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Transcriptome sequencing of *Prunus* sp. rootstocks roots to identify candidate genes involved in the response to root hypoxia

María José Arismendi · Rubén Almada · Paula Pimentel · Adriana Bastias · Ariel Salvatierra · Pamela Rojas · Patricio Hinrichsen · Manuel Pinto · Alex Di Genova · Dante Travisany · Alejandro Maass · Boris Sagredo

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Abstract Root hypoxia in fruit trees affects growth, vegetative development, and reproductive development, which is reflected in low productivity, poor fruit quality, and premature decay of trees. Using Illumina Hiseq2000, we performed transcriptome analysis of roots from two different rootstocks, 'Mariana 2624' and 'Mazzard F12/1,' which are tolerant and sensitive to hypoxia, respectively. Transcriptomes from control and hypoxia-stressed plants (6, 24, and 72 h) were compared, using *Prunus persica* (L.) as reference genome. Hypoxic conditions altered the transcription in both genotypes. There were a high number of common differentially expressed genes (DEG) between the two genotypes for each sampling time, but also exclusive DEG for each genotype, with a few DEG that presented opposite modes of regulations during the hypoxia treatment. An important group of DEGs exclusively upregulated in the tolerant genotype are associated to enzymes of posttranslational protein modifications, such as leucine-rich repeat (LRR), kinases and ubiquitin-protein ligases, regulation of transcription, and process of oxide reduction. Singular enrichment analysis of gene ontology (GO), detected at least 115 GOs involved in the response to root hypoxia in the sensitive and/or tolerant genotypes. At least 25 GOs were identified as part of the baseline differences between the genotypes, most GO were disturbed in the sensitive genotype. The contribution from the baseline gene expression to the differential response between the Prunus genotypes is evidence that the resistant genotype is already "prepared" for a hypoxia event. An example are GO BP:0042221 of response to chemical stimulus; BP:0006979 of response to oxidative stress; MF:0016209 of antioxidant activity; MF:0016684 of oxidoreductase activity, acting on peroxide as acceptor; and MF:0004601 of peroxidase activity, which were disturbed only in the sensitive genotype, but not in the tolerant.

Keywords Hypoxia · *Prunus* · qRT-PCR · RNA-Seq · Rootstock · Transcriptome sequencing

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María José Arismendi and Rubén Almada contributed equally to this work.

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M. J. Arismendi
Programa Doctorado en Ciencias Mención Biología Celular y Molecular Aplicada, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Avenida Francisco Salazar #01145, Temuco, Chile

M. J. Arismendi · R. Almada · P. Pimentel · A. Bastias · A. Salvatierra · P. Rojas · P. Hinrichsen · M. Pinto · B. Sagredo
Centro de Estudios Avanzados en Fruticultura (CEAF), Av. Salamanca s/n, km 105 Ruta 5 Sur, Sector Los Choapinos, Rengo, Chile

P. Hinrichsen · M. Pinto
Instituto de Investigaciones Agropecuarias CRI La Platina, Av. Santa Rosa #11610, La Pintana, Santiago, Chile

R. Almada · P. Pimentel · A. Bastias · A. Salvatierra · P. Rojas · B. Sagredo (✉)
Instituto de Investigaciones Agropecuarias CRI Rayentué, Av. Salamanca s/n, Sector Choapinos, Rengo, Chile
e-mail: bsagredo@inia.cl

A. Di Genova · D. Travisany · A. Maass
Mathomics, Centro de Regulación del Genoma (Fondap 15090007) & Centro de Modelamiento Matemático, Universidad de Chile, Santiago, Chile

Introduction

Stone fruit trees (*Prunus* sp.) are economically important since they produce edible fruits such as peaches, plums, and apricots. As aerobic organisms, stone fruit trees need molecular oxygen for their normal development; however, they experience shortages of molecular oxygen or hypoxia around their roots as a consequence of flooding or over-irrigation in orchards established on poorly drained soils. Excess water saturates the root environment and displaces air from soil pockets. Since water constitutes an important barrier to gas diffusion (oxygen has a ~10,000-fold slower diffusion rate in water than in air), the cellular oxygen level in roots decreases to concentrations that restrict aerobic respiration (Bailey-Serres and Voesenek 2008). The inability of stone fruit trees to tolerate root hypoxia results in substantial yield losses (reduction of flower buds, increase of flower and fruit abscission and fruit cracking) (Sekse 1998, 1995) and decreased fruit quality (reduction of fruit size, altered appearance and chemical composition of fruits) (Horchani et al. 2008; Kozlowski 1997). Root hypoxia also constrains plant vegetative development and induces plant mortality (Kozlowski 1997; Pinochet 2010).

Most stone fruit trees are grafted on rootstocks (seedlings or clonally propagated) that belong to either the same or other *Prunus* species (Webster 2001). Therefore, the stone fruit tree is a biotic unit composed of two parts, the rootstock and the scion. The influences of the rootstock on the scion are profound and partly determine the stone fruit tree's tolerance to many environmental stresses such as drought, salinity, cold, and root hypoxia (Isaakadis et al. 2004; Ranney et al. 1991) and thus, the potential yield of the crop and the fruit quality (Lang 2000). The *Prunus* species commonly used as rootstocks are classified as hypoxia-sensitive, although differences among genotypes regarding their ability to tolerate this stress have been reported (Mizutani et al. 1982; Pinochet 2010; Ranney 1994). Soil inundation results in the rapid death of plants of certain *Prunus* taxa, including *Prunus dulcis* (Mill.), *Prunus armeniaca* (L.), *Prunus mahaleb* (L.), and *Prunus persica* (L.) (Domingo et al. 2002; Nicolás et al. 2005). Plum-based rootstocks like 'Myrobalan 29C' ('M.29C') (*Prunus cerasifera* Ehrh.), 'Mariana 2624' ('M.2624') (*P. cerasifera* × *Prunus munsoniana*) and 'Replantac' (*P. cerasifera* × *P. dulcis*) exhibit higher tolerance to root hypoxia caused by waterlogging and can survive for relatively long periods under this stress condition (Pinochet 2010).

Increasing plant tolerance to root waterlogging or hypoxia is a main goal of *Prunus* rootstock breeding programs (Pinochet 2010). However, the long generation time, along with the extensive space requirements and other limitations constitute important constraints for traditional breeding programs. Greater understanding of the molecular basis of hypoxia tolerance/sensitivity in *Prunus* rootstocks and the identification of genes participating in the response to this stress will be critical steps toward improving hypoxia tolerance in stone fruit plants by molecular assisted selection (MAS) methods. Alternatively, other approaches are possible, based on direct modification of key genes associated with hypoxia tolerance by genetic engineering of *Prunus* species. However, the molecular bases of hypoxia responses are still largely unknown.

Molecular responses to low-oxygen levels (hypoxia or anoxia) have been analyzed in *Arabidopsis thaliana* and some other crop species (Christianson et al. 2010a; Christianson et al. 2010b). Global gene expression studies of *Arabidopsis*, rice, cotton, and poplar have shown complex transcriptional responses to low oxygen involving important changes in ~5–10 % of all the genes examined (Christianson et al. 2010a; Christianson et al. 2010b); Klok et al. 2002; Kreuzwieser et al. 2009; Loreti et al. 2005). The metabolome and transcriptional profiles of plant species with different tolerance to low-oxygen conditions were recently compared and it was found that similar metabolic pathways are activated, such as fermentative metabolism and accumulation of succinate and alanine (Narsai et al. 2011). However, the transcriptional regulation of these pathways apparently varies among the analyzed species. Furthermore, most of the differentially expressed genes identified either had no orthologues or were not affected in the other species, suggesting that these differential transcriptional regulations may explain, in part, the different tolerance levels among species (Narsai et al. 2011). Although there were common responses to hypoxia/anoxia among the compared species, the evolutionary distance among these species, the different experimental conditions used and the different tissues examined make it difficult to identify factors or processes that contribute to survival in low-oxygen conditions. On the other hand, the analysis of contrasting responses of closely related taxa (i.e., rice varieties) to hypoxia has been very valuable for identifying genes associated with hypoxia/anoxia tolerance (Lee et al. 2009; Xu et al. 2006), and seems a more appropriate approach for understanding the molecular basis of plant adaptation to low-oxygen environments.

In order to gain insights into the molecular basis of stone fruit rootstock responses to low-oxygen environments, we performed a large-scale transcriptome sequencing of roots from two different stone fruit rootstocks with contrasting responses to hypoxia using the Illumina technology. Furthermore, we validated the use of the peach genome as a reference for RNA-Seq studies of *Prunus* species.

Material and methods

Plant material

Clonally propagated and virus-free rootstock plants from 'M.2624' (*P. cerasifera* × *P. munsoniana*) and 'M.F12/1' (*Prunus avium*), which are respectively tolerant and sensitive to hypoxia conditions (Amador et al. 2009; Arismendi 2012; Pinochet 2010; Rubio-Cabetas et al. 2011) were acquired from a commercial nursery (Agromillora Sur, S.A., Curicó, Chile). Plants were transplanted to 2-L plastic pots with a mixture of vermiculite:perlite:sand (1:1:1 *v*/*v*) as a substrate. The plants were maintained in the field under a shade net (Raschel sun-shading net with 50 % light transmittance) at the Instituto de Investigaciones Agropecuarias - Rayentué (Rengo, Chile) during the growing season until the hypoxia experiment. Plants were watered three times a week with tap water and fertilized every 2 weeks with 1 g/pot with N:P:K (25:10:10) (Ultrasol™, Soquimich, Chile).

Hypoxia experiments and leaf physiological parameter measurements

Once the plants reached an average height of 30 cm, all the pots with the plants, except the control plants, were placed in 100-L plastic containers. Root hypoxia was generated by filling the plastic containers with water until approximately 4 cm above the pot substrate level and bubbling 100 % gaseous N_2 (1 L/min) through the water to displace dissolved O_2. The oxygen levels immediately next to the plant roots were monitored throughout the experiment with an oxygen-electrode (Extech Instrument, MA, USA). With a total of 15 plants per genotype, roots from three randomly selected plants were collected at 0, 6, 24, and 72 h. Samples collected at time 0 h represented the control without flooding for the RNA-sequencing (RNA-Seq) analysis. To take root samplings, the soil was completely removed and the plant roots were gently washed with tap water, then excised from the plants and immediately frozen in liquid nitrogen. They were stored at −80 °C until RNA extraction. The decrease in net photosynthesis of plants under root hypoxia was measured in control and hypoxia-stressed plants throughout the experiment at 0, 6, 24, 72 h, and 7 days, using a portable photosynthesis system CIRAS-2 (PPSystem, Hitchen, Herts, UK). It was used with a controlled environment CIRAS PLC cuvette (broad windows 2.5 cm^2) warmed at 25 °C. Incident photosynthetically active radiation (PAR) was supplied by a LED light cuvette unit set at 1000 μmol m^{-2} s^{-1} (light intensity that reaches the photosynthesis saturation determined in previous measurements), and the CO_2 concentration in the cuvette was adjusted to 400 ppm and relative humidity at 50 %. The CO_2 assimilation rate (A) was measured outdoors from 0830 to 1230 hours, and the days of measurements were all on a clear day. Measurements were made on young fully expanded leaves (usually fourth from the top) from six plants of each genotypes. The experiment was a completely randomized design. Statistica v4.0 (StatSoft, Inc.) software was used for statistical analysis. Results were expressed as means±standard errors of six measurements. To assess the statistical significance of the treatment differences, a one-way analysis of variance (ANOVA) followed by LSD multiple range test (with $P \leq 0.05$) was employed.

RNA extraction, cDNA library construction, and Illumina sequencing

For RNA-Seq analyses, total RNA was extracted from root samples of control and flooded plants (0, 6, 24, and 72 h of hypoxia), according to (Chang et al. 1993). After a treatment with DNase I (RNase-Free, Ambion, Inc.—Applied Biosystems), total RNA was purified using a RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). The total RNA of each sample was quantified with an Infinite® 200 PRO NanoQuant (Tecan Group Ltd., Swiss). The total RNA samples from three plants at the same time point and treatment were evenly pooled in equal amounts and used for the RNA-Seq experiment. The pooled samples were submitted to Macrogen Inc. (Seoul, Korea) for NGS transcriptome sequencing by Illumina HiSeq 2000. The general procedure was quality control (QC) of RNA samples performed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc. Santa Clara, CA) and then a complementary DNA (cDNA) library was synthesized for each pooled sample using a TruSeq RNA Sample Prep Kit (Illumina, Inc, San Diego, CA) following the manufacturer's instructions. A QC test was performed with an Agilent 2100 Bioanalyzer for each of the eight cDNA libraries. Approximately 5 μg from each of the cDNA library was used for sequencing at the Macrogen Inc. Laboratories on an Illumina HiSeq2000 instrument using 50 bp and a single-end read method.

Bioinformatic analyses

The bioinformatic analysis was performed at the LBMG-CMM (Laboratorio de Bioinformática y Matemática del Genoma del Centro de Modelamiento Matemático) of Universidad de Chile (Santiago, Chile). The raw Illumina data for the eight cDNA libraries consisted of 208 million reads. A trimming process was performed using in-house perl script with a minimum phred-quality of 20 bp and a minimum read length of 30 bp, discarding a total of 56 million reads (27 %). Finally, 152 million single-end 50 bp reads were used for the analysis. The *Prunus persica* genome, gene models, and annotation of version 139 were downloaded from the Phytozome database

(http://www.phytozome.org). The genome size of the current assembly is 227 MB (N50 2.68 MB) arranged in 202 scaffolds, with 27,864 gene predictions and 28,702 coding transcripts with an average size of 1210 bp. A local domain identification was executed using Interpro Scan against PRODOM, COILS, HAMAP, PIR, PFAM, SMART, TIGR, FPRINTSCAN, PATTERNSCAN, PROFILESCAN, and SUPERFAMILY. A total of 120,874 (4960 unique) domains were identified and 154,158 (1529 unique) gene ontology (GoSlim Assignments—http://www.geneontology.org) terms were associated in order to improve further analysis. The genome from *P. persica* was used as sequence of reference. The high-quality Illumina reads were then aligned to this genome using TopHat (Trapnell et al. 2009). TopHat uses the Bowtie algorithm to map the reads to a reference genome. The TopHat parameters used were a maximum of two mismatches when mapping reads to the reference, where a maximum of ten multiple alignments per read was allowed for each library. Using singular enrichment analysis (SEA) according to Du et al. (2010), we evaluated root transcriptome differences in two ways; first, by comparing the level of gene expression between the two genotypes ('M.2624'/'M.F12/1') at baseline (time 0) and at 6, 24, and 72 h after flooding and, second, by determining differentially expressed genes according to their own control plants at 6, 24, and 72 h of treatment, respectively (Table 1, Supplementary Table 1). Differential expression analysis was performed using the Cuffdiff (version 2.0.2) program from the Cufflinks suite (Trapnell et al. 2010). This program constructs a probabilistic model for the expression of genes from reads pool, making it possible to evaluate statistically the differential expression among conditions (Trapnell et al. 2013). Genes abundance were estimated using the FPKM count, which corresponds to fragments per kilobase of exon per million fragments mapped (Trapnell et al. 2010). We turn on multi-reads (-u option) and bias correction (-b option) parameters (Mortazavi et al. 2008; Roberts et al. 2011) to obtain more accurate results. We consider only genes having a minimum of 500 fragment aligned (-c option) as

Table 1 Baseline "altered" GO between roots of 'M.2624' and 'M.F12/1' genotypes of *Prunus* rootstock. Cross-comparison of its analysis results by SEA of level of gene expressions between the roots of 'M.2624'/'M.F12/1'("DEG"), and DEG from 'M.2624' and 'M.F12/1,' respectively, at time of 6, 24, and 72 h of hypoxia treatment. Baseline "altered" GO were established by SEA between "DEG" of 'M.2624'/'M.F12/1' at time of 0 h. *Color scale* indicates the false discovery rate (FDR) probability according to Yekutieli multiple testing correction for GO analysis (Du et al. 2010). Numbers of genes associated to significant GO are indicated in each checking time

GO Information			'M.2624'/'M.F12/1'				'M.F12/1'			'M.2624'		
No	GO Term	Description	0h	6h	24h	72h	6h	24h	72h	6h	24h	72h
1	BP:0055114	oxidation reduction	36	36	32	37	45	73	98	38	63	61
2	BP:0050896	response to stimulus	35	---	28	---	---	---	---	---	---	40
3	BP:0006950	response to stress	31	24	26	---	---	---	---	---	---	37
4	BP:0009607	response to biotic stimulus	9	8	9	---	---	---	---	---	8	13
5	BP:0042221	response to chemical stimulus	17	---	---	---	18	25	36	---	---	---
6	BP:0006979	response to oxidative stress	9	---	---	---	12	15	23	---	---	---
7	BP:0016052	carbohydrate catabolic process	8	7	8	15	---	---	16	---	---	10
8	BP:0000272	polysaccharide catabolic process	5	---	---	---	---	---	---	---	---	---
9	BP:0009308	amine metabolic process	12	---	---	15	---	---	29	---	---	16
10	MF:0003824	catalytic activity	218	212	220	234	225	368	544	214	303	316
11	MF:0016491	oxidoreductase activity	82	79	68	79	81	134	170	65	114	103
12	MF:0008171	O-methyltransferase activity	13	11	5	---	---	7	13	---	---	---
13	MF:0016741	transferase activity, transferring one-carbon groups	25	21	14	---	---	20	28	---	---	---
14	MF:0008168	methyltransferase activity	25	21	14	---	---	20	28	---	---	---
15	MF:0020037	heme binding	23	24	20	21	27	36	50	---	---	---
16	MF:0046906	tetrapyrrole binding	23	24	20	21	27	36	50	---	---	---
17	MF:0016209	antioxidant activity	12	---	---	---	13	18	28	---	---	---
18	MF:0005506	iron ion binding	23	24	21	26	30	39	59	---	---	26
19	MF:0046872	metal ion binding	56	63	60	71	77	103	144	---	84	85
20	MF:0016684	oxidoreductase activity, acting on peroxide as acceptor	9	---	---	---	13	17	25	---	---	---
21	MF:0043169	cation binding	56	63	60	71	77	103	144	---	85	86
22	MF:0004601	peroxidase activity	9	---	---	---	13	17	25	---	---	---
23	MF:0043167	ion binding	56	63	60	71	77	103	144	---	85	86
24	MF:0048037	cofactor binding	19	18	---	---	---	34	38	---	32	30
25	MF:0050662	coenzyme binding	15	15	---	---	---	32	34	---	29	27

P < 1.8E-16

P < 0.05

expressed cutoff. To consider a gene differential expressed a fold changes >1.5 and FDR-corrected *P* values <0.05 were used as filters. GO enrichment analysis was conducted with agriGO (bioinfo.cau.edu.cn/agriGO/) using the singular enrichment analysis (SEA) method and the peach genome protein ID as background of reference (Du et al. 2010). The statistical test method was Fisher and Yekutieli (FDR under dependency) method to perform multi-test adjustment with $P \leq 0.05$ for significance, using five as a minimum number of mapping entries. And SEACOMPARE was used to carry out cross-comparisons of results derived from different data sets of SEA (Du et al. 2010). In the case of gene variant analysis, we called SNP and Indels on genotypes using Samtools (Li et al. 2009) program. Variants having a minimum coverage below eight and a quality score below 100 were discarded. Functional effect of predicted variants were computing using SNPeff (Cingolani et al. 2012) program.

Gene expression analysis by qRT-PCR

Total RNA of root samples from individual plants per treatment was used for gene expression analysis by quantitative PCR (qRT-PCR). There were three control plants (not flooded) for each sampling time. The RNA extraction procedure was as described above. Following the DNase treatment, 5 μg of total RNA was used to synthesize cDNA from each sample, using a Thermoscript RT-PCR System™ (Invitrogen, Inc., Carlsbad, CA). Transcript levels of genes were measured by qRT-PCR using a Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA). All reactions were performed using the Brilliant SYBR Green Master Mix (Stratagene Inc., Santa Clara, CA) according to the manufacturer. For each biological replicate, qRT-PCR reactions were carried out in triplicate (technical replicates) using 2 μL Master Mix, 0.5 μL 250 nM of each primer, 1 μL diluted cDNA and nuclease-free water to a final volume of 20 μL. Controls (with no cDNA and RNA without RT) were included for each run. Fluorescence was measured at the end of each amplification cycle. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 65–95 °C melt. Expression was normalized against the *Prunus* translation enlongation factor two gene (*PpaTEF2*, GenBank database accession number TC3544) due to its consistent transcript level throughout the tissues (Tong et al. 2009). The best primer sequences of each gene (ppa004715, ppa000269, ppa007374, ppa010426, ppa010418, ppa008906, ppa007976, ppa009771, ppa025544, *ADH*1-like, ppa008779, ppa004573, ppa004524) analyzed was defined matching to both the peach genome according to Almada et al. (2013) and ours generated by RNA-Seq (Supplementary Table 2).

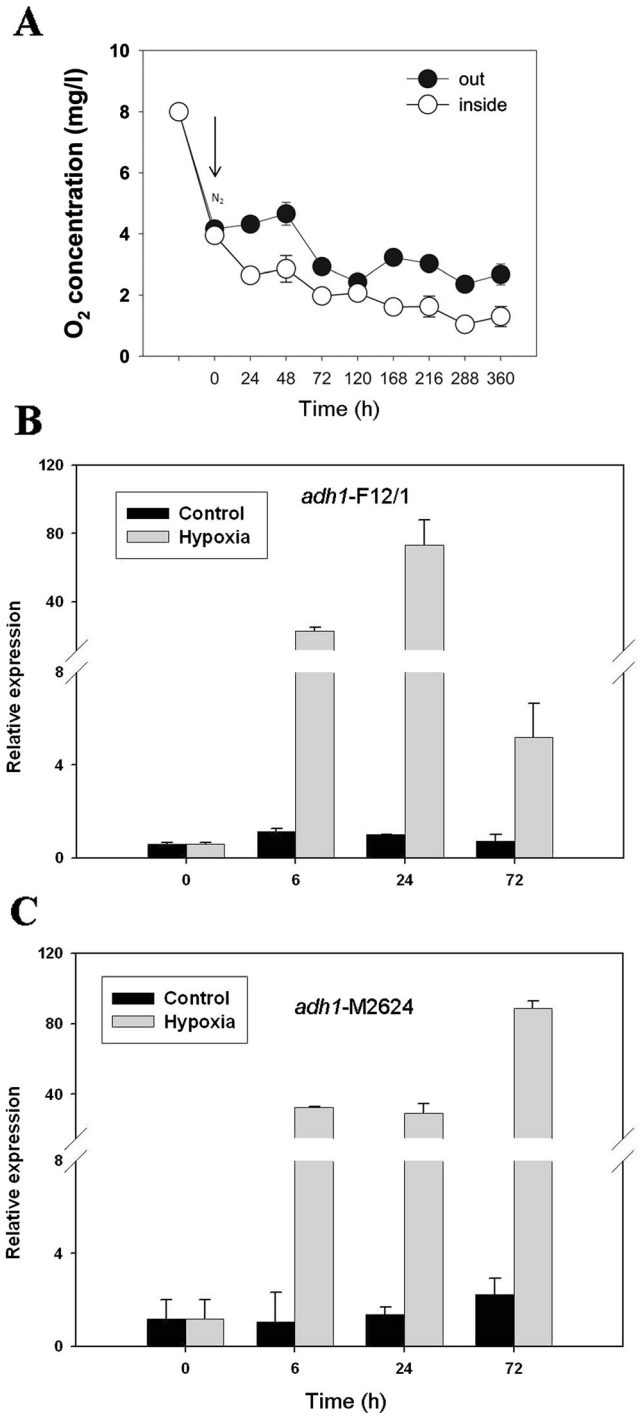


Fig. 1 Effects of root hypoxia caused by flooding on plant CO_2 assimilation, *ADH-like* expression and water oxygen content. **a** Dissolved oxygen in water surrounded the roots (*inside*) and the pots (*out*). **b**–**c**. *ADH-like* expression pattern in plant roots under flooding in sensitive ('M.F12') and tolerant ('M.2624') genotypes, respectively. They are expressed as mean relative expression values +/− standard error (n=3), normalized with TEF2 as a constitutively expressed gene

Results and discussion

Prunus rootstocks genotypes with contrasting response to root hypoxia

The genotypes 'Mariana 2624' ('M.2624') and 'Mazzard F12/1' ('M.F12/1') are respectively tolerant and sensitive to hypoxia conditions (Amador et al. 2009; Arismendi 2012; Pinochet 2010; Rubio-Cabetas et al. 2011). In order to verify that the plants were actually subjected to conditions of oxygen deficiency in their roots, we monitored both the level of O_2 surrounding the roots (Fig. 1a) and the levels of transcription of *alcohol dehydrogenase-like* (*ADH1*) from the roots of flooded plants (Fig. 1b, c). *ADH1* expression is typically used as a hypoxia molecular marker for plants given that it is strongly induced under hypoxic conditions (Kreuzwieser et al. 2009; Liu et al. 2005). The results show that low levels of oxygen in the roots strongly induce *ADH1* expression in 'M.2624' and 'M.F12/1' roots under flooding, irrespective of their differing tolerance to root hypoxia (Fig. 1b, c). At the same time control plants presented very similar pattern of low *ADH1* expression during the treatments at both genotypes. This validates our experimental conditions for inducing hypoxia by flooding. Our results are comparable to those obtained by Liu et al. (2005), who used the same gene as a marker to validate the experimental system for *Arabidopsis*. Liu et al. also found that *ADH1* was strongly induced soon after induction of root hypoxia (30 min), using a hydroponic system and continuous pumping of N_2 gas to remove oxygen. Kreuzwieser et al. (2009) found increased transcript levels of *pyruvate decarboxylase* (*PDC*) and *alcohol dehydrogenase* (*ADH*) genes in poplar roots under hypoxia, using similar experimental conditions to ours. Both genes encode for key alcohol fermentation enzymes, which are part of a pathway that plays an important role in the NADH oxidation during oxygen deficiency (Kennedy et al. 1992). Interestingly, in 'M.F12/1,' the level of expression of *ADH1* gene reached a peak of 80-fold at 24 h, then it decreased to fivefold at 72 h of flooding. However, the tolerant genotype 'M.2624' showed a level of expression of 40-fold at both 6 and 24 h of flooding, reaching a peak of 80-fold at 72 h. Considering the above results and the evident drop in dissolved oxygen levels detected in the water surrounding the roots (Fig. 1a), we established a hypoxia condition to characterize *Prunus* rootstock genotypes with contrasting responses to oxygen depletion in roots. Based on the information above, four times were selected to sample root tissue from control (without flooding) and flooded plants (6, 24, and 72 h under hypoxia) for the RNA-Seq analysis using the Illumina platform.

During the hypoxia treatment, symptoms of leaf wilting, chlorosis, and curling were observed in the sensitive 'M.F12/1' plants, but these symptoms were not observed in the tolerant 'M.2624' plants. As shown in Fig. 2, there are clear differences among symptoms in plant, including leaves, roots, and parameters of CO_2 assimilation. The tolerant genotype 'M.2624' immediately shows a moderate reduction in rate of CO_2 assimilation. However, the sensitive genotype 'M.F12/1' did not show significant differences in CO_2 assimilation with control plants until 24 h after flooding, but later severely fell down. The early response of the tolerant genotype may indicate that it possesses a sensing—probably a signal

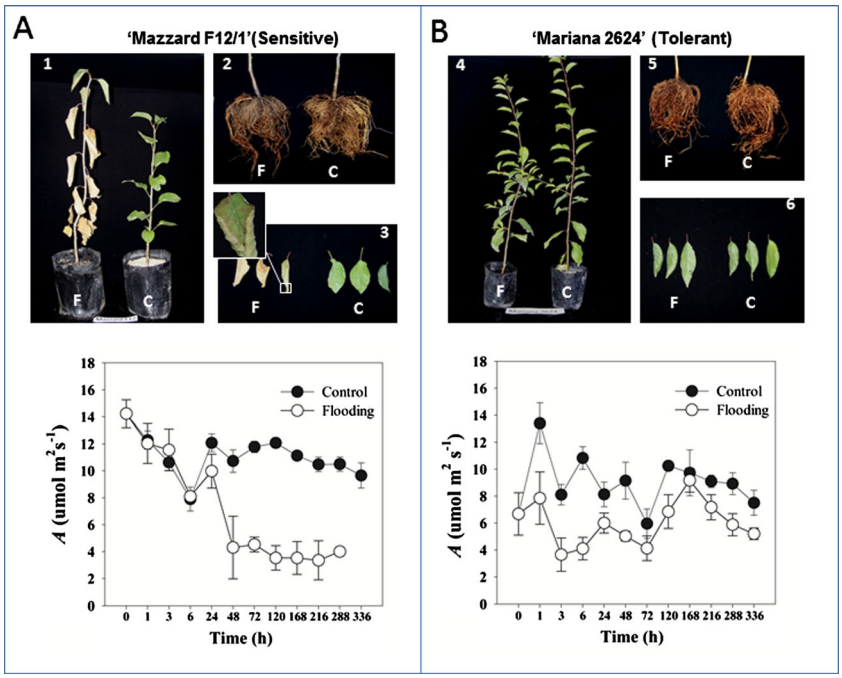


Fig. 2 Effects of flooding for 15 days on plant morphology in *Prunus* sp. rootstocks. The sensitive **a** 'Mazzard F12/1' and the tolerant **b** 'Mariana 2624' *Prunus* sp. rootstocks showed different symptoms after 15 day of hypoxia treatment. In general view of plants (*1*), roots (*2*), and leaves (*3*). Control and flooded plants are indicated as *C* and *F*, respectively. The CO_2 assimilation rates *A* (μmol m^{-2} s^{-1}) was measured during all the experiment for the **a** sensitive and **b** tolerant genotypes, respectively

from roots to leaves—and adaptive mechanism of hypoxia stress, which is not clearly present in the sensitive genotype.

Peach genome as reference for *Prunus* RNA-Seq studies

Regarding the time of sampling (control and flooded plants) four cDNA libraries from pooled RNA samples were constructed for each rootstock genotype, eight samples were sequenced using an Illumina HiSeq 2000 genome analyzer (50 bp single-end reads). From the raw data with 208 million reads, a total of 152 million high-quality reads were obtained, with 81 and 71 million reads from 'M.F12/1' (flooding-sensitive) and 'M.2624' (flooding-tolerant) genotypes, respectively (Supplementary Table 3). These high-quality reads were mapped against the peach reference genome (*P. persica* 139 Genome) available at Phytozome v.9.1 (http://www.phytozome.org), using the Bowtie algorithm (Li and Homer 2010). An 81 % of reads (124,292,720 reads) were aligned and mapped in the *P. persica* genome. Of the total reads mapped, 112,567,951 reads were matched to unique genomic locations, 6,187,201 reads to a maximum of ten genomic locations and 5,537,568 reads were placed on more than ten genomic locations. Reads having more than ten genomic locations were discarded. These alignment results showed high levels of homology among *Prunus* transcriptomes belonging to different subgenus *Amygdalus* (L.) Benth. Hook. (peach group), *Prunus* Focke [= *Prunophora* (Neck.)] Focke (plum group), and *Cerasus* (Adans.) Focke (cherry group), represented by genotypes of *P. persica* (peach genome of reference), *P. cerasifera* × *P. munsoniana* ('M.2624'), and P. *avium* ('M.F12/1'), respectively. High levels of homology of transcriptomes and of synteny of genomes among *Prunus* species were also described by (Martínez-Gómez et al. 2011). The recent results confirm the utility of the peach genome as a reference in RNA-Seq studies in other *Prunus* species. Furthermore, the present study provide a basis for future studies aiming at unraveling the root transcriptomes of *Prunus* species as well as for the development of genomic tools in these economically important tree species.

Hypoxia alters differentially the root transcriptome of the stone fruit rootstocks

Exposure to root hypoxia altered the root transcriptome in both the 'M.2624' and 'M.F12/1' genotypes. The total number of genes with significant differential expression (DEG), including up- and downregulated across all time points (6, 24, and 72 h) varied between the hypoxia-tolerant and hypoxia-sensitive genotypes. Thus, a total of 1406 and 1838 DEG were detected in the roots of the 'M.2624' and 'M.F12/1' rootstocks during the hypoxia treatment, respectively (Fig. 3). Regardless of the genotype, there were more upregulated than downregulated genes during the first 6 h of root hypoxia, and then most DEG were downregulated in the other examined flooding times (Fig. 3). There were considerably fewer DEG in either *Prunus* genotype in this study than reported in woody plants like poplar, with 5250 DEG (Kreuzwieser et al. 2009). It is likely that these differences with previous report are in part explained by the dissimilarities in the statistical power. Anyway, poplar species are considered tolerant to hypoxia, and after 5 h of hypoxia roots, only 195 genes showed altered expression, of which about 85 % were upregulated. The number of DEG increased with long-term hypoxia, with 3728 and 5066 at 24 and 168 h, respectively (Kreuzwieser et al. 2009). However, the number of DEG obtained in our analysis of *Prunus* genotypes, both tolerant and sensitive to hypoxia, was comparable to other reports on rice (*Oryza sativa* L.), cucumber (*Cucumissativus* L.), *Arabidopsis* (*A. thaliana* [L.] Heynh.), and cotton (*Gossypium hirstium* L.) (Christianson et al. 2010a; Christianson et al. 2010b); Lasanthi-Kudahettige et al. 2007; Liu et al. 2005; Qi et al. 2012).

The greatest difference in the number of DEG between tolerant and sensitive genotypes of *Prunus* was observed at 72 h of treatment. At this time, there were twice as many downregulated genes in 'M.F12/1' (1005) as in 'M.2624' (566), but a similar number of upregulated genes in the two

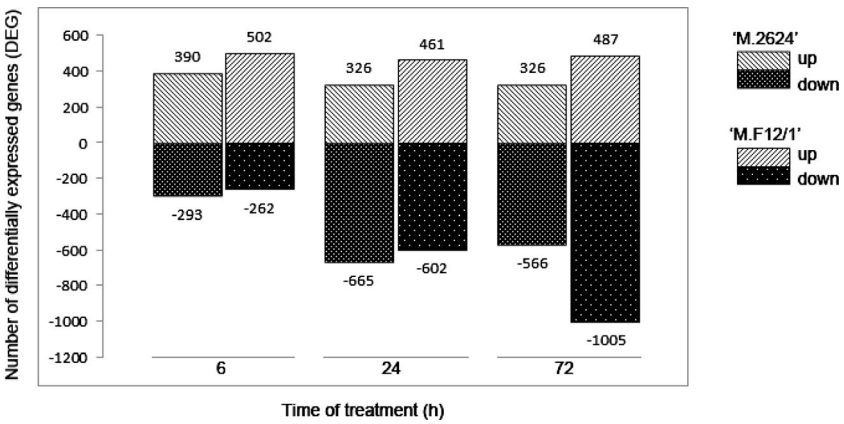


Fig. 3 Number of differentially expressed genes (≥2-fold changes; $q \leq 0.05$) in 'Mariana 2624' and 'Mazzard F12/1' following root hypoxia as determined by RNA-Seq

genotypes (487 and 326, respectively). In *Arabidopsis*, Liu et al. (2005) observed a peak of transcriptional changes, in number and magnitude, 6 h after starting the hypoxic treatment. On the other hand, van-Dongen et al. (2009) reported a steady transcriptional state at 2 h after establishing the stress condition. In poplar, differential expression increased steadily up to 7 days of treatment (Kreuzwieser et al. 2009).

Moreover, in order to examine the transcriptome response to root hypoxia, the identity of DEG was compared in each genotype. Figure 4a shows the overlap in gene expression at different time points in 'M.2624' and 'M.F12/1' genotypes treated by root hypoxia. Some 25.5 % (359 genes) and 24.21 % (445 genes) of the total number of DEG were common to all flooding times analyzed in 'M.2624' and 'M.F12/1, ' respectively. On the other hand, 'M.2624' showed 162, 216 and 227 DEG exclusively at 6, 24, and 72 h of flooding, respectively; whereas that exclusive DEG for 'M.F12/1' were 191, 77, and 534 at 6, 24, and 72 h of flooding, respectively. We also observed DEG at two consecutive times, but not at the third, indicating that DEG were differentially expressed only during two consecutive transition phases. 'M.2624' showed 136 and 280 DEG from 6 to 24 and 24 to 72 h, respectively, while 'M.F12/1' showed 463 and 78 DEG from 6 to 24 h and 24 to 72 h, respectively. Interestingly, there were 26 and 50 DEG, respectively, for 'M.2624' and 'M.F12/1' that only appeared at the first and last sampling times, 6 and 72 h, but not at 24 h.

In order to identify similarities and differences in the transcriptomic response to hypoxia between 'M.2624' and 'M.F12/1,' we compared their transcriptomes regarding their identity and mode of change in gene expression (up- or downregulated) (Fig. 4b). In general, upregulated genes in both genotypes tend to diminish from 228 to 185 and then 138 at 6, 24, and 72 h of sampling, and the downregulated tend to increase from 101 to 306 and then 328 at the same sampling times. We also found the same DEG responding in the opposite manner, with 9, 2, and 6 DEG downregulated in 'M.2624' and upregulated in 'M.F12/1,' and 2, 3, and 4 DEG upregulated in 'M.2624' and downregulated in 'M.F12/1,' at the 6, 24, and 72 h, respectively. The pattern of expression for some of these genes was confirmed by qPCR experiment (see next sections). However, there was also a large number of both up- and downregulated DEG that were exclusive to one of the genotype (Fig. 4b). There were 273, 272, and 349 upregulated and 160, 295 and 675 downregulated DEG exclusive for 'M.F12/1' and 159, 139, 188 upregulated and 385, 357, and 236 downregulated DEG for 'M.2624' at 6, 24, and 72 h, respectively. It is likely that the DEG with opposite responses

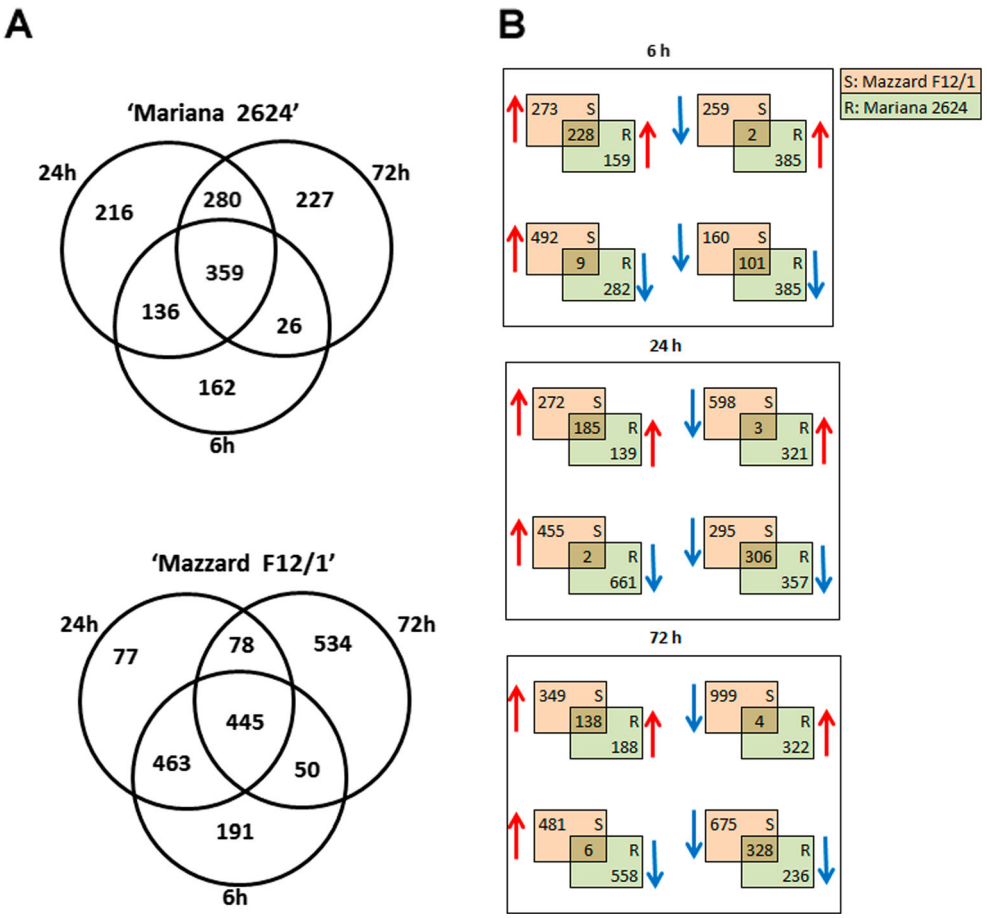


Fig. 4 Defining common and exclusive hypoxia responsive genes in *Prunus* rootstocks with contrasting response to root waterlogging. **a** At different time points within each genotype following root hypoxia. **b** Between the two genotypes following root hypoxia. The identity and number of genes significantly upregulated (*red arrow*) and/or downregulated (*blue arrow*) in 'Mariana 2624' and 'Mazzard F12/1' at different time points under hypoxia conditions were compared. Orange and green boxes contain the DEG exclusively expressed in 'Mazzard F12/1' or 'Mariana 2624' rootstock, respectively. The intersections show the number of DEG common to the both genotypes studied

and those exclusively detected in each genotype might explain the different performances of these rootstocks to hypoxia conditions.

Root baseline gene expression contributes to the differential response between the *Prunus* genotypes

The adaptation of different plant genotypes to root hypoxia depends on both their baseline of gene expression and the distinct response of each plant. The *Prunus* genotypes 'M.2624' and 'M.F12/1' belong to the subgenera *Prunus* and *Cerasus*, respectively, which evolved from a common ancestor (Potter et al. 2007). Therefore, it can be expected to observe different baselines of expression of many genes that determine the distinct performances of the subgenera in the different environments in which they evolved. The analysis of gene ontology enrichment is a powerful bioinformatic tool to study transcriptome changes and has been used to study the transcriptome responses of many species for different purposes (Du et al. 2010; Yi et al. 2013). Using singular enrichment analysis (SEA) according to Du et al. (2010), we evaluated root transcriptome differences in two ways; first, by comparing the level of gene expression between the two genotypes ('M.2624'/'M.F12/1') at baseline (time 0) and at 6, 24, and 72 h after flooding and, second, by determining differentially expressed genes according to their own control plants at 6, 24, and 72 h of treatment, respectively (Table 1, Supplementary Table 1).

Comparison of root transcriptomes from control plants (time 0 h) of the two genotypes (tolerant/sensitive) showed a total of 562 DEGs, of which 308 were "upregulated" and 254 were "downregulated". GO enrichment analysis of the DEGs indicated that there were at least 25 "altered" GOs, which established the difference between the two genotypes at baseline without hypoxia treatment (Table 1). Nine GOs fell into the category of biological processes (BP) and 16 into molecular function (MF). Several of the "altered" baseline GOs showed no differences at 6, 24, and 72 h of hypoxia treatment, such as BP:0042221 of response to chemical stimulus; BP:0006979 of response to oxidative stress; BP:0000272 of polysaccharide catabolic process; BP:0009308 of amine metabolic process; MF:0016209 of antioxidant activity; MF:0016684 of oxidoreductase activity, acting on peroxide as an acceptor; and MF:0004601 of peroxidase activity. In these cases, either one or both genotypes modified their level of gene expression of the aforementioned GOs until reaching similar numbers of transcripts between the genotypes during the hypoxia treatment. However, in the cases of BP:0042221, BP:0006979, MF:0016209, MF:0016684, and MF:0004601, only the hypoxia-sensitive genotype clearly altered its gene expression. No changes were evident in the roots of the tolerant genotype. Altered baseline GOs that maintained the differences in expression during hypoxia treatment either do not participate in the hypoxia response or were modified in one or both genotypes, but do not have comparable levels of expression between the two genotypes. BP:0055114 of oxidation reduction, MF:0003824 of catalytic activity, and MF:0016491 of oxidoreductase activity were in this group. These three GOs showed strong alterations in both the hypoxia-tolerant and hypoxia-sensitive genotypes. However, several GOs were altered only in the sensitive genotype, but they did not reach comparable levels of gene expression with the tolerant genotype. This is the case for MF:0020037 of heme binding, MF:0046906 of tetrapyrrole binding, MF:0005506 of iron ion binding, MF:0046872 of metal ion binding, MF:0043169 of cation binding, and MF:0043167 of ion binding.

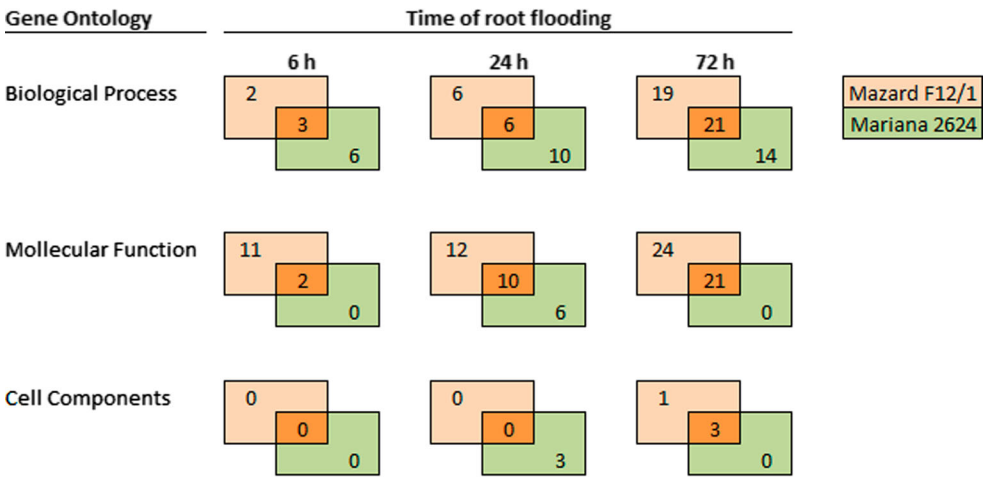
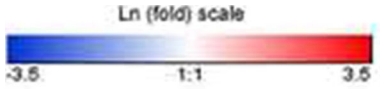


Fig. 5 Comparison of hypoxia responding categories of GO in root of *Prunus* rootstock of 'Mazzard F12/1' and 'Mariana 2624.' Common and distinct GO between the sensitive and tolerant genotypes to hypoxia stress were identified at 6, 24, and 72 h of treatment. *Orange-pale* and *green boxes* contain the GO exclusively perturbed in 'Mazzard F12/1' or 'Mariana 2624' rootstock, respectively. The intersections show the number of perturbed GO common to the both genotypes studied

Table 2 Hierarchical clustering of selected DEG responding to hypoxia treatment. The column 'M.2624'/'M.F12/1' corresponds to ln of FPKM ratio between both genotypes at 0, 6, 24, and 72 h of hypoxia treatment. Columns 'M.2624' and 'M.F12/1' are DEG [ln(fold)] at time of 6, 24, and 72 h of hypoxia treatment, respectively. Heat map color scale was adjusted at interval of −3.5 minimum (*blue*) and 3.5 maximum (*red*)



'M2624'/'F12/1'				'F12/1'			'M2624'			Gene product	Gene ontology;Protein name
0	6	24	72	6	24	72	6	24	72 h		
										ppa006386m	GO:0005975;PRK Phosphoribulokinase
										ppa002531m	GO:0003824;Long chain acyl-CoA synthetase family protein
										ppa015674m	GO:0055114;Peroxidase, putative
										ppa003098m	GO:0003824;Pyruvate decarboxylase, putative
										ppa010224m	GO:0003700;AT-HSFA6B; DNA binding / transcription factor
										ppa025869m	GO:0055114;Unknown protein (oxide reduction)
										ppa021317m	GO:0008150;hypoxia-responsive family protein
										ppa007482m	GO:0055114;AERO1 Oxidoreductase
										ppa007099m	GO:0016491;ADH1 Alcohol dehydrogenase
										ppa023265m	GO:0016491;Stearoyl-ACP desaturase, putative
										ppa007714m	GO:0006096;fructose bisphosphate aldolase, putative
										ppa020991m	GO:0003700;Ovule development protein, putative
										ppa024318m	GO:0003700;WRI1 (WRINKLED 1) transcription factor
										ppa009537m	GO:0055114;Unknown protein (oxide reduction)
										ppa009228m	GO:0016491;ACO1 1-aminocyclopropane 1 carboxylate oxidase
										ppa013087m	GO:0008150;calcium-binding EF hand family protein
										ppa007186m	GO:0016491;ADH1 alcohol dehydrogenase
										ppa007167m	GO:0016491;ADH1 alcohol dehydrogenase
										ppa007154m	GO:0016491;ADH1 alcohol dehydrogenase
										ppa008730m	GO:0003700;AP2 domain-containing transcription factor, putative
										ppa003458m	GO:0003824;Pyruvate decarboxylase, putative
										ppa010330m	GO:0003824;Haloacid dehalogenase like hydrolase family protein
										ppa002723m	GO:0009058;SUS3 UDP-glycosyltransferase
										ppa015602m	GO:0003700;AT-HSFA3 transcription factor
										ppa020646m	GO:0016779;Unknown protein / nucleotidyltransferase activity
										ppa025544m	GO:0003700;bZIP transcription factor family protein
										ppa009346m	GO:0005975;XTR6 xyloglucan:xyloglucosyl transferase
										ppa007374m	GO:0016491;Sorbitol dehydrogenase, putative
										ppa015247m	GO:0016491;Sorbitol dehydrogenase, putative
										ppa007327m	GO:0016491;Sorbitol dehydrogenase, putative
										ppa007343m	GO:0016491;Sorbitol dehydrogenase, putative
										ppa002615m	GO:0004672;CRK6 (CYSTEINE-RICH RLK 6); kinase
										ppa002577m	GO:0005488;Lectin protein kinase, putative
										ppa004703m	GO:0003824;Pyruvate kinase, putative
										ppa006243m	GO:0003700;TEM1 transcription factor
										ppa008787m	GO:0016491;Oxidoreductase, 2OG-Fe(II) oxygenase family protein
										ppa007606m	GO:0003700;AP2 domain-containing transcription factor
										ppa006966m	GO:0055114;LIN2 Coproporphyrinogen oxidase
										ppa017534m	GO:0006950;universal stress protein (USP) family protein
										ppa004715m	GO:0005975;HXK3 hexokinase
										ppa003289m	GO:0008168;Methyltransferase activity
										ppb022158m	GO:0008152;Myrcene/ocimene synthase, putative
										ppa022757m	GO:0005488;Lectin protein kinase, putative
										ppa020571m	GO:0004672;leucine-rich repeat transmembrane protein kinase, putative
										ppa025348m	GO:0016491;Oxidoreductase, zinc-binding dehydrogenase family protein
										ppa003925m	GO:0016763;Initiator tRNA phosphoribosyl transferase family protein
										ppa018203m	GO:0003824;catalytic
										ppa001925m	GO:0016876;Glutamate tRNA ligase, putative
										ppa003561m	GO:0008152;AK3 Aspartate kinase
										ppa000234m	GO:0016020;PDR12 ATPase, coupled to transmembrane movement of substances
										ppa005364m	GO:0005488;PUB9 Ubiquitin-protein ligase
										ppa004353m	GO:0003824;Phosphoribosylamine glycine ligase (PUR2)
										ppa001577m	GO:0004672;lectin protein kinase family protein
										ppa027113m	GO:0000160;ARR6 (Response regulator 6)
										ppa005196m	GO:0016787;Hydrolase
										ppa008027m	GO:0004185;scpl42 serine carboxypeptidase-like 42
										ppa025153m	GO:0004672;Kinase
										ppa011882m	GO:0016209;Fe superoxide dismutase 3, putative
										ppa000253m	GO:0005634;SNL2 (SIN3-LIKE 2)
										ppa022719m	GO:0003700;AP2 domain-containing protein
										ppa005531m	GO:0004672;protein kinase family protein
										ppa003884m	GO:0004672;Leucine rich repeat family protein
										ppa001161m	GO:0004672;Leucine rich repeat family protein
										ppa001122m	GO:0004672;Leucine rich repeat family protein
										ppa007810m	GO:0016491;NADP-dependent oxidoreductase, putative
										ppa013280m	GO:0003700;zinc finger (GATA type) family protein
										ppa023235m	GO:0003700;RRTF1 (Redox responsive transcription factor 1)
										ppa009472m	GO:0005975;Xyloglucan:xyloglucosyl transferase, putative
										ppa018152m	GO:0016491;Short-chain dehydrogenase/reductase (SDR) family protein
										ppa004387m	GO:0005975;AtGH9B13 Arabidopsis thaliana glycosyl hydrolase 9B13
										ppa005447m	GO:0008152;UDP glucosyl transferase family protein
										ppa009513m	GO:0055114;peroxidase, putative
										ppa005700m	GO:0004672;CIPK9 kinase
										ppa008728m	GO:0055114;Peroxidase 17 (PER17)
										ppa003994m	GO:0006096;PFK3 6-phosphofructokinase
										ppa009513m	GO:0055114;peroxidase, putative
										ppa005700m	GO:0004672;CIPK9 kinase
										ppa008728m	GO:0055114;Peroxidase 17 (PER17)

'M2624' requires minor (or moderate) transcriptional changes than does 'M.F12/1' to cope with root stress hypoxia

In addition to the baseline-altered GOs, the enrichment analysis of DEG-based GOs from both the comparison between the tolerant and sensitive genotypes, and changes within their own transcriptomes, revealed another 90 GOs that participated in the response to root hypoxia during the 6 to 72 h of flooding treatment. Of these 90 GOs, 55, 31, and four belong to the categories of BP, MF, and CC (cellular components), respectively. Many of these GO alterations were also detected by comparing DEGs with their control at 6, 24, and 72 h of treatment (Supplementary Table 1). Figure 5 shows the comparison of all the altered GOs from the two genotypes 'M.2624' and 'M.F12/1,' the tolerant and the sensitive to root hypoxia, respectively. For the BP category, there were 3, 6, and 21 altered GOs in both genotypes at 6, 24, and 72 h of hypoxia, with 6, 10, and 14 altered GOs only in the tolerant genotype, and 2, 6, and 19 altered GOs only in the sensitive genotype at 6, 24, and 72 h. In the MF category, the genotypes showed 2, 10, and 21 commonly altered GO at 6, 24, and 72 h of hypoxia treatment, while 11, 12, and 24 GO were altered only in the sensitive genotype at the respective checking times. In this GO category, the tolerant genotype showed only 6 exclusive GOs altered at 24 h of hypoxia. In the case of the CC category, the tolerant genotype modified 3 GOs at 24 and 72 h of hypoxia, which also were altered in the sensitive genotype but only detected at the 72 h of hypoxia treatment, including one GO that was exclusive to this genotype.

The different patterns of altered GOs observed in the roots of the two *Prunus* species, as determined by SEA analysis, likely explain tolerance and/or sensitivity to this stress. Both the groups of "altered-GOs" from the baseline and those that were altered during the response to flooding treatment are good candidates to explain the different responses. Altered GO, such as those associated with biological processes, which were detected exclusively during the response of the tolerant genotype 'M.2624,' could be the most important. However, the altered GOs that were common to the two genotypes could also be involved in the different responses to hypoxia, because having the same GO does not mean having the same genes or that the magnitude of change in expression is the same for both genotypes. In fact, several GOs that were altered in both genotypes did not reach comparable number of transcripts, showing the status of altered GOs in both types of enrichment analysis. Moreover, different alleles for the same genes at the same levels of expression could generate different degrees of response that also produce distinct phenotypes. It is important to note that considering the overall responses of the genotypes, the genotype 'M2624' requires minor (or moderate) transcriptional changes than does 'M.F12/1' to cope with root stress hypoxia. An example of this are the few DEGs from the tolerant genotype associated with transcription factor activity (GO:0003700), which was detected as altered GOs only in the sensitive genotype at 6 h with 25 genes. Although an early response by the tolerant genotype before 6 h of hypoxia cannot be ruled out, the detection of several "altered-GOs" from the baseline is evidence that this resistant genotype is already prepared for hypoxia events. Interestingly, there are some DEGs in this GO category with the opposite response, such as the genes ppa023235 and ppa010647, identified as RRTF-1 (*Redox responsive transcription factor 1*) and ATHB-7 (*Arabidopsis Thaliana Homeobox 7*), respectively (Supplementary Table 4).

Table 3 Hexokinases expressed in roots of *Prunus* sp. during hypoxia treatment. The column 'M.2624'/'M.F12/1' corresponds to ln of FPKM ratio between both genotypes at 0, 6, 24, and 72 h of hypoxia treatment. Columns 'M.2624' and 'M.F12/1' are DEG [ln(fold)] at time of 6, 24, and 72 h of hypoxia treatment, respectively. Heat map color scale was adjusted at interval of −3.5 minimum (*blue*) and 3.5 maximum (*red*)

UniqueID	Name	'M.2624'/'M.F12/1'				'M.F12/1'			'M.2624'		
		0	6	24	72	6	24	72	6	24	72
ppa004715m	HXK3 (HEXOKINASE 3)	-0.51	3.23	3.46	3.44	-0.18	-0.43	-0.68	3.55	3.54	3.26
ppa009797m	HXK2 (HEXOKINASE 2)	-0.29	0.47	1.00	0.45	0.34	0.30	0.65	1.11	1.59	1.38
ppa011439m	HXK2 (HEXOKINASE 2)	-0.43	0.54	0.74	0.37	0.49	0.66	1.01	1.45	1.83	1.81
ppa004471m	HKL1 (HEXOKINASE-LIKE 1)	0.23	0.08	-1.11	-1.49	0.71	2.21	2.72	0.56	0.87	0.99
ppa004637m	HXK1 (HEXOKINASE 1)	0.49	0.10	-1.19	-1.64	0.10	1.49	1.80	-0.28	-0.18	-0.33
ppa008494m	PFK	0.60	0.26	-0.86	-1.35	0.33	1.84	1.76	-0.01	0.39	-0.19
ppa007069m	PFK5, putative	0.39	0.47	0.48	1.17	-0.71	-1.86	-2.63	-0.63	-1.78	-1.86
ppa006628m	PFK5, putative	0.65	0.62	-0.19	0.05	-0.63	0.13	0.01	-0.66	-0.70	-0.59
ppa003690m	PFK2, putative	1.02	0.77	1.31	1.59	0.17	-0.84	-0.99	-0.08	-0.55	-0.42
ppa004431m	PFK2, putative	0.21	0.17	0.51	0.95	-0.55	-1.14	-1.43	-0.59	-0.84	-0.68

Best candidate genes explaining the distinct response to hypoxia

Genes belonging to the altered "GO" and identified at least twice as DEG during the treatment were selected as the best candidate genes (Supplementary Table 4). Table 2 shows a selected list of candidate genes grouped according to their response pattern. The largest group contains upregulated DEGs from both the sensitive and tolerant genotypes, such as alcohol dehydrogenases, sorbitol dehydrogenases, hypoxia-responsive family protein, and universal stress protein (USP) family protein. Interestingly, the transcription of the gene ppa021317 (hypoxia-responsive family protein) is several time stronger in the tolerant than the sensitive genotype. A second group contains DEGs that were upregulated in the tolerant genotype but not significant or downregulated in the sensitive genotype during the hypoxia treatment. Among these genes, several codes for enzymes associated with post-translational protein modification, such as leucine-rich repeat (LRR), kinases, and ubiquitin-protein ligases. The modifications of key enzymes or substrates by these mechanisms have direct effects on pathways synthesis, catabolism, or signaling. This group also includes genes that code for proteins associated with transcription regulation like AP2 domain-containing, ARR6 (Response regulator 6), Sin3-like2, and zinc finger (GATA type) proteins. Also included in this group are genes that code for enzymes that directly participate in the oxide reduction pathway to maintain energy homeostasis and/or protect the cell from reactive oxygen species (ROS). The bottom of Table 2 lists genes that were upregulated in the sensitive genotype but were not significant in the tolerant genotype during the hypoxia treatment. Although most DEGs fell in this category, few genes are shown in Table 2. This table also presents five genes that were upregulated in the hypoxia-sensitive genotype but downregulated in the hypoxia tolerant genotype.

The hexose-phosphorylating enzymes hexokinases (HXK) and fructokinases (FRK) play a key role in coordinating the availability of sugar resources, with major effects on plant physiology and development (Granot et al. 2014). In fact, these enzymes are considered the gateway to most of the organic metabolism in plants, including glycolysis activation (Granot et al. 2014). Therefore, their patterns of expression were analyzed in detail. Seven HXKs and eight FRKs have been annotated in the peach genome, but transcripts of five of each gene were detected in roots of control and flooded plants of both *Prunus* genotypes (Table 3). However, *hexokinase 3* (ppa004715) was strongly upregulated at 6, 24, and 72 h of treatment in the hypoxia-tolerant genotype, but not in the sensitive genotype (Table 3). The other two HXK genes (ppa009797, ppa011439) were also upregulated in the tolerant genotype from 6 to 72 h of flooding. The sensitive genotype upregulated other *HXK* genes detected at 24 h of flooding, but with lower intensity than in the tolerant genotype. In the case of genes that code for PRKs, the tolerant genotype showed a non-significant regulation of four genes, but one downregulated at 24 h of hypoxia treatment. The hypoxia-sensitive genotype showed a similar pattern of PRK gene expression as the tolerant genotype, but one gene was upregulated from 24 h of flooding. Both of these enzymes catalyze irreversible reactions in plants (non-hexose-phosphate phosphatases have been described in plants), providing the initial substrates to generate energy by the glycolytic pathway. However, considering that glucose can only be phosphorylated by HXKs, whereas fructose can be phosphorylated by either HXK or FRK (Granot et al. 2014), it can be hypothesized that because of the early response of the tolerant genotype, which significantly increases the number of HXKs by upregulating the respective genes, it has a better performance than the sensitive genotype. It is should be noted that HXKs can also phosphorylate other sugars like mannose and glucosamine, which may also play roles in generating energy and metabolic signaling (Granot et al. 2014). Morever, HXK activity has been associated with regulation of physiological processes like stomatal closure and photosynthesis in leaves and uptake of minerals in roots (Granot et al. 2014; Lillo 2008). Interestingly, a type of HXK3 has been described in mammalian cells that is regulated by hypoxia and exerts protective effects against oxidative stress, perhaps by increasing ATP levels, reducing oxidant-induced ROS production, preserving mitochondrial membrane potential, and increasing mitochondrial biogenesis (Wyatt et al. 2010).

The generation of reactive oxygen species (ROS) is also typical of hypoxia stress (Blokhina et al. 2003). In this study, the GO associated with responses to the oxidative stress (BP:0006979) of oxidoreductase activity, acting on peroxide as an acceptor (MF:0016684) and peroxidase activity (MF:0004601), were altered in the hypoxia-sensitive genotype, but not in the hypoxia-tolerant one. In addition, determination of malondialdehyde (MDA), a biomarker of oxidative stress, in roots of 'M.2624' and 'M.F12/1' during hypoxia treatment showed significantly higher levels of MDA in the hypoxia-sensitive genotype (Pimentel et al. 2014), suggesting that the hypoxia-tolerant genotype maintains low levels of ROS during hypoxia treatment and therefore requires less gene transcription to prevent ROS damage. Nevertheless, the gene *ascorbate peroxidase 2* (ppa010426) was upregulated in both genotypes (Supplementary Table 4), but with a higher level of over-regulation in the tolerant genotype. A putative peroxidase gene (ppa015674) was upregulated in both genotypes, while C *L-ascorbate oxidase* (ppa003411) was downregulated in both genotypes.

In addition, genes from the response to stress and stimuli category were differentially expressed under hypoxia. Many of them had already been classified in the hypoxia-responsive

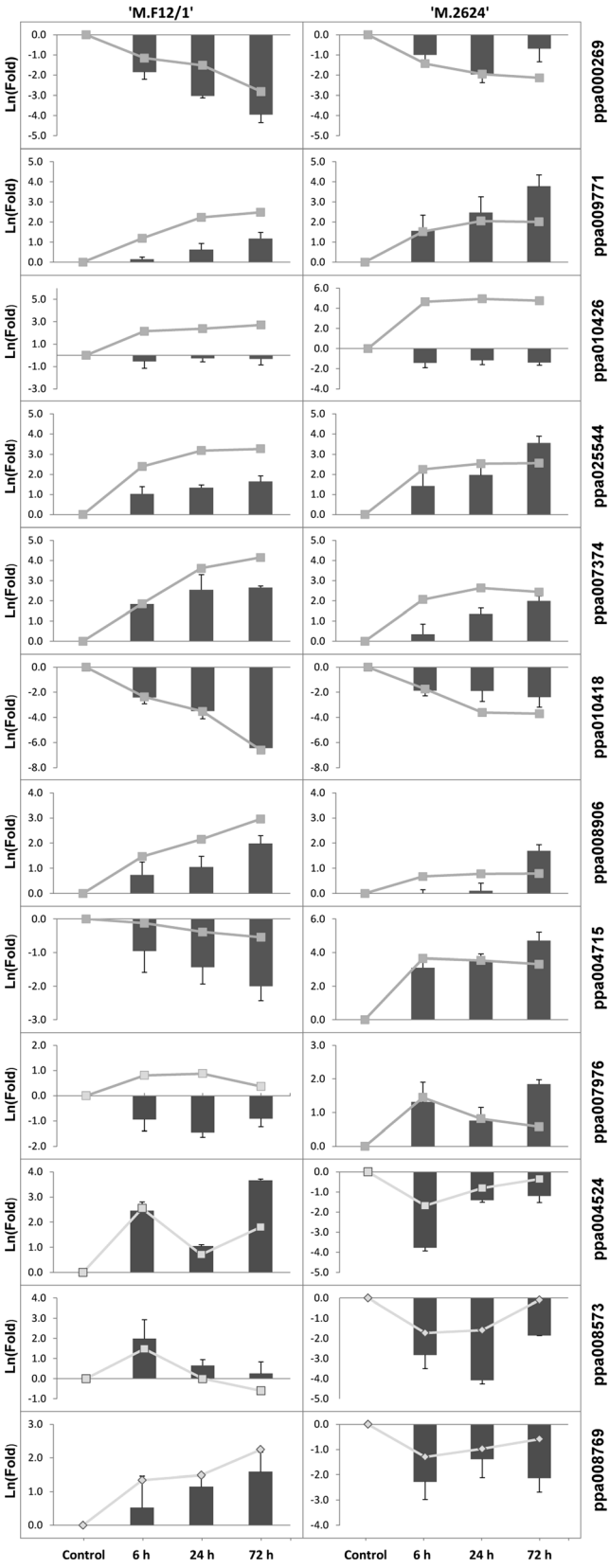


Fig. 6 Validation of the results using real-time PCR. 'Mazzard F12/1' and 'M.2624' are shown in columns, respectively. The continuous lines show the trend of expression obtained by RNA-Seq. The *bars* indicate the gene expression examined by qRT-PCR using total RNA from roots after treatment with hypoxia for 0, 6, 24, and 72 h. They are expressed as mean relative expression values +/− standard error (n=3), normalized with TEF2 as a constitutively expressed gene

protein family. Interestingly, the genes ppa013870 and ppa021317 were upregulated in both genotypes, but 'M2624' presented lower values. Even more interesting, the osmotin-like protein (ppa010410) was upregulated in 'M.2624' and downregulated in 'M.F12/1' (Supplementary Table 4). Ethylene is involved in the response to hypoxia and is considered a major contributor to the formation of adventitious roots and aerenchyma (Shiono et al. 2008). Under anaerobic conditions, *ETR2* (ppa004941m) and *ACC oxidase 1* (ppa009228m) were upregulated in roots of both *Prunus* genotypes under study. These results suggest that ethylene plays an important role in the response to hypoxia in *Prunus*, which is comparable to results obtained with cucumber (Qi et al. 2012), where similar genes were expressed under flooding conditions. As well, the expression of genes that code for *Ethylene Response Factor* (*ERF*) transcription factors were differentially affected by root hypoxia. Two *ERF* genes in *Arabidopsis* were recently identified that are induced by hypoxia, *HRE2* and *HRE1* (Licausi et al. 2010), which belong to group VII of the ERF family in *Arabidopsis* (Nakano et al. 2006). It has been proposed that these genes play a role in plant tolerance to anaerobic stress by enhancing the expression of genes related to anaerobic pathways and ethanol fermentation (Qi et al. 2012). Among genes related to hormonal regulation by auxin, *AXR3* (ppa011570) was upregulated in both genotypes, whereas *ARF4* (ppa001557) was downregulated in the sensitive genotype 'M.F12/1,' but no alteration was detected in 'M.2624.' It is well known that auxin transport is stimulated by ethylene (Morgan and Gausman 1966). Accumulation of auxin in basal stems of plants such as tomato or *Rumex* species induces adventitious root initiation. This response results in the formation of a new root system that is able to replace the original one when tissue is damaged by waterlogging (Vidoz et al. 2010). Our results also suggest that auxin is involved in the response to hypoxia stress in *Prunus* genotypes and plays an important role in signaling.

RNA-Seq genes were confirmed by qRT-PCR

To confirm our results obtained by RNA-Seq from *Prunus* genotypes with contrasting responses to hypoxia, 12 genes were selected from both genotypes to validate their levels of expression by qRT-PCR at the same flooding times (0, 6, 24, and 72 h). The genes selected for this analysis are involved in the fermentative pathway, glycolysis, antioxidant system, and other cellular and metabolic processes (Fig. 6). Considering both genotypes, 'M.2624' and 'M.F12/1,' a total of 24 qPCR assays were performed to validate the RNA-Seq results.

Twenty-one of the 24 qPCR assays showed the same trend of expression obtained from the RNA-Seq experiment, with 11 and 10 for the genotypes 'M.2624' and 'M.F12/1' (Fig. 6), respectively. The genes that did not show consistency between qRT-PCR and RNAseq were the *peroxidase L-ascorbate-2* (APX2, ppa010426) in both genotypes and the gene of L-lactate dehydrogenase, and the putative (ppa007976) only in 'M.F12/1.' Qi et al. (2012) used fewer genes to validate their gene transcriptional analysis (RNA-Seq by Solexa) of cucumber. They included genes associated with antioxidation, glycolysis, and ethylene production. Two genes did not show consistency between the RNA-Seq and qRT-PCR experiments. Two reports of microarray experiments with *Arabidopsis* that used 29 and 17 genes to confirm the results by qRT-PCR found one and four genes, respectively, that were not consistent (Klok et al. 2002; Liu et al. 2005). These small differences among the techniques (qPCR, RNA-Seq, Microarray) might represent different levels of sensitivity. Therefore, with qRT-PCR, we validated the RNA-Seq transcriptional analysis of two *Prunus* rootstock genotypes with contrasting responses to hypoxia.

Conclusions

The genotypes of *Prunus* rootstocks, 'M.2624' and 'M.F12/1, ' showed distinct responses to hypoxic conditions in the roots, the first classified as tolerant and the other as sensitive. During hypoxia treatment, the tolerant genotype 'M.2624' immediately showed a moderate reduction in the rate of CO2 assimilation, while the sensitive genotype 'M.F12/1' did not show significant differences from the control plant until 24 h of flooding and then severely fell down. The early response of the tolerant genotype suggests the existence an early sensing signal from roots to leaves, an adaptive mechanism of hypoxia stress that is clearly not present in the sensitive genotype.

RNA-Seq analysis of the transcriptomes of the roots of the two genotypes identified a large number of DEG. There were many DEG common to 'M.2624' and 'M.F12/1,' and many exclusive to just one of the genotypes. Interestingly, a few DEG presented opposite modes of regulation between the two genotypes at all sampling times. The two classes of DEG, those exclusive to one of the genotypes and those with the opposite mode of regulation, may explain the contrasting responses to root hypoxia. Analysis of gene ontology by SEA detected at least 115 altered GOs that are involved in the response to hypoxia stress in the sensitive and/or tolerant genotypes. Interestingly, 25 of these GO showed differences at the baseline level between the two genotypes when the DEG of control plants were analyzed. Most of these baseline GOs were altered in the sensitive genotype 'M.F12/1' during the hypoxia treatment, while only few GOs in the tolerant M.2624 were altered. This indicates that there is a baseline-DEG group that is important in accounting for the distinct responses of the two *Prunus* genotypes. Gene ontologies like BP:0042221 of response to chemical stimulus; BP:0006979 of response to oxidative stress; MF:0016209 of antioxidant activity; MF:0016684 of oxidoreductase activity, acting on peroxide as an acceptor; and MF:0004601 of peroxidase activity were clearly different at the baseline time (control plants) but were only disturbed in the sensitive genotype. However, they reach similar amount of gene transcripts because no differences were detected between the genotypes during the hypoxia treatment. There were also GOs in the same group of altered baseline that were altered in both genotypes during the hypoxia treatment, like BP:0055114 of oxidation reduction, MF:0003824 of catalytic activity, and MF:0016491 of oxidoreductase activity. Moreover, there are several GOs that were altered only by the sensitive genotype, but did not reach comparable levels of gene expression with the tolerant genotype. This is the case for MF:0020037 of heme binding, MF:0046906 of tetrapyrrole binding, MF:0005506 of iron ion binding, MF:0046872 of metal ion binding, MF:0043169 of cation binding, and MF:0043167 of ion binding. In addition to the baseline-altered GO, there were another 90 GO that participated in the response to root hypoxia during the period 6 to 72 h of flooding. A list was developed of the best candidate genes considering both their belonging to some of these altered GOs and their classification as DEGs at least twice during the hypoxia treatment. Several groups are distinguished according to the patterns of expression. A group that contains upregulated DEGs in both the sensitive and tolerant genotypes includes genes such as alcohol dehydrogenases, sorbitol dehydrogenases, hypoxia-responsive family protein, and universal stress protein (USP) family protein. A second group contains DEGs that were upregulated in the tolerant genotype but not significant or downregulated in the sensitive genotype during the hypoxia treatment. Among these genes, several codes for enzymes associated with posttranslational protein modifications like leucine-rich repeat (LRR), kinases, and ubiquitin-protein ligases. The modifications of key enzymes or substrates by these mechanisms have direct effects on pathway of synthesis, catabolism, or signaling. Hexose-phosphorylating enzymes like hexokinases (HXK) and fructokinases (FRK) are representative of this group. Also belonging to this group are genes that code for proteins associated with transcription regulation like AP2 domain-containing, ARR6 (Response regulator 6), Sin3-like2, and zinc finger (GATA type). Other genes included in this group code for enzymes that directly participate in the oxide reduction pathway and process to maintain energy homeostasis and/or protect the cell from reactive oxygen species (ROS). Another group was identified with upregulated genes in the sensitive genotype but not significant or downregulated in the tolerant genotype during the hypoxia treatment. This result represents a valuable source of information for further studies to identify

the real mechanism and genes that define hypoxia tolerance in *Prunus*.

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Data archiving statement RNA-Seq time series data for *Prunus avium* cv F12 and *Prunus cerasifera* × *Prunus munsoniana* cv. Mariana 2624 can be downloaded from NCBI BioProjects PRJNA215068 and PRJNA215360, respectively.

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