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Differential expression of the genes involved in responses to water-deficit stress in peach trees cv. Chimarrita grafted onto two different rootstocks

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ABSTRACT: Water deficit is responsible for a negative impact on agricultural systems. Several physiological, biochemical, and molecular processes are initiated as a response to water-deficit stress. Quantifying the differential expression of the genes involved in the response to water-deficit stress plays a key role in the development of molecular strategies for engineering water-stress tolerance in plants. Therefore, the present study aims to evaluate the expression of key genes involved in the water-deficit response process in peach trees cv. Chimarrita grafted onto two different rootstocks. The experimental design was completely randomized, with four treatments that correspond to the evaluation periods: day zero (control), the fourth, seventh, and ninth days of water-deficit stress. Leaf samples of each Chimarrita/rootstock combination were analyzed separately. The expression of the genes related to ethylene biosynthesis, osmotic adjustment, and carbohydrate metabolism, namely *ACC oxidase*, *GTL*, *SDH*, *SIP1*, *SOT1*, *S6PDH*, and *P5CS* was quantified by real-time quantitative polymerase chain reaction (RT-qPCR). A differential response was observed in the expression of the *SDH*, *GTL*, *P5CS*, and *SIP1* genes between Chimarrita/rootstock (Aldrighi 1 and Tsukuba 2) combinations. In both combinations of grafting, the *S6PDH* gene presented the highest level of expression at the fourth day of stress. These results show that genes related to carbohydrate and proline metabolism are important molecular markers to identify variability to water-deficit tolerance in *Prunus persica*.

Key words: *Prunus persica*, osmotic adjustment, water stress, RT-qPCR, *S6PDH*, *P5CS*.

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INTRODUCTION

Plants are constantly exposed to biotic and abiotic factors, which trigger different stress responses affecting plant growth and metabolism, consequently, resulting in lower crop yields (Pistelli et al. 2012). Southern Brazil, the leading peach producing region in the country, is characterized by subtropical climate with highly variable environmental conditions within the same season and over the years. One of the problems faced by peach crops in this region is water-deficit stress in key periods such as pre-harvest and harvest, affecting fruit quality and yield of most orchards since the number of farms with irrigation systems is still limited in Brazil (Martinazzo et al. 2013).

Problems of low crop yield in peach orchards in Southern Brazil, related by Rocha et al. (2007), may be reduced by using rootstocks and scion cultivars with certified quality, as well as genotypes adapted to the weather and soil conditions of the region, and proper crop management. Despite the advancement over the last decades of scion cultivars development (adapted to Brazilian weather), there is still no reliable indication of the best rootstock for stone fruit in Brazil.

Among abiotic stresses, water deficit can have a significant negative impact on agricultural systems. In plants, water deficit induces diverse physiological and biochemical responses such as stomatal closure, which in turn reduces photosynthesis and increases respiration, hence considerably affecting plant growth and development (Ripoll et al. 2014).

Within certain limits, plants submitted to water deficit are able to maintain water uptake through their roots by osmotic adjustment in the vacuole and cytosol. Osmotic adjustment involves accumulation of compatible solutes (such as proline, glycine betaine, sucrose, sorbitol, raffinose, trehalose, among others). These solutes reduce the tissue's water potential in relation to the soil solution and, by doing so, the plant is able to maintain cell growth, stomatal opening, and photosynthesis, hence becoming more tolerant to dehydration and more efficient in water uptake by the roots (Krasensky and Jonak 2012; Jiménez et al. 2013). Responses to water deficit are complex because they involve the expression of genes involved in the coordination and integration of several biochemical and physiological pathways which take part in the plants adaptive response to stress conditions (Krasensky and Jonak 2012).

Genes whose expression is increased during water deficit include those which encode key enzymes in the biosynthesis of abscisic acid, ethylene, glycolytic route, proteins involved in osmotic adjustment and cell protection, countless cell signaling proteins such as kinases and phosphatases, and transcription factors (Jiménez et al. 2013). Thus, proteins involved in metabolic pathways which lead to water-stress tolerance are synthesized (Tran et al. 2007).

Understanding how each gene contributes to the final response at the cellular, physiological, and agronomic levels may help identify useful markers for the selection and development of plants with greater water-deficit tolerance (Krasensky and Jonak 2012). Nevertheless, understanding how these events are activated/deactivated and how they interact with each other is essential in the search for more tolerant plants; thus, genotypes that have different levels of tolerance must present qualitative and quantitative differences in gene expression.

The analysis and quantification of mRNAs transcribed in studies of water deficit tolerance has contributed to the understanding of biochemical and molecular processes of plants under stress, therefore, such information may assist in genetic improvement programs (Jiménez et al. 2013). The RT-qPCR technique is accurate in quantifying expression levels of a given gene, making it possible to obtain high-reproducibility data (Mallona et al. 2010). This technique is based on the reverse transcription process followed by the DNA polymerase chain, with the incorporation of fluorescent molecules, the fluorescence signals are generated by fluorescence that are specific for the DNA double strand or marked by fluorescence primers in specific regions. The signal is proportional to the amount of DNA polymerase chain product and the apparatus detects the accumulation of the amplified product during each cycle of the DNA polymerase reaction. Several studies were carried out with the RT-qPCR technique as to verify gene expression in *Prunus* rootstock under water-induced stress, such as those performed by Pistelli et al. (2012) and Almada et al. (2013) in which the molecular responses in plants submitted to flooding were studied, whereas Jiménez et al. (2013) studied the responses in plants under water-deficit stress.

Therefore, the present study aims to verify the expression of key genes involved in the water-deficit response process in peach trees cv. Chimarrita grafted onto Aldrighi 1 and Tsukuba 2 rootstocks.

MATERIALS AND METHODS

Plant materials and water deficit conditions

Two-year-old peach trees cv. Chimarrita grafted onto two *Prunus persica* rootstocks (Aldrighi 1 and Tsukuba 2) were grown in 25-liter pots filled with soil substrate classified as dystrophic yellow-redargisol (Streck et al. 2008). Aldrighi 1 is an old cultivar used as a rootstock that has good grafting affinity for most peach tree cultivars used in Brazil, inducing medium scion vigor and productivity. However, Souza et al. (2016) verified that Aldrighi 1 rootstock induced greater fruit mass and vigor than Tsukuba 2 rootstock. The cultivar is susceptible to root-knot nematodes (*Meloidogyne* spp.). On the other hand, Tsukuba 2 was selected to be used as rootstock for its resistance to root-knot nematodes.

The plants were kept in a greenhouse and the soil was watered on a daily basis up to field capacity until the beginning of the experimental trial at day zero (control) when irrigation was suspended for a 9-day period to induce water-deficit stress. The 9-day period of plant evaluation was based on morphological and physiological characteristics of the leaves, such as decrease in stomatal conductance, epinasty, and early leaf abscission, previously referenced by Martinazzo et al. (2011; 2013) in plants of *Prunus* spp. under water-stress treatments. In the present study, three plants of each Chimarrita/rootstock combination were kept under regular irrigation in order to track the physiological characteristics of the leaves when compared to the plants submitted to water-deficit stress.

For each Chimarrita/rootstock combination, a trial was conducted separately in a completely randomized design with four treatments which correspond to the evaluation periods: day zero (control), the fourth, seventh, and ninth days of water-deficit stress. For each treatment, three biological replicates were used. Leaves of cv. Chimarrita were sampled in each evaluation period, immediately frozen in liquid nitrogen and stored at 80 °C up to RNA extraction.

Total RNA isolation and cDNA synthesis

The total RNA was extracted from 100 mg of leaf tissue which had been ground under liquid nitrogen, poured into 2 mL polypropylene tubes, to which 900 µL of 65 °C pre-heated CTAB buffer solution was then added (Doyle and Doyle 1987). Samples were homogenized in vortex, incubated in a water bath at 65 °C for 30 min and then cooled. The extraction was carried out with successive centrifugation steps, following the protocol proposed by Chang et al. (1993).

The RNA *pellet* was dissolved with 30 µL 0.01% DEPC autoclaved water, homogenized in vortex and stored in an ultrafreezer at –80 °C. The concentration and purity of RNA were measured in NanoDrop ND-1000 (260/280nm) whereas the quality and integrity were verified by electrophoresis in 1.0% agarose gel. Single-stranded cDNAs were synthesized by reverse transcription from 1.5 µg total RNA by using the kit GoScript™ Reverse Transcription System (Promega). The reactions were performed with a final volume of 20 µL containing oligo-dT primers according to the manufacturer's instructions.

Choice and synthesis of primers for RT-qPCR

Seven pairs of primers were synthesized for the analysis of the *aminocyclopropane-1-oxidase* (*ACC oxidase*), *glycosyltransferase-like* (*GTL*), *sorbitol dehydrogenase* (*SDH*), *raffinose synthase* (*SIP1*), *sorbitol transporter* (*SOT*), *sorbitol-6-phosphate-dehydrogenase* (*S6PDH*), and *pyrroline-5-carboxylate synthase* (*P5CS*) genes, whose nucleotide sequences are shown in Table 1.

Table 1. Nucleotide sequence of seven sets of primers used for the RT-qPCR analysis of the target genes.

Gene symbol	Local and number access	T°m (melting)	Forward (5'-3')	Reverse (5'-3')
ACC oxidase[a]	AF129073	63.5 °C	TTCCAGGATGACAAGGTCAGTGGT	GTTTGGGCAATCACTCTGTTGCTCC
GTL[a]	AY354512	62.7 °C	AACAGCTTCAGAGGAGTGGTCGTT	GATTGGTGTCCGGCCATCATCATT
SDH[b]	AF323505	54.8 °C	CGAAGTTGGTAGCTTGGTGAAGA	CTTTGCACTGCTCACATCTCCA
SIP1[b]	AT5g40390	52.8 °C	GGTGCCATCCAGTCCTTTGT	TGCCCTCAATCCTGCAACTT
SOT1[a]	AY924379	62.7 °C	GGTGTCTTAACAATGCCCGAGTCG	TGGGGACCTGAACAACGTCTTCGG
S6PDH[b]	D11080	54.3 °C	ACATGGCACGACATGGGAAAAGAC	AATTGGCTCACTTGAGGCTTGAT
P5CS[b]	AT2g39800	54.1 °C	CGAATTGCTGTGGATGCAAAAGT	GCGAAGGTCAACCACAAGATCA

[a]Pistelli et al. (2012).[b]Jiménez et al. (2013).

Real-time quantitative PCR

The total volume of each RT-qPCR reaction was 12 µL, 6.25 µL SYBR Green fluorophore (Invitrogen®), 0.25 µL (10mM) of each primer (forward and reverse), 1 µL cDNA (dilution 1:5, previously determined), 4.15 µL ultrapure water, and 0.1 µL ROX. The amplification was standardized in a CFX-96 Real Time Thermal Cycler (Bio-Rad) equipment, by using the following amplification conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min with the insertion of the melting curve from 65 °C to 95 °C, increasing 5 °C at every fluorescence measurement. Three biological replicates were used.

The performance of the primers was evaluated through the dissociation curve (melting curve). They all showed a single peak of dissociation of strands of the PCR products. The amplicons were also verified by 1.5% (w/v) gel electrophoretic analysis.

The ΔC_T values were used to calculate the relative expression level (QR) using the $2^{\wedge -(\Delta\Delta CT)}$ method. The threshold cycle (C_T) is the cycle at which the fluorescence level reaches a certain amount (the threshold). This method directly uses the C_T information generated from a qPCR system to calculate relative gene expression in target and reference samples, using a reference gene as the normalizer (Pfaffl et al. 2002). The *tubulin* (*TUA*) gene was chosen as the internal reference standard, among a set of seven candidate reference genes, including *actin* (*ACT*), *elongation factor 1α* (*Ef-1α*), *Cyclophilin 2* (*CYP2*), *glyceraldeyde-3-phosphate dehydrogenase (GAPDH)*, *α-tubulin* (*TUA*), *ubiquitin10* (*UBQ10*), and *18S ribossomal RNA* (*18S*), as described by Rickes et al. (2016).

RESULTS AND DISCUSSION

Abiotic stresses, such as water deficit, trigger a series of biochemical and physiological responses in plants. This process begins with the perception of stress, which leads to the activation of signaling pathways and altered levels of gene expression, hence altering physiology, growth, and development (Yamaguchi-Shinozaki and Shinozaki 2006). Regarding the different genes analyzed in this study, the *SDH*, *GTL*, *P5CS*, *SIP1*, and *S6PDH* genes presented both up-regulated and down-regulated relative expression throughout the stress period, whereas the *ACC oxidase* and *SOT1* genes presented only down-regulated expression.

Among the metabolic changes induced in plants under abiotic stress, the most important one is the accumulation of osmoregulatory molecules composed of organic acids, sugars, and amino acids, which act in the osmotic adjustment, considered to be a typical response to water-deficit in plants (Krasensky and Jonak 2012; Jiménez et al. 2013; Salazar et al. 2015).

The main functions of osmoregulators or compatible solutes are to maintain cellular turgor and concentration gradient so as to uptake extracellular water. This latter function allows the plants to grow continuously, through cell division and expansion, and photosynthesis processes (Krasensky and Jonak 2012; Jiménez et al. 2013). Genes involved in osmolyte synthesis and accumulation are considered osmoprotectants (Jiménez et al. 2013). Among these osmoprotectant molecules, the amino acid proline stands out as it reduces the cellular osmotic potential, maintaining the water balance between the vacuole and the cytoplasm under water deficit, and carbon and nitrogen reserves that are used for restoring the plants growth after stress (Ashraf et al. 2011).

In addition to the processes involved in the coding of proline synthesis, several other genes are expressed in response to water deficit, such as those which encode enzyme that act upon the production and/or transport pathway of other molecules involved in osmoprotection, standing out the *SDH*, *SIP1*, *S6PDH*, and *P5CS* genes, whose expression was evaluated in the present study.

Among the many genes that respond differently to the water-deficit stress, they are certain that relate carbohydrate metabolism.

The raffinose family of oligosaccharides (RFOs) has several functions in plants, e.g., it is used for the transport and storage of carbon and as solutes for plant protection against abiotic stresses, such as water deficit. Raffinose biosynthesis begins when galactinol is formed, subsequently galactinol donates the galactose unit to a sucrose molecule, which forms raffinose, being raffinose synthase responsible for this reaction (Peterbauer and Richter 2001). In our study, the expression of the *SIP1* gene increased 4.27 times at the seventh day of stress in the Chimarrita/Aldrighi 1 combination (Fig. 1F), which suggests the participation of this sugar in the osmoprotection under water deficit. Jiménez et al. (2013) observed a significant increase in the expression of this gene in *Prunus* rootstock leaves submitted to water deficit for a 16-day period. Likewise, Gimeno et al. (2009) witnessed increased expression of this gene in *Citrus* plants when submitted to water deficit for a 5-hour period. →

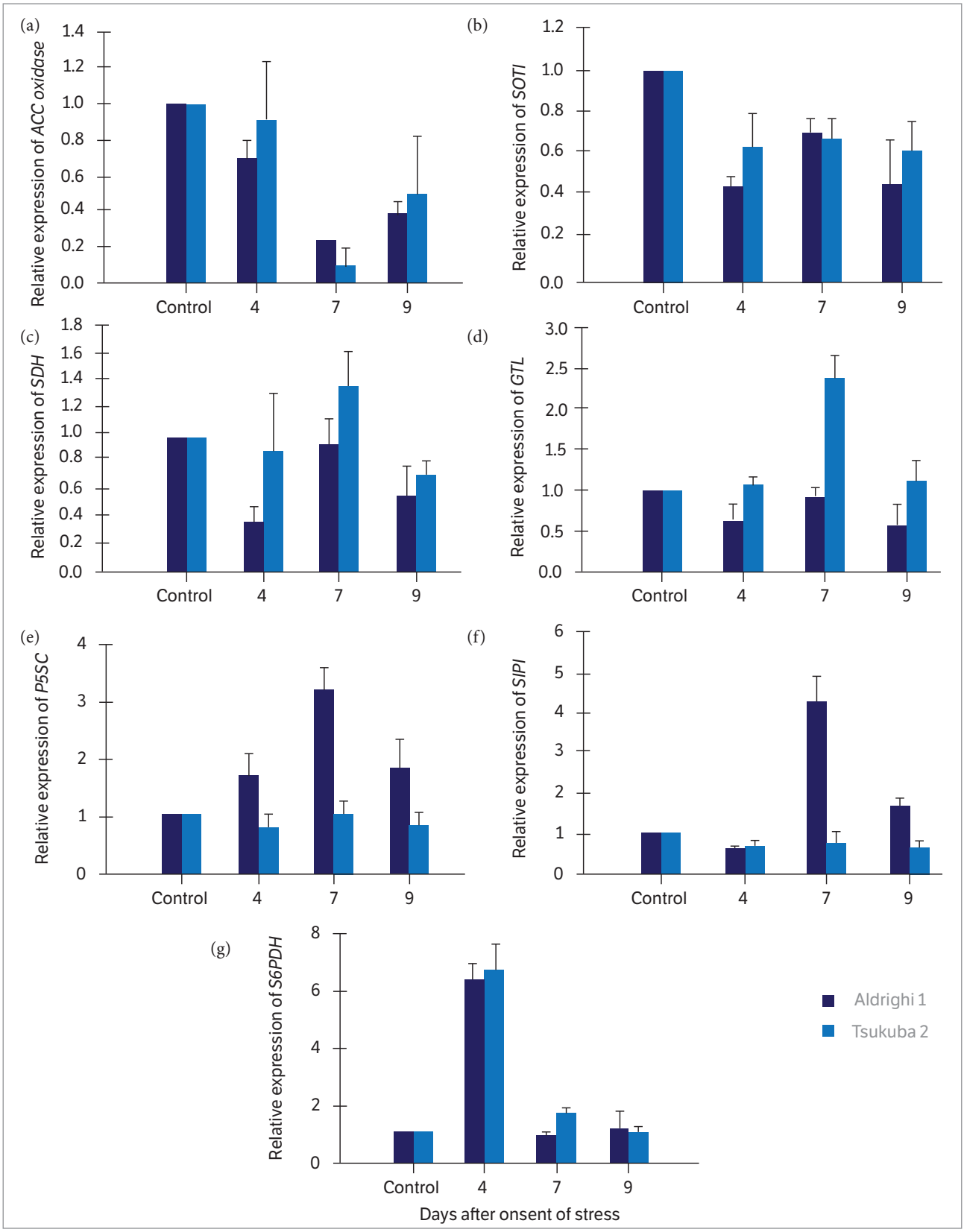

Figure 1. Relative expression of (A) *ACC oxidase*, (B) *SOT1*, (C) *SDH*, (D) *GTL*, (E) *P5CS*, (F) *SIP1*, and (G) *S6PDH* genes in the leaves of peach trees cv. Chimarrita grafted onto Adrighi 1 and Tsukuba 2 rootstocks and submitted to water deficit. Bars indicate standard error (n = 3).

Based on studies with *Arabidopsis* leaves under oxidative stress, Nishizawa et al. (2008) suggest that galactinol and raffinose may act not only as osmoprotectants, cell membrane stabilizers, and eliminators of reactive oxygen species, but also in the protection of cellular metabolism, especially in the reduction of oxidative damage associated to photosynthetic activity under different abiotic stresses.

In the Chimarrita/Tsukuba 2 combination, no significant variations were observed in the expression level of the *SIP1* gene, which was down-regulated throughout the stress period when compared to the control group, showing that there is a differential response at gene expression level in different scion/rootstock combinations underwater-deficit stress.

The *P5CS* gene also exhibited expression level 3.18 times higher than the control in the Chimarrita/Aldrighi 1 combination (Fig. 1E). *P5CS* gene encodes the enzyme that regulates the rate of proline biosynthesis and inhibits its degradation (Kishor et al. 2005; Silva et al. 2010). Based on the expression patterns of the *P5CS* and *proline dehydrogenase* (*PDH1*) genes, Sharma et al. (2011) suggest that proline synthesis is high and its catabolism is suppressed in photosynthetic tissues during stress, whereas catabolism rate remains high in roots and in meristematic tissues.

The regulation of proline metabolism under water stress may be associated with abscisic acid (ABA) as it protects root growth to maximize water uptake while inhibiting shoot growth to prevent water loss (Sharma and Verslues 2010). Although ABA alone may not increase proline levels, which is induced by water deficit, other data suggest that proline metabolism may be linked to redox status, such as tierredoxins (Verslues et al. 2014). Also, there has been evidence that proline synthesis serves to regenerate $NADP^+$ and maintains an adequate NADP/NADPH ratio (Verslues and Sharma 2010; Sharma et al. 2011).

In our study, the *P5CS*gene was down-regulated in the Chimarrita/Tsukuba 2 combination every single day of stress trial period. Jiménez et al. (2013) did not observe changes in the expression levels of the *P5CS* gene in the leaves of four *Prunus* rootstocks under water deficit, while in the roots of the GF 677 rootstock, they registered a significant expression increase (c.a. 2.5 times), as well as a positive correlation with proline levels (2.0 $mg \cdot g^{-1}$ dry weight). Such correlation between transcript increase and proline accumulation has recently been studied and the isoforms of *OsP5CS1* and *OsP5CS2* have been co-expressed in tobacco plants, which have shown accumulation of proline and reduction of oxidative damage in cells under abiotic stress conditions, such as salt stress (NaCl) and metal-induced stress (CuSO4) (Zhang et al. 2014).

The Rosaceae family includes important fruit tree species such as apple, pear, peach, apricot, almond, plum, and cherries, in which the main sugar translocated by phloem is sorbitol (Bieleski 1982; Loescher 1987). In these species, plants are able to passively load the phloem and translocate sorbitol and sucrose by the symplast pathway, hence contributing to the cell osmotic potential as well as avoiding feedback regulation (Slewinski and Braun 2010). In peach trees, sorbitol represents about 60% to 90% of the total carbon exported from the source leaves to the sink organs (Moing et al. 1997). In cherry trees (*Prunus cerasus*), cDNAs of the genes, which encode sorbitol transporters (*PcSOT1* and *PcSOT2*), have recently been identified and both genes showed high expression in the fruit (Gao et al. 2003).

In our study, the expression of the *SOT1* gene which encodes sorbitol transporters was down-regulated in both scion/rootstock combinations throughout the stress period (Fig. 1B), possibly due to an inactivation of the regulation system by carbohydrate synthesis, hence not assisting in the maintenance of the osmotic potential under the imposed stress. Under water-deficit conditions, these transporters are expected to increase their expression so that sorbitol is directed towards root growth. It is possibly a way to increase plant's water uptake ability. However, this effect was not observed in our study, which suggests that possibly at the ninth day, the plants did not present a high water restriction index, i.e., the sorbitol content in the leaves and roots was within levels which did not require a significant change in the expression rate of the transcript transporters. On the other hand, in the leaves and roots of *Prunus* rootstocks submitted to flooding for six days, Pistelli et al. (2012) observed a higher rate of expression of *SOT1* gene in the roots (c.a. 2.5 times) when compared to the leaves of clone S.4. This result demonstrates that there was active transport of sorbitol to the roots, increasing the relative expression of transcripts of the transporters, emphasizing that such sugar may be a source of carbon to →

be used by the cells aiming at temporary energy supply under anoxia condition.

In Rosaceae, sorbitol metabolism in sink tissues occurs mainly through the action of the NAD^+-dependent sorbitol dehydrogenase, which oxidizes sorbitol to fructose. *SDH* is highly active in young leaves and decreases gradually as leaves mature (Loescher 1987). In general, the expression of *SDH* gene was down-regulated compared to the control group in both cultivar combinations (Fig. 1C). However, except for the seventh day of stress, the expression level was up-regulated 1.38 times in the Chimarrita/Tsukuba 2 combination, while in the Chimarrita/Aldrighi 1 combination, the expression was down-regulated (0.94), hence evidencing the differential behavior of the scion/rootstock combination under the imposed conditions.

Some findings suggest that the sensitivity of the gene that encodes *SDH* is up-regulated by the availability of sorbitol in plants, which in turn, may be altered by the effect of stress on the photosynthetic rate and the rate of that sugar transport from the source to the sink tissue. Analyzing the gene expression in the roots of Cadaman and GF 677 rootstocks, Jiménez et al. (2013) found a direct correlation between sorbitol levels and *SDH* expression on the sixteenth day of water deficit.

Another important enzyme that plays a key role in sorbitol biosynthesis is sorbitol-6-phosphate dehydrogenase. In loquat tress (*Eriobotrya japonica*), an increase in sorbitol accumulation induced by low temperatures, has coincided with an increase in *S6PDH* activity (Hirai 1983). The sorbitol accumulation in plants is modified in response to environmental conditions, e.g., in apple leaves photoassimilates are converted to sorbitol instead of sucrose.

In our study, the *S6PDH* gene was highly up-regulated in both Chimarrita/rootstock combinations on the fourth day of stress. The expression level increased 6.74 times in the Chimarrita/Tsukuba 2 combination, and 6.41 times in the Chimarrita/Aldrighi 1, compared to the control (Fig. 1G), following by a drastically decrease in its expression, especially at the seventh and ninth days after the onset of stress. Jiménez et al. (2013) verified that the expression of *S6PDH* in peach leaves was not affected by water deficit at the sixteenth day of stress and this was correlated with sorbitol levels (also at the sixteenth day), in which there was no statistical difference when compared to control. Even though this sugar was not quantified in our study, our findings suggest that the increase in *S6PDH* activity may be closely related to the amount of sorbitol present in the leaf tissue of these plants.

According to Kanamaru et al. (2004), the increase in *S6PDH* activity is closely related to the photosynthetic process because it activates the synthesis of sorbitol by supplying more substrates, i.e., photosynthates, or by increasing the products of the hydrolysis of starch. The high expression of *S6PDH* gene (up to the fourth day of stress), observed in both Chimarrita/rootstock combinations, may be related to a decrease in the expression of the genes that oxidize sorbitol (*SDH* – Fig. 1C), and sorbitol transporters (*SOT1* – Fig. 1B), as verified in the present study. According to Li and Li (2005), the reduction of sorbitol degradation could explain the increased accumulation of sorbitol in leaves under water deficit. Nevertheless, at the seventh day of stress, in the Chimarrita/Tsukuba 2 combination, the expression levels of the *SDH* gene were high (1.38 times), whereas the expression rates of the *S6PDH* gene were 1.71. These results are in agreement with Li and Li (2005), which state that under water deficit the increase of the activity of the gene that catalyzes sorbitol (*SDH*) is inversely proportional to the accumulation of this sugar.

Jiménez et al. (2013) reported low expression of the *S6PDH* gene under water-deficit conditions in the leaves of *Prunus* rootstocks. In turn, Zhang et al. (2011) found a correlation between the high expression of *S6PDH* and the sorbitol content in apple leaves submitted to osmotic stress induced by PEG-6000, which suggests that this gene played an important role in the response to osmotic stress.

There are several glycosyltransferase groups in plants and they partake in sugar transfer reactions for a large group of receptor molecules (Sun et al. 2013). These enzymes are important in the formation and degradation of pectic compounds. In the absence of their activity, the content of galacturonic acids, which are the main constituents of pectin, i.e., lower adhesion between leaf and root cells, may be reduced, hence facilitating dehydration (Bouton et al. 2002). The expression of the *GTL* gene in our study was 2.37 times at the seventh day of stress for the Chimarrita/Tsukuba 2 combination (Fig. 1D), which indicates the participation of this gene in the synthesis of pectic compounds. The possibility of increasing →

water-stress tolerance may be related to increased synthesis of pectic chains, in which these compounds may act as gelling agents as well as anti-desiccants (Leucci et al. 2008). On the other hand, Sun et al. (2013) proposed a possible effect of glycosyltransferases on the specific protection against abiotic stresses. They found that an increased *GTL85A5* gene expression under salt stress was related to the reduction of oxidative damage, as well as lower levels of ionic toxicity when analyzing the levels of malondialdehyde and the ratio of Na^+/K^+ ions in leaves of transgenic tobacco plants.

One of the main defense processes of plants to water deficit is the stomatal closure regulation, which minimizes water loss by transpiration, maintaining the cellular turgor for longer periods. The stomatal closure decreases CO_2 diffusion to the leaf mesophyll, which causes a reduction in the photosynthetic rate, hence affecting the accumulation of photoassimilates (Larcher 2000; Martinazzo et al. 2013). Plants diminish their transpiration area by means of a fast and reversible leaf rolling (which are protected by the cuticle) as well as by means of the stimulus of leaf abscission. Increased levels of phytohormones, such as ethylene and ABA in leaves, induce leaf abscission and stomatal closure respectively, thus allowing a better control of water losses (Salazar et al. 2015).

Ethylene biosynthesis has methionine as its precursor. Methionine is then converted into S-adenosyl-methionine (SAM) with the requirement of one ATP and one H_2O molecule. Oxygen is essential at the end of the reaction to convert 1-amino-chloro-propane-1-carboxylic acid (ACC) into ethylene. Therefore, two enzymes play a key role in ethylene synthesis, *ACC synthase*, which forms ACC, and *ACC oxidase*, which forms ethylene in the presence of oxygen. Under water deficit, a close relationship between ACC expression and ethylene synthesis was demonstrated in *Citrus* (Gómez-Cadenas et al. 1996). ACC moves via xylem, from root to shoot, and a close association has been demonstrated between its expression and the increase in leaf ethylene levels (Liu et al. 2005).

In our study, the expression of *ACC* oxidase was down-regulated when compared to control at the fourth, seventh, and ninth days of water-deficit stress evaluation. At the seventh day, the expression in Chimarrita/Tsukuba 2 (Fig. 1A) was almost zero (0.088), whereas for Chimarrita/Aldrighi 1, it was five times lower (0.222) compared to control (1.0).

Studies have shown that plants submitted to different abiotic stresses have their ethylene levels altered (Gómez-Cadenas et al. 1996; Gil et al. 2009; Pistelli et al. 2012; Larrainzar et al. 2014). In addition, other studies have determined that the action of complex ethylene receptors results in the inactivation of receptor sites involved in the ethylene signaling pathway (Binder and Bleecker 2003). Therefore, the action of ethylene is negatively regulated by the number of available receptor sites, which determines the response to this hormone.

Thus, plants under water stress may exhibit few ethylene receptor sites, which could decrease their sensitivity to this phytohormone, hence not resulting in leaf abscission or postpone abscission as observed in this study (data not shown). This response is related to the down-regulation gene that encodes ACC oxidase, hence evidencing that ethylene may apparently act as a negative regulator in plants under this type of stress.

In Mr.S. 2/5 *Prunus* rootstocks and their somaclones (S.1 and S.4), which differ in their tolerance to flooding, Pistelli et al. (2012) found an up-regulated expression of the *ACO1* gene (c.a. eight times) in leaf tissues of the S.1 clone after six days of exposure to flooding. They also detected that the plants had been severely damaged, showing wilting and strong epinasty, which may be correlated with the high levels of expression verified.

In the present study, the expression of genes involved in water-deficit tolerance processes in different Chimarrita/rootstock combinations was evaluated, and it was possible to assess the rootstock influence on the induced responses in the grafted scion, where the *SDH*, *GTL*, *P5SC*, and *SIP1* genes showed quite different expression patterns for both tested scion/rootstock combinations. Moreover, the *S6PDH* gene presented a quite pronounced expression that was similar for both scion/rootstock combinations at the fourth day of stress, which indicates the participation and importance of sorbitol in the osmotic adjustment for a possible water-deficit tolerance.

Finally, our findings, supported by molecular and genetic bases, indicate that differential genes expression induced by water deficit that may occur in the same species, depend on the period which plants are submitted to stress. Thus, the expression of phenotypic characters is related to gene expression and its interaction with the environment may help to identify critical periods of stress as well as markers

for the selection of more tolerant genotypes to stress conditions that affect crop yield and quality in *Prunus* spp.

CONCLUSION

Water deficit alters differently the expression of genes related to osmotic adjustment, such as, *SDH*, *SIP1*, *GTL*, and *P5CS*, in Chimarrita/rootstock combinations. The expression of genes related to the carbohydrate (*SDH*, *GTL*, *SIP1*, and *S6PDH*) and proline (*P5CS*) metabolism may be used as molecular markers to identify variability to water-deficit tolerance in *Prunus persica*.

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