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Preformed and induced mechanisms underlies the differential responses of *Prunus* rootstock to hypoxia

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ABSTRACT

Analysis of the transcriptomic changes produced in response to hypoxia in root tissues from two rootstock *Prunus* genotypes differing in their sensitivity to waterlogging: resistant Myrobalan 'P.2175' (*P. cerasifera* Erhr.), and sensitive 'Felinem' hybrid [*P. amygdalus* Batsch × *P. persica* (L.) Batsch] revealed alterations in both metabolism and regulatory processes. Early hypoxia response in both genotypes is characterized by a molecular program aimed to adapt the cell metabolism to the new conditions. Upon hypoxia conditions, tolerant Myrobalan represses first secondary metabolism gene expression as a strategy to prevent the waste of resources/energy, and by the up-regulation of protein degradation genes probably leading to structural adaptations to long-term response to hypoxia. In response to the same conditions, sensitive 'Felinem' up-regulates a core of signal transduction and transcription factor genes. A combination of PLS-DA and qRT-PCR approaches revealed a set of transcription factors and signalling molecules as differentially regulated in the sensitive and tolerant genotypes including the peach orthologs for oxygen sensors. Apart from providing insights into the molecular processes underlying the differential response to waterlogging of two *Prunus* rootstocks, our approach reveals a set of candidate genes to be used expression biomarkers for biotech or breeding approaches to waterlogging tolerance.

1. Introduction (shorten by 15% or more)

Land plants have developed a series of physiological, developmental, and biochemical mechanisms that allow them to cope with abiotic stresses (Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009). A number of studies with oxygen-deprived and hypoxic-treated plants (Baxter-Burrel et al., 2003; Branco-Price et al., 2008; Klok et al., 2002; Liu et al., 2005; Takahashi et al., 2004), and even anoxia conditions have been reported in *Arabidopsis* (Pucciariello et al., 2012). These studies have demonstrated that plant responses to full or partial oxygen deprivation are regulated at both transcriptional and post-transcriptional levels (Licausi and Perata, 2009; Licausi et al., 2010, 2011b; Zou et al., 2010). A hallmark shared by many abiotic stresses is the production of reactive oxygen species (ROS) in the chloroplasts, mitochondria or in peroxisomes, which is responsible for the irreversible cellular and tissue damages ensuing. Furthermore, many abiotic stresses like salinity, drought, cold and dehydration (Goggin and Colmer, 2005; Liu et al., 2005) and anoxia/hypoxia (Bailey-Serres and Voesenek, 2010; Branco-Price et al., 2008) have been described as inducers of the plant antioxidant system to control the ROS build-up and allow plant growth and survival (Blokhina and Fagerstedt, 2010). A conserved survival mechanism in hypoxia stress-tolerant plants consists on developing abilities to modify respiration rates, and switch to anaerobic metabolism, mainly fermentative pathways, to obtain energy/reducing power. At least 20 anaerobic polypeptides (ANPs) are newly synthesized as part of the adaptation program to waterlogging (Sachs et al., 1980). The ANPs include enzymes involved in sucrose metabolism, glycolysis, phosphorylated sugar metabolism, anaerobic fermentation, non-symbiotic haemoglobin and cell wall degradation activities needed for aerenchyma formation (Bailey-Serres and Voesenek, 2010; Voesenek et al., 1993). Those ANPs enabling anaerobic fermentation are involved in different metabolic pathways (Bailey-Serres and Voesenek, 2008) that are essential for producing ATP under hypoxia conditions (Dennis et al., 2000; Rocha et al., 2010).

Activation of fermentative pathways, with the resulting accumulation of alanine and succinate levels, is a common feature during hypoxia and is subjected to different levels of transcription control depending on the species (Narsai et al., 2011). During hypoxia, the ethanol produced by ADH in hypoxia-sensitive poplar roots is

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translocated to the aerial plant parts via xylem, where it is metabolized and utilised as carbon source (Kreuzwieser et al., 2004). While flooding tolerant species, such as *Vitis riparia*, are able to maintain enough oxygen (O_2) in the root meristem to guarantee mitosis and nutrient uptake, even in anaerobic soils (Mancuso and Boselli, 2002). The recent discovery of O_2 sensor in plants, support the importance of adapting to low O_2 levels both in normal and under stress conditions. Particularly, the role of different ethylene-responsive proteins, including RAP2.12 (Related to Apetala 2.12), RAP2.2 and RAP2.3, in the modulation of hypoxia tolerance has been demonstrated in *Arabidopsis* (Gibbs et al., 2015; Licausi et al., 2011a).

Prunus spp. trees are mainly grown in Mediterranean climate regions, which are characterized by infrequent rainfalls concentrated in few days and often leading to flooding. The identification and characterization of the adaptation mechanisms developed by waterlogging-tolerant rootstocks is very important to improve the tolerance to flooding of a wider range of genotypes. Among the different species of *Prunus*, Myrobalan plum (*Prunus cerasifera* Ehrh.) and European plum (*P. domestica* L.) are considered waterlogging tolerant (Almada et al., 2013; Amador et al., 2009; Pistelli et al., 2012; Ranney, 1994).

In *Prunus*, as in most plants the response to the hypoxia conditions associated to waterlogging can be conceptually divided into three stages (Dennis et al., 2000). The first stage (0–4 h) consists on the rapid induction of signal transduction elements, which then activates the metabolic adaptation program during the second stage (4–24 h). The third stage (24–48 h) involves the formation of gas-filled air spaces (aerenchyma) in the roots (Dennis et al., 2000). The aim of this work is to characterize the early events of the transition to normoxia to hypoxia conditions, with a focus on the first and the second stages when root cells switch from normal to low-O_2 metabolism. We have performed a transcriptomic analysis of the roots of two *Prunus* genotypes previously identified as differing in their tolerance waterlogging (Amador et al., 2009). Since breeding programs to improve waterlogging tolerance in stone fruit rootstocks and to develop new waterlogging tolerant hybrids are under way (Amador et al., 2009; Xiloyannis et al., 2007), the new insights and candidate genes obtained here could be used to guide these breeding efforts.

2. Material and methods

2.1. Plant materials and stress conditions

Plants of Myrobalan 'P.2175' (*P. cerasifera* Erhr.) (tolerant to waterlogging, A) and 'Felinem' hybrid [*P. amygdalus* Batsch × *P. persica* (L.) Batsch] (sensitive to waterlogging, C), were propagated *in vitro* under aseptic conditions. Explants were established in a 30-mL MS medium (Murashige and Skoog, 1962) with 0.7% (w/v) agar (Cultimed, Panreac, Spain), pH 5.8, with 1.5 mg L^{-1} BAP (6-benzylaminopurine) and kept in a growth chamber at constant temperature (21 ± 1 °C) and a 16 / 8 h photoperiod. Light was provided by cool white fluorescent tubes, 17 µmol m^{-2} s^{-1}. Plants were incubated for a week in MS medium with 1 mg L^{-1} IBA (indole-3-butyric acid) to induce rooting. Rooted plants were transferred to glass jars (70 × 50 mm) containing 30 mL of MS liquid medium provided with a 7-cm diameter #541 Whatman filter paper support. After plants have produced 3–4 roots of 5-cm length, they were allowed to grow in the same glass jars for six weeks before hypoxia experiments.

Hypoxia treatments were carried out by submitting groups of sensitive and tolerant plant genotypes with a low O_2 air mix. A total of 120 plants of Myrobalan 'P.2175' (A) distributed in 15 jars and 72 rooted plants of 'Felinem' hybrid (C) distributed in 9 jars were enclosed in two airtight chambers. The air-flux conditions for treated plants were 3% O_2, 0.03% CO_2 and 97% N_2 gas for 2 h and 24 h (Hypoxia - Y). A second group of plants for each genetic background and developmental stage (Normoxia - Z) was treated similarly, but under normal aerobic oxygen concentration. Root samples were collected at the indicated times after treatment and at 0 h (control). Root samples were collected immediately after the treatment, deep frozen in liquid nitrogen and stored at -80 °C until RNA analysis.

2.2. RNA extraction

Total RNA was isolated from 1 g of root tissue for each biological replicate and two biological replicates were used for each treatment and genotype following the method as described by Meisel et al. (2005), with some modifications. The OD 260/280 ratio was used to assess the quality of the RNA samples. RNA integrity was verified by a denaturing 1.7% agarose gel electrophoresis and ethidium bromide staining.

2.3. Microarray hybridization and scanning

For microarray experiments, equal amounts of RNA samples from ZA0, ZC0, ZA2, ZC2, ZA4, ZC4, YA2, YC2 YA4 and YC4 were pooled to form a reference pool (PR). RNA samples for microarray hybridization were amplified using the method of Van Gelder et al. (1990). For each experimental point, three to four microarray hybridization experiments were performed each using cDNA preparations obtained from different samples of root material representing normal and stress treated tissues. Therefore, biological replicates rather than technical replicates were used (i.e. cDNA samples made from the same RNA). Features, preparation, and hybridization protocols of the peach microarray of ChillPeach were as described in Ogundiwin et al. (2008). Data were normalized in Acuity™ (Axon Instruments, Molecular Device, CA, USA) as described in Tusher et al. (2001).

To generate the raw data for expression analysis (Table A.2), the lowest M Log Ratio was used as expression value and patterns with more than 95% of missing values were filtered. In total, 2465 probes met the threshold for hybridization quality.

2.4. Expression analysis

Differentially expressed genes were identified from the raw dataset using Significance Analysis of Microarray software (SAM package) (Tusher et al., 2001) as described in Pons et al. (2014). Principal component analysis (PCA) and 2D-hierarchical cluster (2D − HCA) wereperformed on significant data using Acuity™ (Axon instruments) as described in Pons et al. (2014). Functional enrichment is performed as described in Pons et al. (2014).

2.5. PLS-DA analysis

To identify genes whose expression most contributed to differentiate tolerant and sensitive genotype groups, and also those genes separating normoxia and hypoxia responses, a Partial Least Squares Discriminant Analysis (PLS-DA) was performed using the software package SIMCA-P (Umetrics Ltd, Windsor, UK). Normalized data were imported and scaled by mean centering. A Variable Importance (VIP) score was generated for each gene based on its ability to explain the separation between groups. In addition, the VIP value (Wold et al., 1993, 2001) was calculated for all genes. The most relevant genes contributing to the separation between the different classes, tolerant vs. sensitive genotypes and between normoxia and hypoxia conditions were selected so as to have a minimum VIP score of 2.5.

2.6. Quantitative real time PCR analysis

One microgram of total RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for quantitative Real-Time PCR (qRT-PCR) (Invitrogen, Life Technologies, Carlsbad, CA, USA) in a total volume of 20 µL. Two microliters of a 40× diluted first strand cDNA was used for each amplification reaction in a final volume of 20 µL. qRT-PCR was performed on a StepOnePlus Real-Time PCR System