rootstocks on vegetative growth and qualitative profiles of grape berries were evaluated by using an omics approach. A scheme of all selected rootstocks, that belongs to the "M" series, were showed in figure 2.

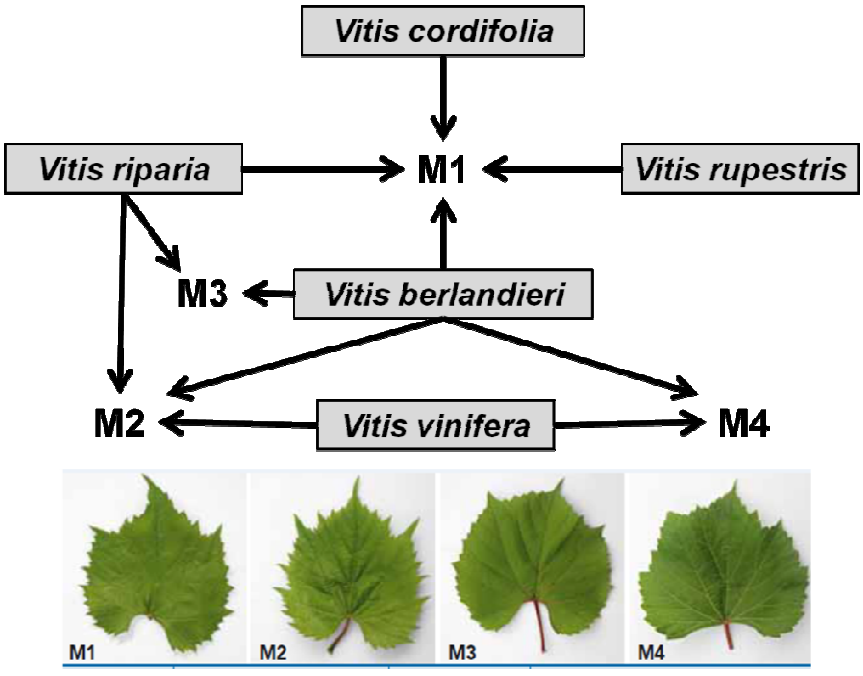

Figure 2. Grapevine rootstocks of the M series and their parental.

Improving the knowledge about the molecular, biochemical and physiological bases of stress resistance is an absolute requirement for the selection of genotypes able to cope with stress conditions without any negative consequences on the vegetative growth and production of high quality grape. The eco-physiological techniques of analysis, together with genomic, transcriptomic (the subject of this thesis), proteomic and metabolomic approaches used in this project may give a valuable contribution to the understanding of the syndrome kinetics, as well as the progressive deterioration of plant performances paralleling the onset of the stress.

2. Study of genes expression by using a transcriptomic approach

In order to evaluate the grapevine responses to abiotic stresses we use a whole-genome approach at transcriptomic level.

In the field of functional genomics, transcriptome analysis has always played a central role in the studying of gene expression at a whole-level and to unravel gene-networks regulation (Nookaew *et al.*, 2012). In order to study expression levels of thousands of genes simultaneously, in 1995 the microarray technology was introduced (Schena *et al.*, 1995). A DNA microarray is a collection of microscopic DNA spots attached to a solid surface. Each DNA spot contains pico-moles of a specific DNA sequence, known as probes. These can be a short section of a gene or other DNA element that are used to hybridize a cDNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

However, microarray technology has some limitations: (i) background levels of hybridization (*i.e.*, hybridization to a probe that occurs irrespective of the corresponding transcript's expression level) limit the accuracy of expression measurements; (ii) probes differ considerably in their hybridization properties; (iii) hybridization results from a single sample may not provide a reliable measure of the relative expression of different transcripts; (iv) arrays are limited to interrogating transcripts with relevant probes on the array (Marioni *et al.*, 2008).

In recent years Next Generation Sequencing (NGS) technology, which is a novel high-throughput sequencing technology producing millions of sequences per single sequencing run, has emerged (Fu *et al.*, 2009). Among NGS techniques, RNA-seq is the most used for transcriptomics studies. This is due to the fast development of the technology, together with the decrease in the running cost and the possibility to uncover novel transcriptional-related events.

RNA-seq gave us several advantages: (i) analysis of novel transcripts, small RNA and alternative splicing events; (ii) the higher resolution in comparison to the microarray technology; (iii) definition of quantitative level of genes expression; (iv) less technical variation; (v) permit to avoid the problem of the "false positive", observed with the microarray probes (Nookaew *et al.*, 2012; Fu *et al.*, 2009). A detailed comparison among RNA-seq and other transcriptomic methods is given in Table 1 (Wang *et al.*, 2009).

Technology	Tiling microarray	cDNA or EST sequencing	RNA-Seq
Technology specifications			
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Resolution	From several to 100 bp	Single base	Single base
Throughput	High	Low	High
Reliance on genomic sequence	Yes	No	In some cases
Background noise	High	Low	Low
Application			
Simultaneously map transcribed regions and gene expression	Yes	Limited for gene expression	Yes
Dynamic range to quantify gene expression level	Up to a few-hundredfold	Not practical	>8,000-fold
Ability to distinguish different isoforms	Limited	Yes	Yes
Ability to distinguish allelic expression	Limited	Yes	Yes
Practical issues			
Required amount of RNA	High	High	Low
Cost for mapping transcriptomes of large genomes	High	High	Relatively low

Table1. Advantages of RNA-Seq compared with other transcriptomics methods. *Modified from Wang et al., (2009).*

After an RNA-seq course, reads have to be mapped onto a genome. There are two strategies to do that: the first one involve the alignment of RNA-seq reads onto a reference genome (Trapnell *et al.*, 2010) (Figure 3), the second one is a de novo assembly of the short reads and is used when an annotated genome is absent (Robertson *et al.*, 2010).

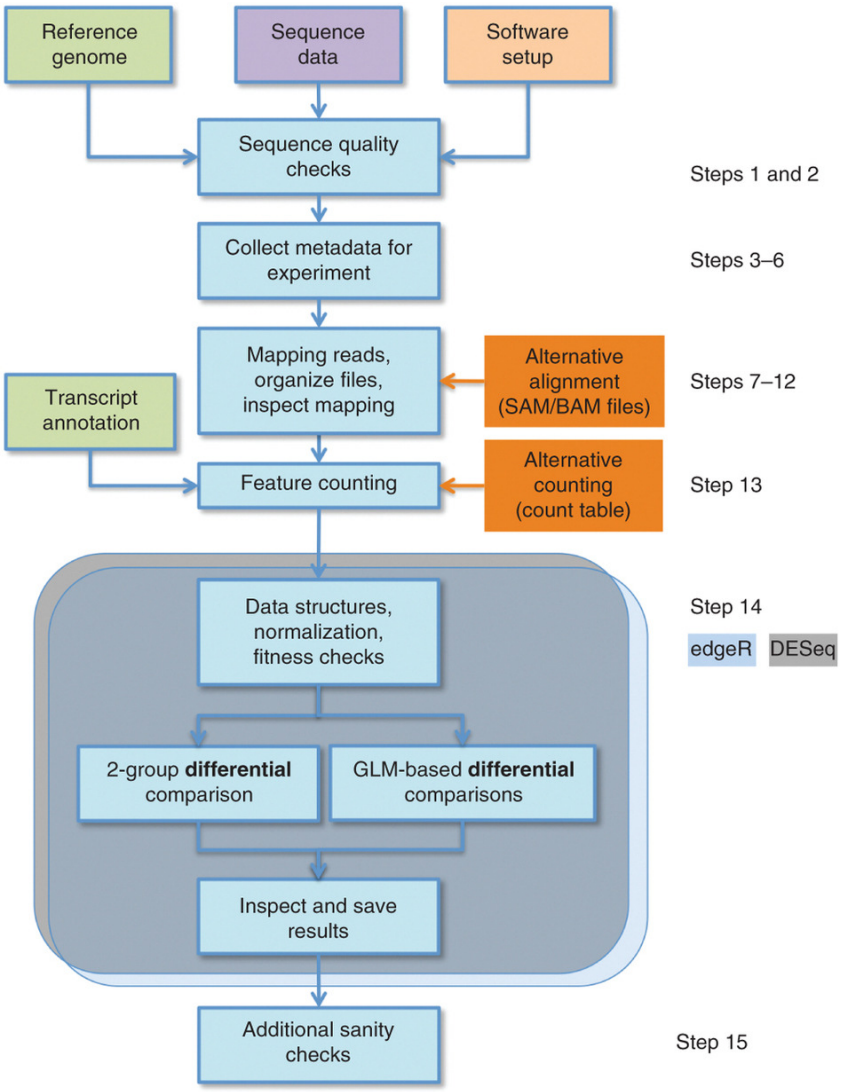

Figure 3. Count-based differential expression pipeline for RNA-seq data using edgeR and/or DESeq. *Modified from Anders et al. (2013).*

In order to statistically analyze RNA-seq data and identify Differentially Expressed Genes (DEGs), several statistical methods have been developed. Among these, edger (Robinson *et al.*, 2010) and DEseq (Anders and Huber, 2010) methods are the most used. These methods take similar strategies to perform differential analysis for count data. However, these R packages differ in the strategies to estimate the dispersion: edgeR moderates feature-level dispersion estimates toward a trended mean according to the dispersion-mean relationship, while DESeq takes the maximum of the individual dispersion estimates and

the dispersion-mean trend. In practice, this means DESeq is less powerful, whereas edgeR is more sensitive to outliers. Recent comparative studies have highlighted that no single method dominates another across all settings (Anders *et al.*, 2013) (Figure 3).

3. Grapevine and its genome

Since 2000, forty-nine plant species have been sequenced and fifty-five genomes have been published (Figure 4).

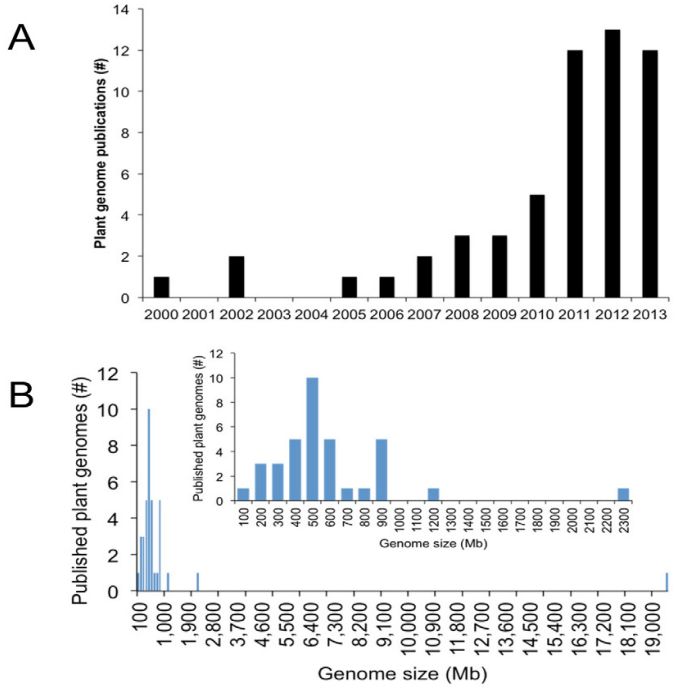

Figure 4. Published plant genome statistics. (A) Number of plant genomes sequenced since Arabidopsis thaliana in 2000 by year. (B) Published plant genome size distribution with insert focused on median genome size between 77 and 2300 Mb. *Modified by Michael et al. (2013).*

Publications of these genomes allowed the scientific community to investigate about intriguing aspects of plant genome biology. These observations were enabled not only by high quality genome assemblies but also by a greater number of genomes available for comparisons (Michael and Jackson, 2013). The large amount of produced information, helped to define the roles of hundreds of genes and provided access to sequence-based markers for breeding.

As concerns grapevine, the complete genome sequence was obtained in 2007 (Table 2) by two independent projects (Jaillon *et al.*, 2007; Velasco *et al.*, 2007). The availability of the genomic sequence gave the opportunity to conduct several genome-wide studies focused on different aspects of grape biology such as berry development and response to different biotic and abiotic stresses (Bottcher *et al.*, 2013; Castellarin *et al.*, 2007; Chaves *et al.*, 2010; Ziliotto *et al.*, 2012; Vannozzi *et al.*, 2012; Dal Santo *et al.*, 2013; Fasoli *et al.*, 2012), as already described in the above chapter.

Paper	Size (Mb)	Assembled (Mb)	Assem (%)	gene (#)	repeat (%)	scaffold N50 (kb)	contig N50 (kb)	Sequencer types	PMID
Jaillon et al. (2007)	475	487	103	30,434	41	2,065	66	Sanger	17721507
Velasco et al. (2007)	505	477	95	29,585	27	1,330	18	Sanger, Roche/4454	18094749

Table 2. Grape published genomes. Abreviations: kb, kilobases; Mb, megabases; Chr, chromosome; PMID, PubMed ID.

The sequencing of the grapevine genome represented the fourth genome of the flowering plants, the second one among wood plants and the first one concerning fruit producting plants (Michael and Jackson, 2013). The French-italian sequencing (Jaillon *et al.*, 2007) was obtained by the selection of the PN40024 line, a particular Pinot Noir clone characterized by a high degree of homozygosity (approximately 84%) and obtained through multiple auto-fecundation cycles in order to by-pass the high heterozygosis that characterize grapevine. The other grape genome, published by the Institute of S. Michele all'Adige (IASMA, Velasco *et al.,* 2007), was obtained by sequencing the heterozygous clone ENTAV115 of Pinot Noir cultivar.

The actual genome sequence available on line is the 12X assembly coverage of PN40024 (http://genomes.cribi.unipd.it/grape/), the last annotation of the genome was carried out by the CRIBI institute (University of Padova, Italy) by using data retrieved from the PN40024 clone.

As concern PN40024, the genome size is approximately 475 Mb and 30,434 genes have been identified. On the other hand, genome sequence obtained by the ENTAV115 clone, is slightly larger, with a size for the haploid genome estimated at 505 Mb and a total

number of 29,585 gene prediction (http://genomics.research.iasma.it/gb2/gbrowse/grape/). More detailed information about PN40024 and ENTAV115 are indicated in Table 2.

The public release of these genomes represents a remarkable goal, but also a formidable starting point for a vast range of studies aimed at improving our knowledge about gene function and genetic variability in this species. Transfer and interpretation of results obtained in model organisms on molecular mechanisms involved in the determination of important agronomical characters is now feasible together with the new opportunity for a molecular breeding in grapevine.

The advent of high-throughput re-sequencing technologies (Bentley, 2006),we have entered an exciting era in which we can finally learn what differences are found among individuals within a species at the DNA sequence level. Recent data obtained from different plant species have shown us how plastic, dynamic and variable plant genomes are. Comparison of genomic sequences related to different cultivar of *Vitis vinifera* species highlighted the presence of a core genome containing genes that are present in all strains and a dispensable genome composed of partially shared and strain-specific DNA sequence elements. Morgante *et al.*(2007) introduced this new concept called 'pangenome' (from the Greek word pan, meaning whole).

In recent years, the number of *Vitis vinifera* cultivar and other *non-vinifera* species re-sequenced has significantly increased, due to the lower cost and the higher speed of analysis of new generation sequencer (e.g. SOLiD, Illumina, Roche/454, Proton) (citazione). This should allow us to extend the concept of pan-genome to the genus *Vitis*, by indentifying a core genome which contains genes shared among *V. vinifera* and *non-vinifera* species (*e.g.* V. rupestris, V. berlandieri, V. riparia and intra-specific hybrids).

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Chapter II

Transcriptomes comparison of a susceptible and a putative tolerant grapevine rootstocks to drought give insight into the mechanisms of water stress tolerance

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1. Introduction

Before the devastation of European viticulture caused by of the introduction of phylloxera (*Daktulosphaira vitifoliae*) from North America in the nineteenth century, varieties of V. vinifera used commercially for wine production in Europe were traditionally grown on their own roots. Subsequently, the use of rootstocks from the pest's origin was introduced to provide resistance to this and other deleterious diseases and to save the fate of European viticulture. Although, at first, the replacement of the entire root system of "vinifera" varieties with "non vinifera" or "American" species was seen as a sort of contamination of the purity and quality of wine, subsequently, the use of grapevine rootstocks spread also over those countries where viticulture constituted a young and new challenge and where phylloxera did not represent a menace yet (Arrigo and Arnold, 2007; Gregory *et al.*, 2013). The explanation of this trend relies on the fact that the use of rootstocks was quickly found not only to confer resistance to diseases, but to imply a larger range of advantages, altering numerous physiological processes at the level of scion such as biomass accumulation (Gregory *et al.*, 2013), fruit quality (Walker *et al.*, 2002, 2004), and the ability to respond to many biotic and abiotic stresses (Meggio *et al.*, IN PRESS; Marguerit *et al.*, 2012). In fact, apart from conferring resistance to phylloxera, rootstocks were found to have many other desirable attributes, such as tolerance to calcareous soils (Covarrubias and Rombolà, 2013), salinity (Fisarakis *et al.*, 2001), nematodes (McCarthy and Cirami, 1990) and drought (Marguerit *et al.*, 2012; Gambetta *et al.*, 2012). All these characteristics make the use of rootstocks and the development of new genotypes of crucial importance in the contemporary viticulture.

Water availability is one of the major environmental factors contemporary viticulture has to cope with (Flexas *et al.*, 2009; Cramer *et al.*, 2007; Chaves *et al.*, 2010). Most of the word's wine-producing regions are subjected to seasonal drought, and, based on the global climate models, which predict an increase in the aridity in the next future (IPCC, 2007), water deficit may became the major limiting factor in wine production and quality. Moreover, the enhanced pressure on water resources increased the global perception of the need to reduce the "water footprint" for irrigated crops (Cominelli *et al.*, 2013) and in addition to a product enjoyable in all sensorial aspects, consumers expect wines to be healthy and produced in an environmental sustainable manner (Bisson *et al.*, 2002).

Grapes are well adapted to semi-arid climate such as that of Mediterranean and are generally considered relatively tolerant to water deficit. The large and deep root system, together with physiological drought avoidance mechanisms, such as stomatal control of transpiration, xylem embolism (Tramontini *et al.*, 2013) and the ability to adjust osmotically, make these plants able to grow also in sub-optimal water conditions. However, considering that a large proportion of vineyards are now located in region where seasonal drought coincides with the grapevine growing season, the combined effect of soil water deficit, air temperature and high evaporative demand is known to limit grapevine yield and to delay the vintage date (Walker *et al.*, 2004; Flexas *et al.*, 2009; Chaves *et al.*, 2009), with a negative effect on grape berry and, consequently, wine quality. Water deficit leads to many morphological and physiological changes across a range of spatial and temporal scales (Chaves *et al.*, 2002), including reduced expansion of aerial organs (Cramer *et al.*, 2007), maintenance of root growth (Sharp and Davies, 1979), decrease in transpiration and photosynthesis (Chaves *et al.*, 2010), accumulation of osmotic compounds and ions (Cramer *et al.*, 2007), activation of detoxifying processes and, in parallel, the transcriptional regulation of a large number of genes (Cramer *et al.*, 2007; Tattersall *et al.*, 2007; Tillett *et al.*, 2011). Stomata closure is one of the early plant physiological responses upon moderate to mild water deficits conditions and this phenomenon is often related to a metabolic limitation (Chaves *et al.*, 2002): CO2 diffusion is lowered by stomata closure and thus results in a lower rate of net carbon (C) assimilation. In grapevine, it has been reported that photosynthetic process is quite resistant to water stress (Chaves *et al.*, 2009; Souza *et al.*, 2003). Under low to moderate deficit irrigation, maintenance of the activity of the Calvin cycle and of the maximum rates of carboxylation and electron transport has generally been observed (Souza et al. 2005a). However, as the water stress becomes severe, net CO2 assimilation (An) and other metabolic processes operating in the mesophyll are inhibited and water use efficiency thus declines. The imbalance between the light capture and its utilization results in a down-regulation of photosystem II (PSII) activity, phenomenon known as photo inhibition (Reddy et al., 2004) and the changes in the photochemistry of chloroplasts in leaves result in the dissipation of excess light energy in the PSII core and antenna with the generation of active oxygen species (ROS) such as H2O2, O2-, -OH, 1O2, and NO. These ROS are

responsible for most of the oxidative damages in biological systems (Apel and Hirt, 2004) and cellular components. Thus, a strict control of ROS levels is mandatory for plant survival (Kar, 2011) and the cross-talk between ROS accumulation and redox state is integrating part of a fine homeostasis control that plays a pivotal role in the plant response to stresses (Suzuki *et al.*, 2012). Together with the accumulation of ROS scavenging compounds and related genes, such as superoxide dismutases (*SOD*), catalase (*CAT*), ascorbate peroxidase and glutathione peroxidase (Kar, 2011; Mittler, 2002), the photorespiration provides an important strategy to cope with the effect of light energy excess as observed in a recent expression study conducted by Cramer *et al.* (2007).

Phytohormones play a central role in the ability of plants to adapt to abiotic stresses by mediating a wide range of adaptive responses, which often alter gene expression by inducing or preventing the degradation of transcriptional regulators via the ubiquitin–proteasome system (Kelley and Estelle, 2012; Peleg and Blumwald, 2011; Santner and Estelle, 2009). Abscisic acid (ABA) is one of the most studied stress-responsive hormone in plants, especially as concerns the plant response to water deficit (Fujita *et al.*, 2011; Qin *et al.*, 2011). Its synthesis is one of the fastest plants responses to abiotic stresses, triggering ABA-induced gene expression and inducing stomatal closure to reduce water loss and eventually limiting cellular growth (Peleg and Blumwald, 2011). Physiological studies showed that under abiotic stresses, especially drought and salinity, plants accumulate high levels of ABA accompanied by major transcriptome changes, including (Qin *et al.*, 2011) genes involved in its sensing (*PYR/RCAR*), signal transduction (type 2C protein phosphatases, *PP2Cs*; *SnRK2s* kinases), regulation (e.g. *AREB/ABFs*, *DREB, MYB*), and response (e.g. response to dehydration-like, *RD-like*; *LEA proteins; HIS*) (Fujita *et al.*, 2011; Qin *et al.*, 2011; Novikova *et al.*, 2009).

Although ABA remains the best-studied hormone for plant stress response, a growing number of studies are revealing that many other hormones, such as auxins, ethylene, jasmonates (JAs), gibberellins (GAs), Salicylic acid (SA), and Brassinosteroids (BRs) are involved in the plant response to water stress (Peleg and Blumwald, 2011). Thus, plant adaptation to water limiting conditions, as well as other abiotic stresses, has to been seen as the concerted action of all the above cited plant hormones throughout a fine tuned cross-talk (Kohli *et al.*, 2013). JA, for instance, accumulates, together with JA-responsive

genes, under many types of stress conditions including drought. This was observed in soy-bean (Creelman and Mullet, 1995), *Pinus pinaster* (Pedranzani *et al.*, 2007), tomato (Pedranzani *et al.*, 2003) and rice (Kiribuchi *et al.*, 2005). Although the precise function of JA in drought response is still unclear there are strong evidences of the cross-talk between JAs with ABA and other plant hormones. In rice, the ectopic expression of *AtJMT,* a gene which converts JA to MeJA, not only let to an higher accumulation of MeJA in young panicles, but also to a dramatic loss in grain yield substantial increase in ABA levels, suggesting a cross-talk between the two hormones. Moreover the JASMONATE ZIM-DOMAIN (JAZ) protein, a repressor of the MYC2 transcription factor and thus of the JA-signaling pathway, was recently found to interact with other partners beside MYC2, including the GA-signaling genes *DELLAs*, suggesting also a synergic JA-GA cross-talk (Wasternack, 2007). Plant hormones highlight an interesting cross talk also with ROS, which act as secondary messengers of these regulators (Kar, 2011). It is well known that H_2O_2 regulates ABA-mediated stomatal closure by acting on Ca^{2+} levels and inactivating PP2Cs (Meinhard *et al.*, 2002). Stomatal closure is also mediated by ethylene via ETR1, an hormone receptor, which is involved in H_2O_2-sensing (Desikan *et al.*, 2005). Finally, in Joo *et al.* (2001) is reported an induction of ROS in roots mediated by auxins, suggesting a role downstream of transport in auxin signaling and gravitropism.

The decrease of osmotic potential (π) in response to water stress is another well-known mechanism by which many plants adjust to drought conditions. Apart from the passive solute concentration resulting from dehydration, plants can accumulate solutes in an active and genetically regulated manner. The compounds involved in this adjustment widely between species: in the majority of perennial woody species they seem to be organic compounds as observed in peaches, apples and cherries, which accumulate sorbitol (Escobar-Gutiérrez and Gaudillére, 1994; Wang *et al.*, 1996; Arndt *et al.*, 2000), or in *Morus alba*, which accumulate amino acids (Ramanjulu *et al.*, 1994). *V. vinifera* cv Cabernet Sauvignon shoot tips of water-deficit-treated plants were found to accumulate malate, proline and glucose concentration.

Recently, a biochemical and physiological study of novel candidate genotype to be used as rootstock in grapevine was performed (Meggio *et al.*, IN PRESS). This genotype, designed as M4 [(V. vinifera x V. berlandieri) x V. berlandieri x cv Resseguier n.1] and

established from 1985 by the DiSAA research group operating at the Milan University, was selected for its high tolerance to water deficit (WS) and salt exposure (SS). In comparison with the 101.14 commercial genotype, M4 un-grafted plants subjected to water and salt stress showed a greater capacity to tolerate WS and SS maintaining photosynthetic activity also under severe stress conditions and accumulating, especially at the root level, osmotic compounds and ions. Here we report results obtained from a large scale whole transcriptome analyses performed on leaf and root tissues of both M4 and 101.14 genotypes under the same WS experimental conditions exposed in Meggio et al. (2013). The absolute novelty of this study relies on the fact that, differing from previous studies performed on plants subjected to an instantaneous decline in water availability, in our experiment, water deprivation was accomplished gradually, miming as far as possible those conditions that might occur in field

2. Materials and methods

2.1 Experimental design and plant material.

Two-years-old glass-house grown plants of the susceptible and tolerant grapevine rootstocks under study, respectively the widely used 101.14 genotype (*V. riparia* x *V. rupestris*) and the experimental M4 one ((*V. vinifera* x *V. berlandieri*) x *V. berlandieri* cv. Resseguier n. 1), were subjected to drought stress with the aim of understanding the genetic determinism of tolerance and susceptibility in both genotypes. A total number of 108 glasshouse-grown plants from each genotype were subdivided into three groups: plants grown under well-watered (WW) conditions, plants grown under water-deficit conditions (WS, Water-Stress) and plants grown under high salinity (SS, Salt-Stress), not discussed in the present paper. WW plants, which were used as controls for water stress, were grown in pots with a water-availability equal to the 80% of the field capacity. The water-stress treatment (WS) was imposed by growing 101.14 and M4 plants in limited water-availability conditions in respect to the control. This was accomplished gradually decreasing the water-availability in pots from 80% to the minimum level of 30% of field capacity. The whole drought experiment had a duration of 10 days during which, leaf physiological measurements were performed on fully expanded leaves, immediately before sampling. Leaf water potential (Ψ_{leaf}) was determined using a Scholander-type pressure chamber (model PMS-1000, PMS Instruments, Corvallis, Oregon, USA), net assimilation rate (A_n) and stomatal conductance (g_s) measurements were performed using a LI- 6400 portable photosynthesis system (Li-Cor Inc. Lincoln, Nebraska, USA), as reported in Meggio et al. (2014). In addition, we hereby report unpublished data of leaf transpiration rate (E, mmol H_2O $m^{-2}s^{-1}$), performed in the same experimental conditions: 600 μmol of photons $m^{-2}s^{-1}$, a CO_2 concentration of 380 μmol mol^{-1}, 1.5 kPa of vapor pressure deficit (VPD) and a block temperature of 25 °C.

Both the genotypes experienced a similar degree of water deficit, allowing a robust comparison of their physiological and molecular responses as reported in Meggio *et al.* (IN PRESS). Four time points, designed as T1 to T4 were considered for both leaf and root tissues sampling. Five leaves and root tissues were collected from pools of at least 6 plants for each genotype (101.14 and M4) and each condition (WW and WS), respectively at 2 (WS-T1), 4 (WS-T2), 7 (WS-T3) and 10 (WS-T4) days after the beginning of drought

treatment. The starting point of the kinetic (T0), considered as common for both the treatments, was constituted by 101.14 and M4 plants grown in WW conditions at the beginning of treatments. All samplings were performed in two biological replicates producing a total of 40 samples originated from leaves and 40 samples originating from roots for a total of 80 samples.

2.2 Whole transcriptome analysis.

Total RNA was extracted from frozen grapevine tissues using the "SpectrumTM Plant total RNA Kit" (Sigma) according to manufacturer instructions. mRNA was extracted from the total RNA using the Dynabeads mRNA Direct kit (Invitrogen pn 610.12). A variable quantity of mRNA ranging from 0,4 to 1,6% respect to the amount total of RNA was obtained. Samples for Ligation Sequencing were prepared according to the SOLiD Whole transcriptome library preparation protocol (pn 4452437 Rev.B). The samples were purified before RNase III digestion with Purelink RNA micro kit columns (Invitrogen, pn 12183-016), digested from 3' to 10' according the starting amount of mRNA, retro-transcribed, size selected using Agencourt AMPure XP beads (Beckman Coulter pn A63881) and barcoded during the final amplification. Obtained libraries were sequenced using Applied Biosystems, SOLiD™ 5500XL , which produced paired end reads of 75 and 35 nucleotides for the forward and reverse sequences respectively. Reads were aligned to the reference grape genome using PASS aligner, a program able to perform several alignments on a sequence subset trying different parameters to trim the low quality bases and select for those values that maximize the number of aligned reads (Campagna *et al.*, 2009). The percentage identity was set to 90% and one gap was allowed whereas the quality filtering parameters were set automatically by PASS. Moreover, a minimum reads length cutoff of 50 and 30 nt was set for the forward sequences and reverse reads respectively. The spliced reads were identified using the procedure described in PASS manual (http://pass.cribi.unipd.it). The forward and the reverse reads were aligned independently on the reference genome. We used PASS-pair program in the PASS package to perform the pairing between the forward and the reverse reads and we selected only those sequences that are uniquely aligned. As gene reference we used the v1 grape gene prediction available at http://genomes.cribi.unipd.it/grape and htseq-counts program

(http://www-huber.embl.de/users/anders/HTSeq/doc/count.html) to quantify gene abundance.

2.3 Multifactorial and pairwise statistical analysis.

DEseq R package (http://www.r-project.org/) was used to perform the statistical analyses for discovering differentially expressed genes (DEGs) by using both a multi-factor and a pairwise-comparison approaches (Maza *et al.*, 2013). In order to evaluate the single effects of the genotype (R: 101.14 and M4), of the type of stress imposed (Treatment, T: WW, WS, and SS for control, water stress and salt stress experiment, respectively) and of the time point considered (P: T1-T4 and T1-T3 for WS and SS, respectively) on gene expression, a multifactorial analysis was carried out by using the multi-factor designs method of DEseq R package (Anders and Huber, 2012; http://bioconductor.org/packages/release/bioc/html/DESeq.html). This method allows to evaluate the weight of each factor considered in the analysis (R, T and P) and its impact on gene expression and, consequently on DEGs, according to a false discovery rate (FDR) < 0.05 for both experiments and tissues. This procedure consist of the following passages: create a count data set with multiple factors (with the three components described above), estimate size factor and dispersions of the data, fit generalized linear models (GLMs) according to the two models (full model regresses the genes' expression on both the library type and the treatment condition, the reduced model regresses them only on the library type), and then compare them in order to infer whether the additional specification of the treatment improves the fit and hence, whether the treatment has significant effect (Anders and Huber, 2010).

Pairwise tests (Anders and Huber, 2010) between stressed (WS) and unstressed (WW) tissues were also performed. In this case the analysis was performed considering each genotype, tissue and time point singularly.

2.4 Ontology and Differential Clustering Analyses (DCA).

In order to functionally classify the genes affected by drought treatment, the Gene Ontology (GO) terms were retrieved, imported in the Blast2GO software v2.5.0 (Götz *et al.*, 2008) and increased of about 16% by means of the Annex function (Myhre *et al.*,

2006) as previously reported by Botton *et al.* (2008). DEGs resulted from multifactorial analysis, which were affected by the effect of all components (common DEGs between R, T, and P) and those ones in common between rootstock and treatment components, were associated to GO categories. Within the more representative GO categories, those DEGs associated to GO terms related to plant hormones, secondary metabolism, sugars, stresses, cell wall and transcription factors (TFs) were selected for the following Differential Clustering Analysis (DCA).

The DCA analysis was performed by using an R script, which is a slightly modified version of the original method carried out by Ihmels *et al.* (2005), Lelandais *et al.* (2008) and Cohen *et al.* (2010).

The DCA analysis is carried out in three steps that we develop here. (i) The correlation values of all DEGs belonging to the GO categories related to a reference rootstock (rr) and a target rootstock (tr) were initially calculated with the 'cor' R function. (ii) Correlation values of the rr were subsequently clustered by applying the 'kmeans' R function. The number of clusters related to the rr were selected in accordance to an average correlation value, which was heuristically chosen higher than 0.65 for each cluster. The same order chosen for the rr was used to arrange the tr DEGs of the GO-selected categories. Hence, the transcripts from each cluster were co-expressed in the rr (correlation > 0.65) but not necessarily in the tr one. (iii) DEGs related to each cluster of the tr were subsequently grouped into two sub-clusters (a and b) by using a hierarchical clustering method (with 'hclust' R function). The average of the correlation values belonging to each sub-cluster (Ca and Cb) and the average of the correlation values between the two clusters a and b (Cab) were eventually calculated.

Correlation values of tr and rr matrices are graphically represented in white, yellow and red colors for strongly correlated, weakly correlated and anti-correlated genes, respectively. DCA results were finally presented as a unique distance matrix between gene expression measurements in which rr and tr rootstocks were respectively represented in rows and columns. Clusters of each rootstock were compared and assigned to "full", "partial", "split" or "no" conservation categories after comparing Ca, Cb and Cab values with the threshold T, which is chosen equal to 0.5 in this study. Specifically, if (Ca and Cb) < T the cluster was assigned to the "no conservation" category, if (Ca or Cb) > T the

cluster was assigned to the “partial conservation” category, if (Ca and Cb) > T and Cab < T the cluster was assigned to the “split conservation” category and if (Ca, Cb and Cab) > T the cluster was assigned to the “full conservation” category.

3. Results

3.1 Physiological traits analyses

The present study was performed in order to shed light into the genetic determinism of tolerance and susceptibility to water stress in two grapevine rootstocks characterized by different levels of tolerance. The water-stress treatment (WS) was imposed gradually decreasing the water-availability in pots from 80% to the minimum level of 30% of field capacity whereas well-watered (WW) plants, which were used as controls for water stress, were grown in pots with a water-availability equal to the 80% of the field capacity.

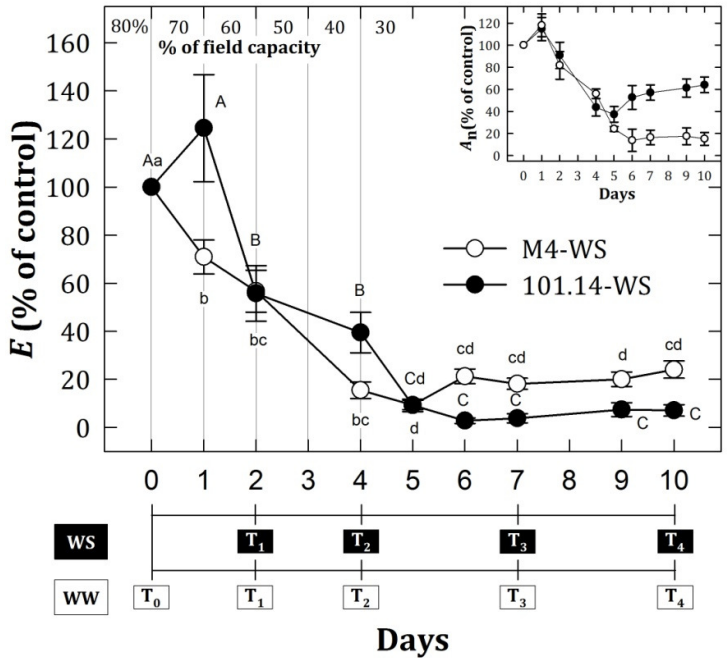

Figure 1. Average ± SE values of net CO2 assimilation (An) and leaf transpiration (*E*) for water stress plants. Results are expressed as a percentage of control. T1-4 represent sampling time points throughout the experimental period after control (T0). Values indicated with the same letters do not significantly differ according to Duncan's test ($P < 0.01$).

E measured in WW conditions was 2.73 ± 0.3 and 2.4 ± 0.2 mmol H_2O $m^{-2}s^{-1}$ for M4 and 101.14 genotypes and maintained similar values throughout the whole experiment. On the other hand upon WS, as drought gradually proceeded leading to severe stress conditions (around 30% of field capacity), 101.14 plants showed an almost complete stomatal

closure, whereas a transpiration rate of approximately 20 % with respect to its control was maintained in M4 (Figure 1).

3.2 mRNA-seq sequencing and mapping of reads to the grapevine genome

To gain a better understanding of the molecular mechanisms underlying the drought tolerance of the M4 genotype compared to the susceptible 101.14 one, an mRNA-seq analysis using Next Generation Sequencing (NGS) technologies was performed. The whole transcriptome sequencing was performed on a Solid 5500XL platform. The analysis was accomplished on all the 80 samples previously described, in order to obtain a detailed screenshot of the transcriptome changing of the two rootstocks within the entire stress kinetics. The whole experiment produced approximately 4.8 billion of paired-end reads (75 and 35 bp length for forward and reverse reads respectively), with the total number of reads produced for each time point ranging from 29 to 82 million paired-end reads and a median of 45 million reads. An average percentage of 90% of total reads passed the quality control test (filtered based on reads length after the trimming of the low quality bases) and were mapped to the PN40024 12Xv1 reference genome (Jaillon *et al.*, 2007) producing a number of unique mapping reads ranging from 10 to 37 million depending on the sample.

3.3 Multi-factor design and discovering of differentially expressed genes

For the evaluation of Differentially Expressed Genes (DEGs) upon water stress in the two rootstocks under study, a main step in the statistical analysis was estimating the influence of different independent components (*i.e.* variables) on transcriptome. Amongst these were the effect of genotype, indicated as "R" (101.14 and M4 respectively), the type of treatment imposed, indicated as "T" (well watered, WW; water stress, WS) and the time point considered during the kinetic of stress, indicated as "P" (T1, T2, T3, T4).

Thus, a multi-factor analysis was carried out on mRNA-seq data sets obtained from WS root tissues and WS leaf tissues, in order to evaluate both the singular (R, T, P) and combined (R:T, R:P, T:P, R:T:P) impacts of each component on DE genes according to a

p-value lower than 0.05. The Venn diagram shown in Figure 2A summarizes the impact of each component indicating the number of genes specifically influenced by a single component and those ones influenced by more than one variable. In root tissues undergoing water stress treatment, the total amount of DEGs influenced by each single component R, T and P was 7408, 7905 and 5839, respectively (Figure 2A), whereas in leaf tissues, DEGs were 3794 for R, 3476 for T and 2284 for P component (Figure 2B). In other words, considering for example WS roots (Fig 1A), 2887 genes were differentially expressed only because of the effect of rootstock, i.e. of the different genotype, regardless of the effect of treatment (WW or WS) and time point considered (T1-T4). Conversely 2077 genes were exclusively influenced by the application of treatment, independently by the genotype (101.14 or M4) and the time point considered (T1-T4). Finally there were only 551 genes which appeared to change their expression just because of the effect of the experiment kinetic.

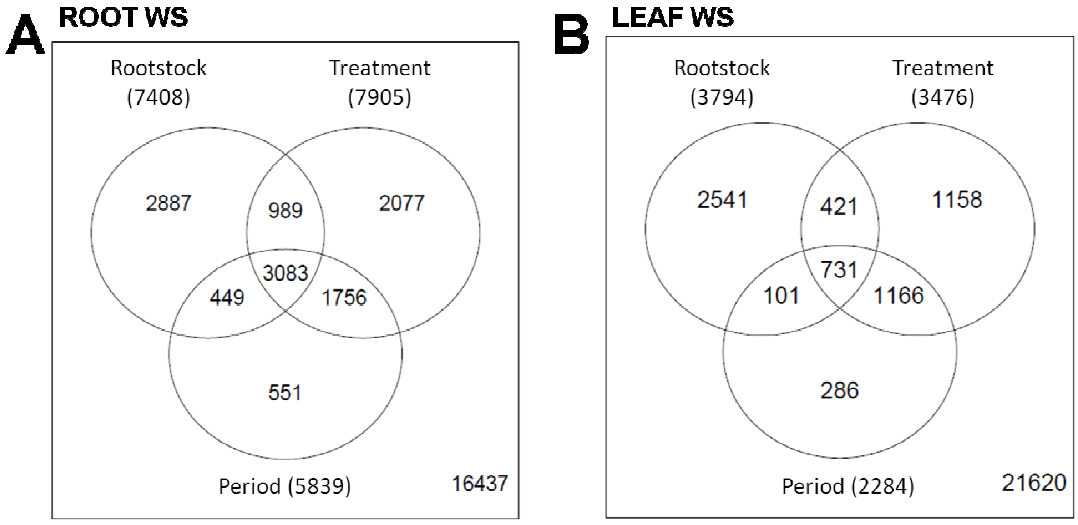

Figure 2. Venn diagram with DEGs resulted from Multifactorial analyses carried out on root and leaf tissues upon WS treatment, in according to a $p<0.05$. Total number of DEGs influenced by each component are given in bracket.

The multi-factorial analysis highlighted a strong influence of the organ considered (root or leaf) on the final number of DEGs. Indeed, WS root showed a significantly high number

of DEGs, with 11792 transcripts, whereas in WS leaf the amount of DEGs was approximately 50% less, with 6404 transcripts.

Interestingly, whereas in roots the treatment (T) component seemed to have the major influence on expression, showing the highest number of DEGs (7905) in comparison to all the other components, in leaves, the genotype (R) appears to have the highest impact on the modulation of transcripts, with 3794 DEGs. As concerns the effect of the stress kinetic on DEGs, its influence appeared to be less important for both root and leaf tissues. In addition to the Multifactorial analysis, a pairwise comparison between WS and WW data f both tissues and genotypes was carried out from T1 to T4. Taking into account that treatment and genotype were the variables showing the major impact of on transcriptome pairwise comparisons, performed separately for the two genotypes under study (101.14 and M4), were accomplished between the water stressed tissues, respectively leaves and roots, and the non-stressed ones at each time point (T1-T4) (Figure 3). Apart from M4 leaf tissues, which showed a peak of DE genes at T2 (4 days after stress imposition, DASI) followed by a decrease (T3) and a new increase at T4, all other comparisons revealed an increased amount of differentially expressed genes within the first 7 days (T1-T3), with a peak at T3 probably related to the increasing stress levels (Figure 3A, B). Except for M4 leaf tissues, T4, which corresponds to 10 DASI, showed a sensible decrease in the number of DE genes respect to T3 (Figure 3A, B). This could be due to the fact that all plants undergone a sort of pot-effect depression or, in the case of the susceptible genotype, that the plant metabolism is inhibited because of the stress. A first general observation is that, in both 101.14 and M4 rootstocks, water deficit affected an higher number of DEGs in root tissue (Figure 3B) in comparison to the leaf one (Figure 3A).

Regarding the leaf tissues, there was a relatively low amount of DEGs between stressed and control plants on day 2 (T1), when stress levels were very low (Figure 3A). This was observed both in 101.14 and in M4 rootstocks, with respectively 29 and 44 DE genes detected. As the stress increased with time, so did the number of stress-responsive transcripts. Indeed, on day 4 (T2) there were massive changes in gene expression with over 1200 transcripts, which increased or decreased significantly in both genotypes (1232 DEGs in 101.14 and 1223 DEGs in M4) (Figure 3A). As previously mentioned, for WS leaf data, a first macroscopic difference between the two rootstocks (for WS leaf) was

detected at T3 (7 DASI), with an increased number of DEGs in 101.14 in comparison to T2 (1475 DEGs at T3) and a drastic decrease of M4-related DEGs (677 DEGs; Figure 3A). Amongst those DEGs identified at each time point, there were genes found to be common to both 101.14 and M4 rootstocks and other genes, which appeared to be genotype-specific.

As concerns data related to leaf tissue at 4 DASI (T2), there were 619 DEGs common to both 101.14 and M4 that were significantly different from the relative control and 604 specific genes that were up or down regulated exclusively in M4 rootstock (613 were 101.14-specific). Indeed, 4 days after the imposition of stress, the two genotypes appear to respond in a quite similar manner, at least based on the raw transcriptional data. Interestingly, at T3, the number of M4-private DE genes decreased, with only 168 accessions that were significantly different from the control only in M4 and a much higher number (966) of 101.14-private DEGs. The opposite pattern was observed at T4, with a higher number of M4-private genes (715) against those (300) registered in 101.14. Approximately 21% to 31% of 101.14 rootstock genes were significantly different from controls both at T2 and T3 and T4 whereas 21% to 44% of DE genes were common for T2-T3-T4 in M4 (data not showed).

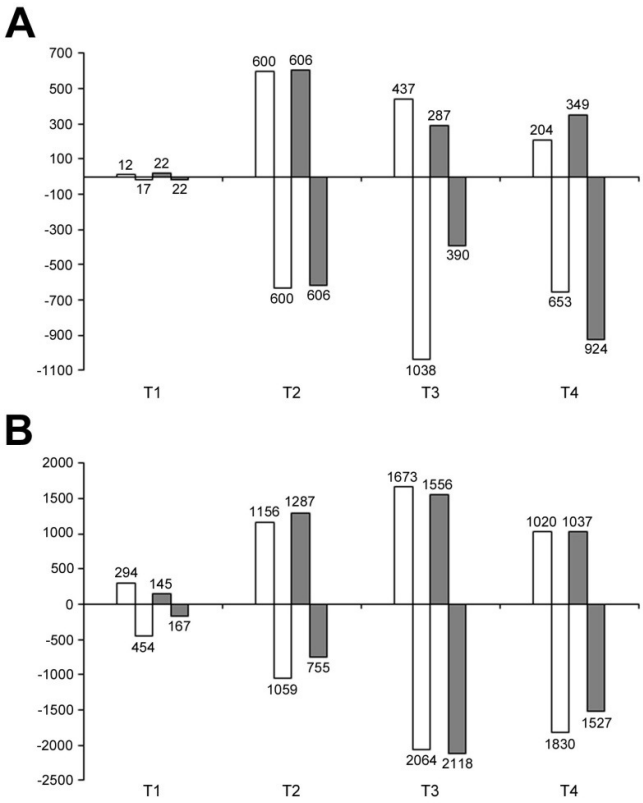

Figure 3. Pairwise statistical analyses. DEGs were identified calculating the log2-ratio (WS versus WW) of the counts detected for each gene in M4 and 101.14 rootstocks. The pairwise comparisons were accomplished at each time point, in according to a $p<0.05$.

When compared with the root tissues expression data, the leaf transcriptome appeared to be less drought-responsive, which might reflect, in part, the higher sensitivity of an actively growing tissue to water deprivation, as already observed in other plant species such as poplar (Cohen et al., 2010). Figure 3B showed the number of differentially up- and down-regulated genes detected in pairwise comparisons between stressed (WS) and unstressed (WW) root tissues. T1 was characterized by a much higher response in 101.14 genotype compared to M4, with up to 748 DE genes in the first one compared to 312 in the latter. Similarly to what observed in leaves, as the stress severity increased so did the number of DE genes in both the rootstocks, reaching a peak at 7 DASI (T3), with 3737 DE genes detected in 101.14 and 3674 in M4. An interesting observation that characterizes both the roots and leaves of stressed tissues is that the susceptible genotype

always appears to modulate the expression of more transcripts than the tolerant one, which might reflect the minor degree of perturbation undergone by the M4 rootstock.

A subgroup of DEGs we considered of particular interest were those ones influenced by both the effect of the rootstock and of the treatment, further validated by pairwise analyses, and by the effect of all the three components. In this case, common DEGs between R and T were 989 and 421 for WS roots and WS leaves respectively, whereas common ones between R, T , P were 3083 for WS roots and 731 for WS leaves (Figure 2). These two groups of DEGs were associated to their respective GO terms. Genes considered were 4072 and 1152 for roots and leaves gene-set, respectively. GO terms were grouped into different macro-categories as follows: "*plant hormones*", "*antioxidant responses*", "*sugars*", "*cell wall*", "*secondary metabolism*" and "*transcription factors*" (TFs) (Figure 4).

Amongst those macro-categories related to root tissue, "*Transcription factors*" was the one counting the highest number of DEGs, with 307 genes corresponding to 7.5% of all DEGs considered (4072 in total). As far as concerns "*secondary metabolism* and *sugars*", DEGs related to these category were 209 and 223, respectively, corresponding to 5.1% and 5.5%. A lower number of DEGs was associated to "*plant hormones*", "*cell wall*" and "*antioxidant responses*" categories, corresponding to 3.7%, 3.3% and 3%.

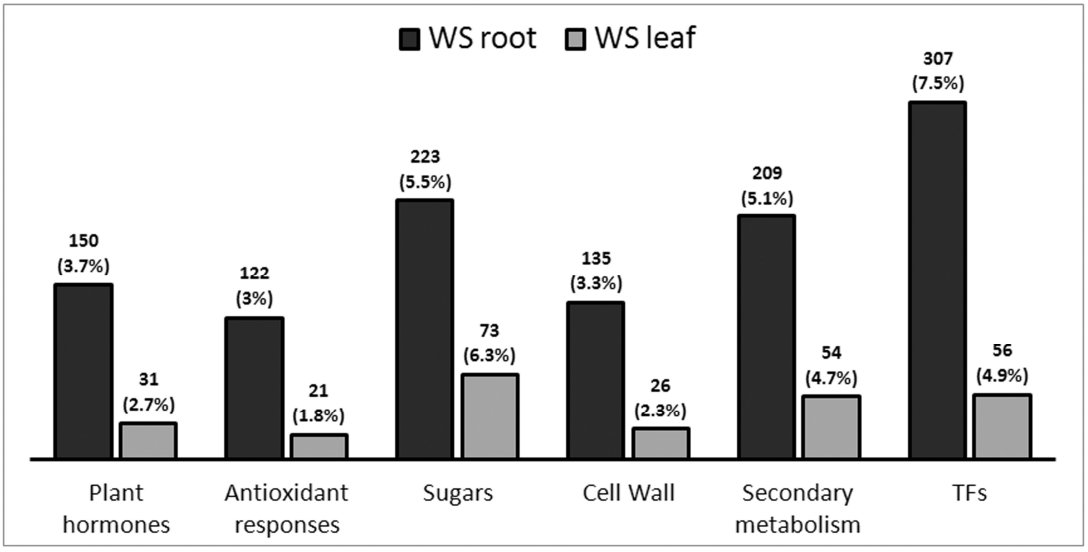

Figure 4. Ontologies analysis of plant-hormones, antioxidant responses, sugars, cell wall, secondary metabolism ant TFs –related GO terms. Number and percent of DEGs belonging to each category are given.

On the other hand, DEGs identified in WS leaves highlighted a different weight of ontology categories in comparison to the root tissue. In this case the highest percentage of transcripts was related to "*sugars*", with 73 DEGs (6.3%). "*Transcription factors*" and "*secondary metabolism*" categories were less represented, with respectively 56 (4.9%) and 54 (4.7%) genes. Finally *"Plant hormones", "antioxidant responses*" and "*cell wall*" categories showed 31 (2.8%), 21 (2%) and 26 (2.3%) DEGs, respectively. For each tissue and ontology category, a DCA analysis was carried out.

To better investigate these common DE gene subsets, an additional analysis (DCA) was carried out in order to identify those transcript that showed different behavior between M4 and 101.14 rootstocks.

3.4 Differential cluster analysis (DCA)

Results obtained from the multifactorial analysis and the pairwise analyses were used together in a Differential Cluster Analysis (DCA). Previous studies have demonstrated that generally, genes belonging to the same GO category and sharing a similar function, are significantly co-expressed (Eisen *et al.*, 1998; Kim *et al.*, 2001; Ihmels *et al.*, 2004) showing high level of pair-wise correlation. Nevertheless, comparison of transcriptomes belonging to different organisms can highlight differences in the patterns of gene co-regulation within individual GO categories. These differences are likely to reflect differences in the physiology, or in the adaptation to different environments. To better capture differential expression patterns between 101.14 and M4, and to systematically characterize the conservation or divergence of co-expression between genes with a related function, we implement and used a recently developed approach, termed Differential Cluster Algorithm (DCA). This analysis was performed on gene groups defined by membership in the same GO categories previously described (i.e. those ones detected in the subgroup of R-T and R-T-P common DEGs by the multifactorial analysis) by correlating their expression values obtained from the pairwise analysis (provided as $\log_2$ of the ratio between read counts obtained from WS and WW tissues). The DCA algorithm allowed us to identify co-expression clusters embedded within these gene sets and to assign each of these clusters to one of four categories indicating the level of conservation

between the two genotypes: “full”, “partial”, “split”, or ”absent”. A full conservation class is supposed to contain DEGs which are co-regulated in a similar manner in both genotypes, whereas a “partial conservation class” or a “split conservation” class identifies, together with subsets of genes that show similar co-regulation in the two genotypes, also sub-clusters of genes which display independent or even inversely correlated patterns. Those clusters that showed the most interesting results both in root and leaf tissues are reported in Figures 5 and 6. Again, roots and leaves highlighted different responses upon stress.

In root tissue, the most interesting results concerned *“Transcription factors*” and *“secondary metabolism*” ontologies. Within TFs category (Figure 5A), reference DEGs (101.14) were subdivided into three primary clusters designed as R-TF1, R-TF2 and R-TF3. Each of these clusters were uniformly co-expressed in 101.14. In contrast, in M4 they were split in two distinct secondary clusters, one of which was similar in both 101.14 and M4 indicating a “partial conservation”, the other one showing a different behaviour of several genes. Amongst them, of particular interesting were those belonging to the *WRKY* family for what concerns cluster R-TF1, *MYB* family for R-TF2 and *NAC* family for R-TF3. In Figure 5A we listed these genes together with their pattern of expression within the whole stress kinetic in both the genotypes considered. Looking at the picture it’s clear that the totality of WRKY TFs show a different behaviour between the two genotypes, being strongly up-regulated at T2 (4 DASI) limitedly to the M4 rootstock. Regarding the other gene families belonging to the TFs ontology which highlighted particularly interestingly results, clusters R-TF2 and R-TF3 embed five MYBs and three NACs transcription factors that were induced at T1 (2 DASI) in M4 and, conversely were significantly down-regulated in 101.14 (Figure 5A). It is worthy to note that *VvMYB14* (VIT_07s0005g03340) and *NAC83-like* (VIT_14s0068g01490) TFs, which do not belong to the primary clusters R-TF1, R-TF2 and R-TF3 (the list of all primary clusters identified is reported in Supplementary table Z), have been included in the list since they are particularly interesting, in fact although highly expressed in both the genotypes analysed, they show patterns of expression totally different between them. *VvMYB14* showed an high induction in stressed M4 roots at T2, T3 and T4 whereas its up-regulation in 101.14 was limited to T2 (Figure 5A). *VvNAC83* was induced at T2 only in M4 WS roots, while

in 101.14 it showed an unchanged expression from T1 to T3, and a significant down-regulation at T4.

As previously mentioned, "secondary metabolism" (SM) is another category which gave interesting results for DCA (Figure 5B). Within this ontology, most transcripts were related to the stilbene synthase (STS) and gluthatione-S-transferase (GST) gene families (clusters R-SM4, 2 and 3). Based on the nomenclature proposed by Vannozzi et al. (2012), *VvSTS12* (VIT_16s0100g00800), *VvSTS24* (VIT_16s0100g00940), *VvSTS13* (VIT_16s0100g00810), *VvSTS16* (VIT_16s0100g00840), *VvSTS17* (VIT_16s0100g00850), *VvSTS18* (VIT_16s0100g00860) and *VvSTS27* (VIT_16s0100g00990) were found to be significantly up-regulated in only in M4 stressed roots at T2, whereas they were generally down-regulated in 101.14 roots (Figure 5B) for both R-SM4 (no conservation) and R-SM3 (partial conservation) clusters. Regarding GST-related transcripts two distinct gene expression kinetics were identified. Indeed, *VvGST29-like* (VIT_01s0026g01380) and *VvGST8* (VIT_05s0051g00110) showed an up-regulation at T1 only in M4 stressed roots (cluster R-SM2, partial conservation) and *VvGST7-like* (VIT_07s0005g04890) and a *VvGST-like* gene (VIT_14s0060g02170) were strongly induced in M4 WS root at the second time point (cluster SM3).

The last category considered for DCA analysis on roots is the one related to plant hormones (PH). For this category two interesting primary clusters were identified: R-PH1 and R-PH2: R-PH1 belongs to the "no conservation" classand contains transcripts that were up-regulated only in M4 at T1. R-PH2 primary cluster, which display a "split conservation" contains genes induced exclusively in 101 at T2, T3 and T4 (Figure 5C).

R-PH1 counts for genes related to auxin transport (Pin-formed, *PIN1*), auxin signal transduction (Small Auxin Up RNA genes, *SAUR-like*), jasmonates biosynthesis (jasmonates-O-methyltransferase-like, *JAOMe-like*) and gibberellins (GA) signal transduction (GA-Insensitive, *GAI1;* Repressor of Ga1-3-Like 1, *RGL1*). As concerns R-PH2, it is noteworthy that two abscisic acid 8'-hydroxylase –related genes were present in this cluster.

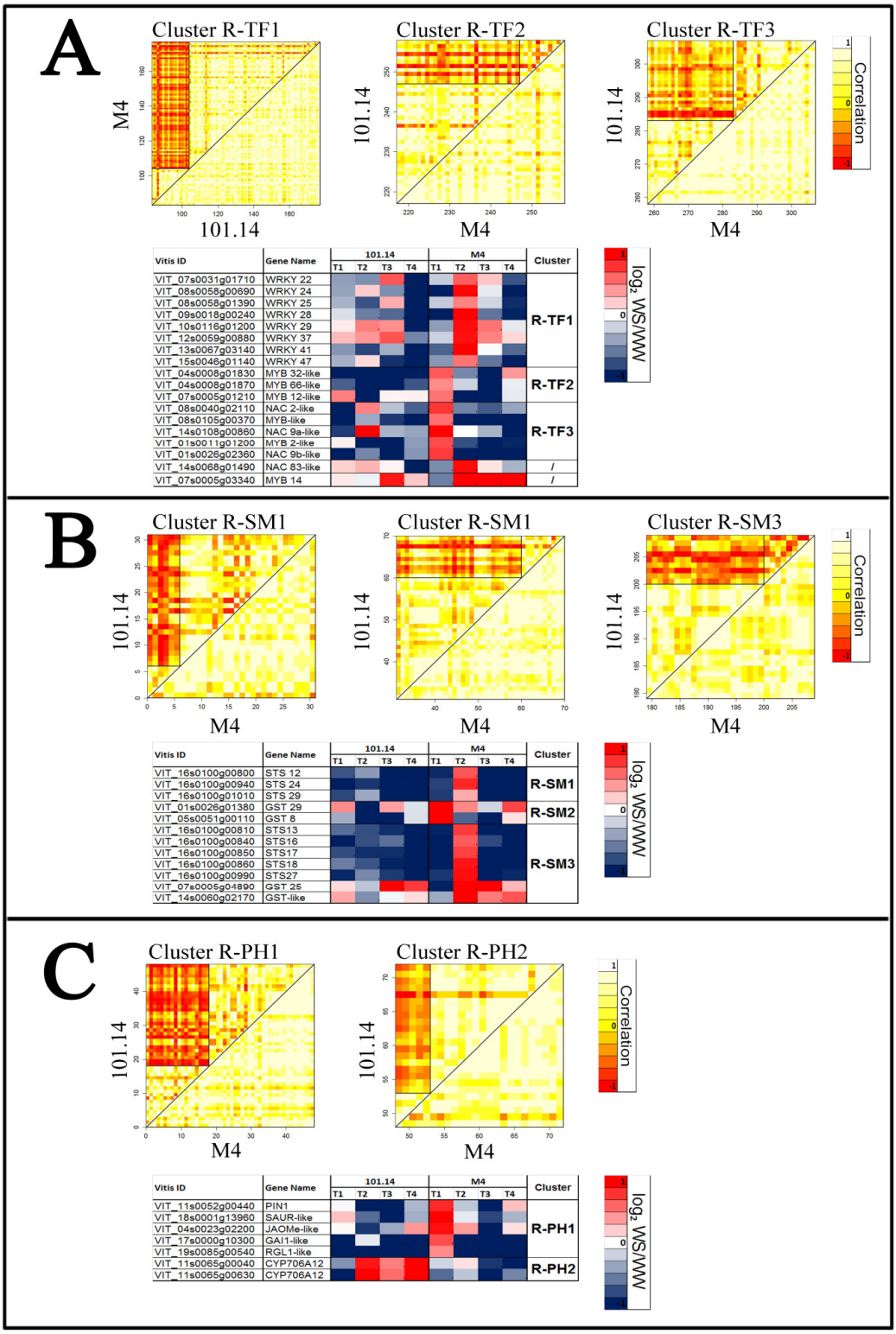

Figure 5. Differential cluster analysis results of root tissue. For correlation matrix (DCA), white (value = 1), yellow (value = 0), and red (value = -1) indicate a complete correlation, no correlation, or anti-correlation,

respectively, among transcripts. As concerns tables with list of considered genes, red and blue represent up- and down-regulation, respectively, of genes upon WS treatment.

Leaf tissue highlighted different results in comparison to root tissue. Within all considered categories, sugars (L-SG), secondary metabolism (L-SM) and transcription factors (L-TF) showed the most interesting results in the DCA. As previously described in the ontology analyses, sugars category is the most represented in terms of DEGs. Six transcripts contained in L-SG1 (partial conservation) cluster (Figure 6A) showed interesting expression kinetics. In particular the enolase, Pyruvate Kinase-like (*PK-like*) and 1,3-β-glucananase sugar-related genes were slightly induced at T1 and strongly up-regulated at T2 in M4 stressed leaves. On the other hand, Glucose-1-phosphate adenylyltransferase 1-like (*AGPase 1-like*) and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase -like (*PGK-like*) showed a severe induction in both T1 and T2 time points in the M4 rootstock. Finally, sugar transporter 13-like (*SUC 13-like)* was induced in M4 leaf at time points T2 and T3 (Figure 6A). On the contrary, these genes were down-regulated over all the kinetic in 101.14 leaves, considering $\log_2$ WS/WW values (Figure 6A).

The second considered category in the DCA analysis is secondary metabolism. This group highlighted very specific results if root and leaf tissues were compared. As previously described, root tissue –related clusters contained several genes mainly belonging to stilbene synthase and gluthatione-S-transferase families. In contrast, leaf tissue L-SM4 cluster included 12 interesting genes related to the flavonoid metabolic pathway, which were all induced at T1 and strongly expressed and induced at T2 in the M4 stressed leaves (Figure 6B). It is worthy of note that these genes are involved in most of the reactions which start from phenylalanine and lead to the biosynthesis of flavonols and anthocyanins –related compounds. Specifically L-SM4 (partial conservation) cluster contained: (i) Phenilalanine Ammonio Lyase (*PAL*), Chalcone Synthase (*CHS*) and Chalcone Isomerase (*CHI*) genes, that catalyzed the early reactions of the phenylpropanoids pathway; (ii) flavonoid 3-hydroxylase-related genes (*F3'5'H 2-like* and two *F3H-like*); (iii) Flavonol Synthase 4 (*FLS4*) and *FLS5*, which lead to the flavonols biosynthesis; (iv) Dihydroflavonol 4-Reductase (*DFR 4-like*), Leucoanthocyanidin Dioxygenase (*LDOX*) and Anthocyanidin Reductase (*ANR*), which lead to the anthocyanins biosynthesis.

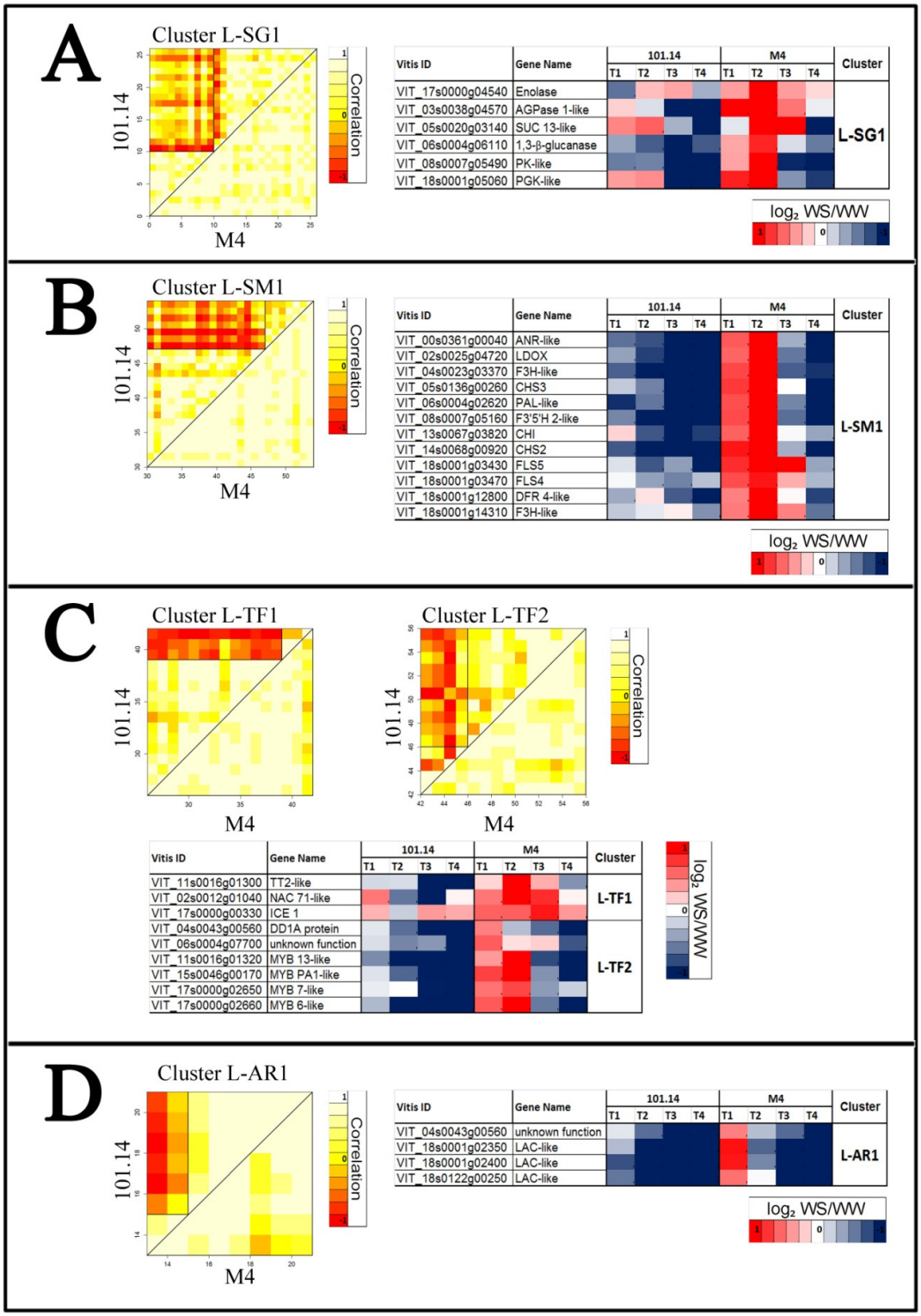

Figure 6. Differential cluster analysis results of leaf tissue. For correlation matrix (DCA), white (value = 1), yellow (value = 0), and red (value = -1) indicate a complete correlation, no correlation, or anti-correlation,

respectively, among transcripts. As concerns tables with list of considered genes, red and blue represent up- and down-regulation, respectively, of genes upon WS treatment

The third relevant category which comes from DCA is related to plant TFs (L-TF). Within this category, MYB family was the most represented gene family which showed opposite expression kinetic among M4 and 101.14. In L-TF2 (partial conservation) cluster (Figure 6C) four MYB TFs were identified, which were induced 2 and 4 days after stress imposition (T1 and T2) only in M4 rootstock. On the contrary all these TFs showed an higher expression in the 101.14 unstressed leaf in comparison to the stressed one (Figure 6C). In addition to these genes, L-TF2 cluster contained a rice DD1A *(DD1A-like)* gene and another gene with unknown function, which were both up-regulated at T1 in M4 leaf subjected to water stress. Transparent testa 2 (*TT2*-like) and *NAC 71-like* genes, that belongs to L-TF1 (partial conservation) cluster, were both up regulated in M4 (log_2 WS/WW) until 7 days after stress imposition (T3), while, Inducer of CBF expression 1 (*ICE1*) stress-related genes was strongly induced in M4 rootstock over all the kinetic considered in this experiment (Figure 6C).

Finally, the last considered category was the antioxidant responses (L-AR). Interestingly, in this group (split conservation) there were three genes belonging to the Laccase (LAC) family that highlighted an induction in M4 leaves in the early phase after water stress imposition (T1) (Figure 6D).

4. Discussion

In this study the transcriptome profiles obtained from leaves and roots of a drought-tolerant and a susceptible grapevine rootstock upon water stress were compared in order to clarify the molecular mechanisms explaining, at least at the transcriptional level, the different degrees of susceptibility observed. The novelty of the present work relies on two main aspects: firstly, to our knowledge this is the first report of a comparison between transcriptomes of a susceptible and a putative-tolerant grapevine rootstock, secondly, this work also introduces a comparison between the basal (roots) and the aerial (leaves) parts of the plants upon stress.

In order to reproduce as accurately as possible real open-field conditions and thus study an adaptive instead of a shock response to water stress, drought imposition was progressively applied to M4 and 101.14 plants by gradually reducing water addiction to desired field capacity from 80 % to 30 % during a 10 days treatment (Figure 1). This was a different experimental design compared to what observed in Cramer *et al.* (2007), where authors completely stopped water addiction from the beginning of water stress experiment.

During the first stages of water deprivation leaf physiological parameters decreased in both 101.14 and M4 genotypes, but after 6 days, when the stress became more severe (30% of field capacity), leaves transpiration was almost completely inhibited in 101.14 plants, whereas maintained *E* values of about 24% with respect to its control in M4 (WW plants). In the same experimental condition, Meggio *et al.* (IN PRESS) observed a concurrent decrease of g_s and A_n in both genotypes in the early stages of WS,, but at later time points, a different physiological response to water stress took place among the two genotypes. Indeed, an almost complete inhibition of both assimilation and transpiration rates was observed in 101.14 as stomatal conductance drop to values of 5 % with respect to its control. On the contrary M4, maintaining g_s values of 20 % with respect to its control, allowed higher transpiration rates (24 %) partially recovered A_n to values of approximately 60 % compared to control.

All these data indicates that, after a concurrent decrease of all physiological parameters observed in both genotypes in the early stages of drought (Meggio *et al.,* IN PRESS), as stress conditions became severe, M4 was able to maintain higher transpiration and net

assimilation rates demonstrating a much better ability to acclimatize in comparison to the susceptible genotype.

RNA-seq analysis helped us to clarify the molecular mechanisms which explain these differential responses. Aligning reads obtained from transcriptomes deriving from different grapevine species such as *V. riparia*, *V. rupestris*, *V. Berlandieri* and *V. vinifera* to the reference genome obtained from a high homozygous Pinot noir line, could be hazardous since structural variation and variability had been described even within the *vinifera* species, as observed by Venturini *et al.* (2013) aligning *V. vinifera* cv Corvina against the PN40024 12v1 reference genome. Citing other examples, in a large scale study in which the assemblies of 18 genomes coming from 18 natural *A. thaliana* accessions were compared (Gan *et al.*, 2011), was demonstrated that one-third of protein-coding genes predicted in Col-0 are disrupted in at least one of the other arabidopsis accessions and in some way restored under the form of alternative gene models. Moreover it was shown that gene expression of different ecotypes in the same conditions differs for nearly half of expressed genes and is associated with *cis* variants due to SNPs (approximately 0.5 to 0.8 Mln between Col-0 and the other ecotypes) and in/del (about 1,2 Mln).

Based on these observations seems to be necessary sequenced and reannotated each individual genomes to avoid bias interpreting the consequence of genetic variation and expression. Nevertheless, despite alignment of 101.14 and M4 transcriptomes against that PN40024 reference led to a "lost" of a remarkable number of reads which did not map to any prediction within the reference genome (approximately 50%), the high throughput of the instrument used for sequencing allowed to reach a suitable coverage of the grape transcriptome ranging from approximately 20 to 60 fold. Moreover, as we were dealing with a comparative approach, aligning reads to a common reference genome rather than making de novo assemblies for each single genotype was considered essential to establish comparisons and relations between transcriptional data obtained from the two genotypes. In these sense the PN40024 reference genome was utilized as a sort of common denominator for interpreting a part of the phenotypic variability observed between the two rootstocks.

Multifactorial analyses (Figure 2) on the whole leaf and root transcriptome datasets allowed us to better define the weight of the genotype (*i.e.* the rootstock) and of the

treatment on the transcriptome layout and to filter out those DE genes whose expression is only liked to the contribute of a single component. In other word we excluded those genes which are differentially expressed just because of differences amongst genotypes or because of treatment and we only consider those ones which were affected by the contribute of both the variables (McCarthy *et al.*, 2012).

A first general observation comparing results of the multifactorial analyses performed on leaves and roots is that in root tissue (Figure 2A) the "treatment" factor seems to be the main variable explaining differential gene expression (7905 DEGs) depend on the kind treatment imposed) whereas in leaf tissue (Figure 2B) the weight of the genotype (rootstock) appear to be the highest (3794). This observation is not surprising, considering that the root system is the first organ perceiving the water deprivation stress and the main one actively responding to it. In this case it's clear the kind of treatment imposed represent the main variable influencing expression whereas the effect of the genotype is less determinant on differential expression of genes. The opposite is true on the aerial part of plants: in leaves, the genotype factor appeared to have a major effect when compared to the other components. Frensch *et al.* (1997) observed that in maize plants undergoing water deprivation, roots and leaves use different strategies in response to stress and there is a preferential growth of root system in respect to shot. These differential responses were partially explained by the "cable theory", according to which the elongation zones in roots and shot are hydraulically separated and use different strategies to control water potential gradients. A possible explanation of this event is that, after a first stress perception mediated by roots, secondary signals which caused perturbations were perceived by leaves and the effect of the genotype in countering these perturbations is of primary importance.

As a general observation, the number of DEGs in roots was always higher compared to leaves, as reported in Figure 2, showing the Venn diagrams obtained from the multifactorial analysis, but also in Figure 3 showing the pairwise comparison within each timepoint considered. If we only compare those DEGs considered for the ontologies analysis (those ones common to R-T, and R-T- P components), there were 4072 DE genes detected in roots and only 1152 in leaf.

In root, TFs category was the most represented one with 7.5% of total DEGs belonging to it. On the other hand, in leaf tissue, the majority of DEGs belong to "sugars" category.

This is not unexpected considering that sugars accumulation during osmotic stresses has been reported in numerous species and tissues (Wingler and Roitsch, 2008; Sun *et al.*, 2010; Castellarin *et al.*, 2007; Valluru and Van den Ende, 2008).

As concerns plant hormones category, it was showed an induction of auxin, JAs and GAs related-genes at T1 in M4 stressed roots (Figure 5C), whereas a up-regulation of these transcripts in unstressed root was observed in 101.14. Regarding JAs metabolism, VvJAOMe-like (VIT_04s0023g02200) is a key enzyme in jasmonates biosynthetic pathway and is involved in the formation of methyl jasmonate (MeJA) from jasmonic acid (Wasternack, 2007; Seo *et al.*, 2001). MeJA mediates diverse developmental processes and defence responses against biotic and abiotic stresses in plants (Ismail *et al.*, 2012). Some authors showed, in rice and chickpea (root), a strong correlation between drought and MeJA biosynthesis and they hypothesize its role in water stress mediated response. They speculate that plant produced MeJA in response to water scarcity, which in turn stimulated the production of the "stress hormone" ABA (De Domenico *et al.*, 2012).

In figure 5C was observed an induction at T1 in M4 WS root of GAI1 and RGL1, which are essential components of GAs signal transduction. It is well known that, under environmental stresses, DELLA protein, such as RGL1, were accumulated and conferred more tolerance to plants (Achard *et al.,* 2008). Achard *et al.* (2008), showed that DELLA have an important role in oxidative stress responses by reduced ROS species; indeed, they observed that DELLAs delayed H_2O_2-induced cell death, thereby promoting stress tolerance (Achard *et al.*, 2008).

On the other hand, in leaf, DEGs related to sugars were the most represented in the ontology analysis (Figure 4) and they were strongly up-regulated in M4 leaves upon WS, conversely to what observed in 101.14 (Figure 6A). Among this category, VvAGPase1-like (VIT_03s0038g04570) where highly induced in M4, indeed it showed an expression which is three and ten times higher upon WS at T1 and T2, respectively (Figure 6A). AGPase is a key enzyme in starch biosynthesis and its expression is related to threalose accumulation (O'Hara *et al.*, 2013). In *Arabidopsis thaliana*, Kempa *et al.* (2008) showed that, in presence of osmotic stress, ADP-glucose pyrophosphorylase (AGPase1 and 2), which provides ADP-glucose, were induced. In addition they hypotesize a role of genes belonging to both starch mobilisation and starch synthesis processes when plants were

subjected to osmotic stresses (Kempa *et al.*, 2008). In addition, Gamm *et al.* (2011) found that AGPase activity was increased after infection of grapevine leaves with the oomycete *Plasmopara viticola*, together with accumulation of trehalose and the induction of trehalase.

Another category which showed particularly interesting results was the "antioxidant responses" one (Figure 4), in particular genes belonging to the laccase family were strongly induced at T1 only in M4 stressed leaves (Figure 6D). Laccase are part of another large family of enzymes, called the multicopper enzymes. They are present in both plants and fungi but their role in stress responses are still unclear (Mayer and Staples, 2002). As concerns abiotic stresses, Liang et al. (2006) demonstrated that maize *LAC1* expression was enhanced upon high salinity and they suggested an involvement of this class of enzymes in response to salt stress (Liang *et al.*, 2006).

"Secondary metabolism" ontology category was the only one in common among considered organs but involved different responses between root and leaves. It is worthy of note that root (Figure 5B) and leaf (Figure 6B) highlighted a strong specificity of DEGs expression kinetics, as observed in DCA analysis. In particular, in M4 root tissue was observed a strong up regulation of several *STS* transcripts (e.g. VvSTS18/24/27) after 4 DASI (T2) and an induction of four GST-related genes at T1 (GST8 and 29) and T2-T4 (GST25 and GST-like). It is well known that genes involved in resveratrol biosynthesis (*STSs*) increased their expression in response to several biotic and abiotic stresses (UV, high salinity, drought) but their role in these processes is still matter of debate (Versari *et al.*, 2001; Höll *et al.*, 2013; Vannozzi *et al.*, 2012). Stilbene and resveratrol synthase were expressed in several organs in response to abiotic stresses. A strong tissue specificity of resveratrol synthase was observed in peanut plants during development, indeed this compound was present at relatively high levels in both roots and pods organs, but at below the detection limit in leaves (Chung *et al.*, 2001). As concerns *Vitis* species, an induction of STSs genes in response to treatment was observed in *Vitis rotundifolia* hairy root after H_2O_2 treatment (Nopo-Olazabal *et al.*, 2014). Hydrogen peroxide is a ROS which have been suggested to play a key signaling role in plant responses to several abiotic stresses (such as extreme temperatures, drought, radiation, ozone, and wounding) treatment. In the above described work they highlighted a strong specificity in root tissue of these

transcripts in response to abiotic stresses perturbations (Nopo-Olazabal *et al.,* 2014). As showed by Wang *et al.* (2010), stilbenes synthases expressions were higher in both young root and leaves of *Vitis vinifera* cv. Cabernet sauvignon, but STS was highly expressed only in leaves when an UV treatment was carried out. In the present paper it was reported for the first time in *Vitis* a strong specificity of *STSs* genes expression in root tissue after water stress treatment, considering the induction of these transcripts observed in various and specific organs in response to other kind of abiotic (UV light in particular) and biotic stresses (Vannozzi *et al.*, 2012; Nopo-Olazabal *et al.*, 2014; Wang *et al.*, 2010; Shi *et al.*, 2014) we can hypnotized that the expression of this class of transcripts, listed in figure 5B, should be strongly related to drought treatment. In addition to STS, also GSTs transcripts (figure 5B) showed an up-regulation in M4 roots. Plant GSTs have also long been associated with responses to biotic and abiotic stress, hormones and developmental change; GSTs role in response to abiotic stress is due to their capability of normalizing ROS level (Kar, 2011; Dixon *et al.*, 2010).

On the other hand, in leaf tissue expression of genes related to secondary metabolism highlighted different responses to what observed in root. In this case it was observed a strong induction in M4 stressed leaves at T1 and a strongly induction at T2 (log_2 WS/WW of 2-4) of transcripts leading to flavonoids biosynthesis, such as CHS, F3H, FLS, LDOX and other genes involved in this pathway (Figure 6B). The up-regulation of transcripts and metabolites in response to drought in the aerial part of grapevine and other species were reported in other studies (Castellarin *et al.*, 2007; Ramakrishna and Ravishankar, 2011). Castellarin *et al.* (2007) showed that the majority of genes committed to the flavonoid pathway (*i.e.* CHS, F3'5'H, F3'H and UFGT) showed patterns of increased transcript accumulation in WS plants. In addition, drought often causes oxidative stress and was reported to show increase in the amounts of flavonoids and phenolic acids in willow (*Pisum sativum*) leaves (Chalker-Scott, 1999) and it was observed that plant tissues containing anthocyanins are usually rather resistant to drought (Larson, 1988).

As previously described, upon WS, roots and leaves of the tolerant genotype M4 exhibit an higher induction of stilbenes and flavonoids biosynthetic genes, respectively. But, why plants choose to synthesize these compounds which are metabolically expensive? A possible explanation is related to their roles antioxidant compounds.

Water stress, which can be define as “primary stress”, induces the accumulation of several compounds and increase the production of reactive oxygen species (Kar, 2011). Indeed, metabolic pathways in plant organelles are sensitive to changes in environmental conditions (such as drought and other stresses), and metabolic imbalances can induce a “secondary” oxidative stress, which caused oxidation of cellular components and may damage organelle integrity (Apel and Hirt, 2004; Suzuki *et al.*, 2012; Mittler, 2002). It is clear that ROS control and scaveging is mandatory for plant survival in presence of abiotic stresses. To protect themselves against oxidative damage and control ROS levels, plants evolved defence mechanism to scavenge ROS by producing a large numbers of molecules which acts as scavenger (*i.e.* SOD, CAT, APX) (Kar, 2011; Sozuki *et al.,* 2012). In addition to these scavengers, there are other compounds with antioxidant activity in plant. Stilbenes and flavonoids are claimed to have this important function in these processes. Stilbenoids (resveratrol in particular) are a powerful defence antioxidant molecules founded in several species and their accumulation is particularly higher in grapevine species (Tillett *et al.*, 2011; Höll *et al.*, 2013; Vannozzi *et al.*, 2012; Stuart and Robb, 2013). The chemistry of stilbenes in grape compounds is very complex. These antioxidant compounds act as phytoalexins, synthesized by plants in response to biotic and abiotic stress (Flamini *et al.*, 2013). Their antioxidant activity was extensively studied in humans (de la Lastra and Villegas, 2007; Hosoda *et al.*, 2013) but how stilbenes act as antioxidant in plants are still object of experiments. Also flavonoids, which were significantly induced in M4 leaves upon WS (Figure 6B), have been suggested to play an important function as antioxidant in plant response to oxidative stresses (Ramakrishna and Ravishankar, 2011; Brunetti *et al.*, 2013).

Indeed, they contrast the oxidative stress related to an excess of excitation energy in the chloroplast by absorb solar wavelengths (Agati *et al.*, 2012). Oxidative damage is strongly induced by environmental perturbations which limit CO_2 assimilation and its diffusion to the carboxylation sites (Brunetti *et al.*, 2013; Agati *et al.*, 2012; Hernández *et al.*, 2009). These events are exacerbated when plants were subjected to environmental stresses, such as drought or high salinity. This function of flavonoids may reduce the activity of enzymes which are claimed to acts as “primary” ROS scavenger (*i.e.* SOD and CAT) in the chloroplast (Brunetti *et al.*, 2013; Mullineaux and Karpinski, 2002). In addition,

flavonoids were capable of quenching H_2O_2 and other free-radicals species and to protect the chloroplast membrane from oxidative damage thanks to their capability to stabilizing membranes that contain non-bilayer lipids (Agati *et al.*, 2012).

Taking into accounts the above discussed data, transcripts related to stilbenoids, together with GSTs and DELLAs related-genes (figure 5B, 5C), and flavonoids (figure 6B) biosynthesis have, respectively, a root and leaf tissue-specific expression upon drought. In presence of water stress, M4 rootstock may acts differential mechanisms in root and leaves which leads to the production of molecules, such as resveratrol and flavonoids. These events may constitute a secondary antioxidant system in these plant tissues. The higher resistance of M4 rootstock to water stress, in comparison to what observed in 101.14, should be related to these events. Indeed, a possible explanation is that grapevine species which are drought-tolerant act, in addition to "primary mechanisms" of ROS scaveging, also other "secondary mechanisms" which leads to the biosynthesis of different secondary compounds in root and leaves. In this way, M4 (like other grapevine stress-tolerant genotypes) have a greater capability to control ROS homeostasis and prevent oxidative damages than the susceptible genotypes.

M4 and 101.14 showed an interesting differential regulation of flavonoids/stilbenoids-related genes in root and leaf, but how is regulated the transcription of these gene? Transcription factors could have an important role in these processes. Role of stress-responsive TFs is well documented, they interact with *cis*-elements in the promoter regions of several stress-related genes and thus up-regulate the expression of many downstream genes resulting in imparting abiotic stress tolerance (Agarwal and Jha, 2010).

Transcriptomic data suggest that plant TFs are involved in a large number of metabolic processes in response to abiotic stresses and that controlled the expression of a large number of genes by an intricate regulatory network (Lata *et al.*, 2011).

As showed in figure 5A, in root, three classes of TFs showed the major changes in transcript abundance between M4 and 101.14 in response to water deficit: WRKY, MYB and NAC families. Among these, WRKY were the most represented. WRKY proteins represent an important class of transcriptional regulators involved in plants biotic and abiotic stress (e.g. drought, high salinity, UV) induced pathways. In this experiment, a relatively large group of *VvWRKY* genes were strongly induced in M4 root 4 days after

stress treatment, these included *VvWRKY24*. Despite what observed by Wang et al., (IN PRESS), which hypotized a role of *VvWRKY24* only in response to cold stress, it was one of the most expressed in our dataset in response to drought. Interestingly, Li *et al.,* (2010) showed that *AtWRKY54,* which is ortholog to our *VvWRKY25* (Figure 5A), is significantly induced by cold and drought treatments, and by *E. necator* infection. These result suggests that *VvWRKY25* may mediating plant defense response in *V.vinifera* and also play a role in abiotic stress responses.

In our work, *VvWRKY24/28/29/37/41* exybith a co-expression with STSs genes (figure 5A), indeed they were all strongly induced at T2 only in M4, althought are ncessary more evidences,, it is might hypothesized that some WRKY are actively involved in the regulation of STSs gene expression . In addition to the WRKY family, also *VvMYB14* transcript were strongly induced in M4 stressed roots and its up-regulation paralleled those observed for STSs genes. As already observed by Höll *et al.* (2013), *VvMYB14* (figure 5A) control the expression of grapevine *VvSTS25/27/29* under UV-C and wounding conditions. In our experimental conditions the co-expression analysis reveals a cooperation between that*VvSTS27/29* and *VvMYB14* (Figure 5A) suggesting for the latter a possible role also in drought stress response. Except for *VvMYB2* (Figure 5A), whose *Arabidopsis* ortholog cooperate with *AtMYC2* as transcriptional activators of genes (as rd22) able to increase tolerance to drought (Abe *et al.*, 2003), the other stress-induced *MYBs* are not already indicated as stress-related TFs. However, it is worthy of note that they are co-expressed with *GST29-like* and *GST8-like* (Figure 5B), two genes involved, as described above, in the response to oxidative stress.

In WS root, last identified family of TFs in DEGs (Figure 5A), was that of NAC . Within this family, the involvement of *NAC2* (Figure 5A) in the regulatory network governing responses to stresses is well documented. In transgenic rice plants for *Os01g66120/OsNAC2/6* gene were showing an enhancement of drought tolerance (Nakashima *et al.*, 2007) or *AtNAC2* play a central role in the cross-talk among several hormonal metabolisms in response to salinity stress (He *et al.*, 2005).

In leaf tissue were observed a lower number of DEGs TFs-related, compared to root tissue, and most of them belong to the MYB-family. As well as in root, also in leaf MYB TFs are known to control several pathways in response to stress through the activation of a

large number of stress-responsive genes (Lata *et al.*, 2011; Ambawat *et al.*, 2013; Czemmel *et al.*, 2012). In this work, all *MYBs* identified as DEG were induced at T1 and T2 (Figure 6C) in the putative-tolerant rootstocks and their expression paralleled that observed for DEGs coding for flavonoids biosynthetic enzymes (Figure 6B). A strong connection between MYB and flavonoids is well documented (for a review see Czemmel *et al.*, 2012). R2R3-MYB proteins have been identified to be the key determinants in regulatory networks controlling not only the allocation of specific gene expression patterns during flavonoid biosynthesis, but also diverse aspects of development and responses to biotic and abiotic stresses which are not related to production of secondary metabolites (Ravaglia *et al.*, 2013; Stracke *et al.*, 2001). Among *VvMYB* genes (Figure 6C), *VvMYBPA1-like* was one of the most abundantly expressed (Figure 6C). *MYBPA* genes are known to induce the expression of biosynthetic genes (*CHS, CHI, DFR, LDOX* and *ANR*)involved in proanthocyanidins (PA) biosynthetic pathway, indeed PA accumulation has been shown to be regulate by the presence of *MYBPA1* and *MYBPA2* (Czemmel *et al.*, 2012). Together with *VvMYBPA1-like*, also *MYB12* is known to enhance the expression of several flavonoids biosynthetic genes, such as *CHS, CHI, F3H* and *FLS* (Ambawat *et al.*, 2013), which are all induced at T1-T2 in M4 WS leaf (Figure 6B). Furthermore, *MYB2* gene, which also is reported in our dataset, in *Arabidopsis* is known to be induced upon dehydration or salt stress (Ambawat *et al.*, 2013). In order to confirm the differences observed among M4 and 101.14 in the control of secondary metabolism – related genes by TFs, the next step in this work will be the analysis of the cis-elements present in the promoter region of both genotypes.

In this work we exhaustively described the transcriptomic responses to drought of two genotypes in root and leaf tissues. Despite to what observed in other studies (Cramer *et al.*, 2007; Tattersall *et al.*, 2007; Tillett *et al.*, 2011), we did not considered responses to water stress which were in common among susceptible and tolerant plants (e.g. ABA, photosynthetic and sugars –related genes) but only the expression of those genes which were strictly related to the tolerant rootstock. This allowed us to identify novel putative gene networks carried out by grapevine which showed an higher resistance to osmotic stress. In the near future, the expression of selected genes has to be evaluate on a large range of genotypes which showed differential responses upon drought in order to

understand if these genes can be used as functional markers for WS-tolerant grapevine plants.

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Chapter III

Grapevine rootstocks differentially affected ripening inception and auxin-related genes of Cabernet sauvignon berries

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1. Introduction

Grape berry development exhibits a double-sigmoid pattern characterized by two phases of rapid growth separated by a lag phase, during which little or no growth occurs (Coombe and McCarthy, 2000). The onset of ripening (named véraison) occurs at the end of this lag phase and involves the accumulation of sugars (hexoses) in the vacuoles of mesocarp cells, anthocyanins in the berry skin, catabolism of organic acids, and the development of other compounds related to flavour and aroma (Conde *et al.*, 2007; Symons *et al.*, 2006; Kuhn *et al.*, 2013). Several hormones may participate and interplay in the control of grape berry development and ripening, such as ethylene, auxin, abscisic acid (ABA), gibberellins (GAs), cytokinins (CKs) and brassinosteroids (BRs) (Davies and Böttcher, 2009). The early stages of berry development, from fertilization to the formation of the fruit (fruit set), are mainly driven by auxins, CKs and GAs that promote cell division and expansion. Although these hormones have a pivotal role in grape berry development, they are produced mostly by the seeds (Giribaldi *et al.*, 2010). The changes occurring from pre-véraison to full ripening are accompanied by significant increases in ABA content, this hormone claimed to be a ripening determinant in non-climacteric fruits such as grape berry (Kuhn *et al.*, 2013; Giribaldi *et al.*, 2010; Sun *et al.*, 2010). In addition to ABA, endogenous levels of BRs and ethylene transiently increase at pre-véraison and véraison, respectively. Exogenous applications of these hormones positively modulate many ripening-related processes such as the accumulation of anthocyanins, most likely by enhancing the transcription of *CHS, F3H, UFGT,* and *MYB1* genes (Symons *et al.*, 2006; Giribaldi *et al.*, 2010; Chervin *et al.*, 2008; Jeong *et al.*, 2004; Ziliotto *et al.*, 2012). These treatments can also induce the uptake and storage of sugars in the berries (Giribaldi *et al.*, 2010; Böttcher *et al.*, 2011). On the other hand, it is well known that exogenous application of auxin at the pre-véraison stage cause a shift in grape berry ripening and a repression of several ripening-related genes (Ziliotto *et al.*, 2012; Davies *et al.*, 1997).

Several studies pointed out that auxin levels increase during the early development of the grape berry (pre-véraison), and then decrease at the véraison stage. The decrease of auxin content is necessary, as demonstrated by the exogenous application of auxin at véraison that negatively affected the expression of genes encoding enzymes related to sugars and anthocyanins (*CHS, F3'H* and *UFGT*) biosynthesis and caused a delay in the reduction of

chlorophyll and acidity (Davies and Böttcher, 2009; Ziliotto *et al.*, 2012; Böttcher *et al.*, 2011). However, it was also observed that treatments with this hormone are positively correlated with the induction of genes related to ethylene biosynthesis (ACC-oxidase, *ACO*, and ACC-synthase, *ACS*) (Ziliotto *et al.*, 2012). This effect was not unexpected taking into account that high levels of auxin are required for the transition to auto-catalytic ethylene biosynthesis, the key event in the ripening of climacteric fruit (El-Sharkawy *et al.*, 2010; Tatsuki *et al.*, 2013).

Böttcher et al. (2010) speculate that auxin levels might be controlled throughout conjugation by GH3 proteins; in particular they showed that GH3 protein catalyzed auxin conjugation to amino acids, such as aspartate, and they observed that low levels of free auxin content correspond to high levels of conjugated auxins (Böttcher *et al.*, 2010).

Despite the obvious economic implications of controlling these processes, the mechanisms that regulate auxin homeostasis and its control during grape berry ripening remain unclear. In order to clarify this aspect, it is necessary to well investigate the molecular control of auxin homeostasis based on genes involved in biosynthesis (*TRYPS*), conjugation (*GH3*), action (*ARF* and *AUX/IAA*) and transport (*PIN*). In a previous paper (Ziliotto et al., 2012), it was hypothesized that exogenous application of the synthetic auxin NAA caused an excessive availability of auxin, most likely counterbalanced by homeostatic mechanisms which are activated after the treatment.

As above described, grape berry ripening is regulated by a complex regulatory networks in which also microRNA (miRNA) play a pivotal role. miRNAs are a class of non-coding RNA molecules with an important role as negative regulators of gene expression. In recent experiments (Carra *et al.*, 2011; Carra *et al.*, 2009; Wang *et al.*, 2012; Wang *et al.*, 2011), several conserved and novel miRNA, which showed a tissue specificity and have a putative role in grape berry ripening, has been characterized. Despite the large number of information given in the last years, miRNAs' roles in the regulatory network linked to grape berry ripening are only partially understood.

In addition to endogenous signals (i.e. hormones and miRNAs), grape berry ripening is modulated by exogenous stimuli such as environmental factors. Light, water availability and temperature severely affect grape berry ripening and quality, and may cause a shitf in the date of vintage (Kuhn *et al.*, 2013). Among these, water deficit has been the most

studied for its negative impact on grapevine yield and wine quality (Chaves *et al.*, 2010; Qin *et al.*, 2011). Generally, drought cause an accumulation of secondary (phenylpropanoids, especially anthocyanins and stilbenoids) and primary (sugars and organic acids) metabolites and their related transcripts in grape berries (Castellarin *et al.*, 2007; Deluc *et al.*, 2009). Grapevine responses to water deficit are heavily affected by the genotype; indeed, as observed by Deluc et al. (2009), under water stress, Cabernet sauvignon (CS) grape berries showed an higher accumulation of the "stress hormone" abscissic acid (ABA) and better responses to drought, in comparison to the white cultivar Chardonnay.

In order to counteracts these problems, in lasts years the use of rootstocks and the development of new genotypes has assumed greater importance in modern viticulture (Corso et al., chapter I of this thesis). Rootstocks provide tolerance to exogenous limiting factors, both biotic (e.g., soilborne pests) and abiotic (e.g., salinity, water or oxygen deficit), while influencing the ecophysiological behaviour of the scion and its berry quality (Tramontini *et al.*, 2013).

As above mentioned, an acceleration of ripening may negatively affect grape berry and wine quality. Consequently, further investigations about rootstocks and genotypes with improved tolerance to abiotic stresses are essential.

In order to evaluate the effect of rootstock on grape berry quality and development, a detailed investigation was performed at the transcriptomic level (mRNA-seq and microRNA-seq) on development and ripening of berries of *Vitis vinifera* L. cv Cabernet Sauvignon (CS) grafted either onto the "commercial" (i.e. the most commonly used) rootstock 1103P or onto a new rootstock, called M4 [(*V. vinifera* x V. berlandieri*) x V. berlandieri* xcv Resseguier n.1], the latter showing high tolerance to water deficit and salinity. Results pointed out differences in terms of berry ripening kinetics and a differential regulation of genes involved in auxin action (ARF and AUX/IAA), conjugation (GH3) and transport (PIN) among CS/M4 and CS/1103P.

2. Materials and methods

2.1 Plant Material and experimental design

Experiments were performed on *Vitis vinifera* L. cv Cabernet Sauvignon (CS) grafted onto 1103P and M4 rootstocks (Pasqua vigneti e cantine, Novaglie VR, Italy). Grapevines were grown in well-watered conditions.

Whole berries were collected from both CS/1103P and CS/M4 bunches, at 45, 59, 65 days after full bloom (DAFB). During this phase most berries reached véraison. Additional samples (skin and pulp separately) were collected at 72, 86 and 100 DAFB (Figure 1). All tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until use. Two biological replicates were collected, each made up of one hundred berries collected from fifty bunches (two berries per bunch) and chosen according to the CIRG index proposed by Carreño et al. (1995) at the same position within the cluster (median position). Sampling was performed in 2011 and 2012.

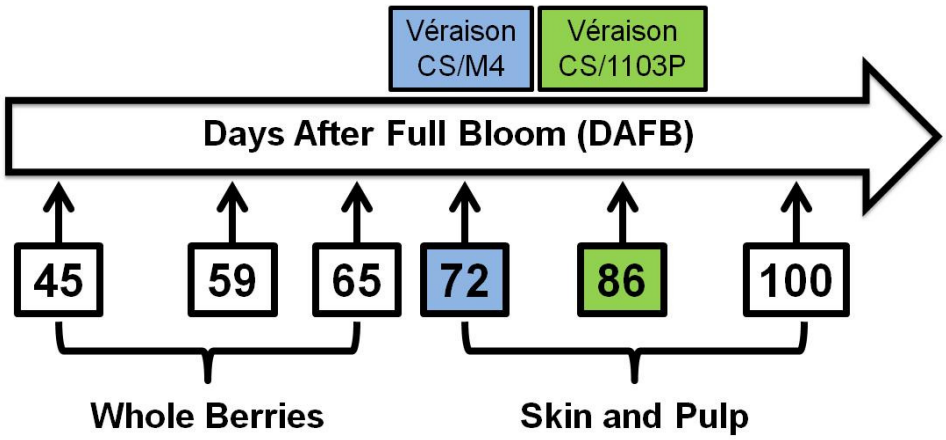


Figure 1. Schematic representation of experimental trial. The sampling dates of both CS/rootstocks berries were at 45, 59, 65, 72, 86 and 100 DAFB. Véraison of CS/M4 (72 DAFB) and CS/1103P (86 DAFB) are also indicated.

Physical (berry diameter and volume) and biochemical (Total Soluble Solids, Brix°) parameters were determined on 80 berries at each time point considered in the experiment. In order to better define grape berry ripening evolution in the two CS/rootstock combinations, colorimetric analyses were performed on 100 berries at 45, 59, 65, 72 and

86 DAFB, using the CR-10 colorimeter (Konica-Minolta Holdings Inc., Tokyo, Japan). Color was measured according to the L*a*b* space, defining brightness (L*, from white to black) and the chromatic coordinates (a*, from red to green; b*, from yellow to blue).

2.2 RNA-seq and qPCR analyses.

Total RNA for both mRNA-seq and real-time PCR experiments was extracted from either whole berry samples or from skin and pulp separately, using the perchlorate method as reported by Ziliotto et al. (2012). Small RNAs for microRNA-seq analyses were extracted from all samples following the CTAB method (Chang *et al.*, 1993), with few modifications.

mRNA was purified from the total RNA using the Dynabeads mRNA Direct kit (Invitrogen pn 610.12). A variable quantity of mRNA ranging from 0.4 to 1.6% with respect to the total RNA was obtained.

Samples for Ligation Sequencing were prepared according to the SOLiD Whole transcriptome library preparation protocol (pn 4452437 Rev.B). Samples were purified before RNase III digestion with Purelink RNA micro kit columns (Invitrogen, pn 12183-016), digested from 3 to 10 minutes according the starting amount of mRNA, reverse-transcribed, size selected using Agencourt AMPure XP beads (Beckman Coulter pn A63881), and barcoded during the final amplification. Obtained libraries were sequenced using Applied Biosystems SOLiD™ 5500XL , which produced paired-end reads of 75 and 35 nucleotides for the forward and reverse sequences, respectively. Reads were aligned to the reference grape genome using PASS aligner, a software able to perform several alignments on a sequence subset, trying different parameters to trim the low quality bases and select the best ones to maximize the number of aligned reads (Campagna *et al.*, 2009). The percentage identity was set to 90% with one gap allowed whereas the quality filtering parameters were set automatically by PASS. Moreover, a minimum reads length cut-off of 50 and 30 nt was set for the forward sequences and reverse reads, respectively. The spliced reads were identified using the procedure described in PASS manual (http://pass.cribi.unipd.it). Forward and reverse reads were aligned independently on the reference genome. PASS-pair was used from the PASS package to perform the pairing between forward and reverse reads and select only those sequences that are uniquely

aligned. The version 1 of grape gene prediction available at http://genomes.cribi.unipd.it/grape was used as a reference genome, whereas htseq-counts program (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html) was adopted to quantify gene transcripts abundance.

RNA-seq was carried out in three specific phases of berry development: pre-véraison, véraison and maturity. Specifically, pre-véraison. (45 DAFB) and traditional CS harvest date (100 DAFB) were the same for both combinations, while full véraison stage was at 72 and 86 DAFB for CS/M4 and CS/1103P, respectively (Figure 1). mRNA and small RNA pre-véraison (whole berries), véraison and harvest (skin and pulp) for both CS/rootstocks combinations were used for mRNA-seq and microRNA-seq analysis, performed at CRIBI, University of Padova.

For quantitative real-time PCR analysis (qPCR), cDNA was synthesized using 2 µg of total RNA, 2.5 µM $(dT)_{18}$ primer, 200 Units of M-MLV Reverse Transcriptase (Promega) and 1 Unit of RNAguard (Amersham Biosciences), at 37°C for 90 minutes in a final volume of 20 µL. qPCR was carried out in triplicate on two biological replicates for each sample with StepOne Plus Real-Time PCR System (Applied Biosystems) by using specific primers listed in Supplementary table 1.

For microRNAs quantification, cDNA synthesis and qPCR experiments were carried out using the TaqMan® MicroRNA Assays (Life Technologies) according to the manufacturer's instructions.

2.3 Statistical and bioinformatics analyses on mRNA-seq and microRNA-seq data

The R package DEseq (Anders and Huber, 2010) was used for the statistical analyses of both mRNA-seq and microRNA-seq data. A false discovery rate (FDR) of 0.05 was set up as a threshold for identifying differentially expressed genes (including those encoding miRNAs). A pairwise comparison between M4 and 1103P genotypes was accomplished for each couple of samples at each time point (Pre-véraison, Véraison, Harvest).

In order to cluster together transcripts and microRNAs with complementary behaviors, a Time Course Cluster analysis using the Mfuzz R package (Kumar and Futschik, 2007) was performed.

The Gene Ontology terms were retrieved and imported in the Blast2GO software v2.5.0 (Götz *et al.*, 2008). Enrichment analysis was performed for each set of differentially expressed genes with the built-in Fisher's exact test function with $P \leq 0.01$ and FDR correction.

2.4 Phylogenetic analyses of *ARF and AUX/IAA* auxin-related gene families

A characterization of grape ARF and AUX/IAA gene families and the consequent association with the gene expression data were carried out.

For the characterization of these gene families, all *Solanum lycopersicom* and *Arabidopsis thaliana* ARF (Guilfoyle *et al.*, 1998; Kumar *et al.*, 2011) and AUX/IAA (Reed, 2001; Wu *et al.*, 2012) proteins were blasted and aligned against *Vitis vinifera* PN40024 12X v1 proteome (http://genomes.cribi.unipd.it/DATA/), by using MEGA4 software (http://www.megasoftware.net/mega4). To confirm the presence of the protein domains related to ARF (B3 + ARF + AUX/IAA domains, the last one is optional) and AUX/IAA (AUX/IAA domain), all putative proteins related to these families were checked by using InterProScan software (http://www.ebi.ac.uk/Tools/pfa/iprscan/). To check sequence similarities and genetic distance between *Vitis vinifera*, *Solanum lycopersicom* and *Arabidopsis thaliana ARF* and *AUX/IAA* families. The ARF and AUX/IAA aminoacids sequences of tomato, Arabidopsis and grape were aligned by using a pairwise (Gap opening penalty = 10; gap extension penalty = 0.1) and a multiple (Gap opening penalty = 10; gap extension penalty = 0.2) alignments with ClustalW (MEGA4 software; Tamura *et al.,* 2007). The phylogenetic tree was constructed by using the neighbor-joining method of MEGA4 software. Finally, expression level of the respective transcripts were checked on the mRNA-seq data.

3. Results

3.1 Physical and biochemical analyses

In order to evaluate the differences in grape berry ripening and quality parameters between CS/1103P and CS/M4, physical (volume and color) and chemical (Soluble Solids Concentrations; SSC) parameters were determined (Figure 2 and 3). Measurements were carried out on one hundred berries collected from fifty bunches (two berries per bunch) sampled in 2011 and 2012.

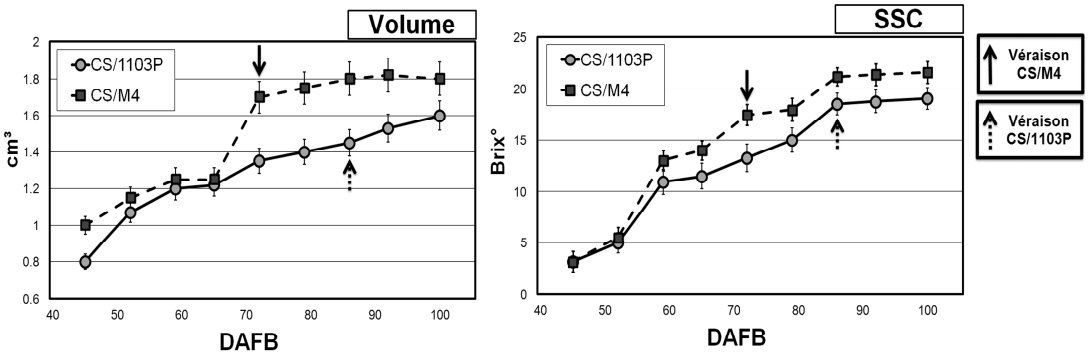


Figure 2. Berry volume and soluble solids content in CS/1103P (circle) and CS/M4 (square) throughout fruit development. Solid and dotted arrows indicate véraison of CS/M4 and CS/1103P, respectively. Bars represent the SD.

Grape berry volume followed different kinetics in CS/1103P compared to CS/M4 (Figure 2). At pre-véraison (45 GDF), the volume of CS/M4 (1 cm^3) berries was greater than that observed for CS/1103P (0.8 cm^3), while in the subsequent stages (52-65 GDF) there were no significant differences among rootstocks. At 72 DAFB, when most of CS/M4 berries reached véraison, volume values measured for CS/M4 were significantly higher (+0.35 cm^3) compared to those observed for CS/1103P. These differences were maintained up to 86 DAFB, when CS/1103P berries showed a significant increase in volume. At harvest time (100 DAFB) volume was similar in both rootstocks, indicating a partial recovery for CS/1103P berries.

The above mentioned differences among CS/rootstock combinations were confirmed also for SSC (Soluble Solids Concentrations) values (Brix°) (Figure 2). SSC measurements indicated that, at the pre-véraison stages (45-65 DAFB), content of total soluble solids (related to the sugars content) was similar between rootstocks. From 72 DAFB, CS/M4

displayed a higher increase for SSC. CS/1103P berries increased their soluble solids content at 86 DAFB (18.55), when it reached values closed to those of CS/M4 berries at véraison (72 DAFB; brix° = 18). Thereafter, SSC contents were similar between CS/rootstock combination, although CS/M4 showed slightly higher values.

Concerning the colorimetric measurements (Figure 3), at pre-véraison all samples were in the same situation in terms of ripening evolution (diamond indicator), while later on there were different kinetics related to pigmentation evolution between 1103P and M4 rootstocks, as also observed in terms of berry volume. Indeed, CS grafted on 1103P showed a delay, in terms of pigmentation evolution, in comparison to the other CS/rootstock combination (square indicator). At post-véraison (circle indicator) all samples reached the same pigmentation levels, confirming the recovery of CS/1103P showed also for volume and SSC parameters (Figure 2)

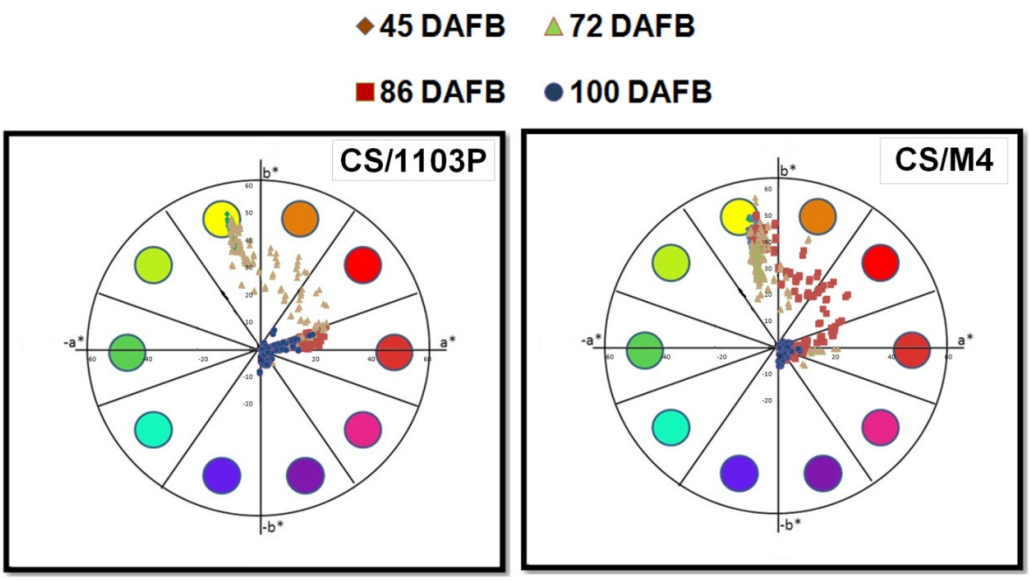


Figure 3. Colorimetric analyses, conducted on four time points, of CS/1103P and CS/M4 rootstocks, corresponding to different development conditions.

Considering these results, rootstocks seem to induce a different ripening kinetics, affecting the degree of pigmentations and physical/chemical parameters, such as volume and sugars content (SSC). Thus, to better evaluate the effect of rootstock on berry ripening is

mandatory to “synchronize” ripening onset in the two CS/rootstocks, combinations. For this goal among samples collected in 2011 were identified two common phases (indicated as pre-véraison and véraison) between CS/1103P and CS/M4, by combining physical, chemical, colorimetric data and a preliminary expression profiles analysis, performed via qPCR technique (Figure 4), of key genes of flavonoid (chalcone synthase 3; CHS3; flavonol synthase 1, FLS1; Leucoanthocyanidin reductase 1 and 2, LAR1 and LAR2) and flavone and flavonols (UDP-glucose:flavonoid 3-O-glucosyltransferase, UFGT) pathways (Figure 4) to perform RNA-seq analyses. An additional transcriptome analysis was performed close to the commercial CS vintage date (100 DAFB) to evaluate how the expression of genes related to late ripening phase was evolved in the two combinations. This sampling date was chosen taking into account that at the commercial vintage no significant differences ($p < 0.05$) for traditional ripening parameters (brix°, pH and titratable acidity) were observed between the CS/M4 and CS/1103P ripe berries. No significant statistical differences ($p < 0.05$) at commercial CS vintage were observed also when the analysis of traditional ripening parameters was extended to an historical series of seven years (data not showed). However, samples collected close to the commercial CS vintage can give us information about differences, still present, at transcriptomic level between the two combinations.

Considering these findings, pre-véraison was confirmed to occur for both rootstocks at 45 DAFB, while véraison appeared to be different for CS/M4 (72 DAFB) and CS/1103P (86 DAFB) and full ripening was the same for all samples (100 DAFB).

Samples of both CS/rootstock combinations corresponding to pre-véraison, véraison and full ripening phenological phases were chosen for the following mRNA-seq and microRNA-seq analyses.

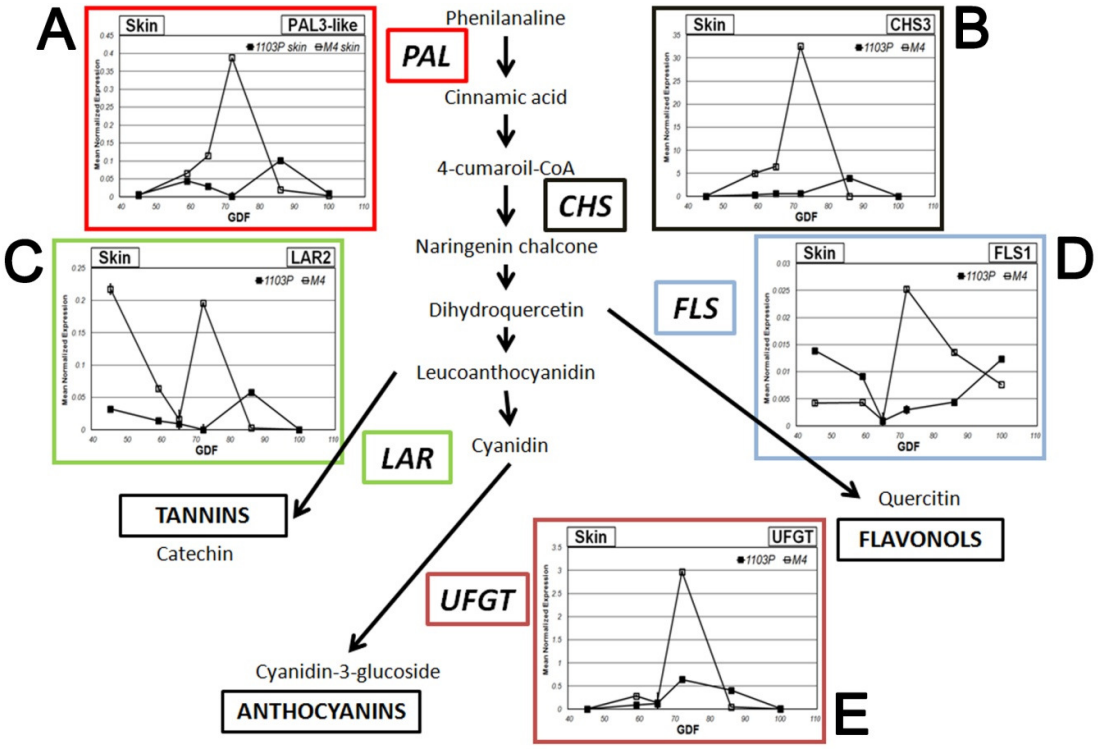


Figure 4. Preliminary qPCR analyses conducted on all samples during the entire ripening phase. Graphs related to *PAL3*-like, *CHS3*, *LAR2*, *FLS1* and *UFGT* genes are shown. Bars represent standard error.

PAL3-like (Figure 4A), *CHS3* (Figure 4B), *LAR1* (data not showed) and *LAR2* (Figure 4C) showed similar expression kinetics. In particular, a peak of expression was observed at 72 DAFB in CS/M4 showed and at 86 DAFB in CS/1103P, confirming, as discussed above, a delay in ripening inception and skin pigmentation evolution for the latter combination. For *FLS1* (Figure 4D) and *UFGT* (Figure 4E) genes, only M4 showed a peak at 72 DAFB (Figure 4D, 4E).

3.2 Differentially expressed (DE) genes, DEmiRNA and time course clustering analyses

As previously described, mRNA-seq and microRNA-seq analyses were performed on CS/1103P and CS/M4 berries at pre-véraison, véraison and harvest phases. A detailed description of samples used for these analyses was given in table 1.

Table 1. Samples used for mRNA-seq and microRNA-seq analyses. DAFB, sampling date, phenological phases and sampled tissues are given. Two biological replicates of all samples were used for RNA-seq analyses

DAFB	Date	Phenologycal phase	Sampled tissue
45	13-lug	Pre-véraison	Whole berry
72	09-ago	Vèraison CS/M4	Skin and pulp
86	23-ago	Vèraison CS/1103P	Skin and pulp
100	06-set	Harvest	Skin and pulp

As concerns statistical analyses, the pairwise comparisons between M4 and 1103P genotypes were accomplished at each time point (Pre-véraison, Véraison, Harvest), in according to a $p<0.05$. Differentially expressed genes (DEG) or Differential expressed miRNA (DEmiRNA) were identified calculating the $\log_2$-ratio (M4 versus 1103P) of the counts detected for each gene (or miRNA) in CS/M4 and CS/1103P combinations. Among comparisons, DEG were: 662 at pre-véraison; 3603 and 3412 at véraison in skin and pulp, respectively; 738 and 2618 at harvest in skin and pulp, respectively. To classify DEGs in relation to their molecular function and cellular processes, a Gene Ontology (GO) term enrichment analysis was performed (data not showed). As regards genes more expressed in CS/M4 combination (positive $\log_2$-ratio), DEGs belonging to "photosynthesis" (GO:0015979), "photosystems" (GO:0009521), "thylakoids" (GO:0009579), " cell wall organization and biogenesis" (GO:0071554) and "pectate lyase activity" (GO:0030570) were over-represented in both tissues at véraison stage (72 DAFB), whereas at harvest (100 DAFB) this over-representation was observed only in pulp tissue. As concerns plant hormones, at véraison several significant GO categories were found in CS/M4, specifically the GO terms "response to ethylene stimuli " (GO:0009723, GO:0009873), "responses to auxin stimuli " (GO:0009733), "auxin signaling" (GO:00009734) were over-represented in pulp. At harvest, GO categories related to "abscisic acid -mediated signaling pathway" (GO:0071215) and "cellular responses abscisic acid" (GO:0009738) were over-represented only in pulp.

Among DEGs observed in skin and pulp of CS/1103P berries (negative $\log_2$-ratio), sampled at pre-véraison and véraison stage, were over-represented that annotated in GO categories related to "cell wall organization and modifications" (GO:0005618,

GO:0071554, GO:0042545). As regards transcripts related to secondary metabolism, an over-representation of GO terms related to “metabolism and biosynthesis of phenylpropanoids and flavonoids” (GO:0009698, GO:0009699, GO:0009812 , GO:0009813) was observed at pre-véraison and harvest in pulp tissue. Categories related to stress responses, such as "response to biotic stimulus" (GO:0009607), "defenses responses" (GO:0006952) and "response to stress" (GO:0006950), were significantly over-represented at pre-véraison. At harvest, it was observed an over-representation of GO terms related to plant hormones, specifically those related to "cellular responses to auxin stimuli" (GO:0071365) and "signal transduction pathway auxin mediated" (GO:0009734).

For what concerns miRNA-seq, only 30 novel and conserved DEmiRNA were identified (in at least one time-point) with statistical analysis. This result is not unexpected considering that the analysis was carried out with a total of 471 miRNA.

In order to shed light about differences in CS/M4 and CS/1103P ripening regulation mediated by miRNA, DEmiRNA and the respective target genes were identified in pulp and skin gene datasets. To obtain this information, firstly DEGs and DEmiRNA, separately for each tissue, were subjected to a Time Course Cluster analysis, using the Mfuzz R package (Kumar and Futschik, 2007).,. This analysis allowed us to identify six and seven clusters for the DEG with different kinetic in skin and pulp, respectively, and six clusters for differentially expressed miRNA in both skin and pulp. The last step step was to identify target genes of differentially expressed miRNA and to check if these targets were significantly differentially expressed in our experiment.

In skin tissues, miRNAs that showed an opposite behaviour in comparison to the respective genes-target were associated with metabolic categories mainly related to plant growth (figure 5A). In particular miR396, which has Growth-regulating factor 9 (*GRF9*) as target, showed a significant up-regulation at véraison and harvest in CS/M4 berries.

On the other hand, in pulp tissue genes which seem to show a control mediated by miRNA belonged to growth regulation, auxin transcription factors, Homeobox-leucine zipper transcription factors and detoxification -related metabolic categories. Indeed, miR167 (figure 5B) and miR166 (figure 5E) were strongly up-regulated in CS/M4 at harvest, and their relative gene-target *ARF3/ARF6/ARF8* and *REVOLUTA-PHABULOSA* transcription factors (TFs), were down-regulated at the same phenological phase. Among pulp-related

clusters, other miRNAs showed an interesting behaviour; in particular miR160 (figure 5C), which repressed *ARF10A* and *ARF16*, and miR171 (figure 5D), which control the expression of *SCARECROW 6 (SCL6)* and *SCL-like* transcripts, where both down-regulated at pre-véraison and significantly induced at véraison stages. The last two considered miRNA were miR172 and miR159a (figure 5F, G). The first one repressed an *AP2-like* transcription factor (TF) showed and high up-regulation in CS/M4 at véraison and a slightly induction at harvest; conversely, the second one was induced in CS/1103P at pre-véraison and véraison stages and negatively regulated the expression of *GLIOXALASE 1 (GLX1)*. From these results and available literature (Ziliotto *et al.*, 2012; Davies *et al.*, 1997) is emerging the critical role of auxin in the modulation of grape berry ripening thus we decide to analyse in deep genes involved in the regulation of auxin action.

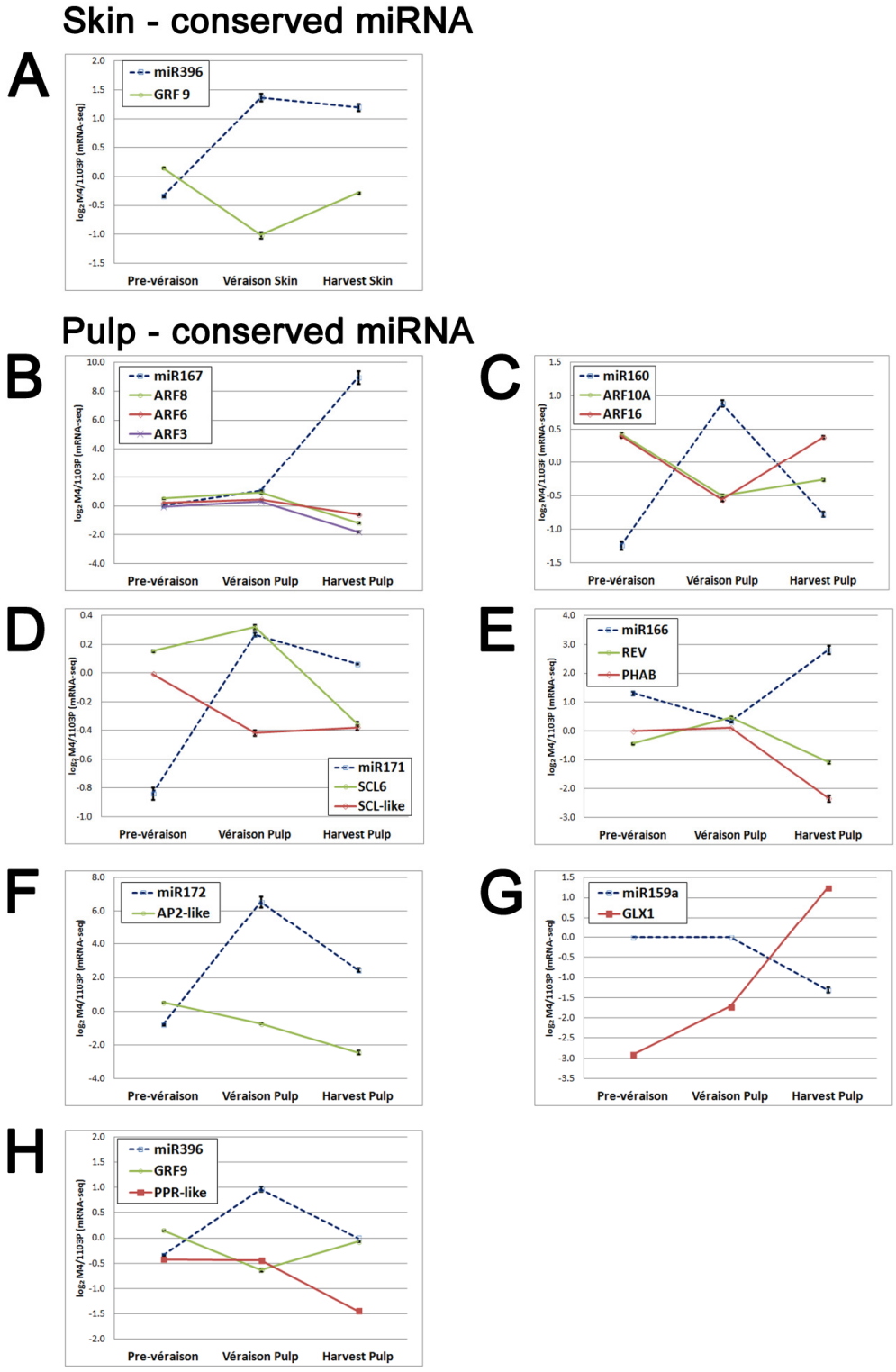


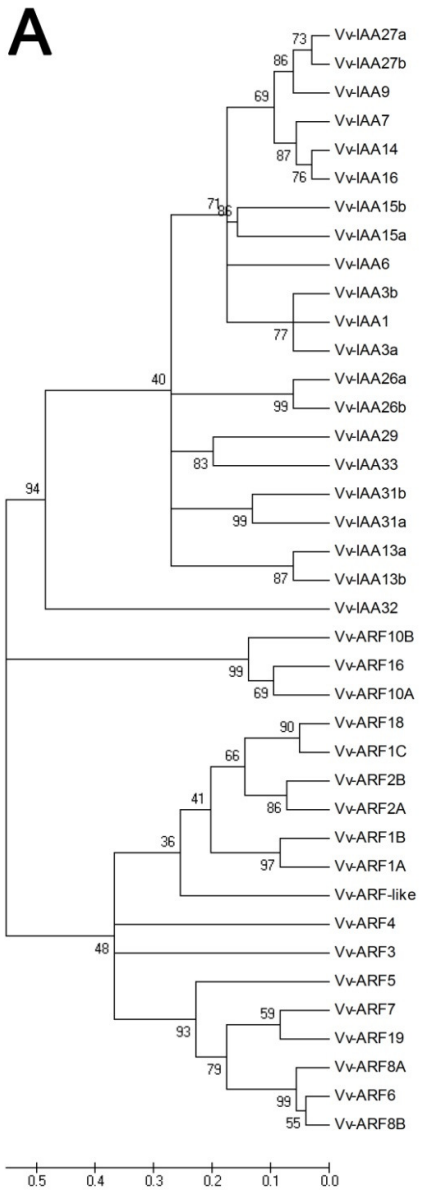
Figure 5. Expression pattern of miRNA (microRNA-seq data) and their respective genes target (mRNA-seq data. Expression values of RNA-seq data were al given as $\log_2$ (M4/1103P) ratio. Bars represented SE.

3.3 Characterization and phylogenetic analyses of grape AUX/IAA and ARF auxin-related families

In order to characterize AUX/IAA and ARF putative grapevine protein, a phylogenetic analysis was carried out. *AUX/IAA* family is characterized by the AUX/IAA protein domain, while *ARF* proteins contains B3, ARF and the optional AUX/IAA domains. It is well known that aux/iaa inactivate arf proteins, with the formation of an heterodimer. All putative proteins which exhibit the typical domains of arf and aux/iaa proteins were blasted again family members previously identified in *Arabidopsis thaliana* and *Solanum lycopersycom* (Supplementary figures 1 and 2).

In total were identified 18 and 21 proteins of grape belonging to *ARF* and *AUX/IAA* gene families (figure 6A, B), respectively; all proteins sequences contains domain related to these gene families, so it can be concluded which they are good candidates to be member of these categories of auxin transcription factors. In a previous work, 26 grape AUX/IAA putative proteins were identified (Çakir *et al.*, 2013). Given the different results obtained, in this work we re-checked all the putative proteins identified in the other experiment and we found several identified sequences which contains with B3 and ARF domains, typical of the ARF proteins and not of the AUX/IAA ones. So, it can be concluded that the previous analysis conducted by Çakir et al. (2013) was not exhaustive and contained some mistakes , thus we decided to use our phylogenetic characterization.

Arf putative proteins were distributed among 13 chromosomes and highlighted a varied sequences length among its members. Indeed, the longest sequence was 1155 aa-longer (VvARF19) and the lowest was 623 aa-longer (VvARF16) (Figure 6B). On the other hand, aux/iaa putative proteins were characterized by a distribution among a fewer number of grape chromosome (eight in total) and more uniform length. Between these family, the length of the sequences were lower in comparison to those observed for *ARF* family, with an average of 254 aa (Figure 6B). Expression data derived from mRNA-seq analyses were associated to annotated ARF and AUX/IAA putative transcripts identified in the version V1 grape annotation. In order to selected the transcripts for qPCR experiments, each transcript was associated with the number of RNA-seq counts and an heat-map with all values ($\log_2$ M4/1103P) was created (Supplementary figures 3 and 4).



B

V1 12X	Name	Lenght (aa)	Chr	Domain(s)
VIT_01s0244g00150	Vv-ARF2B	769	1	B3 - ARF - AUX/IAA
VIT_02s0025g01740	Vv-ARF1C	682	2	B3 - ARF - AUX/IAA
VIT_04s0079g00200	Vv-ARF8B	707	4	B3 - ARF - AUX/IAA
VIT_06s0004g02750	Vv-ARF10A	711	6	B3 - ARF - AUX/IAA
VIT_06s0004g03130	Vv-ARF4	798	6	B3 - ARF - AUX/IAA
VIT_07s0104g01230	Vv-ARF-like	696	7	B3 - ARF - AUX/IAA
VIT_08s0040g01810	Vv-ARF16	623	8	B3 - ARF - AUX/IAA
VIT_10s0003g00420	Vv-ARF3	779	10	B3 - ARF
VIT_10s0003g04100	Vv-ARF6	908	10	B3 - ARF - AUX/IAA
VIT_11s0016g00640	Vv-ARF19	1155	11	B3 - ARF - AUX/IAA
VIT_11s0065g00310	Vv-ARF7	1115	11	B3 - ARF - AUX/IAA
VIT_12s0028g01170	Vv-ARF8A	927	12	B3 - ARF - AUX/IAA
VIT_12s0035g01800	Vv-ARF1B	678	12	B3 - ARF - AUX/IAA
VIT_13s0019g04380	Vv-ARF10B	679	13	B3 - ARF - AUX/IAA
VIT_15s0046g00290	Vv-ARF18	702	15	B3 - ARF - AUX/IAA
VIT_17s0000g00320	Vv-ARF2A	862	17	B3 - ARF - AUX/IAA
VIT_18s0001g13930	Vv-ARF5	953	18	B3 - ARF - AUX/IAA
VIT_18s0089g00910	Vv-ARF1A	649	18	B3 - ARF - AUX/IAA
VIT_01s0011g04070	VvIAA32	202	1	AUX/IAA
VIT_04s0008g00220	VvIAA26a	345	4	AUX/IAA
VIT_04s0008g05560	VvIAA29	227	4	AUX/IAA
VIT_05s0020g01070	VvIAA31b	198	5	AUX/IAA
VIT_05s0020g04680	VvIAA3b	168	5	AUX/IAA
VIT_05s0020g04690	VvIAA14	243	5	AUX/IAA
VIT_05s0049g01970	VvIAA13a	321	5	AUX/IAA
VIT_07s0005g02550	VvIAA31a	175	7	AUX/IAA
VIT_07s0005g04380	VvIAA13b	306	7	AUX/IAA
VIT_07s0141g00270	VvIAA1	186	7	AUX/IAA
VIT_07s0141g00290	VvIAA7	238	7	AUX/IAA
VIT_09s0002g03410	VvIAA26b	439	9	AUX/IAA
VIT_09s0002g04080	VvIAA27b	320	9	AUX/IAA
VIT_09s0002g05150	VvIAA6	192	9	AUX/IAA
VIT_09s0002g05160	VvIAA15b	294	9	AUX/IAA
VIT_11s0016g03540	VvIAA27a	320	11	AUX/IAA
VIT_11s0016g04490	VvIAA15a	216	11	AUX/IAA
VIT_11s0016g05640	VvIAA33	184	11	AUX/IAA
VIT_14s0030g02310	VvIAA3a	199	14	AUX/IAA
VIT_14s0081g00010	VvIAA16	170	14	AUX/IAA
VIT_18s0001g08090	VvIAA9	410	18	AUX/IAA

Figure 6. A. Phylogenetic tree of all putative aux/iaa and arf proteins. **B.** List of arf and aux/iaa putative proteins. V1 12X identifier (V1 12X), protein name (name), length (aminoacids; aa), chromosome (Chr) and domain(s) were given.

3.4 CS/1103P and CS/M4 berries highlights differential regulation of auxin-related genes.

Expression patterns of some selected *ARF* and *AUX/IAA*-related genes were investigated along the whole berry developmental kinetic by using qPCR (Figure 6). In addition to those, some selected transcripts belonging to *GH3* (Böttcher *et al.*, 2011) *PIN* and *ABCB* gene families were also tested by qPCR.

These analyses highlighted that transcripts expression was strongly affected by the scion/rootstock combination, tissue and grape berry ripening kinetic (Figure 7, 8, 9).

As concern *ARF* gene family, qPCR analyses were conducted on *VvARF1A* (VIT_18s0089g00910), *VvARF5* (VIT_18s0001g13930), *VvARF8A* (VIT_12s0028g01170), *VvARF8B* (VIT_04s0079g00200), *VvARF10A* (VIT_06s0004g02750), VvARF10B (VIT_13s0019g04380) (Figure 7A-F).

A first general observation is that ARF-related genes were highly induced in CS/M4 at 45 DAFB (pre-véraison stage), in comparison to what observed in CS/1103P. This is true for all ARF transcripts, except for *VvARF5* which showed a slightly induction at 45 DABF in CS/M4 (figure 7B). On the other hand, in CS/1103P berries was observed a delay in the induction of these transcripts, indeed a peak was registered at 65 DAFB. This observation was true for the most of ARF studied, in particular for *VvARF1A, VvARF5, VvARF8A* and *VvARF10B* (Figure 7A, B, C, F). In the sequent developmental stages (after 65 DAFB) 1103P caused a general induction of these genes in CS berries, in comparison to M4, with some differences related to the timing and the analyzed tissue (skin or pulp). In particular, *VvARF5* (figure 7B) was induced in CS/1103P at 86 and 100 DAFB in both tissues, highlighting a involvement for this genes in the late phases of ripening. Expression of *VvARF8B* (figure 7D) was related to tissue and developmental stages, indeed it was slightly induced at 72-86 DAFB and strongly up-regulated at 100 DAFB only in pulp tissue of CS/1103P. In addition, this transcript was induced in skin tissue of CS/M4 only at 86 DAFB. *VvARF8A* (figure 7C) showed an expression-specificity which is related to tissue, stages and CS/rootstock combination, with an earlier induction in the skin of CS/M4 (72 DAFB) in comparison to that observed for CS/1103P (86 DAFB). Finally, it is worthy of note that *VvARF10B* (figure 7F) transcript was always induced by 1103P rootstock in both CS tissues and during the entire grape berry ripening kinetics.

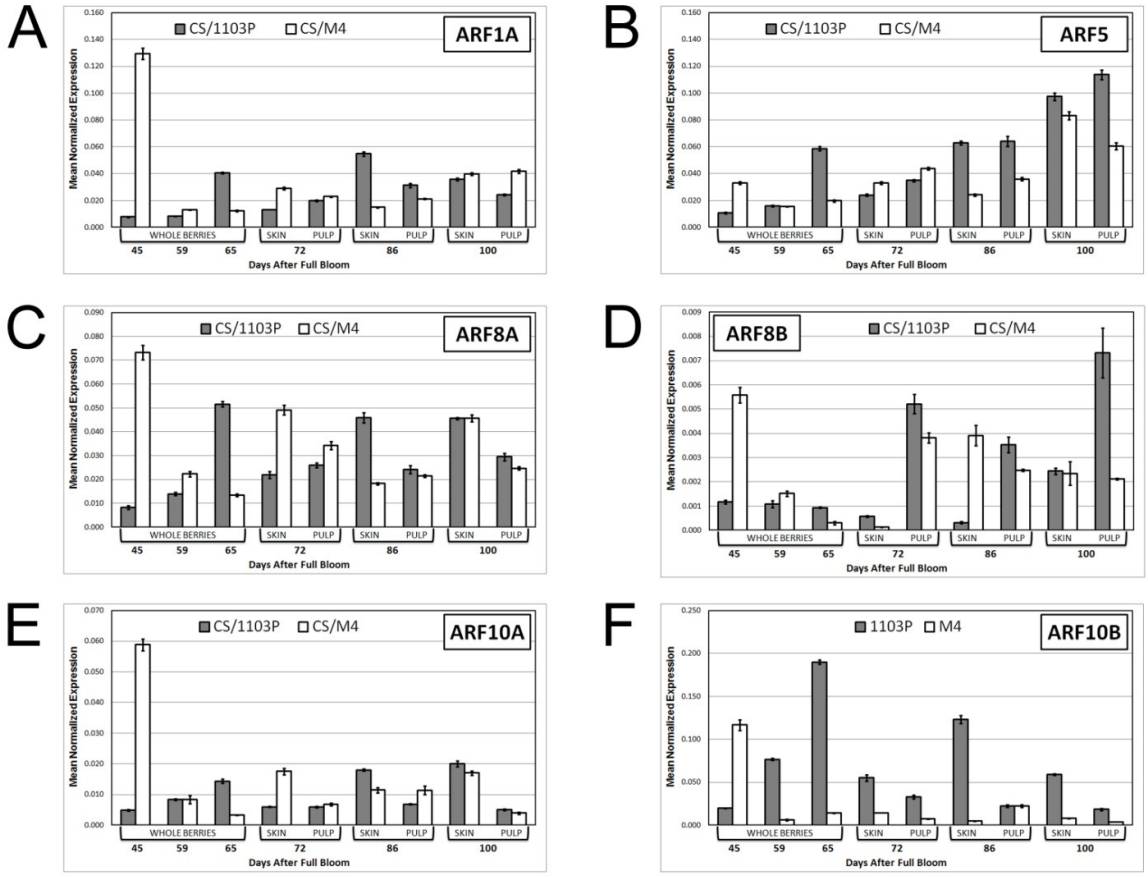


Figure 7. Expression pattern, evaluated by qPCR, of genes related to Auxin Response Factors (ARF). Expression pattern, evaluated by qPCR, of following auxin-related genes: VvARF1A (VIT_18s0089g00910, A), VvARF5 (VIT_18s0001g13930, B), VvARF8A (VIT_12s0028g01170, C), VvARF8B (VIT_04s0079g00200, D), VvARF10A (VIT_06s0004g02750, E), VvARF10B (VIT_13s0019g04380, F) in CS/1103P (grey) and CS/M4 (white) berries. Transcript levels are measured as means of normalized expression ±SE of three technical replicates. Two biological replicated were considered for this analysis.

The second analyzed category of regulators of auxin action was the AUX/IAA one. In this case the expression of *VvIAA1* (VIT_07s0141g00270), *VvIAA6* (VIT_09s0002g05150), *VvIAA9* (VIT_18s0001g08090), *VvIAA15A* (VIT_11s0016g04490), *VvIAA15B* (VIT_09s0002g05160) and *VvIAA16* (VIT_14s0081g00010) was evaluated by qPCR in all samples (Figure 8A-F).

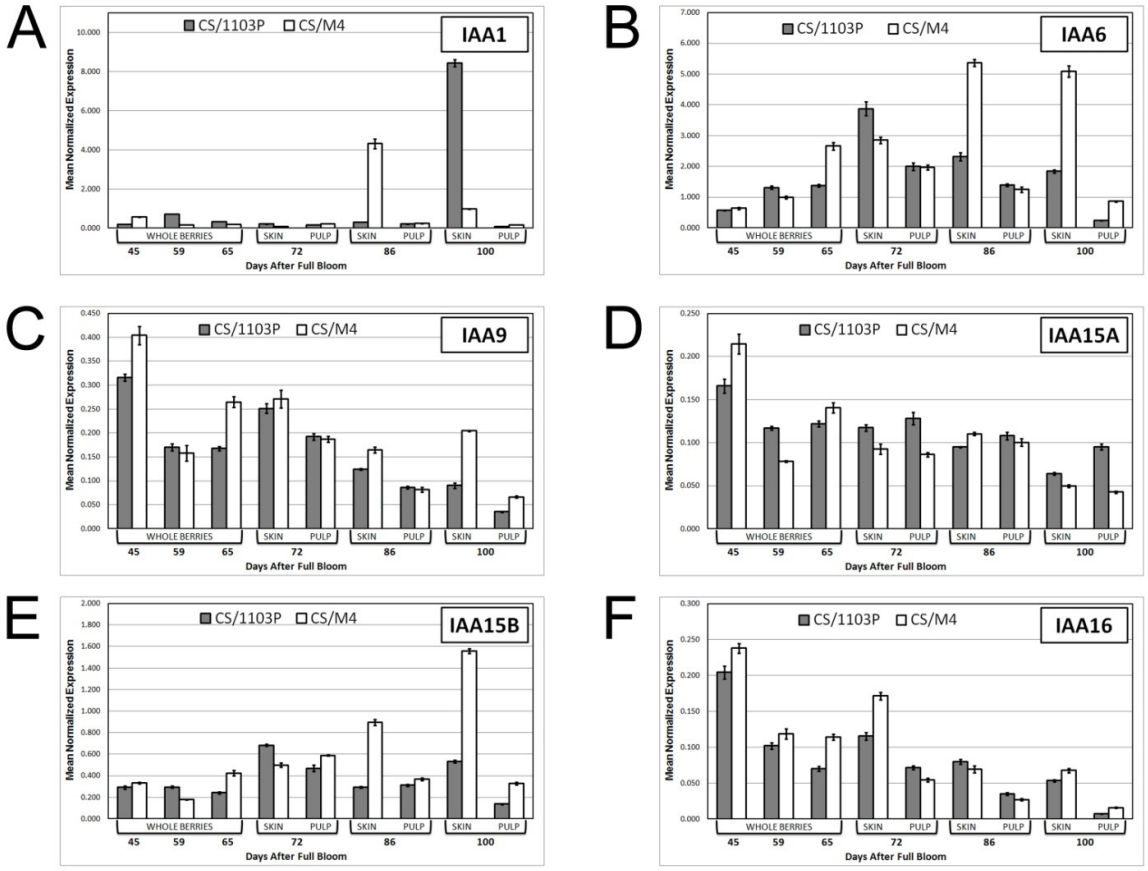


Figure 8. Expression pattern, evaluated by qPCR, of genes related to AUX/IAA transcription factors.
Expression pattern, evaluated by qPCR, of following auxin-related genes: VvIAA1 (VIT_07s0141g00270, A), VvIAA6 (VIT_09s0002g05150, B), VvIAA9 (VIT_18s0001g08090, C), VvIAA15A (VIT_11s0016g04490, D), VvIAA15B (VIT_09s0002g05160, E) and VvIAA16 (VIT_14s0081g00010, F) in CS/1103P (grey) and CS/M4 (white) berries. Transcript levels are measured as means of normalized expression ±SE of three technical replicates. Two biological replicated were considered for this analysis.

On the contrary to that reported for *ARF*, *AUX/IAA*-related genes showed an higher expression in CS/M4 in comparison to what observed in the other scion/rootstock combination, with some exceptions. In particular in CS/M4, the accumulation of *VvIAA9* (figure 8C), *VvIAA15A* (figure 8D) and *VvIAA16* (figure 8F) transcripts slightly peaked at 45 DAFB, therefore at the beginning they were co-expressed with the majority of ARF-related genes (figure 7). These AUX/IAA genes were induced in CS/M4 also in the subsequent phases of grape berry development, especially at 72 and 100 DAFB for *VvIAA16* and *VvIAA9* genes, respectively, in skin tissue (figure 8C and F). *VvIAA1* (figure

8A), *VvIAA6* (figure 8B) and *VvIAA15B* (figure 8E) showed the higher differences among rootstocks in the last phases of grape berry ripening (86-100 DAFB). This behaviour was particularly evident for *VvIAA6* (figure 8B) and *VvIAA15B* (figure 8E) being their significantly up-regulated at 86 and 100 DAFB in skin tissue of CS/M4 (3 times higher in comparison to CS/1103P). *VvIAA1* (figure 8A) showed a strong induction at a specific time-point and tissue for both rootstocks, with differences related to the developmental phase. Indeed, the expression of this gene was induced in skin tissue at 86 DAFB in CS/M4 (20 times higher in comparison to CS/1103P), while it was also strongly induced (10 times higher) in CS/1103P but at the last stage of grape berry ripening (100 DAFB).
The last two considered categories were *GH3* and *PIN* –related transcripts (figure 9).

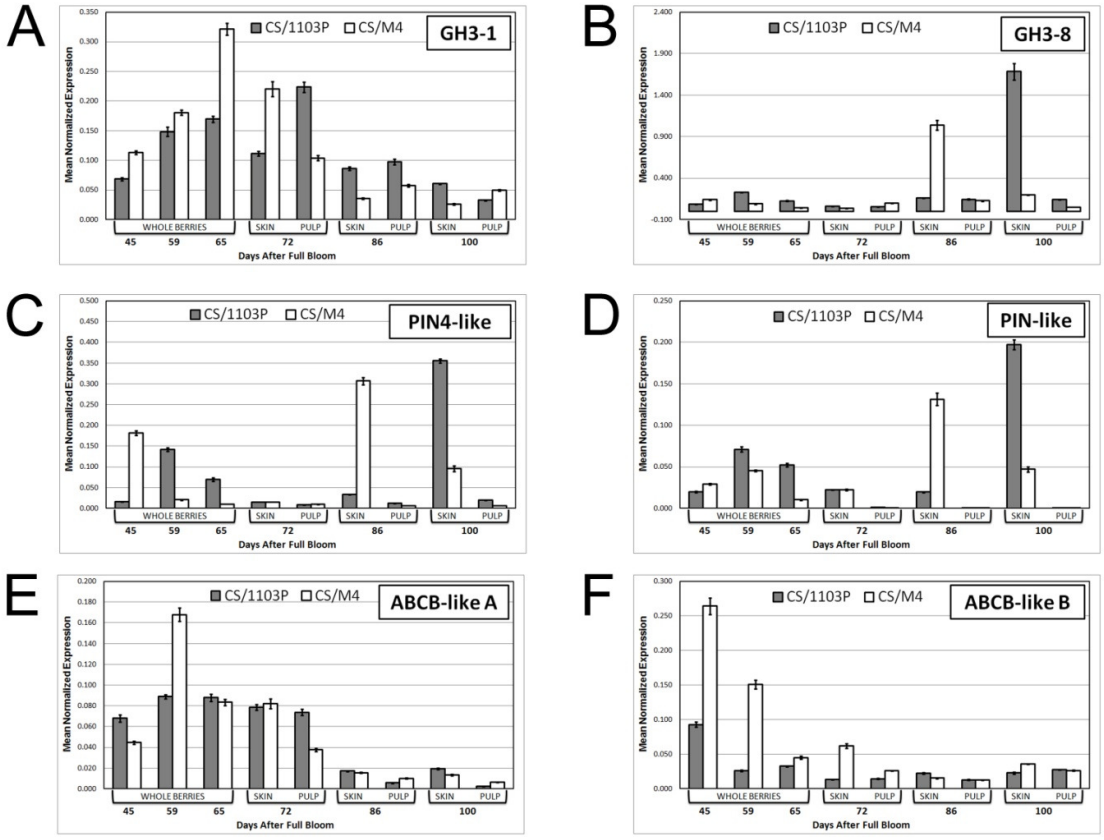


Figure 9. Expression pattern, evaluated by qPCR, of genes related to auxin conjugation (GH3) and transport (PIN). Expression pattern of following auxin-related genes: VvGH3-1 (VIT_03s0091g00310, A), VvGH3-8 (VIT_07s0104g00800, B), VvPIN4-like (VIT_01s0011g04860, C), VvPIN-like (VIT_05s0062g01120, D), VvABCB-like A (VIT_04s0044g01860, E) and VvABCB-like B (VIT_07s0031g02200, F) in CS/1103P

(grey) and CS/M4 (white) berries. Transcript levels are measured as means of normalized expression ±SE of three technical replicates. Two biological replicated were considered for this analysis.

For these categories transcripts corresponding to *VvGH3-1* (figure 9A), *VvGH3-8* (figure 9B), *VvPIN2* (figure 9C), *VvPIN*6 (figure 9D), *VvPIN*9 (figure 9E) and *VvPIN*10 (figure 9F) were monitored along the berry ripening by qPCR.

GH3 genes, involved in auxin conjugation and homeostasis, showed different expression kinetic among the two CS/rootstocks combinations. *VvGH3-1* (figure 9A) was significantly induced in CS berries grafted onto M4 rootstock in the first phases of grape berry development, in particular at 65 and 72 DAFB. Conversely, it showed a delayed and lower induction in CS/1103P (86-100 DAFB). *VvGH3-8* (figure 9B) exhibited a different expression pattern in comparison to *VvGH3-1*, in particular it was strongly induced in skin tissue at 86 and 100 DAFB in CS/M4 and CS/1103P, respectively. This results indicate again a strong specificity in the expression of genes belonging to these family, with a delay observed for CS/1103P.

Also *PIN*-related genes, responsible for auxin transport, were differentially regulated among rootstocks. In fact, *PIN*-related transcripts were accumulated earlier and at higher level in CS/M4, as already observed for the other two (*AUX/IAA* and *GH3* genes) negative regulators of auxin-related metabolisms. In both rootstocks *VvPIN4-like* (figure 9C) showed an interesting double peak but with some differences related to the developmental phase. Indeed, this genes was induced in CS/M4 at 45 and 86 (skin tissue) DAFB, and in CS/1103P at 59, 65 and 100 (skin tissue) DAFB. Analysis gene expression of *VvPIN-like* (figure 9D) highlighted similarities to what observed for *VvPIN2* but only at last stages.. ABCB-like A (figure 9E) was significantly induced only in CS/M4 in the last phases of pre-véraison (59 DAFB).The last studied transcript was ABCB-like B (figure 9F), which was strongly induced by M4 rootstock, up to 65 DAFB.

The above described results highlighted interesting differences induced by grapevine rootstocks in the regulation of genes belonging to several auxin metabolisms. In the next paragraph we will investigate about some microRNA which regulated auxin-related genes.

3.5 Effect of rootstock on miRNA expression and their control of auxin metabolisms.

In order to dissect the effect of microRNA on auxin metabolism during grape berry ripening, some selected miRNAs and their targets (already describe previously, figure 5) were selected and validated by qPCR.

Selected miRNAs were miR160 (figure 10A) and miR167 (figure 10B), which have as target *VvARF16* and *VvARF3*, respectively; and miR171 (figure 10C) and its targets SCL6-like and another SCARECROW-like (data not showed), two genes regulating both shoot branching (Wang *et al.*, 2010) and control cell division and auxin transport in root and leaves of several species (Dhondt *et al.*, 2010; Gao *et al.*, 2004).

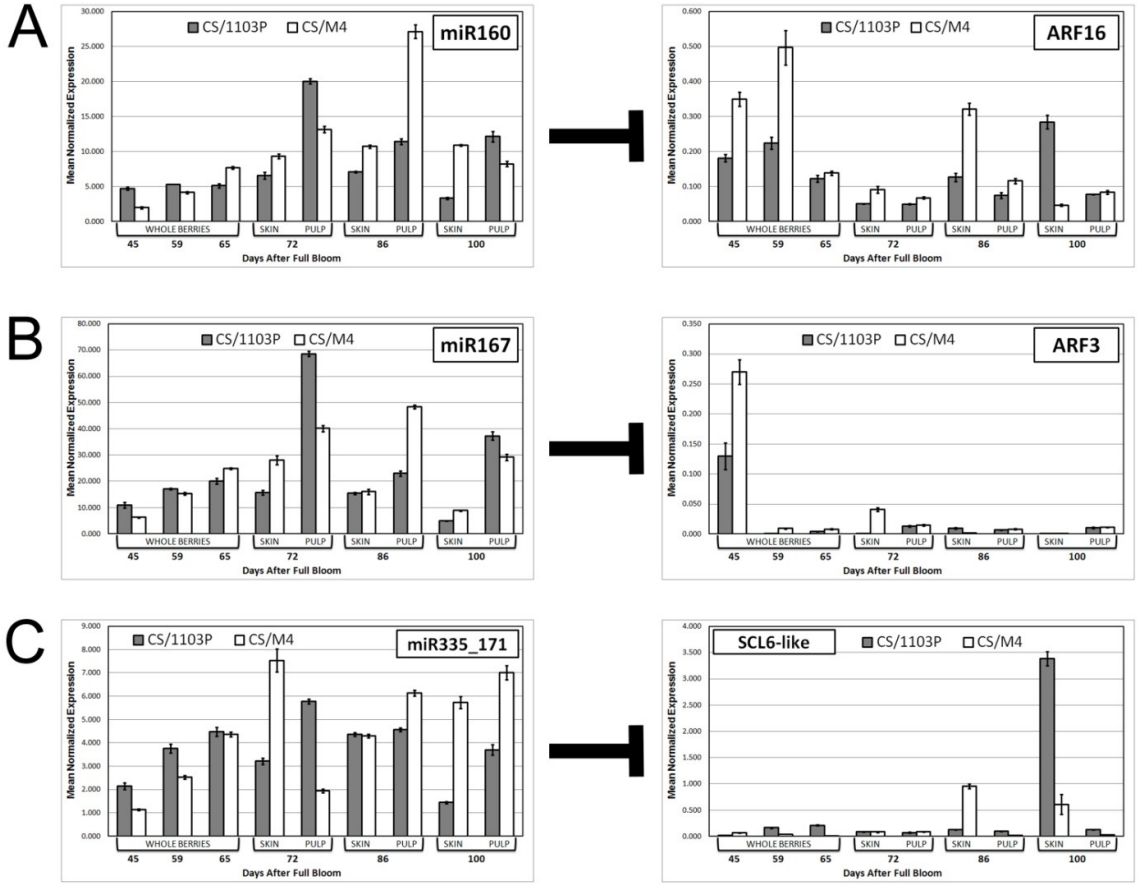


Figure 10. Expression pattern, evaluated by qPCR, of miRNAs and their respective genes targets related to auxin signal transduction and transport. Expression pattern of following auxin-related miRNAs and genes: **A.** miR160 and VvARF16 (VIT_08s0040g01810); **B.** miR167 and VvARF3 (VIT_10s0003g00420); **C.** miR171 and VvSCL6-like (VIT_02s0154g00400). Transcripts and miRNAs expression was evaluated in

CS/1103P (grey) and CS/M4 (white) berries. Expression levels are measured as means of normalized expression ±SE of three technical replicates. Two biological replicated were considered for this analysis.

miR160 and miR167 have an important role in the control of *ARF* and consequently in the control of auxin action. As showed in figure 10A and 10B, expression level of the above cited miRNAs at 45 DAFB was higher in CS/M4 (2 times higher) in comparison to that observed for CS/1103P. On the other hand, the expression of *VvARF16* and *VvARF3* was significantly lower in CS/1103P in comparison to that observed in corresponding CS/M4 samples (figure 10A and 10B), as already described in the previous paragraph. As concerns CS/M4, at 86 DAFB the accumulation of miR160 was significantly induced in pulp tissue and parallel a decrease in *VvARF16* transcripts amount occurred (figure 10A).
miR167 seems to have an earlier induction, with some differences among CS/rootstock combinations. Indeed, it was induced at 72 and 86 DAFB in pulp tissue of CS/1103P and CS/M4, respectively; the *VvARF3* target, after an initial induction in CS/M4, was repressed in both rootstocks during all the kinetic (figure 10B).
The last considered miRNA seems to have a major effects on CS/1103P berries. Indeed, when the expression levels of miR171 were significantly lower in skin tissue (100 DAFB), target transcripts *SCL6-like* were strongly induced. As concerns the other considered time points, miR171 was expressed at high level in both CS/rootstocks combinations and the target was consequently repressed (figure 10C).

4. Discussion

In this work, for the first time the effect of two grapevine rootstocks on CS grape berry quality and development/ripening was evaluated by using a whole genome transcriptomic approach (mRNA-seq and microRNA-seq).

It is well known that rootstocks, in function of their ability in the nutrients (like potassium or nitrogen) uptake, can significantly affected grape berry development and fruit composition (Gambetta *et al.*, 2012; Walker *et al.*, 2000; Stockert and Smart, 2008). The first massive use of rootstocks in viticulture was a consequence of the accidental introduction of phylloxera (*Daktulosphaira vitifoliae*), a root-feeding aphid, to Europe in the 1800s. Phylloxera epidemic was counteract by grafting grapevines of the species *Vitis vinifera*, including international varieties as Cabernet Sauvignon and Chardonnay, on American wild type grapes. Later on rootstocks were used for other purposes as the increment of vine performance or the adaptation to specific soil types and adverse environmental conditions (Gambetta *et al.*, 2012; Covarrubias and Rombolà, 2013; Gregory *et al.*, 2013; Meggio *et al.*, IN PRESS). The impact of rootstocks on fruit composition and, therefore, on wine organoleptic properties is less clear but can be important to such an extent that grafting is desirable even in the absence of the above-mentioned stresses. Rootstocks can have a direct effect on wine quality influencing content in nutrients, sugars and anthocyanins of the grape berry, or an indirect effect by controlling vigour and thus affecting berry size and as well as fruit ripening time (Cortell *et al.*, 2007a, b). Generally speaking, rootstocks that are able to control/reduce vigor also can induce an acceleration of fruit ripening. However, in some cases very vigorous rootstocks were found to advance fruit maturation. In this work we showed that berry development and ripening of CS grapes was deeply altered when CS scions were grafted onto 1103P and M4 rootstocks. Indeed, 1103P delayed the véraison of CS berries (about 14 days) from the date recorded for M4. These results were in according to that reported in the literature, indeed, it is well known that 1103P is a very vigorous rootstock which has a long vegetative cycle and delays ripening (Gambetta *et al.*, 2012; Koundouras *et al.*, 2008), while M4 is considered a medium or high vigorous rootstock (Meggio *et al.*, IN PRESS), but to a lesser extent in comparison to the 1103P one. This should partially explained the differences observed in terms of grape berry ripening and development

among the two CS/rootstocks combinations (Figure 2). One of the most important factors in determining the quality of the wine is the heterogeneity of grape ripening progression at the bunch level. At véraison the level ripening asynchrony is strongly influenced, beside the cultivar and the environmental conditions, by the hormonal content (Deluc *et al.*, 2009). In this context, the relationship between plant hormones and the effects of the hormonal treatments on grape berries have been extensively studied at the transcriptomic levels (Symons *et al.*, 2006; Davies and Böttcher, 2009; Chervin *et al.*, 2008; Ziliotto *et al.*, 2012), while transcriptomic studies regarding the effect of the rootstock are almost absent.

Physical and biochemical analyses carried in this work (figure 2), showed differences in terms of ripening kinetics among two CS/rootstocks combinations. In particular, the véraison for CS/M4 was identified at 72 DAFB, where samples showed a SSC of about 18 brix°. In CS/1103P berries the same SSC value was reached only two week later (at 86 DAFB) . These results were in according to those reported by other authors that recorded a significant delay in developing berries of Shiraz when grafted on 1103P instead of onto other less vigorous rootstocks (Walker *et al.*, 2000).

mRNA-seq results, enrichment analysis and qPCR (Supplementary figure 5) pointed out an early induction of genes involved in sugar metabolism in CS/M4. Hexose sugars accumulation begins at véraison (Grimplet *et al.*, 2007), whereas in the earlier stages these solutes are not stored in the vacuoles but metabolised (Davies and Robinson, 2000). Flow of solutes from the phloem cells to the berry mesocarp occurs via apoplastic and is mediated by transporters (named HT1 and HT2) ,that allow the accumulation of hexoses, in particular fructose and glucose (Grimplet *et al.*, 2007), in vacuole. *HT1* expression in CS/M4 skin was similar to what observed by Fillion et al. (1999) and Davies and Robinson, (2000). In particular two peaks were observed, at 59 and 86 DAFB which correspond to pre and post –véraison stages, respectively (supplementary figure 5C).

VvGIN1 (supplementary figure 5A) and *VvGLU-INV* (supplementary figure 5B), two genes coding for a vacuolar invertase involved in the hydrolysis of sucrose to fructose and glucose, were expressed both in CS/1103P and CS/M4 at pre-véraison stage (45-65 DAFB), as already reported by Davies and Robinson, (2000), but to a significantly higher level in CS/M4 berries. All these data indicate that in CS/M4 a higher accumulation of

soluble solids accumulation and sugars-related genes transcripts than that observed in CS/1103P occurred (Figure 2; Supplementary figure 5).

Sugars concentration is positively correlated with the amount of anthocyanins during grape berry ripening and negatively correlated with others phenolics compounds because the latter offer another substrate for synthesis of anthocyanins (Castellarin *et al.*, 2011; Gambetta *et al.*, 2010; Liang *et al.*, 2011). Genes involved in phenylpropanoids biosynthetic pathway were expressed earlier and to a higher level in CS/M4 (Figure 4). This pattern was in according with the earlier véraison (14 days) observed in CS/M4 berries (Figure 3). Indeed, it is well known that accumulation of anthocyanins and induction of genes involved in their biosynthesis are strongly related to the onset of ripening in grape berries (Kuhn *et al.*, 2013; Grimplet *et al.*, 2007; Chen *et al.*, 2006). The early expression of genes related to anthocyanins pathway is also positively related to the greater intensity of colour observed in CS/M4 berries in comparison to that observed in CS/1103P (data not showed), as already suggested by other authors (Pérez-Magariño and González-San José, 2004; Ryan and Revilla, 2003).

Expression of *PAL3*-like genes (Figure 4A) was higher in skin tissue as observed by Grimplet *et al.* (2007) and Castellarin *et al.* (2011). As concerns *PAL* gene (*VvPAL3-like*) we observed a strong correlation between its expression and berries staining intensity. This fact was expected considering that PAL is the first enzyme of phenylpropanoid pathway and is therefore producing also precursors for the of phenolic compounds, thus PAL is indicated as one of responsible for rate-limiting steps in anthocyanins biosynthesis (Guillaumie *et al.*, 2011). Similarly, the increased expression of *VvCHS3* and *VvUFGT* genes (Figure 4B, E) had a positive impact on anthocyanins accumulation (Liang *et al.*, 2011; Lijavetzky *et al.*, 2012). The increase of anthocyanins-related genes occurred during the ripening phase was paralleled by anthocyanins accumulation in the berry (Davies and Robinson, 2000). The same trend was also observed for the gene coding for the synthesis of flavonols (*FLS1*; Figure 4D), while the *LAR2* gene (Figure 4C), which encodes for an enzyme involved in tannins biosynthesis, showed a induction in CS/M4 at pre-véraison (45 DAFB) and in both rootstocks at véraison (72 and 86 DAFB in CS/M4 and CS/1103P, respectively). The increased expression of *VvLAR2* at pre-véraison should

be linked to an induction of tannins accumulation that, as observed by Grimplet *et al.* (2007), were typically produced in the first phase of berry growth.

The above discussed data showed significant differences in berry composition when CS scions are grafted onto M4 or 1103P and support the fact that rootstocks exert a strong influence on berry development and ripening-related processes. But, what are the molecular mechanisms that control these events? A possible explanation could be related to auxin metabolism. Considering the large numbers of DEGs related to auxin identified using Mfuzz cluster and enrichment analyses, and the relevant role of auxins during grape berry development(Ziliotto *et al.*, 2012), a characterization of grape ARF and AUX/IAA gene families and the consequent association with the gene expression data was carried out (Figure 6; supplementary figures 3 and 4). Level of the synthetic auxin indole-3-acetic acid (IAA) reached its maximum in flowers and young berries, and then decreased until véraison (Bottcher *et al.*, 2012). The decrease in IAA levels is thought to be a prerequisite for the onset and progression of ripening in a large range of fruit, including grapes (Ziliotto *et al.*, 2012; Böttcher *et al.*, 2011; Davies *et al.*, 1997; Schaffer *et al.*, 2013; Symons *et al.*, 2012). In this work, we observed that the delayed véraison of CS/1103P berries was paralleled by a delayed induction and a lower expression of *VvGH3-1* gene (Figure 9A) when these two events were compared to those occurring in CS/M4 ones. These results were in according to that observed by Böttcher *et al.* (2010 and 2011), which demonstrate that the increasing levels of conjugated form of IAA in grapes might be linked to the low levels of active IAA recorded at, and after, the onset of ripening. Conjugation of IAA to amino acids, catalyzed by GH3, have an important role in the control of auxin homeostasis and the pivotal role of GH3 proteins in control of fruit ripening is still under investigation (Pattison *et al.*, IN PRESS). As already observed in rice, GH3-8 is linked to the repression of expansin-related genes and their expression is skin-tissue specific (Ding *et al.*, 2008). Also the induction of *VvGH3-8* (Figure 9B) detected at post-véraison phase of CS/M4 (86 DAFB) and CS/1103P (100 DAFB) was paralleled by a decrease in expansin-related transcripts expression as already observed by Ziliotto *et al.,* 2012.

ARFs and AUX/IAAs genes regulation was essential for several metabolisms related to grape berry development and ripening. Our data highlighted that ARF genes, which are

actively involved in the auxin action, showed an earlier induction at pre-véraison stage in CS/M4 berries (figure 7; supplementary figure 3), when auxin level reached its maximum (Davies *et al.*, 1997). As concerns CS/1103P most of ARF transcripts, *VvARF8A/10B* in particular (Figures 7C, F), reached a peak (65 DAFB) in their expression later than occurred in CS/M4. Taking into accounts the differences observed between CS/rootstocks combinations in the regulation of ARF transcripts, a role in grape fruit development of these transcripts was suggested. Several genes belonging to this family were showing similar expression kinetic indicating that their function can overlap during fruit development, as already observed in tomato (Kumar *et al.*, 2011). In particular expression of *VvARF8A* (Figure 7C) suggested, in both CS/M4 and CS/1103P, its involvement in fruit development and ripening. An involvement of ARF8 in fertilization and fruit s et was suggested (Goetz *et al.*, 2007; Seymour *et al.*, 2013), but its role during fruit ripening is still unknown. *VvARF1A* and *VvARF10A* (Figures 7A, E) were significantly induced at the pre-véraison stage, especially in CS/M4, suggesting a function in cell expansion during berry green phase and thus a role in the early phases of fruit development. Indeed, a recent work demonstrated a role of tomato ARF10 in the regulation of the size and shape of the fruit (Hendelman *et al.*, 2012). As concerns ARF1, it was observed an induction at the mature green phase in tomato, suggesting its involvement in fruit development too (Kumar *et al.*, 2011).

VvARF5 was the only one highly expressed in the latter phases of berry ripening, especially at 86-100 DAFB period. Its high expression during fruit ripening was already observed by Kumar *et al.* (2011), in tomato fruit, but role of this genes during fruit developmental processes is still unknown.

Several AUX/IAA-related genes showed an earlier and higher induction throughout all grape berry development in CS/M4 samples (Figure 8; supplementary figure 4). It is well known that in absence of auxin, AUX-IAA sequester ARF by protein-protein interaction and inhibit the binding between the latter and the promoter region of auxin-responsive genes. In this context AUX/IAA genes have been claimed as regulators of auxin response during fruit development and ripening (Pattison *et al.*, IN PRESS; Wang *et al.*, 2005; Woodward and Bartel, 2005).

Among this family, *VvIAA9* and *VvIAA15A* were more induced during the green phase of grape berry development (Figure 8C, D), as already observed in tomato by Audran-Delalande *et al.* (2012), so, their expression can be related to the control of auxin responsive genes in the early phases of berry ripening. Conversely, *VvIAA6* and *VvIAA15B* (Figure 8E, F) were significantly induced in CS/M4 at 86-100 DAFB suggesting their role in control of auxin responsive genes in the latter phases of grape berry ripening. It is worthy of note that *VvIAA1* showed a strong tissue specificity in both CS/rootstocks combinations, with differences among rootstocks related to the expression pattern, as already described in the results section (Figure 8A).

Genes coding for cellular auxin transporter enzymes, were the lasts one considered in this experiment (Figure 9). Auxin concentration in fruit, as well as in other organs, is strictly related to its polar transport, which contributes to the formation of local auxin maxima and minima that control various aspects of fruit development and ripening development (Pattison *et al.*, IN PRESS). Auxin efflux is mainly due to PIN and ABCB enzymes, while auxin influx is controlled by AUX/LAX proteins (Pattison *et al.*, IN PRESS; Geisler and Murphy, 2006). mRNA-seq (data not showed) and qPCR (Figure 9) analyses highlighted interesting expression patterns in these genes families. As showed in figure 9C and 9D, *PIN*-like genes were mainly expressed in the final phases of grape berry ripening in skin tissue, with an early induction in CS/M4 in comparison to what observed in CS/1103P. *ABCB*-like genes were expressed in the pre-véraison stage (45-65 DAFB) and showed an higher expression in CS/M4 (Figure 9E, F). *PIN* and *ABCB* genes were suggested to control auxin intracellular levels and homeostasis in different species and tissues (Carraro *et al.*, 2012; Forestan *et al.*, 2012; Pattison and Catalá, 2012). Our results suggest a specific tissue and temporal -expression of both *PIN* and *ABCB* families and their involvement in the reduction of auxin levels and control of auxin homeostasis in grape berries.

Taken together, these data suggest an important involvement in the control of grape berry development/ripening of genes that are related, on one hand to auxin signal transduction (*ARF* and *AUX/IAA*) and, on the other hand, to homeostasis of this hormone through the expression of genes involved in conjugation (*GH3*) and transport (*PIN* and *ABCB*). Analysis of the expression patterns of genes involved in auxin transport and metabolism

are an important first step to elucidate the control of auxin levels and gradients in the fruit, and are being performed in an increasing number of various fruit species (Ziliotto *et al.*, 2012; El-Sharkawy *et al.*, 2010; Dal Cin *et al.*, 2009; Kang *et al.*, 2013). In addition to these mechanisms, miRNAs seems to be important in the control of fruit development and ripening and their role is currently emerging (Carra *et al.*, 2011; Wang *et al.*, 2012). An important role in the modulation of auxin-related genes after pre-véraison stage was indeed played by miR160 and miR167 which repressed *VvARF16* and *VvARF10*, respectively, at véraison stage (Figure 10). The higher expression of miR160a/b-1, which corresponds to those considered in this word (figure 10A), during early and late stages of grape berry ripening was reported also by Wang et al. (2014), which showed an induction of this miRNA at young and mature berry of the table grapevine 'Summer Black'. Despite what observed by Carra et al. (2009), which hypotized a role of only before grape ripening, we can speculate about its involvement also during the following phenological phases. As observed for miR160, also miR167a (Figure 10B) expression was induced during ripening, this should be related to its role in the repression of genes involved in growth and development, which were expressed in the pre-véraison phases (Liu *et al.*, 2012). Another important issue of transcriptional control mediated by miRNA, is related to their mobility. Indeed, in this study it was observed changes in conserved miRNA expression, together with the presence of novel miRNA genotype specific (data not showed), which is probably due to a production of these miRNA in the root zone and their transport to the aerial part. Long-distance

transport of small interfering RNA and microRNA has been proven in several studies. For, example, Yoo et al. (2004) showed that small RNA corresponding to authentic regulatory RNAs (siRNA and miRNA) can enter and move through the phloem of several plant species. Furthermore,

these authors identify a novel protein, *Cucurbita maxima* PHLOEM SMALL RNA BINDING PROTEIN1 (CmPSRP1), and show that it likely plays a role in trafficking of small RNA through

the phloem. So, in both leaf and root development, miRNAs establish intercellular gradients of gene expression that are essential for cell and tissue differentiation (Gursanscky *et al.*, 2011) but their translocation from the basal (root) to the aerial

(leaves/berries) part of the plant has still to be proven. It, therefore, remains a distinct possibility that proteins may facilitate or inhibit the movement of small RNAs not only from cell to cell, but to distal locations in plants via the phloem.

In another paper, we observed that, after an NAA-treatment at the pre-véraison stage, Merlot grape berries counterbalanced the excessive availability of auxin controlling auxin homeostasis through the modulation of genes involved in biosynthesis, breakdown, conjugation and transport (Ziliotto *et al.,* 2012). Data presented here confirmed that auxin level (in this case due to different scion/rootstock combination) can be a key factor in the onset and progression of grape berry ripening. The ability of rootstock to induce high auxin levels in scion buds was postulated Sorce et al. (2006) to explain the positive effect of vigorous peach rootstocks on scion branching. In the case of fruit ripening, auxin acted as a positive regulator of genes that control grape berry size (*e.g.* expansin-related genes) before the véraison stage; it was indeed observed the up-regulation at the pre-véraison stage, which was different for CS/M4 and CS/1103P, of transcripts that control auxin-responsive genes (*e.g. VvARF8A* and *VvARF1A*). The induction of genes that belonged to ARF family was paralleled by the expression of transcripts that control auxin levels (*e.g. VvGH3-1, VvIAA9, VvIAA15A, VvIAA16*), highlighting an accurate regulation of auxin homeostasis in grape berries at these phases. Moreover, control of auxin levels in grape berry seems pass through other mechanisms involving the control of transport-related genes in early (e.g. ABCB) and late (PIN) phases of berry development (figure 9).

Although several studies emphasize the function of auxin as a repressor of genes involved in the ripening process (Daminato *et al.*, 2013; Sundberg and Ostergaard, 2009), a more complex role of auxin regulating this process is currently emerging from studies carried out both in climacteric fruit as peach (Tatsuki *et al.*, 2013; Trainotti *et al.*, 2007a) and in climacteric-suppressed fruit as plums (El-Sharkawy *et al.*, 2010). These studies demonstrated that auxin can play an autonomous role in the ripening and, at the same time, to govern the ripening interacting with ethylene (Trainotti *et al.*, 2007b). The interaction between auxin and ethylene in the controlling of ripening is supported by the ability of auxin treatment to restore a correct progression of ripening in fruit in which this event is partially impaired as in Stony-Hard peaches (Tatsuki *et al.*, 2013) and on Shiro, a

suppressed climacteric plum (El-Sharkawy *et al.*, 2010). These results suggest that a scarcity of auxin might negative affect the levels of autocatalytic ethylene production.

In the case of grape, auxin treatment performed at véraison induced the expression of genes involved in the ethylene biosynthesis although the ripening progression was deeply delayed (Ziliotto *et al,* 2012). In this contest, auxin acted as negative regulators on some genes related to grape berry ripening but its induction at the pre-véraison stage could be necessary to triggers other metabolism involved in ripening processes. Auxin seems to be accumulated rapidly and in higher levels in CS berries grafted onto the M4 genotype and high levels of ripening-related genes resulted in the early transition of the green fruit into the ripening stage as already observed in other fruit (El-Sharkawy *et al.*, 2010; Pattison and Catalá, 2012; Trainotti *et al.*, 2007b; Exposito-Rodriguez *et al.*, 2011).

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Chapter IV

Grape berry ripening delay induced by a pre-véraison NAA treatment is paralleled by a shift in the expression pattern of auxin- and ethylene related genes

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**Equal contribution*

1. Introduction

A large number of physiological and molecular events are known to occur during grape berry ripening, but the regulatory mechanisms controlling this critical developmental phase are still poorly understood. The onset of ripening (termed véraison) is accompanied by significant changes, at both physical (pulp firmness) and chemical (accumulation of sugars and flavor compounds, synthesis of anthocyanins and reduction of organic acids concentration) levels (Nunan et al., 1998; Robinson et al., 2000), concurrently with the modification of the transcription rate of a large number of related genes (Deluc et al., 2007; Pilati et al., 2007). Auxin, ethylene, abscisic acid (ABA) and brassinosteroids (BRs) are actively involved, throughout a complex network of interactions with other mobile signals, in the regulation of grape berry ripening (Davies et al., 2009). Interestingly, the highest levels of auxin are observed at early berry development, then its concentration decreases rapidly before véraison, becoming undetectable after two weeks (Deytieux-Belleau et al., 2007; Böttcher et al., 2010).

On the other hand, another study showed no dramatic changes in auxin concentration during berry growth and development (Symons et al., 2006). Application of synthetic auxins before véraison delays ripening, as seen in several ripening related physiological processes (Davies et al., 1997; Böttcher et al., 2010a; Böttcher et al., 2010b), and heavily modifies the transcription of key genes involved in the sugars metabolism, cell wall turn-over and biosynthesis of phenylpropanoids (Jeong et al., 2004). Among the latter, the expression of genes encoding chalcone synthase (CHS), flavanone hydroxylase (F3H), UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT), and MYB transcription factors (Davies et al., 1997; Davies et al., 2009;) is negatively affected by auxin. Davies et al. (1997) showed that treatments with the synthetic auxin BTOA (benzothiazole-2-oxyacetic acid) were able to modify the hexose accumulation mechanisms by altering the expression of the related genes. NAA applications at véraison also inhibited genes belonging to cell wall structure, such as GRIP4 coding for a proline-rich protein, and negatively affected ABA metabolism (Davies et al., 2009). Endogenous levels of ethylene, ABA and BRs increase at véraison, and exogenous applications of these hormones accelerate the initiation of the ripening phase, concurrently stimulating the accumulation of anthocyanins, most likely by enhancing the transcription of CHS, F3H, UFGT, and MYB1

genes (El-Kereamy et al., 2003; Jeong et al., 2004; Symons et al., 2006; Chervin et al., 2008; Giribaldi et al., 2011). These treatments can also induce the uptake and storage of sugars by berries (Giribaldi et al., 2011). In addition, low doses of ethylene at véraison stimulated grape berry expansion, enabling cell elongation in pulp and skin, and inducing genes encoding aquaporins (AQUAPORIN1 and AQUAPORIN2) and cell wall hydrolase/esterase, such as Polygalacturonase (PG1) Expansin (EX), and Pectin-methyl esterase (PME) (Chervin et al., 2008; Sun et al., 2010).

Since mutants with impaired ripening are not available in grapevine, the best alternative way to investigate the role of hormones during berry development consists in altering the specific process by means of exogenous applications of plant growth regulators. Transcriptome studies dealing with the effects of exogenous hormone treatments in grapevine have focused on ethylene (Chervin et al., 2008), referring to the pivotal role of this hormone in the transcriptional regulation of its biosynthesis and signal transduction during grape berry development. In particular, ethylene treatments were shown to induce the transcription of ARF8 (auxin response factor) and NCED (9-cisepoxycarotenoid dioxygenase) genes, the latter encoding a key enzyme of ABA biosynthesis (Chervin et al., 2008). Auxin treatments were also investigated, showing an increase in ethylene due to the stimulation of the expression of genes encoding its biosynthetic key enzymes (Chae et al., 2000; El-Sharkawy et al., 2008) and signal transduction elements (El-Sharkawy et al., 2009).

In order to shed light on the hormone interactions occurring at véraison, a specific transcriptomic study was carried out on NAA treated berries. This study confirms the capacity of NAA to delay grape berry ripening at the transcriptional level. The duration of this delay may be associated with the recovery of a steady state of auxin concentration. In the presence of altered levels of auxin, the crosstalk between hormones involves diverse specific mechanisms, acting at both the hormone response and biosynthesis levels, thus creating a complex network of transcriptional responses.

2. Materials and methods

2.1 Plant materials and treatment

Experiments were performed on Vitis vinifera L. cv. Merlot berries collected at a commercial vineyard (Vini e vigne, Monselice PD, Italy). One-hundred bunches from fifty homogeneous plants (two bunches per plant) were treated in planta with a synthetic auxin (naphtalenacetic acid, NAA, 200 mg/L; SIGMA-N640) at the pre-véraison stage corresponding to fifty-three days after full bloom (DAFB), as suggested by Jeong et al. (2004). Whole berries from treated and untreated bunches were collected at 57, 60, 70, 95, and 110 DAFB (see Additional file 10), and either immediately used for biochemical analyses or frozen in liquid nitrogen and stored at -80°C for RNA isolation and transcriptomic evaluations. Because of a delayed ripening observed upon the treatment, additional samples were collected from NAA-treated bunches up to 160 DAFB. The sample at 148 DAFB was chosen ex post as being representative of the harvest date of treated berries, according not only to the Color Index for Red Grape (CIRG), but also to the biochemical parameters that were similar to the control samples at harvest (see Results section for a detailed description). At each time-point, three biological replicates were sampled for the biochemical analyses and two for transcriptomic assessments. Each replicate was collected from five to seven bunches and was made up of at least fifty berries chosen according to the CIRG index proposed by Carreño et al. (1995) at the same position within the cluster (median position). The juice from each replicate was used to assess the biochemical indicators (titratable acidity, pH, tartaric acid, malic acid, soluble solids) using a WineScan FT 120 multiple-parameter analyser (FOSS, Denmark), while anthocyanin content was determined as described by Mattivi et al. (2006). A colorimetric index was chosen since gene expression analyses in individual grape berries during ripening initiation revealed that pigmentation intensity could be assumed as a valid indicator of developmental staging within the cluster (Lund et al., 2008).

2.2 RNA extraction, microarray analysis and quantitative real time PCR

Total RNA for both microarray and real-time PCR experiments was extracted from whole berries stored at -80°C using the perchlorate method as reported by Rizzini et al. (2009).

Microarray experiments were carried out using the grape AROS V1.0 platform (http://www.operon.com), as described by Rizzini et al. (2009). The following samples were hybridized: NAA-treated berries at 60 DAFB versus untreated berries at 60 DAFB (N1/C1), NAA-treated berries at 110 DAFB versus untreated berries at 110 DAFB (N2/C2), and NAA-treated berries at 148 DAFB versus untreated berries at 110 DAFB (N3/C2). For each of the three comparisons, three slides were hybridized using targets corresponding to two biological replicates (at least one biological replicate was dye-swapped, except for the N1/C1 comparison for which both replicates were dye-swapped and thus four slides were hybridized). Raw hybridization data were quality-filtered, background subtracted, and intra-array normalized with the loess method. The above calculations were all carried out with the package limma and other basic statistical functions of R for Mac OS X v2.13.1 (http://www.r-project.org/). The same package was also used for discovering differentially expressed genes by means of the linear modelling approach (lmFit) and the empirical Bayes statistics (eBayes), both implemented in limma (Smyth et al., 2004). All the experimental procedures comply with minimum information about a microarray experiment (MIAME) standards for array data (Brazma et al., 2001). Gene expression data have been submitted to Gene Expression Omnibus (GEO) (accession no. GSE37341) at NCBI (https://www.ncbi.nlm. nih.gov/geo/). For quantitative real-time PCR analysis (qPCR), cDNA was synthesized using 2 μg of total RNA, 2.5 μM (dT)18 primer, 200 Units of M-MLV Reverse Transcriptase (Promega) and 1 Unit of RNAguard (Amersham Biosciences), at 37°C for 90 minutes in a final volume of 20 μL. qPCR was carried out in triplicate, on two biological replicates for each sample, with StepOne Plus Real-Time PCR System (Applied Biosystems) by using specific primers listed in Additional file 11. The specificity of amplification was assessed as indicated by Botton et al. (2011). Data were acquired, elaborated, and exported with the StepOne Software version 2.1 (Applied Biosystems), whereas all the final calculations were carried out with the automated Excel spreadsheet Q-Gene designed by Simon (2003) using the modifications of the delta cycle threshold method suggested by Pfaffl (2001). Gene expression values were normalized to the housekeeping gene UbiCF (Ubiquitin Conjugating Factor; CF203457) already used by Castellarin et al. (2007) and reported as arbitrary units of mean normalized expression, using equation 2 of Q-Gene.

2.3 Microarray annotation and enrichment analysis

The sequences of the oligos spotted onto the AROS V1.0 microarray were matched by means of the Blastn algorithm against the transcripts of the 12X genome assembly obtained at the CRIBI Centre of the University of Padova and publicly available at the website http:// genomes.cribi.unipd.it/. The Gene Ontology terms were retrieved, imported in the Blast2GO software v2.5.0 (Götz et al., 2008) and increased of about 16% by means of the Annex function (Myhre et al., 2006) as reported by Botton et al. (2008). Enrichment analysis was performed for each set of differentially expressed genes with the built-in Fisher's exact test function with $P \leq 0.01$ and FDR correction.

2.4 HORMONOMETER analyses

The HORMONOMETER tool (Volodarsky et al., 2009; http://genome.weizmann. ac.il/hormonometer/) was used by following the same pipeline adopted in peach by Bonghi et al. (2011). Since this bioinformatic tool accepts only Arabidopsis gene expression data, the probes spotted onto the grape microarray were matched against the 12X genome assembly as reported above and, in turn, the genes predicted in the latter release were matched with those of Arabidopsis by blasting the respective protein sequences against each other (grape deduced proteins vs TAIR10 proteins). In this way, an association 'array probe-grape gene-Arabidopsis gene' was obtained, allowing to use as input data for HORMONOMETER the grape gene expression data coupled with the respective locus names and Affymetrix probe IDs of the putative Arabidopsis orthologs. In the case in which different grape genes matched a single Arabidopsis gene, their expression values were averaged and considered just once. In addition to the whole set of grape genes spotted onto the microarray, three subsets were submitted to HORMONOMETER: i) genes with hormone-specific responsiveness (i.e. that are not multiple targets of hormones), ii) hormone-responsive genes encoding transcription factors (TFs), and iii) genes encoding TFs with hormone-specific responsiveness (an intersection between the two previous groups). A short description of the basic principles of functioning of the HORMONOMETER tool is given by Bonghi et al. (2011).

3. Results

3.1 Biochemical analyses

Physical (berry volume) and chemical (total anthocyanins content, soluble solids concentration, titratable acidity) parameters were assessed in both control and NAA-treated berries (Figure 1), in order to verify the actual efficacy of the treatment.

Untreated berries showed an increase of volume after the time of the treatment (53 Days After Full Bloom, DAFB) until reaching a temporary lag phase (from 60 to 70 DAFB) during which this parameter did not vary significantly. Thereafter, it increased and reached its maximum at harvest (110 DAFB). The volume of NAAtreated berries showed a significant increase up to 70 DAFB, when their lag phase began. At this time, the volume of treated samples was about half that of the control. Moreover, the lag phase of NAA-treated berries was more than 50% longer (from 70 to 95 DAFB) with respect to the control. Thereafter, the volume increased, until reaching, at harvest (148 DAFB), a value similar to that observed in untreated samples (Figure 1A).

Anthocyanins content of whole berries in control samples increased very rapidly up to five days following the lag phase, and then gradually decreased until harvest (110 DAFB). The accumulation of anthocyanins was significantly inhibited in NAA-treated berries to almost undetectable levels up to 80 DAFB. Thereafter, a constant increase was observed until harvest (148 DAFB), finally reaching a level similar to that measured in the control (Figure 1B).

Soluble solids concentration (SSC) of control berries constantly increased throughout the whole experiment, especially during the lag phase of berry growth. NAA treatment showed an inhibitory effect also on this parameter, similar to that of anthocyanins. In fact, no increase of SSC was observed up to 80 DAFB, whereas a constant rise was measured thereafter, reaching at harvest the same values as the control (Figure 1C).

In control berries, a constant decrease of titratable acidity, well correlated with both malic and tartaric acid degradation, was observed during ripening evolution. On the other hand, NAA-treated berries always showed significantly higher levels of total acidity than the control, except for samples at harvest, whose acidity was similar to that assessed in untreated samples. However, in treated berries a clear correlation was observed only

between titratable acidity and malic acid content. Additionally, a significant increase of tartaric acid was observed immediately after the NAA treatment (65 DAFB), followed by a constant but less rapid decrease compared to control fruit (Figure 1D).

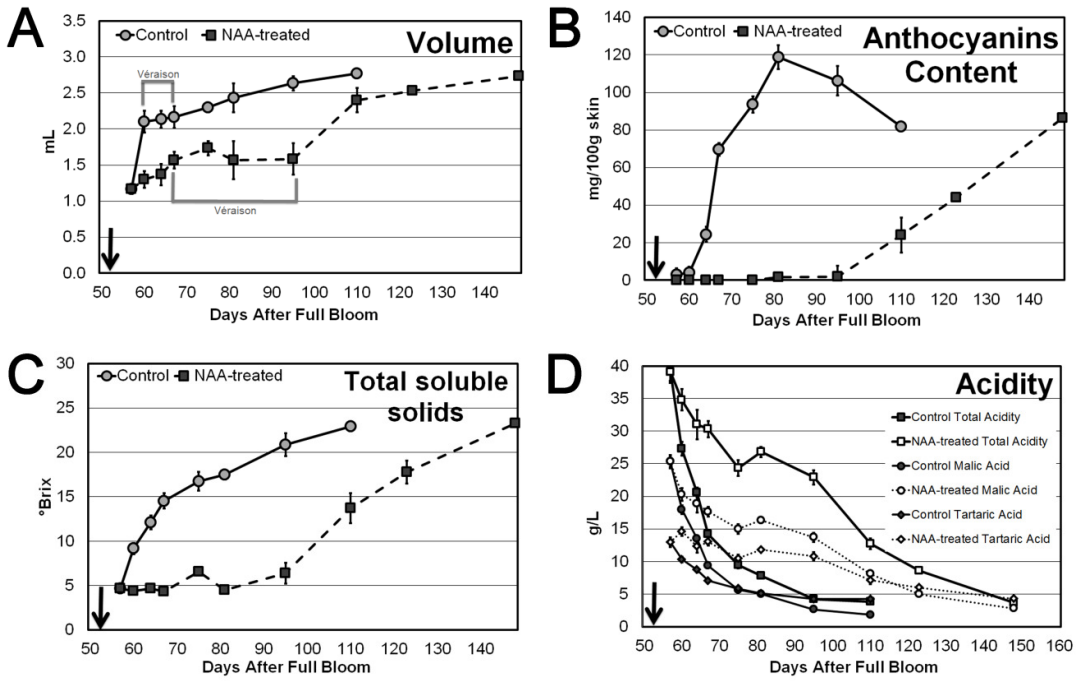

Figure 1. Biochemical analysis. Evolution of physical (berry volume) and chemical (anthocyanins content, solid soluble concentration, titratable acidity) parameters in control (circle) and NAA-treated (square) berries throughout fruit development. NAA treatment (arrow) was performed at 53 DAFB. Data concerning volume are the average of values obtained by fifty berries. Soluble solid concentration, tritatable acidity, malic acid, tartaric acid and anthocyanin contents are given by the average values of three biological replicates. Bars represent the SE.

3.2 Differentially expressed genes and enrichment analysis

Three comparisons were carried out by means of microarray experiments. The samples to be compared were chosen in order to achieve as much information as possible about the effect of the auxin treatment at the transcriptional level, its duration, and the implications in terms of physiological changes and technological relevance (see Additional file 1A and B). The first comparison was carried out between NAA-treated and control fruits at 60 DAFB (N1/C1) in order to identify genes differentially expressed at 3 days after the auxin treatment, in correspondence with the onset of véraison in the control. The second comparison was made on NAA-treated and control berries at 110 DAFB (N2/C2) in

correspondence of the harvest of untreated berries, to point out the effects of the treatment on ripening evolution. The third comparison (N3/C2) highlighted transcriptional differences present in treated berries, which had biochemical and phenotypic parameters similar to the control at harvest.

Among the three comparisons, genes with significant ($P < 0.05$) differential expression were 1,511 in N1/C1, 1,016 in N2/C2, and 1,136 in N3/C2 (see Additional file 2). Among the genes differentially expressed in N1/C1, N2/C2, and N3/C2, 239 (15.8%), 289 (28.4%), and 74 (6.5%) genes, respectively, showed a fold-change variation of at least 2-fold in terms of down- or up-regulation. It is noteworthy that treated samples at harvest (148 DAFB) showed an almost complete transcriptional recovery with respect to the control at 110 DAFB.

Microarray data were validated by means of qPCR experiments performed on a subset of selected genes, revealing similar expression patterns as confirmed by the significant correlation (Pearson coefficient = 0.77; $P = 0.0007$) pointed out between them (see Additional file 3).

In order to functionally classify the genes affected by the auxin treatment, Gene Ontology (GO) term enrichment analysis was performed, as described by Blüthgen et al. (2004) and Botton et al. (2008), in each of the three comparisons against the whole array background. A complete list of the enriched GO terms resulted from Fisher's exact test can be found in Additional file 4, Additional file 5 and Additional file 6. In the first comparison (N1/C1), no significant enrichment was found when a $Q < 0.05$ was considered as a threshold value, although GO terms related to protein synthesis (ribonucleoprotein complex, translation, ribosome, ribonucleoprotein complex biogenesis, ribosome biogenesis, structural constituent of ribosome) were significantly over-represented ($P < 0.01$). It is noteworthy that also the terms "protein transport" and "establishment of protein localization" were those with a higher significance and shown to be under-represented. At the second comparison (N2/C2), few terms showed a significant Q. However, considering the $P < 0.01$, terms related to the cell wall (external encapsulating structure organization, cellular cell wall organization or biogenesis) appeared to be overrepresented (Additional file 5). In the last comparison (N3/C2), GO terms related to development (developmental process, anatomical structure development, multicellular organismal development) were

significantly over-represented with Q < 0.05. It is worthy to note that among the terms with a significant P value, particularly enriched are those related to 1,3-β–glucan (1,3-beta-glucan biosynthetic process, beta-glucan metabolic process, betaglucan biosynthetic process, 1,3-beta-glucan metabolic process, 1,3-beta-glucan synthase activity, 1,3-beta-glucan synthase complex). Among the hormone-related terms, the "jasmonic acid mediated signaling pathway" was overrepresented.

3.3 MapMan analysis

To investigate the main metabolic pathways affected by the NAA treatment, a MapMan analysis (Thimm et al., 2004) was performed on N1/C1 comparison based upon differentially expressed genes chosen according to P < 0.084, which was shown to be an acceptable threshold according to array validation analyses carried out with qPCR. This specific threshold was chosen in order to enlarge the number of genes to be used as input data for the Map- Man software.

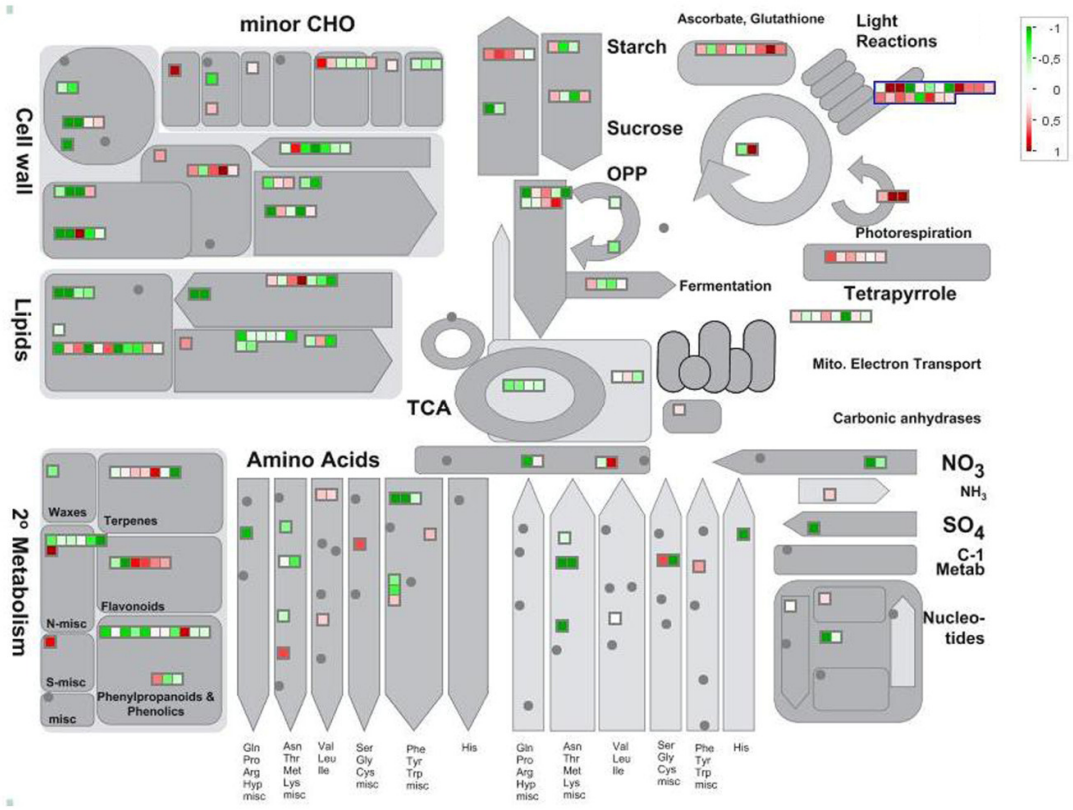

Figure 2. MapMan analysis. MapMan visualization of differences in expression of genes involved in metabolic processes. Classification into bin categories were done by using a mapping file of the grape

AROS V1.0 platform (http://mapman.gabipd.org/web). Heat maps show genes with statistically significant (P value < 0.084) differential expression identified by comparing NAA-treated and control berries at 60 DAFB (N1/C1). A conventional red-to-green scale was used to indicate up-regulation (red) or down-regulation (green).

MapMan pointed out that several metabolisms were down-regulated in NAA-treated berries, such as those involving cell wall metabolism, carbohydrates, lipids, secondary metabolites, and amino acids, with the only exception of the light reactions pathway that showed a general up-regulation (Figure 2).

The cell wall and secondary metabolism bin categories, which were linked to the above described biochemical parameters, were investigated. The cell wall category included genes coding for pectin methyl esterase, endo- transglycosylase, polygalacturonase, and expansin-like protein (Figure 2), whereas the secondary metabolism included genes encoding alcohol dehydrogenase, phenylalanine ammonium lyase (phenylpropanoids and phenolics pathway) and chalcone synthase (flavonoid pathway). Within this secondary metabolism category, genes coding for ß-carotene hydroxylase (terpenes pathway) and cynnamoyl-CoA reductase (flavonoid pathway) showed an up-regulation in NAA-treated berries (Figure 2). Expression patterns of key genes involved in cell expansion and phenylpropanoids pathway were validated in qPCR experiments carried out in all samples (see Additional file 7). This validation analysis pointed out that the expression profiles of selected genes (anthocyanins: CHS1, Vv_10010748; CHS3, Vv_10004167; F3H, Vv_10003855; UFGT, Vv_10004481, MYB31, Vv17s0000g06190 and MYB4, Vv4s0023g03710; cell wall metabolism: PG1, Vv_10003791, and EX1, Vv_10000426; water uptake: TIP;2-like, Vv_10003817 and AQUA1, Vv_10003711), paralleled the kinetics of anthocyanins content and berry volume (Figure 1), showing an early inhibitory effect of the auxin treatment, followed by a recovery at harvest, when the treated samples showed transcripts levels similar to the control.

A detailed list of genes with the respective bin codes belonging to each MapMan category is reported in Additional file 8.

3.4 HORMONOMETER analysis

To understand the hormone-related transcriptional response of the berry to the auxin treatment, a HORMONOMETER analysis was carried out relying upon putative hormone indexes whose transcript levels were measured by means of the microarray. This tool allows to describe, in terms of correlation (or anti-correlation), the similarity (or dissimilarity) between a query transcriptional response and a transcriptional response typically assessed upon a certain hormone treatment as defined by known hormone indexes in Arabidopsis. Separate runs of this tool were carried out with different subsets of genes as input, as performed by Bonghi et al. (2011). The subsets are: i) all the hormone indexes (H), ii) genes with hormone-specific responsiveness (sRG), iii) hormone-responsive genes encoding TFs (TFs), and iv) genes encoding TFs with hormone-specific responsiveness (sTFs). Along with this analysis, mean log ratios (weighted according to the P level of significance) of genes belonging to biosynthesis (BS), metabolism (MET), transport (TR), perception (PER), signal transduction (ST) and hormone-responsiveness (HR) categories were calculated for each of the eight hormones considered by the HORMONOMETER. The categorization was made according to the Arabidopsis Hormone Database 2.0 (AHD) web site (http://ahd.cbi. pku.edu.cn/). Both analyses were carried out in the three comparisons made with microarrays and the resulting heat maps were focused on hormones involved in grape berry ripening with a primary role (i.e. auxin, ethylene, abscisic acid and brassinosteroids) (Figure 3).

The proportion of hormone responsive genes in Arabidopsis ranges between 3.8 and 9.4% of the whole transcriptome (TAIR 10 version; 27,416 genes) according to the hormone considered, whereas in grape the percentage ranges between 5.5 and 10.1% of the whole gene set (12X genome assembly, see Materials and Methods section). As far as the grape microarray is concerned (14,562 genes), the proportion of hormone responsive genes are similar to that of Arabidopsis, ranging from 4.3 to 8.9% with values for each hormone comparable to those calculated for Arabidopsis (See Additional file 9). A minimal bias may therefore be assumed to exist when grape expression data are used as input for HORMONOMETER, as hypothesized in a recent work on peach (Bonghi et al., 2011).

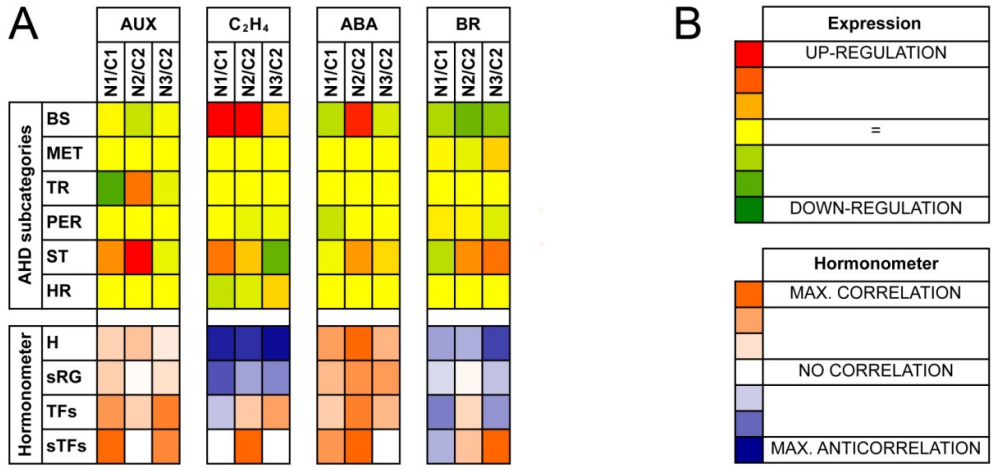

Figure 3. HORMONOMETER. Heat maps showing the expression of AHD subcategories (top) and the HORMONOMETER results (down). A. The heat map was produced by considering the genes encoding elements of hormone biosynthesis (BS), metabolism (MET), transport (TR), perception (PER), signal transduction (ST), and response (HR), for auxin (AUX), ethylene (C2H4), abscisic acid (ABA) and brassinosteroids (BR), which are the hormone primarily involved in grape berry ripening. HORMONOMETER data were grouped into hormone-responsive genes (H), genes with hormone-specific responsiveness (sRG), hormone-responsive genes encoding TFs (TFs), and genes encoding TFs with hormone-specific responsiveness (sTFs). For each hormone, the following comparisons have been analyzed: N1/C1, N2/C2, and N3/C2. See the Materials and Methods section for a detailed description. B. Colour codes for the two heat maps. For the AHD subcategories, red and green represent up- and down-regulation, respectively. In the HORMONOMETER, orange (value = 1), white (value = 0), and blue (value = -1) indicate a complete correlation, no correlation, or anti-correlation, respectively, in terms of direction and intensity of the hormone index with the queried experiment.

Within the AHD subcategories related to auxin, significant variations in genes encoding TR and ST elements were observed. In the first comparison (N1/C1), the auxin treatment repressed the transport of the hormone, at least at the transcriptional level, along with the significant up-regulation of its ST elements. The other AHD subcategories did not show any significant variations. These data were paralleled by a substantial correlation in the HORMONOMETER results, more significant when only the TFs were considered in the analysis, especially the auxin-specific ones (sTFs). The second comparison (N2/C2) reflected a situation typical of an auxin related transcriptional response. The AHD subcategories indicated that the BS elements were slightly repressed and that both the TR-

and ST-related genes were significantly up-regulated. This may be interpreted as a typical homeostatic response, confirmed by the HORMONOMETER results, which indicated a general unspecific correlation between auxin target expression and the typical auxinrelated transcriptional response. In the last comparison (N3/C2), the AHD categories were very stable and the HORMONOMETER analysis still pointed out an active transcriptional response to auxin, with a significant correlation for the TFs and sTFs subsets, suggesting that the response to the hormone may have involved mainly auxin specific transcription factors.

As concerns ethylene, interesting data were observed regarding both the AHD subcategories and the HORMONOMETER results. As far as the biosynthetic genes are concerned, a strong up-regulation was found in the second comparison (N2/C2), whereas data in the other two cases were less significant. The ST-related transcription showed a significant variation in all comparisons, being stimulated in the first and second (N1/ C1 and N2/C2, respectively), and repressed in the third (N3/C2). Significant variations were observed also in the HR genes, which were down-regulated in all cases except the N3/C2 comparison. The HORMONOMETER analysis showed a strong and broad anti-correlation in all situations when all genes and the ethylene-specific ones (sRG) were considered. An almost reversed situation was observed in the other subsets (TFs and sTFs), except for the first comparison (N1/C1) that still showed an anti-correlation and no correlation, for TFs and sTFs, respectively. In N2/C2, a stronger correlation was found for sTFs than for TFs, whereas in the third comparison no correlation was found for the hormone-specific TFs.

Genes coding for BS elements of abscisic acid (ABA), were down-regulated at N1/C1 comparison, while in the N2/C2 comparison they were up-regulated. A weak transcriptional repression was found for genes encoding PER elements in the first comparisons, although with low significance. A stimulation of transcription was found in ST-related genes that paralleled that of BS. The HORMONOMETER showed a general correlation in all subsets and all situations, without, however, any ABA-specificity.

The brassinosteroids category showed significant data in both the analyses (AHD subcategories and HORMONOMETER). BS-related genes varied significantly in all three comparisons, with a down-regulation trend in all cases. Slight, but not significant,

variations were also observed with respect to the genes encoding MET elements. A down-regulation was reported for genes coding for PER elements in the third comparison (N3/C2). Genes related to ST were down-regulated in N1/C1 and clearly up-regulated in all the other cases. Finally, the HORMONOMETER analysis evidenced an extensive anti-correlation, with the only exceptions of all TFs and sTFs in the second and third comparisons, respectively. In particular, the latter case pointed out a significant correlation.

3.5 Expression of auxin-, ethylene-, and abscisic acid related genes

Expression patterns of selected auxin-, ethylene-, and ABA-related genes were validated by qPCR experiments. As far as the former genes are concerned (Figure 4), the NAA treatment negatively affected the expression of Tryptophan Synthase beta-subunit 1 (TRYPS-like, Figure 4A), a gene involved in the biosynthesis of tryptophan, an auxin precursor. The treatment also induced the accumulation, up to 95 DAFB, of transcripts of genes responsible for auxin perception (Transport inhibitor response 1, TIR1-like; Figure 4B), polar transport (PIN3-like; Figure 4C) and irreversible conjugation (Indole-3-acetic acid amido synthetase, GH3-like; Figure 4D). Concerning the signal transduction, two AUX/ IAA genes (IAA4-like and IAA31-like; Figure 4E and F) and Auxin response factor 8 (ARF8-like; Figure 4G) were upregulated in treated berries one week after NAA application (60 DAFB), whereas later on and up to harvest the accumulation of their transcripts was higher in control berries.

ACC synthase (ACS6) and ACC oxidase (ACO2) genes, encoding the key enzymes of ethylene biosynthesis, were strongly up-regulated in treated berries during véraison (Figure 5A and B). Two genes encoding ethylene receptors, i.e. Ethylene insensitive 4 (EIN4-like) and Ethylene response sensor 1 (ERS1-like), had similar expression levels in both control and NAA-treated fruits until the inception of ripening in the control (60 DAFB), when a significant increase was registered earlier in untreated berries than NAA-treated ones (Figure 5C and D). Three Ethylene response factors genes (ERF3-like, ERF-AP2-like and ERF5-1), involved in the regulation of ethylene response, were all positively affected by the NAA treatment, although with different timings (Figure 5E, F and G).

Since the HORMONOMETER analysis showed some significant variations also in the expression of abscisic acid (ABA) targets, two ABA-related genes were also investigated.

The 9-cis-epoxycarotenoid dioxygenase3 (NCED3), which is a key gene involved in ABA biosynthesis, was significantly down-regulated in NAA-treated samples one week after the treatment (60 DAFB) (Figure 5H) and Abscisic acid insensitive 3 (ABI3), involved in ABA perception, was down-regulated in NAA-treated berries up to 95 DAFB. Thereafter, its mRNA levels in NAA-treated samples reached those observed in untreated samples (Figure 5I).

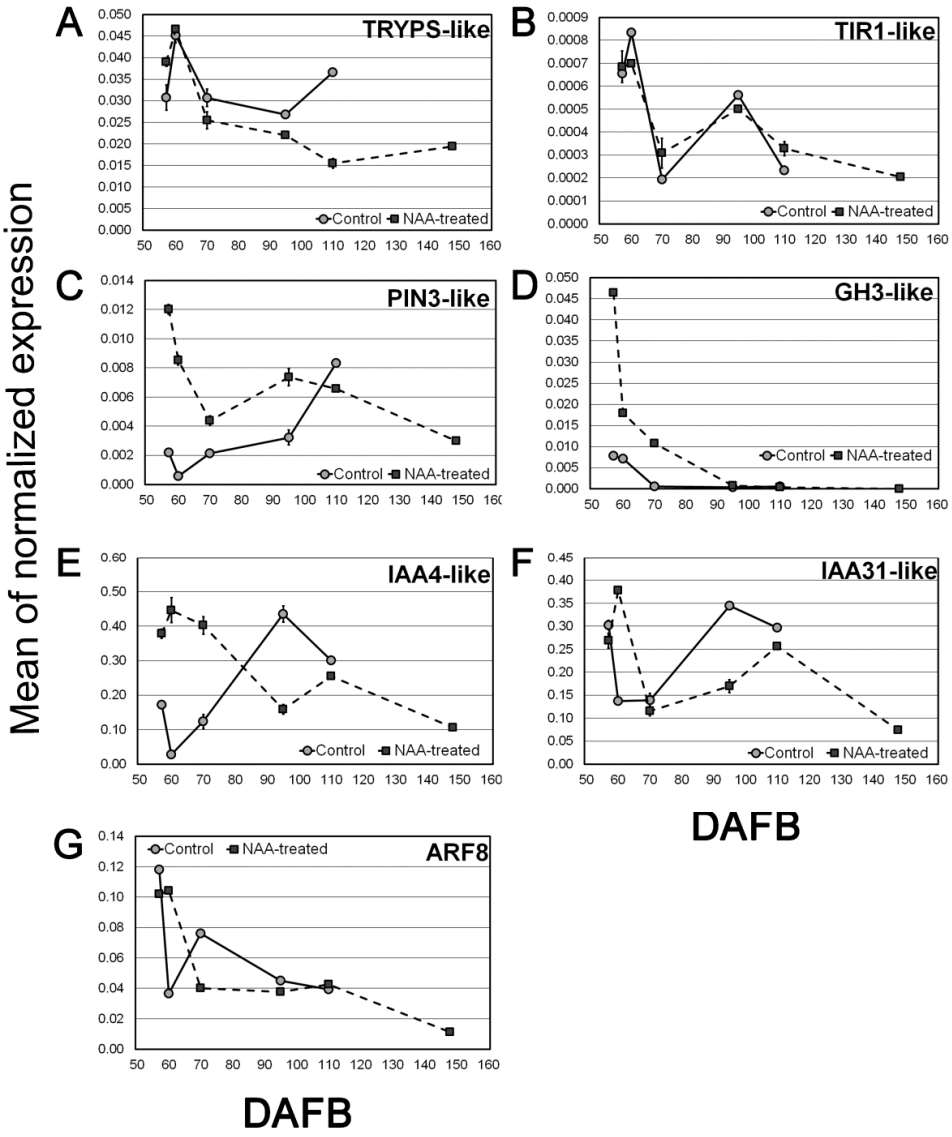

Figure 4. Expression pattern, evaluated by qPCR, of genes involved in auxin biosynthesis, conjugation, transport and signal transduction. Expression pattern, evaluated by qPCR, of the following auxin-related genes: TRYPS-like (Vv_1007514, A), TIR1-like (Vv_10005087, B), PIN3-like (Vv_10007217, C), GH3-like (Vv_10007966, D), IAA4-like (Vv_10002615, E), IAA31-like (Vv_10000794, F), ARF8-like

(Vv_10003009, G). Transcript levels in NAA-treated (square) and control (circle) berries are shown as means of normalized expression ±SE.

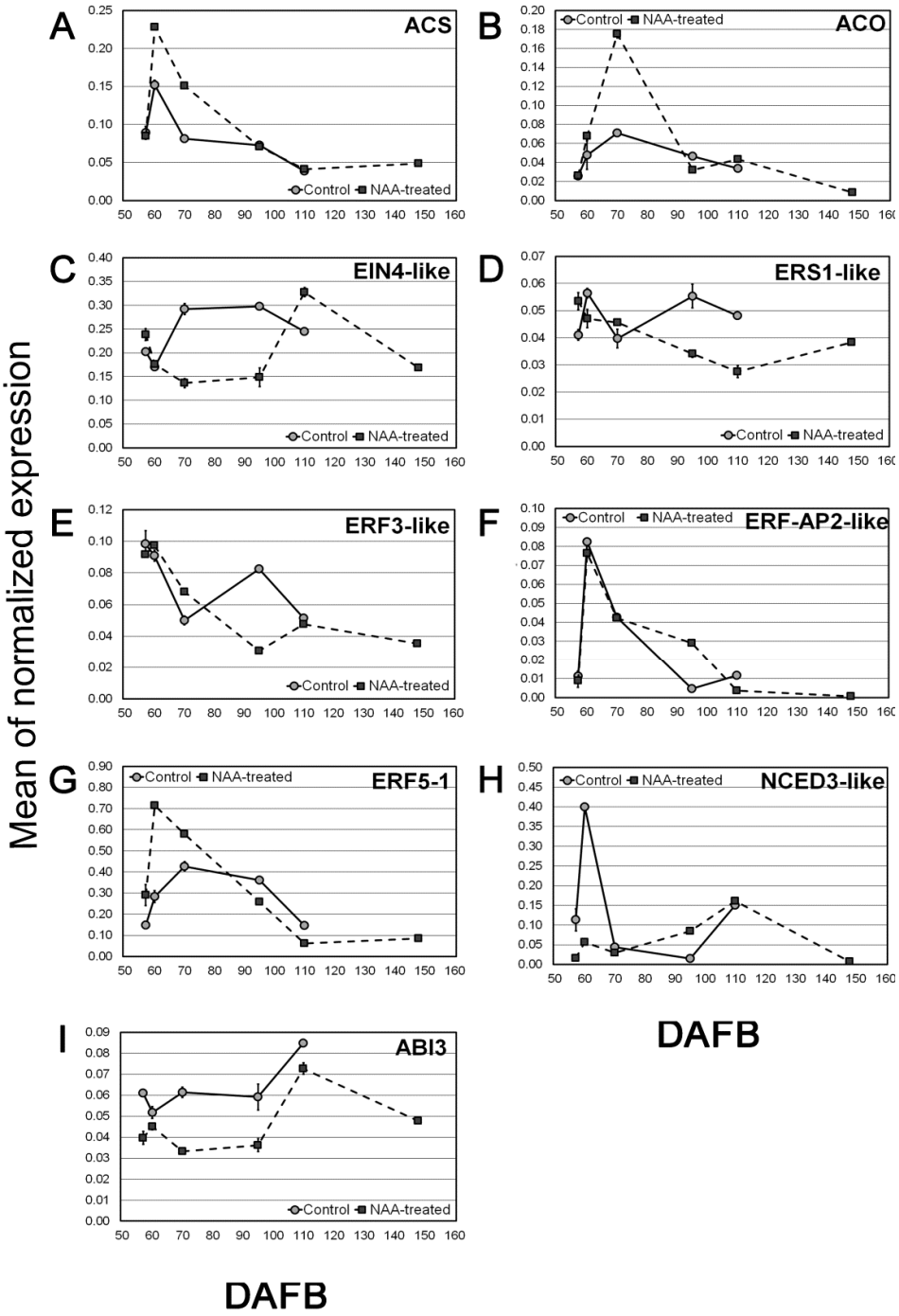

Figure 5. Expression pattern, evaluated by qPCR, of genes involved in ethylene and ABA biosynthesis and signal transduction. Expression pattern, evaluated by qPCR, of the following ethylene- and ABA-related

genes: ACS6 (Vv_10001614), ACO2 (Vv_10004370), EIN4-like (Vv_10010357), ERS1-like (Vv_10007917), ERF3-like (Vv_10001775), ERF-AP2-like (Vv_10000332), ERF5-1 (Vv_10001287), NCED3 (Vv_10009127), ABI3 (Vv_10001065). Transcript levels in NAA-treated (square) and control (circle) berries are shown as means of normalized expression ±SE.

4. Discussion

A ripening delay caused by the application of auxins has been previously recorded both in climacteric and nonclimacteric fruits (Cohen et al., 1996; Davies et al., 1997). In grapevine, a ripening delay induced by the application of natural or synthetic auxins, including NAA, to berries before véraison was observed in a large range of cultivars (Jeong et al., 2004; Detyeux-Belleau et al., 2007). Results presented in this study confirmed that NAA, applied at the prevéraison stage, strongly delays ripening inception in cv. Merlot (Figure 1). All the parameters used to monitor the ripening progression (in particular berry volume, SSC, and titratable acidity), with the exception of the initial delay occurring in the treated berries, showed overlapping kinetics in both treated and untreated fruit. These data suggest that the auxin treatment caused just a shift in the initiation of ripening, as already hypothesized by Böttcher et al. (2010). This observation is also confirmed by the microarray data analysis, which showed a decreasing number of differentially expressed genes throughout the experiment (see Additional file 2). At the véraison of control fruit (60 DAFB), MapMan analysis clearly shows that NAA application down-regulated genes involved in cell expansion (cell wall metabolism and water uptake) and secondary metabolism, in particular those responsible for flavonoids biosynthesis (Figure 2), consistently with the biochemical analyses. This repressive effect remained well evident up to 110 DAFB, whereas a partial recovery was observed thereafter, as already reported by Davies et al. (1997) and Jeong et al. (2004). At harvest of NAA-treated berries (148 DAFB), the transcription level of genes involved in the flavonoids biosynthetic pathway was still enhanced with respect to the control fruit at harvest, while a full recovery was observed for those involved in cell wall metabolism and water uptake (see Additional file 7). These observations show that NAA is more effective in counteracting the accumulation of flavonoids rather than berry expansion, as demonstrated by Böttcher et al. (2011).

Both in control and treated fruits there is a clear coordination of the transcriptional regulation of genes determining cell expansion (i.e. EX1 and PG1) and turgor (i.e. Pip1), in agreement with the model for cell expansion proposed by Cosgrove (1997), especially during the early post-treatment phases and up to 95 DAFB. During this phase, the NAA treatment clearly repressed the genes involved in both processes, which was consistent

with the berry volume measures and thus reflecting an almost exclusive transcriptional control of berry expansion. An inversion of this trend and a complete recovery to the levels of the control was observed thereafter (after 95 DAFB), although not correlated with the faster volume increase occurring in treated berries to reach a final volume at harvest equal to that of the control fruits. This may be due to different mechanisms controlling berry expansion other than the auxin-controlled transcription, most likely at posttranscriptional level, as previously demonstrated for aquaporins whose gating behaviour can be affected by phosphorylation, heteromerization, pH, Ca2+, pressure, solute gradients and temperature (Chaumont et al. 2005). Regulation of aquaporin trafficking may also represent a way to modulate membrane water permeability. Taken together, these data indicate that the berry expansion process is under the control of multiple regulatory pathways, involved according to a well-defined developmentally-programmed chronological sequence.

To shed light on the role of auxin and its cross-talk with other hormones in the regulation of berry ripening, a specific analysis was carried out on hormone-related genes by using the HORMONOMETER bioinformatic platform (Volodarsky et al., 2009). This was paralleled by a merged analysis of specific gene categories (i.e. the AHD categories). This approach allowed to set up a hypothetical model describing what happened in terms of auxin-related response after the NAA treatment (Figure 4). The application of NAA caused an excessive availability of auxin, most likely counterbalanced by homeostatic mechanisms involving synthesis, breakdown, conjugation and transport (Perrot-Rechenmann and Napier, 2005; Woodward and Bartel, 2005). However, at 60 DAFB auxin biosynthesis and metabolism gene categories did not differ significantly between control and treated samples, transport was generally repressed, and an auxin-specific transcriptional response was seen along with a general activation of signal transduction elements. Therefore, it is likely that the homeostatic mechanisms had already been activated within the first 7 days after the treatment. This hypothesis is supported by qPCR expression data, especially those related to GH3-like, IAA4-like, and IAA31- like genes (Figure 5D, E, F). In NAA-treated berries at 57 DAFB, the first of these three auxin-related genes was expressed 6-fold higher compared to the control, then its expression decreased to just 2.5-fold at 60 DAFB, followed by a constantly decreasing trend leading

to the same levels measured in the control at 95 DAFB. GH3 (Gretchen Hagen 3) genes, specifically those belonging to group II (Staswick et al., 2005), encode enzymes that conjugate IAA to amino acids. Interestingly, it has been recently shown that GH3.1 plays a role in the formation of IAAAspartate at the onset of grape berry ripening, and it positively responds to the combined application of ABA and sucrose, and to ethylene, linking it to the control of ripening processes (Böttcher et al., 2011). Nevertheless, both the IAA genes showed well-correlated diverging trends from 57 up to 60 DAFB, with the highest differences pointed out in the latter time point, coinciding also with the highest level of their expression in NAA-treated berries. Also the ARF8-like gene showed the largest divergence at 60 DAFB and the HORMONOMETER data indicate a very active transcriptional control compatible with an auxinspecific response. The expression patterns of these four genes along with the HORMONOMETER data and the overall physiological response indicate that biologically active concentrations of auxin were achieved throughout a homeostatic recovery occurring within 7 days after the treatment, during which the physiological response is mainly unspecific and due to a likely pharmacological effect of NAA. During this period, conjugation and transport may contribute to a decrease in the auxin levels, leading to the same range of concentration that can be found before ripening inception, thus generating a developmental block. This block is most likely mediated by a primary auxin signaling, whose main players include the IAAs and the ARFs, as their expression patterns indicate. At 110 DAFB, an overall repression of biosynthetic genes along with a stimulation of those coding for TR and ST elements was observed in NAA-treated samples.

The HORMONOMETER indicates the activation of specific gene targets that were not auxin-specific, although they were compatible with still biologically active auxin levels. In this phase, a likely secondary homeostatic response was occurring, mainly at the level of biosynthesis as shown by the repression of upstream auxin BS genes such as TRYPS-like. The primary transcriptional response achieved within 110 DAFB triggered the recovery cascade that was active also thereafter, as demonstrated by biochemical parameters. However, at this stage the biological meaning of the homeostatic recovery is different from that occurring before 60 DAFB. It is likely that the early homeostatic reaction was just aimed at detoxifying from high auxin concentrations, whereas that

occurring at 110 DAFB was a symptom of a normal ripening progression resembling the natural ripening inception during which auxin levels were shown to decrease (Böttcher et al., 2011). Some auxin-specific targets, mainly TF encoding, were shown to be active up to 148 DAFB, most likely triggering the transcriptional regulation of genes, such as CHS1 and F3H that were shown to be down-regulated (see Additional file 7, A and C). At this stage, however, the overall transcriptional response was scarce since berry ripening was definitely accomplished, as shown by the physiological and biochemical parameters.

Fluctuations in auxin levels and response were shown to be correlated with ripening progression and a possible mechanism was hypothesized to explain how the berry reacts to the NAA treatment, but how does auxin action link to other hormones, such as ethylene, ABA, and brassinosteroids, that are known to regulate the same developmental processes? The HORMONOMETER analysis may help to explain this aspect, especially considering the first comparison (N1/C1), in which the existence of a strong antagonistic effect between auxin and ethylene and, to a lesser extent, a substantial 'synergism' between auxin and ABA were shown. Both these aspects were quite marked for both the whole subset of transcriptional indexes (H) and the specific ones (sRG). The transient positive effect of NAA on the transcription of ACS6 and ACO2 genes (Figure 5A and B), already measured in other fruits (Barry et al., 2000; Trainotti et al., 2007; El-Sharkawy et al., 2008), may be interpreted as a part of the secondary homeostatic reaction to the auxin treatment, as described above. As such, the transient increase of ethylene biosynthesis specifically induced by biologically active auxin concentrations would counteract the excess of auxin by activating downstream mechanisms, in this case related to the biosynthesis of the hormone (i.e. the TRYPS gene), thus releasing the berry from the developmental block.

According to the Arabidopsis model of ethylene signaling, reduced expression and activity of receptors increase sensitivity to ethylene, whereas increased receptor expression and activity decrease sensitivity (Lin et al., 2009). It is also known that ethylene receptors act in cooperation, according to mutual, but often unique roles, thus differentially regulating ethylene responses and giving diverse outputs according to the receptor complex combination (Liu et al., 2012). Furthermore, in Arabidopsis, EIN4 was shown to have a unique role in ethylene signaling (Hua *et al.,* 1998; Cancel *et al.,* 2002) and a synergistic

effect on ers1 function, as it is required to maintain ethylene insensitivity in an ers1 background (Liu et al., 2012). Taking into account these data, a relevant role during grape berry ripening may be played by the putative AtEIN4 orthologue, as the corresponding gene was expressed in a ripening-dependent manner, with increasing levels after véraison, measured both in the control and NAA-treated samples (Figure 5C). Also an ERS1-like gene showed similar expression patterns, although shifted ahead (Figure 5D). Similar transcriptional behaviors were reported also by Deluc et al. (2007) and Chervin and Deluc (2010) along with a peak of ethylene biosynthesis, and may be consistent with a higher sensitivity to the hormone at véraison (delayed by the auxin treatment), which decreases thereafter throughout ripening.

The effect of auxin on genes involved in ethylene response was very weak, as seen in both the AHD and the HORMONOMETER analyses (Figure 3), with the exception of an ERF5-1 gene, which was significantly up-regulated at 60 DAFB (Figure 5G). A significant correlation was observed between the expression patterns of this gene and ACS6, leading to the hypothesis that ERF5-1 may mediate the auxin-induced up-regulation of ethylene biosynthetic genes in grape. This hypothesis is currently being investigated with dedicated experimental trials in order to shed light on the crosstalk between these two hormones, which is crucial for grape berry development and ripening.

Although the NAA treatment caused a general stimulation of ethylene biosynthesis and action, a negative effect on the transcription of genes involved in flavonoids biosynthesis, cell wall metabolism and water uptake, previously shown to be ethylene-related (El-Kereamy et al., 2003; Chervin et al., 2008), was observed. Several studies have examined the interactions between auxin and ethylene at the transcriptional level and different models were proposed (Stepanova et al., 2007; Lewis et al., 2011; Muday et al., 2012). Taking into account this information, the effect of NAA may have bypassed the primary level of crosstalk between the two hormones, resulting into the activation of only some targets in common with ethylene that may belong to the secondary crosstalk. Consistent with this possibility, the upstream regulatory regions of many genes induced by auxin and ethylene were shown to contain putative auxin response element (AuxRE) and ethylene response element (ERE) sequences, which are sites for ARF and EIN3/EIL binding, respectively (Lewis et al., 2011). Future studies should specifically address this aspect.

The existence of a synergism between auxin and ABA was unexpected taking into account the opposite roles previously claimed for these hormones in the regulation of grape berry ripening (Davies et al., 2009). These data, however, may indicate that the HORMOMETER analysis is able to reveal a previously unappreciated selectivity of auxin towards the regulation of ABA-related processes, as already reported by Volodarsky et al. (2009) for salicylic acid and auxin. In fact, data presented here pointed out that auxin down-regulated the genes involved in ABA biosynthesis (Figure 5H), while the signal transduction pathway elements were substantially unaffected or stimulated (see Additional file 2). These ambiguous outcomes were already pointed out in previous studies revealing that ABA and auxin signaling pathways belong to a very complex regulatory network with unexpected features (Nemhauser et al., 2006).

5. Conclusions

Taking into account the available data concerning the hormonal regulation of the ripening syndrome in grape and tomato, a putative model was herein assembled to better understand the hormonal cross-talk occurring during our experiments (Figure 6).

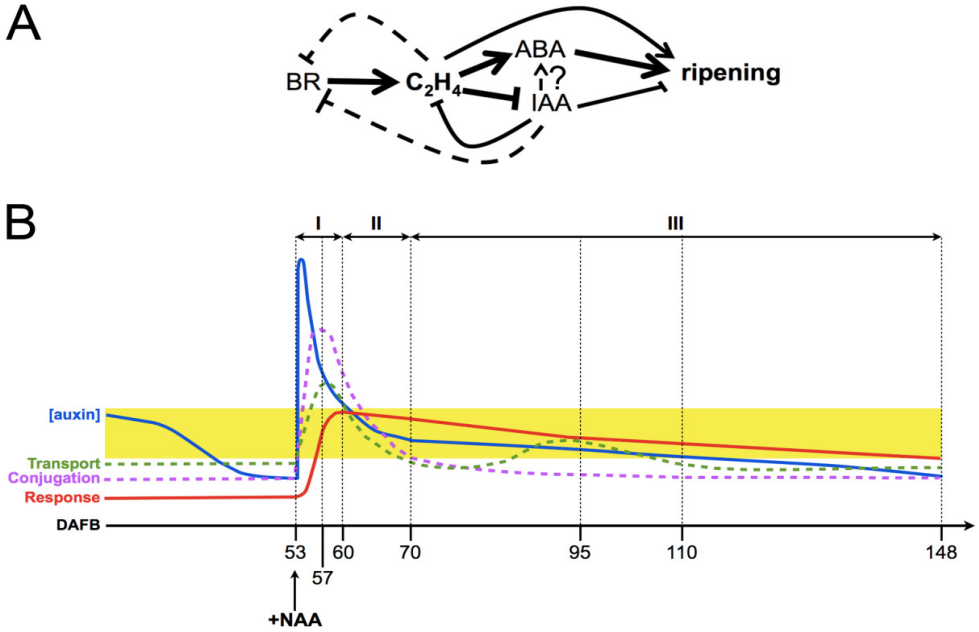

Figure 6. Hypothetical model summarizing the interactions occurring between the hormones mainly involved in the regulation of ripening inception and progression. A. Brassinosteroids and ethylene may trigger the first molecular events associated with ripening inception, with the latter hormone involved in the developmental shift preceding véraison. Ethylene would also negatively regulate auxin action by repressing its biosynthesis and trigger ABA-related genes in order to enable the progression of ripening-associated biochemical changes. A direct positive effect of ethylene on ripening may also be postulated based upon available data. Conventional symbols are used to describe positive and negative interactions. The thickness of the lines indicates the preferential ways of interactions determining ripening inception and progression, whereas dotted lines indicate possible feedback interactions. Interactions occurring between auxin and ABA are complex and still under investigation. B. Hypothetical model explaining auxin-related events occurring upon the NAA treatment (+NAA). This model was assembled based upon the expression of auxin-related genes. The yellow-shaded area indicates a likely range of auxin concentration compatible with its biological activity. Three main responsive phases were identified according to this model: phase I (53-60 DAFB), during which the berry displays a primary homeostatic response most likely due to an unspecific pharmacological reaction; phase II (60-70 DAFB), during which a biologically active concentration of auxin is recovered and a secondary homeostatic response is triggered, and phase III (70-148 DAFB), in which a normal ripening progression is observed.

According to this working model, which is currently being validated, brassinosteroids (BR) may start the cascade of events leading to ripening by increasing ethylene levels, as reported in tomato (Vardhini et al., 2002). It is known that a dramatic increase in endogenous BR levels occurs at the onset of fruit ripening in grape (Symons et al., 2006) and that also an ethylene peak is measurable just before véraison (Chervin et al., 2004). Moreover, ethylene seems to repress BR-regulated genes once ripening is triggered (Tonutti et al., unpublished data), thus indicating a possible feedback mechanism allowing a time progression of the syndrome through the coordination of the downstream events. According to this view, ethylene may play a central role in ripening inception. On one hand, it acts independently and directly on the activation of ripening-associated processes, such as those related to cell wall modifications (Chervin et al., 2008), and on the other hand it cooperates with ABA to indirectly trigger several biochemical changes associated with ripening, such as berry coloration (El-Kereamy et al., 2003; Sun et al., 2010). It also represses auxin biosynthesis, thus releasing the berry from the developmental block exerted by this hormone (Böttcher et al., 2010). When the NAA treatment was performed, the berry was most likely undergoing this developmental shift controlled by ethylene, which was still reversible. Therefore, the transient increase in auxin levels imposed by the exogenous treatment caused a reversion by counterbalancing the developmental control exerted by ethylene, thus leading the berry back to the pre-véraison stage with a consequent delay of the ripening progression.

6. Acknowledgements

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Chapter V – General conclusions

Viticulture and wine producing are important practices for the economy of several countries in Europe, and also in others continents. Despite its importance, global wine production decreased 6% in 2012 to 252 million hectolitres (Figure 1; Organization of Vine and Wine, OIV, 2013).

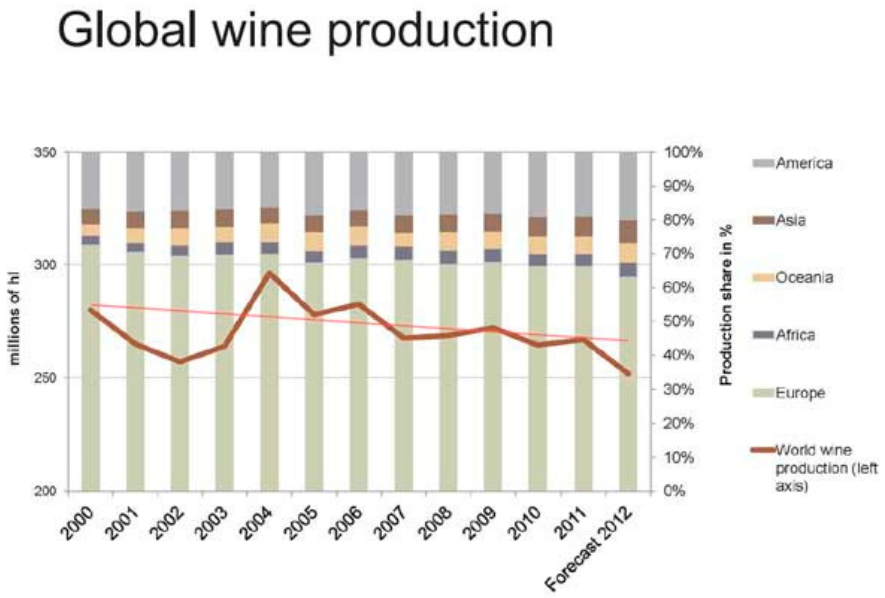

Figure 1. Global wine production 2000-2012, source OIV

This was partially due to a lower harvest in Europe during lasts years but also a longer term trend. France, Italy, and Spain are still the biggest producers, while it is worth noticing that China, Chile, and New Zealand recorded the largest increases in production over the last years. In spite of the downward trend in vine surface area, grape production underwent an upward trend over the last few years (Fraga *et al.*, 2012).

Viticulture and winemaking are influenced by a large number of factors, among which climate, soils, and grown varieties/genotypes are the most important (Fraga *et al.*, 2012). Climate is a key factor in the present viticulture (Figure 2). Grapevine physiological change, together with grape berry development and ripening, are high related to the clime. As showed in Figure 2, the inception and the duration of each phenological stages is mostly related to environmental conditions (Jones and Davis, 2000).

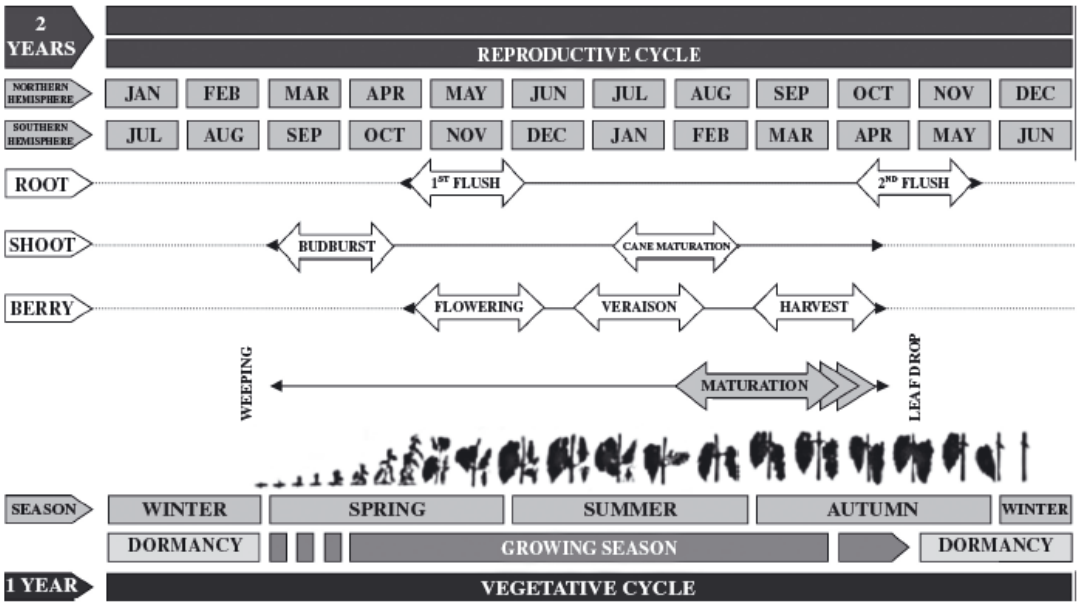

Figure 2. Vegetative and reproductive cycles and vine phenological stages. *Modified from Fraga et al. (2013)*

The duration of the growing season of a particular cultivar is affected, together with the climate which strongly influences the development of this crop and the yield and wine quality, also by the combination of these factors: soil moisture, air temperature, and crop-management practices (Webb *et al.*, 2012). Within this context, climate changes is the most important factor in which the viticulture have to cope with (Hannah *et al.*, 2013). As described in the first chapter of this thesis, breeding of new grapevine genotypes, which can better deal with the environmental changes, is essential for Italian and European viticulture. Indeed, development of new grapevine rootstocks with an higher tolerance to environmental stresses, drought in particular, should be a successful strategy to overcome climate limitations (Hannah *et al.*, 2013) and maintain the traditional Mediterranean grapevine growing area. This strategy have several advantages compared to the breeding programs associate to grape cultivar, mainly related to the handiness to confer desired carachteristic (e.g. drought tolerance) to the vine. Taking into account results presented in this thesis and in another work (Meggio *et al.*, IN PRESS), M4 rootstock well comply with this requisite. Indeed it showed an higher resistance to drought in comparison to 101.14 susceptible genotype, acting different strategies related to the regulation of different metabolism and pathways (e.g. plant hormones, sugars, flavonoids and

stilbenes). In addition to their capability to overcome climate limitations, grapevine rootstocks greatly influenced grapevine reproductive performances (Koundouras *et al.*, 2008; Kidman *et al.*, 2013) fruit development, ripening and quality (Walker *et al.*, 2002, 2004). So, together with the induction of an higher tolerance to environmental disturbance to the scion, viticulture need new rootstocks which did not alter quality of grape berry and wine or, better, which increase their qualitative characteristics.

The second part of this thesis showed that M4, in addition to the higher tolerance to drought, did not significantly alter grape berry quality of Cabernet sauvignon cultivar. Indeed, it was showed that it cause an advance in the onset of ripening in comparison to the 1103P rootstock, which is a more vigorous rootstock (Gambetta *et al.*, 2012). Within this background, M4 not only enhance water stress tolerance, but also positively influenced grape berry development and ripening throughout the control of different metabolism, among which auxins seem to play a pivotal role.

So, considering new scenario for the European and Italian vine growing and the climate changes which can alter quality of grape berries and wine on a global scale, development of new rootstocks with desirable traits, together with those belonging to the "M series", it will be one of the main goal of the future viticulture.

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Additional Chapter

Sensorial, biochemical and molecular changes in Raboso Piave grape berries applying "Double Maturation Raisonnée" and late harvest techniques

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**Equal contribution*

1. Introduction

At ripening Raboso Piave grape berries have high acidity level and an unbalanced polyphenols profile, in which condensed tannin precursors (e.g. catechin) are prominent (De Rosso et al., 2009). These two features cause the austere aroma (De Rosso et al., 2010; Mattivi et al., 2006), which is the main contributor in the sensory profile of the Raboso Piave wine.

To alter the composition of the berries, and consequently improve wine quality, viticulture strategies can be adopted based on a delay of traditional harvest (TH) using Late Harvest (LH) or withering carried out both on- or off-plant. LH produces significant changes in wine composition by inducing levels of proline, sugar, alcohol and total acid content in the decanted must and base wine (Guillaumie et al., 2011). Off-plant withering causes the accumulation of abscisic acid (ABA) and proline, as well as an increase in alcohol dehydrogenase (ADH) and lipoxygenase enzyme activity (Costantini et al., 2006). As an alternative to these techniques, a new practice called "Double Maturation Raisonnée" (DMR) has been developed (Cargnello G., 1995; Cargnello et al., 2006). DMR consists of a type of on-plant withering in which the heads bearing fruit and/or shoots are cut in a reasoned or well thought out, manner. In fact, cutting the cane produces two cluster populations: the first is located above the cut where the connection between cluster and vine is still active, while the second is located below the cut and is composed of clusters for which any connection with the trunk is severed. In the first population, the profile of metabolites is similar to that observed in LH berries as a vascular connection is maintained and over-ripe processes are not always characterized by major dehydration events (Guillaumie et al., 2011). In contrast, significant biochemical changes do occur in the second population, and these changes are primarily related to water loss (e.g. a strong increase in sugar content caused by the cellular juice concentration), as has been described previously in berries subjected to withering (Rizzini et al., 2009). In addition, polyphenols are "more mature" in DMR berries, titratable acidity remains high because malic and tartaric acid catabolism is slowed, and the susceptibility to pathogen attack is reduced (Carbonneau A., 2008). All these changes improve sensorial parameters of the wine obtained from DMR berries.

Metabolic profiles observed in LH and withered-berry techniques are paralleled by significant changes in the expression of genes involved in key ripening processes (Guillaumie et al., 2011; Rizzini et al., 2009; Bonghi et al., 2012; Zamboni et al., 2010). These genes belong to functional categories involved in abiotic and biotic stresses (e.g. desiccation, pathogen susceptibility), primary and secondary metabolisms (e.g. sugars, aroma and polyphenols biosynthesis and transport) as well as regulators of development (e.g. hormones and transcription factors). The effect of water loss on berry transcriptome is strongly dependent

on genotype; however, it is well documented that water loss strongly affects the expression of several genes involved in the phenylpropanoids pathway (Versari et al., 2001) and causes the accumulation of phenols (e.g. stilbenes) that have been assumed as putative biomarkers of the withering process (Bonghi et al., 2012; Zamboni et al., 2010; Versari et al., 2001; Zamboni et al., 2008). Similarly, the (+)-valencene synthase gene, encoding an enzyme involved in sesquiterpene biosynthesis, has been proposed as putative marker of LH berries because its expression was up-regulated in both white (Chardonnay) and red (Cabernet Sauvignon) cultivars (Guillaumie et al., 2011). Much informations are already available concerning changes in must sensory profiles and biochemical indicators of the grape ripening processes (Carbonneau A., 2008) in berries subjected to DMR, but molecular data are totally lacking.

The goal of this research was to evaluate, by using a microarray approach, the impact of LH and DMR techniques on Raboso Piave berry transcriptome, focusing attention on differentially expressed genes related to sensory and biochemical parameters. Our data demonstrated that DMR Raboso Piave berries subjected to DMR, in comparison to TH and LH, maintained high titratable acidity and showed re-equilibration of polyphenols profile, mainly due to an increase of flavonol and a reduction of tannin precursors. These modifications were accompanied by significant changes in the transcription of genes involved in primary and secondary metabolism.

2. Materials and methods

2.1 Plant Material, treatment and biochemical analysis

The experiments were performed on Vitis vinifera L. cv. Raboso Piave berries collected in a commercial vineyard (Giorgio Cecchetto), located in the Piave DOC area (Treviso, Italy) in 2008. One-hundred bunches from sixty homogeneous plants were collected at different times to form six thesis: a) Traditional Harvest (TH), usually performed at the end of October (in this case at October 29th), b) Late harvest (LH) berries over-ripened on the plant and harvested 20 days after TH (DATH), and c) DMR berries collected from clusters located both above (DMR-A) and below the cut (DMR-B), each harvested at 36 and 45 DATH. Every week representative berries were separated into two groups: the first was used for biochemical and sensory analyses, the other berries were frozen in liquid nitrogen and stored at -80 °C.

In order to evaluate biochemical parameters, the juice from fifty homogenized berries was used to measure total acidity (g/L), malic and tartaric acid (g/L), pH and sugar content (°brix) , using a WineScan™ Basic (FOSS, Italia) analyzer following the manufacturer's instructions.

Total phenolic content in the grapes was determined using the Folin-Ciocalteu colorimetric method modified by (Yang *et al.*, 2004). Anthocyanins content was evaluated using the protocol described by (Ribéreau-Gayon *et al.*, 2006), while high-performance liquid chromatography (HPLC) was used to determine skin flavonoids (catechin and quercitin) and stilbenes (cis-piceids, cis-resveratrol and trans- resveratrol) amounts, following the protocol of (Sun *et al.*, 2006).

Biochemical data were statistically analyzed (one-way ANOVA and post hoc LSD test) by using the software package SPSS release 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

Sensorial analyses were conducted on musts obtained from TH-, LH-, DMR-A- and DMR-B berries using the protocols described by (Cargnello, 2009).

2.2 RNA extraction, microarray analysis and qPCR

Total RNA for both microarray and real-time PCR experiments was extracted from whole berries using the perchlorate method as described by (Ziliotto *et al.*, 2012).

Microarray experiments were conducted using the grape AROS V1.0 platform (www.operon.com), as described by (Rizzini *et al.*, 2009). The following samples were hybridized: LH versus TH, DMR-A versus TH and DMR-B versus TH only for the 36 DATH.

For each of the three comparisons, three slides were hybridized using targets obtained from two biological replicates.

Raw hybridization data were quality-filtered and background-subtracted using TIGR Spotfinder software (www.tm4.org/spotfinder) as described by (Ziliotto *et al.*, 2012). Resulting data were intra-array normalized using the median method (Yang *et al.*, 2002).

MeV software (www.tm4.org/mev) was used for discovering differential gene expression among the comparisons (t-test) and a Venn diagram with differentially expressed genes (DEG) among three comparisons was created using GeneVenn software (www.genevenn.sourceforge.net).

Gene expression data have been submitted to Gene Expression Omnibus (GEO) (accession no. GSE43385) at NCBI (https://www.ncbi.nlm.nih.gov/geo/).

PageMan software (Usadel *et al.*, 2006) was used to perform an enrichment analysis based on a Fisher exact test. This analysis was conducted on each set of common and specific differentially expressed genes among three comparisons, with a $p<0.05$ ($z > 1.96$).

In order to find patterns and highlight similarities and differences in data, a Principal Component Analysis (PCA) (Soumya Raychaudhuri, 2000) was performed on biochemical and transcriptomic data, and metabolites. For biochemical and metabolites PCA analysis, a log2 ratio of LH and DMRs versus TH were used as input data. For transcriptomic data, gene ontology (GO) categories related to acidity, sugars and phenylpropanoids metabolism were identified. Later on, transcripts of the grape AROS V1.0 platform related to each category were mediated and used as input data. For qPCR data, a PCA analysis with log2 ratio of LH and DMRs versus TH of transcripts studied with real-time PCR were used as input data.

cDNA synthesis and quantitative real-time PCR (qPCR) analyses were performed following methods described by (Ziliotto *et al.*, 2012). Primers used in qPCR experiments are listed in Supplementary Table 1.

3. Results and discussion

3.1 Biochemical and Sensorial Analysis

FOSS analysis of berries subjected to LH, DMR-A and DMR-B, in comparison to those sampled at TH, showed statistically significant differences in terms of titratable acidity, malic and tartaric acids as well as sugar content and total polyphenols (Table 1). In particular DMR-B berries, maintained a titratable acidity similar to that observed in TH-berries (reduction of 9%), while DMR-A and LH berries showed a reduction of 18 and 21% in comparison to TH-berries, respectively, as has been previously observed in other grapevine cultivars subjected to a late harvest dehydration (Guillaumie *et al.*, 2011; Bellincontro *et al.*, 2004). The malic acid content in DMR-B berries was very similar to TH berries; while in DMR-A and LH there was a decrease of 19% and 32%, respectively. In contrast, none of the late harvest techniques used significantly affected tartaric acid content.

Variations of tartaric and malic acid levels in grape berries led to a significant increase in pH only in LH-bunches (+0.26% in comparison to TH), while significant changes were not observed for DMR berries (+0.1% for DMR-B). These data demonstrated that DMR was effective in maintaining the titratable acidity measured in Raboso Piave berries at TH. This positive effect was absent when DMR berries were harvested later (45 days after TH, data not shown). In this case, a 40% and 45% reduction in malic acid level was observed in DMR-A and DMR-B, respectively. This strong reduction could be due both to an increase in malate oxidation and its transformation into soluble sugars, as suggested by (Terrier, 2001). To confirm this hypothesis it is necessary to verify if the increase in sugar content in DMR berries is due simply to dehydration or if it is also a result of the catabolism of malic acid.

Total Soluble Solids (TSS) and total polyphenols were higher in LH, DMR-A and DMR-B in comparison to TH berries (Tab.1). This result was expected considering that a xylem backflow occurs in berries subjected to a delay in harvest time, with the concomitant reduction in the functionality of phloem leading to weight (water) loss (~7% of berry volume per day) (Tilbrook and Tyerman, 2009). The altered water potential magnifies the

changes occurring during ripening, in particular those concerning to sugar concentration, organic acid evolution and aroma and polyphenols profiling.

Table 1 – Tritatable acidity, malic acid, tartaric acid, pH, sugar content and total polyphenols changes in must obtained from 200 Raboso Piave berries at traditional harvest (TH), late harvest (LH), Double Reasoned Maturation 36DATH above (DMR-A) and below (DMR-B) the cut. Data are the average of three biological replicates. x: average; SD: standard deviation; ns: not significant; average values followed by the same letter, in the same column, are not significantly different (LSD, 0.05).

* Significant ($p < 0.05$).

** Highly significant ($p < 0.01$).

*** Very highly significant ($p < 0.001$).

Harvest		**Titratable acidity**	**Malic acid**	**Tartaric acid**	**pH**	**Sugar**	**Total polyohenols**
TH	x	12.15a	7.34a	5.93a	3.07b	16.5c	4.63d
	SD	0.61	0.37	0.29	0.05	0.82	0.38
LH	x	9.63bc	5.06c	4.66b	3.31a	17.6b	5.82c
	SD	0.32	0.25	0.33	0.04	0.96	0.19
DMR-A	x	10.05b	6b	4.98b	3.11b	18.9b	6.18bc
	SD	0.49	0.3	0.28	0.07	0.7	0.49
DMR-B	x	11.06a	7.06a	5.34ab	3.17b	20.9a	6.94a
	SD	0.67	0.35	0.26	0.08	1.15	0.29
effect harvest time		**	**	n.s.	*	***	***

Sensorial analyses of Raboso Piave musts obtained from TH, LH and DMRs berries revealed significant organoleptic differences among the three techniques (Fig. 1), in accordance with the biochemical parameters. DMR musts (Fig. 1C, D), in comparison to those obtained from TH (Fig. 1A) and LH (Fig. 1B) berries, were less snappish and angry, more well-rounded and exhibited better phenolic maturity.

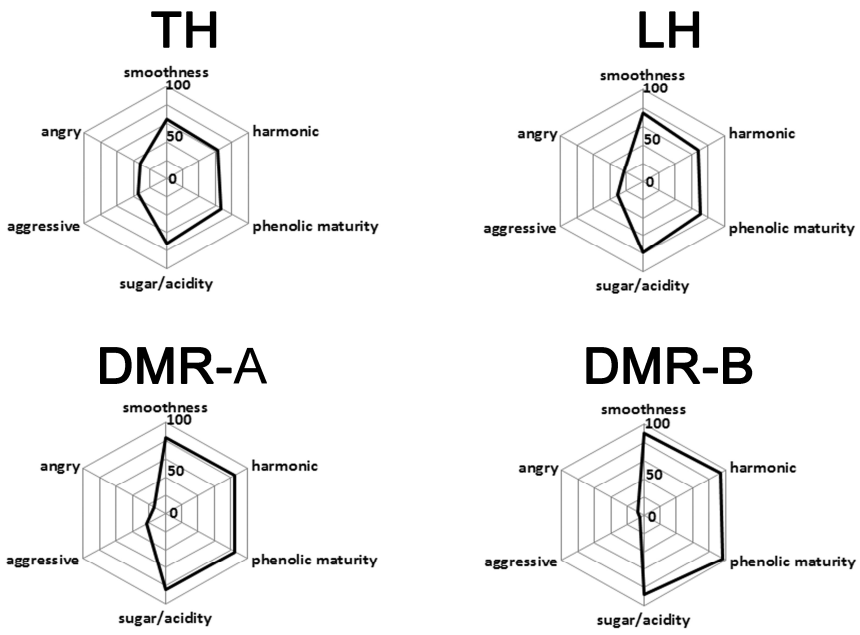

Figure 1. Sensorial analysis. Evaluation of sugar/acidity ratio, smoothness, angry, harmonic, phenolic maturity, aggressive sensorial parameters of Raboso Piave berries at TH, LH, DMR-A and DMR-B. For each category a score is given (100 is the maximum score).

3.2 Transcriptome profiling of TH, LH and DMR berries

LH, DMR-A and DMR-B transcriptome profiles were compared to those obtained from berries sampled at TH. Among LH/TH, DMR-A/TH and DMR-B/TH comparisons, genes differentially expressed were 662, 1000 and 1556, respectively (p value <0.05) (Figure 2).

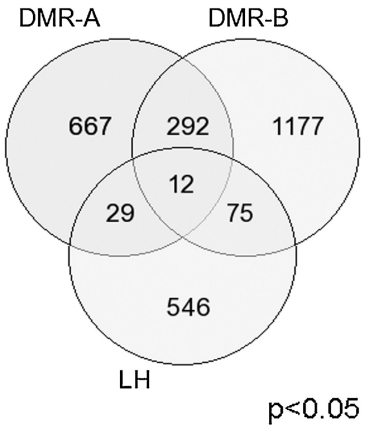

Figure 2. Venn diagram of differentially expressed genes. Specific and common differentially expressed genes ($p < 0.05$) resulting from statistical analysis of LH/TH, DMR-A/TH and DMR-B/TH comparisons.

A list of common and specific genes can be found in Supplementary Table 2.
Microarray data were validated by qPCR techniques performed with a set of 12 random genes, showing similar expression patterns among microarray and qPCR techniques (r= 0.56; R2= 0.75) (Supplementary Figure 1).

The Venn diagram (Fig. 2) showed that 12 significant genes, belonging mainly to primary metabolism and stress responsive gene networks, are in common among the three comparisons (Supplementary Table 2). It is well known that grape berry dehydration is paralleled by an accumulation of abiotic and biotic stress related transcripts, as well as an up-regulation of sugar-related genes (Deluc *et al.*, 2009).
To identify significantly overrepresented metabolic pathways, a PageMan analysis was performed on common and specific genes. Metabolic pathways were categorized by applying the MapMan ontology vocabulary and the complete list of the enriched BIN (i.e. functional classes) terms resulting from Fisher's exact test can be found in Supplementary Table 3. No significant enriched BIN terms were found in PageMan analysis performed on LH/TH comparison.
For PageMan enrichment analysis, BIN terms related to simple phenols (secondary metabolism.simple phenols, BIN 16.10) were significantly overrepresented in both DMR-A/TH and DMR-B/TH up-regulated genes. In the BIN category 16.10, the majority of up-regulated genes are coding for laccase (data not shown), a class of enzymes responsible for flavonoids oxidation, which is involved in plant protection from biotic and abiotic stresses (Bonghi *et al.*, 2012; Zamboni *et al.*, 2010; Pourcel *et al.*, 2007). Taking into account that the laccase family is, along with other polyphenol oxidase gene families, massively expanded in grapevine with respect to Arabidopsis (>60 genes in V. vinifera against 17 in Arabidopsis) (Mica *et al.*, 2009) it is worthy to note that VIT_18s0001g00680 and VIT_18s0117g00550 were up-regulated in both DMR-bunches, while the transcription of VIT_18s0122g00420 was induced only in DMR-B bunches (Supplementary Table 2).
Among down-regulated genes, a significant over-representation was observed for genes belonging to the BIN category related to water transport (transport.Major Intrinsic Proteins.PIP, BIN 34.19.1), in particular aquaporins, in both DMR-A/TH and DMR–B/TH

comparisons. Aquaporins are a family of transmembrane proteins considered to be largely responsible for the high permeability to water exhibited by plasma membranes thus the down-regulation of aquaporins transcripts during ripening or LH has been suggested as a strategy for reducing water loss (Guillaumie *et al.*, 2011). In this work, we observed that an higher number of aquaporin isoforms (six isoforms) were down-regulated in DMR-B bunches, on the other hand only two isoforms were differentially regulated in the DMR-A ones (two isoforms) (Supplementary Table 2). This difference could be explained considering that ripening grape berry is not hydraulically isolated from the parent plant by xylem occlusion but, rather, is "hydraulically buffered" by water delivered via the phloem (Choat *et al.,*2009). In the case of DMR-A bunches (still attached to the plant), the delivery of excess phloem water could be related with the less number of down-regulated aquaporin isoforms.

For DMR-A/TH comparison, specific down-regulated genes belonging to categories regarding cell wall (cell wall.cell wall proteins.LRR, BIN 10.5.3 and cell wall.degradation.mannan-xylose-arabinose-fucose, BIN 10.6.2) and lipid metabolism (lipid metabolism.exotics.methylsterol monooxygenase, BIN 11.8.2, lipid metabolism.FA synthesis and FA elongation.acyl coa ligase, BIN 11.1.8) were overrepresented. Metabolisms related to cell wall degradation and reduction of lipid biosynthesis (mainly sterols) were stimulated in the case of a prolongation of berry ripening on the plant (Le Fur *et al.,* 1994). Concerning terms related to up-regulated genes those associated to "cell wall.modification" and lignin biosynthesis (secondary metabolism.phenylpropanoids.lignin biosynthesis, BIN 16.2.1) were overrepresented. Activation of the lignin pathway is likely a reaction of skin cells to an advanced stage of dehydration (Guillaumie *et al.*, 2011; Bonghi *et al.*, 2012; Zamboni *et al.*, 2008), which could represent a barrier for the gas exchange between cells and the surrounding environment. In still metabolically active fruit tissues (skin, in particular), this stress condition activates specific reactions aimed at stress adaptation and the minimization of microbial attacks. It has already been reported that the application of DMR is particularly effective in indirect grey mold control on grapevine (Cargnello *et al.*, 2006).

For DMR-B specific genes, terms related to fermentation (fermentation.aldehyde dehydrogenase, BIN 5.10) and stress (stress.abiotic.touch/wounding, BIN 20.2.4) were significantly overrepresented in the up-regulated genes.

Among BIN categories over-represented in DMR-A/TH and DMR-B/TH comparison, those related to sugars/acidity and polyphenols were selected, for which the GO terms were retrieved to implement the information present in the MapMan ontology vocabulary. These GO categories were used to perform a PCA analysis to better identify those specifically affected by the application of different harvest techniques (Fig. 3). For sugars/acidity (Fig. 3A) and polyphenols (Fig. 3B), most of the GO terms were separated by the first principal component, with a variance of 58.8% and 83.3%, respectively, while second principal component showed a variance of 28.4% and 11.5%, respectively.

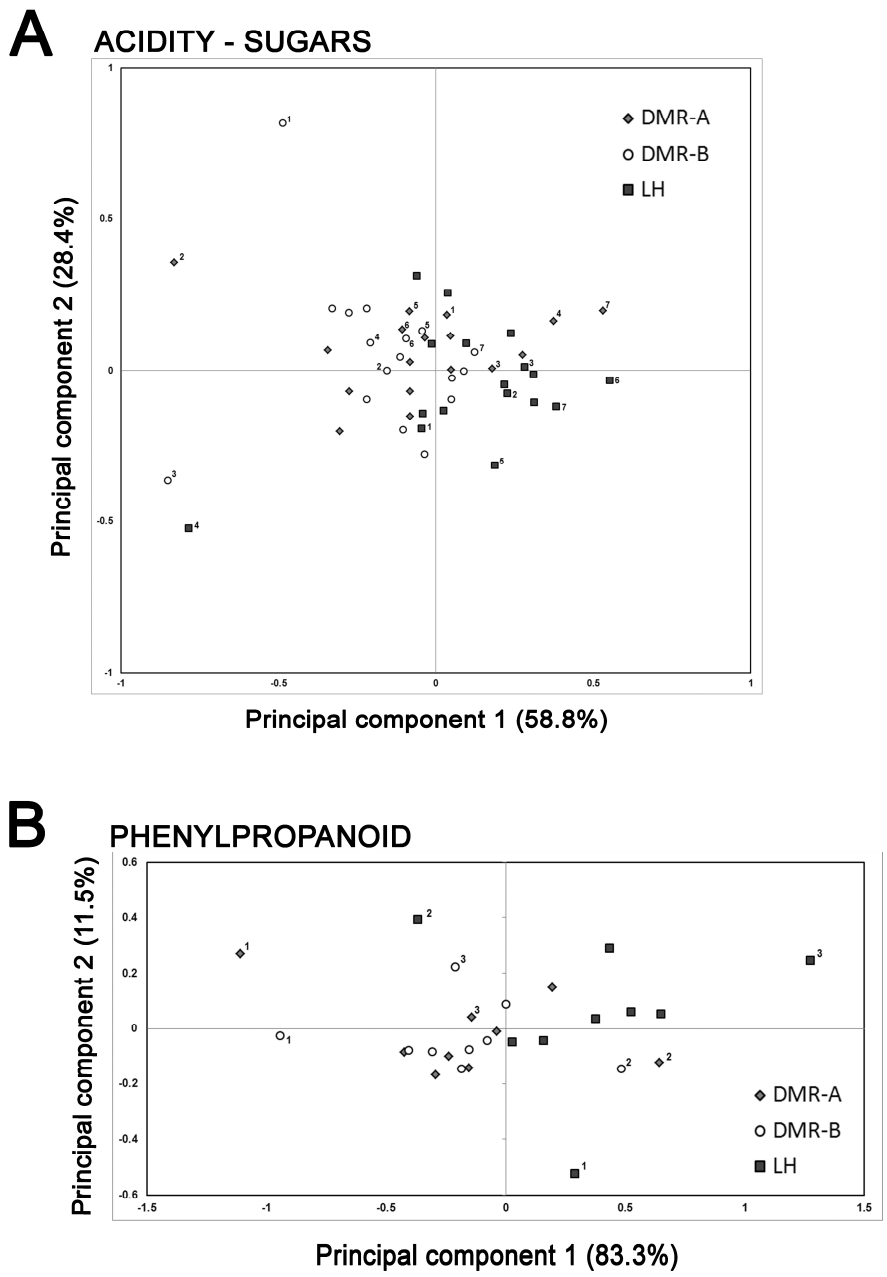

Figure 3. PCA analysis of sugar-acidity and polyphenols GO categories PCA analyses with sugar-acidity and phenylpropanoids GO category using LH/TH, DMR-A/TH and DMR-B/TH microarray data; number of described GO terms are given in brackets. A. PCA analysis of following sugar/acidity GO terms: transcription activator activity ATHB-12 (1), citrate transport (2), malate metabolic process (3), fructose 2,6-bisphosphate metabolic process (4), galactose metabolic process (5), alcohol dehydrogenase (NAD) activity (6), gluconeogenesis (7), pyruvate metabolic process, fumarate metabolic process, phosphoenolpyruvate-dependent sugar phosphotransferase system, beta-fructofuranosidase activity (invertase), sucrose metabolic

process, sucrose biosynthetic process, glucose metabolic process, sucrose transport, nucleotide-sugar transport. B. PCA analysis of the following polyphenols GO terms: L-phenylalanine catabolic process (1), leucoanthocyanidin reductase activity (2), naringenin-chalcone synthase activity (3), L-phenylalanine biosynthetic process, laccase activity, flavonoid 3',5'-hydroxylase activity, anthocyanidin 3-O-glucosyltransferase activity, flavonol synthase activity, leucocyanidin oxygenase activity.
As shown in figure 3, DMR-B/TH, DMR-A/TH and LH/TH comparisons exhibited different distribution of microarray transcriptomic data. Specifically, DMR-B/TH data were located on the left side in both graphs (Fig. 3A, B), data regarding LH/TH were on the opposite side and DMR-A/TH showed an intermediate position (between the DMR-B/TH and LH/TH comparisons).

For sugars/acidity categories, PCA data calculated as cumulative expression of genes belonging to "malate metabolic metabolism" (GO:0006108) obtained from DMR-B/TH berries were clearly separated from those of DMR-A and LH, as highlighted also in the PageMan results (Supplementary Table 3). These results confirm that malate metabolism is a process specifically affected in the grape cluster located below the cut of the vine, when the DMR technique is applied. As pointed out by biochemical parameters (Tab. 1), malate content in DMR-B berries showed levels similar to those observed in TH berries, highlighting a slowing down in its catabolism. On the other hand, changes in malate/tartrate ratios and the rapid consumption of malic acid observed in LH and DMR-A berries reflect the modulation of the metabolism that acts in response to the decrease in cell water potential (Bellincontro *et al.*, 2004; Tonutti and Bonghi, 2013).
For sugar metabolism, LH/TH "fructose 2,6-bisphophate metabolic process" (GO:0006003, nr. 4), "galactose metabolic process" (GO:0006012, nr. 5) and "gluconeogenesis" (GO:0006094, nr. 7) GO terms were separated from those of DMR/TH by the first principal component for GO:0006003 and by both components for GO:0006012 and GO:0006094. In DMR/TH comparison, genes belonging to fructose and galactose metabolic GO categories showed a slight up-regulation, in comparison to LH.
DMR/TH comparisons showed different regulation of genes belonging to alcohol dehydrogenase (AHD), the enzyme which catalyzes the conversion of acetaldehyde into ethanol. Indeed GO category "alcohol dehydrogenase (NAD) activity" (GO:0004022, nr. 6) of DMR/TH data was separated by the first principal component from LH/TH comparisons, emphasizing modification of sugar metabolism. Alcohol dehydrogenase 1 (ADH 1) transcripts was up-regulated in DMR/TH berries, in comparison to LH/TH

(Supplementary Table 2), due to an activation of the fermentative pathway, already observed in several experiments with an increase in ethanol and acetaldehyde levels following dehydration (Bellincontro *et al.*, 2004; Chkaiban *et al.*, 2007; Cirilli *et al.*, 2012). The induction of aerobic fermentation is probably related to an excess of sugars concentration, which may also be the case for DMR berries that showed higher levels of total soluble solids in comparison to LH berries (Table 1). In this case increasing levels of ADH 1 may be a necessary step in removing acetaldehyde related to an increasing concentration of sugars. In PCA analysis, GO terms related to sugar transport (nucleotide-sugar transport, GO:0015780, and sucrose transport, GO:0015770) did not show significant differences among three comparisons. It is well known that severe water stress affects phloem unloading, inhibits the accumulation of sugars and negatively regulates sugar transporters in grape berries (WANG *et al.*, 2003), as evidenced for genes coding for sugar transporters that showed low expression values (data not shown).

Taken together, these data highlighted that dehydration did not influence sugars biosynthesis and transport; therefore it can be concluded that the increase in sugar levels in DMR grape berries is due to the excess of concentration which occurs after grape berry dehydration.

For the polyphenols category, DMR/TH "L-phenylalanine catabolic process" (GO:0006559) terms, related to the Phenylalanine ammonia-lyase (PAL) gene, were significantly separated in the LH/TH comparison. Besides, genes that act in the next steps of the phenylpropanoids pathways ("naringenin-chalcone synthase activity", GO:0016210, and "leucoantocyanidin reductase activity", GO:0033788) also showed different distribution in PCA graphs of DMR/TH and LH/TH comparisons. These data highlighted significant differences between samples in the regulation of phenylpropanoids metabolism, with the adoption of different routes in DMRs and LH berries. This category will be discussed in more detail in the next section.

3.3 Phenylpropanoids metabolomics and transcriptomic analysis

Biochemical and sensorial analyses pointed out that DMRs grape berries reached a balanced polyphenols profile (Table 1) and complete phenolic maturity (Figure 1), two of the major factors affecting red wine quality. Metabolites analysis revealed that the amount

of polyphenols in DMRs berries is significantly higher than that measured in berries harvested at TH and LH (Supplementary table 4).

To better understand changes in polyphenols metabolism, a PCA analysis was performed on data related to the content of resveratrol, quercitin, catechin and anthocyanins, normalized against TH berries values, measured in LH, DMR-A and DMR-B berries (Figure 4A). Quercitin and catechin of DMRs were significantly separated from LH data by the first principal component accounting for 96.8% of variance. Trans-resveratrol value was also separated among the three delayed harvests by the first principal component, in this case with more marked differences. Instead, cis-resveratrol of LH was separated from DMR-A and DMR-B by the second principal component accounting for 3.2% of variance. Anthocyanins data did not show significant differences among the three harvest techniques.

Detailed data on metabolites quantification in TH, LH and DMRs are listed in Supplementary Table 4.

Transcript amounts of the main genes involved in polyphenols biosynthesis (listed in Supplementary Table 1) were analyzed using the procedure described for metabolites (Figure 4B).

PCA analysis highlighted that most of data can be explained by the first and second principal component, with a variance of 97.0% and 2.0%, respectively.

Data distribution observed for PAL gene (Nr. 2) was the same as that observed for PCA conducted with GO terms data (Figure 3) The ratio between the level of PAL transcripts measured in DMR-B- and TH berries was clearly separated from that recorded for DMR-A and LH samples by the first principal component with a variance of 97.0%, confirming the existence of a different regulation of PAL gene expression among the three harvests. Stilbene synthase gene (STS, nr.1) is involved in resveratrol production and showed significant differences between DMRs and LH. PCA pattern shown by STS transcripts parallels those obtained for trans-resveratrol in the PCA analysis conducted using phenol

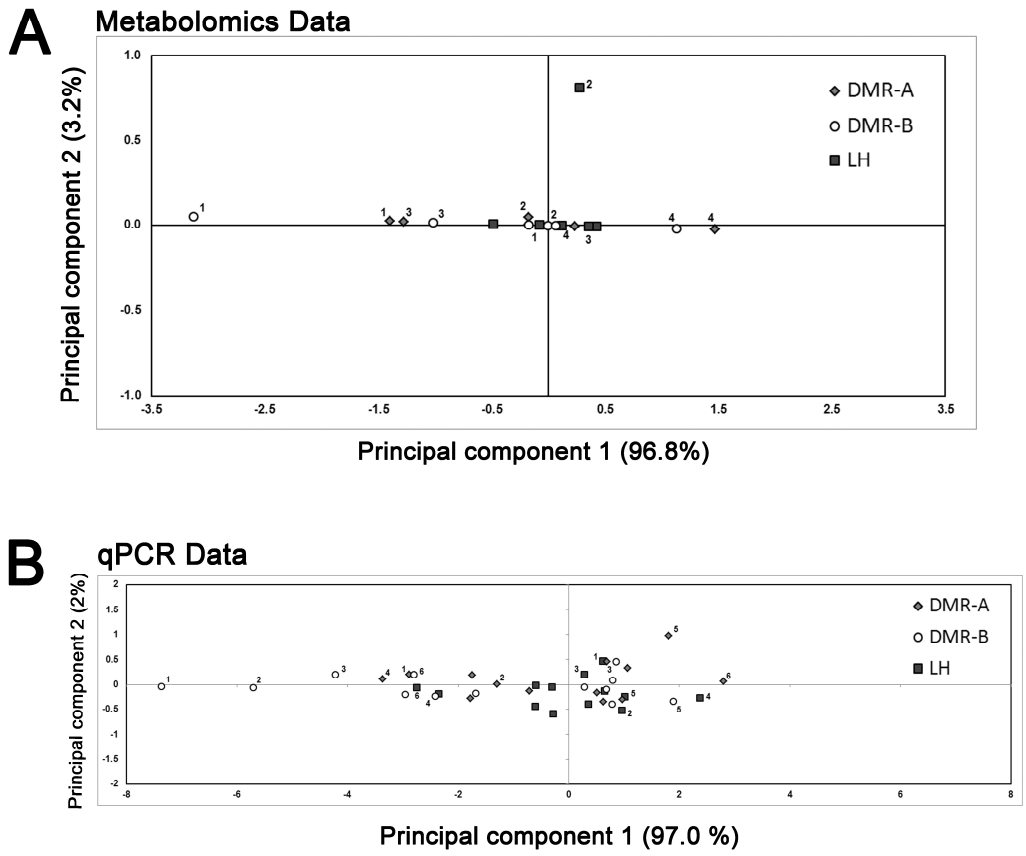

Figure 4. PCA analysis of polyphenols metabolomics-qPCR data and heat map of qPCR genes expression

PCA analyses using LH/TH, DMR-A/TH and DMR-B/TH polyphenols metabolomics and transcriptomic data; number of described terms are given in brackets. A. PCA analysis of the following polyphenols metabolomics data: trans-resveratrol (1), cis-resveratrol (2), quercitin (3), catechin (4), trans-piceid, total polyphenols. B. PCA analysis of following polyphenols qPCR genes: STS (1), PAL (2), MYB-B (3), FLS 1 (4), LAR 2 (5), LAC (6), F3H, PPO2, UFGT, CHS3, MYB-A, LAR1-1, LDOX. C. qPCR visualization of genes involved in polyphenols metabolisms. Heat map showing LH/TH, DMR-A/TH and DMR-B/TH genes expression. Black (4) and White (Unchanged) indicate respectively maximum and unchanged log2 ratio of the qPCR genes of the experiment.

metabolites (Figure 3). Similar results, although to a lesser extent, were obtained for flavonol-synthase 1 (FLS 1, nr. 4) and MYB-B (nr. 3), involved in flavonol biosynthesis and its regulation, respectively, and leucoanthocyanidin-reductase 2 (LAR2, nr 5), which is involved in catechin biosynthesis. No significant differences were observed for the expression of genes involved in anthocyanins synthesis (e.g. leucoanthocyanidin oxidase and UDP-glucose:flavonoid 3-O-glucosyltransferase) as well as flavonoids catabolism (e.g. polyphenol oxidases and laccases) by comparing the three harvest strategies.

Taken together, metabolites and transcripts analyses revealed that there are different regulation mechanisms of polyphenols metabolism in DMR and LH samples. DMR showed high expression of PAL gene (Figure 4C), confirming that this gene is strongly activated during both over-ripening and postharvest dehydration (Rizzini *et al.*, 2009; Bonghi *et al.*, 2012; Zamboni *et al.*, 2010). However, two members of the PAL gene family were shown to be down-regulated in withering Raboso Piave berries (Bonghi *et al.*, 2012). This suggests that the expression regulation of the numerous members of the V. vinifera PAL multigene family is complex and may represent a key step in the multiple physiological responses to postharvest stress. The increasing availability of polyphenols precursors was used for synthesizing stilbenes and some categories of flavonoids. As far as stilbenes are concerned, DMR and LH berries, showed higher content of trans-resveratrol (e.g. 3 times more in DMR-B than in TH, Supplementary Table 4), as already reported by (Carbonneau A., 2008), paralleled by an up-regulation of STS 1 gene (more than 40 times higher in DMR-B than that observed in TH, Fig. 4C). This result was not unexpected, indeed senescence (Vannozzi *et al.*, 2012) and abiotic stresses, such as dehydration, are able to induce STS expression (Bonghi *et al.*, 2012; Zamboni *et al.*, 2010; Versari *et al.*, 2001). Among flavonoids, the application of DMR-B induced the accumulation of quercitin, a flavonol-related metabolites (Supplementary Table 4), while catechin was significantly reduced. These trends were consistent with the induction of transcripts encoding for FLS 1 and MYB-B in comparison to TH (Fig. 4C) suggesting a shift in DMRs samples (DMR-B in particular) towards a flavonol pathway and a concomitant repression of tannins biosynthesis. To the contrary, in LH berries flavonoids composition remained almost similar to that registered in TH berries characterized by a low level of quercitin and FLS1 transcripts as well as an accumulation of catechin and

LAR 2 mRNAs (Fig. 4C), which means that the LH technique was less efficient than DMR in the reallocation of dehydroquercitin toward quercitin instead of leucoantocyanidin and then catechin. The increase of quercitin observed in DMR-B berries has an important impact on quality parameters considering that this flavonol contributes to the bitter taste and color of red wine by stabilizing anthocyanin pigments (Cheynier *et al.*, 2006). The decrease of catechin results in reduced synthesis of tannins. This event is correlated to the reduction of astringency registered in Raboso Piave musts obtained from DMR berries, as demonstrated by sensorial analysis. A decrease in total anthocyanins was observed for DMR berries, in comparison to TH and LH berries. This could be due to the diversion of a common precursor towards flavonols instead of anthocyanins.

In DMR berries harvested later (45 days after H), the positive effect on polyphenols balance disappeared (data not shown). This event, associated with a drop in titratable acidity (Tab. 1), suggests that an excessive delay in the harvest of DMR berries negatively affects the global quality of Raboso berries.

The unique traits of wines obtained from overripe and/or dehydrated berries are the result of processes and events, some of which are strictly regulated in terms of gene expression. Several pathways, related to wine quality traits, appear to be positively modulated by delaying the harvest and, in particular, when berries undergo DMR. However, the effectiveness of DMR is strongly influenced by the technical expertise of the wine grower, who should modify the duration of DMR in relation to climate and perform sensorial analyses on grapes in order to establish the best vintage time. The DMR technique can successfully modify grape berry quality only when these factors are taken into consideration in a well thought out manner.

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