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

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* Ehrh.) (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 Super-Script 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

(Applied Biosystems by Life Technologies, Paisley, UK), using the Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and primers. The temperature cycling protocol consisted of 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C for denaturation, and 1 min at 60 °C for annealing and extension. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve after amplification and by size estimation of the amplified product by agarose electrophoresis. Relative expression was measured by the relative standard curve procedure. Results were the average of two independent biological replicates repeated twice. The sequences obtained from the Peach Genome Database (www.peachgenome.org) were used to design specific primers to be used in qRT-PCR analysis. Primers were designed using PRIMER3 software 1. The expression of *actin* (Gene Bank accession N AB046952) was used as a control.

The following primers were used: for the *vacuolar H+-pyrophosphatase* (*V-PPase*) [PPN028B06 ID: *ppa001776m* fragment (113 bp long)] as forward 5′-TTTGGTCTCAAGGGTGAAGG-3′ and as reverse 5′-ATTTCTATTGGGGCGACCTC-3′; for the *alanine aminotransferase* (*AlaAT*) [PPN049F10 ID: *ppa003850m* fragment (172 bp long)] as forward 5′-GGCAATTAAAGCAGCAGAGG-3′ and as reverse 5′−CCACAA GTCATTCATGGACG-3′.

Moreover, 31 Chillpeach unigenes and 17 *Arabidopsis* hypoxia responsive genes (Table A.1) were selected for medium throughput qRT-PCR analysis. To select oligo pairs, Chillpeach transcript sequence was updated using the *Prunus persica* genome v2.0.a1 for all transcript coding sequences (CDS). In the case of *Arabidopsis* genes, peach orthologs were first identified by using BLASTN. Oligo pairs for selected genes were designed using the Primer-BLAST tool (Ye et al., 2012). In the design of oligo primers, the following conditions were imposed: Tm 58–60 °C, GC content 20–80%, primer length 20–22 bp and an amplicon size of 140–150 bp. *P. persica* genome v2.0.a1 all transcript CDS was used to screen non-specific amplifications. When more than one specific oligo was obtained for a gene the oligo pair which mapped most of the 3′ end of the gene was selected. A VIP gene was considered validated if the Pearson correlation coefficient between the expression results obtained in the microarray time course and the qRT-PCR time course was higher than 0.60. The genes that were selected from Arismendi et al. (2015) were validated by comparing the profile of expression from our and their qRT-PCR experiment.

3. Results

3.1. Differential hypoxia response in Myrobalan 'P.2175' and 'Felinem' Prunus rootstocks

The induction of the root response to hypoxia was verified at the molecular level by measuring the expression of *AlaAT* and *V-PPase* genes by qRT-PCR (Fig. 1). These genes are known to be induced in plants during hypoxia (or low O_2 conditions) and are essential for plant survival (Park et al., 2005; Rocha et al., 2010). In agreement with this role, both *AlaAT* (Fig. 1A) and *V-PPase* (Fig. 1B) were induced by hypoxia treatment in the tolerant Myrobalan 'P.2175', but not in the sensitive 'Felinem'. These results demonstrate that our hypoxia system is appropriated to study waterlogging response of *Prunus* rootstocks at the transcriptomic level.

3.2. Global changes in transcriptome of Prunus rootstocks caused by waterlogging stress at short time

In order to identify the molecular mechanisms underlying the differences in waterlogging response of the two genotypes, pools of plants from Myrobalan 'P.2175' and 'Felinem' subjected to normoxia (Z) or hypoxia (Y) conditions were analysed with an expression microarray at 2 and 24 h stress exposure. A total of 2442 genes were identified as being differentially expressed in at least one condition, using a cut-off FDR < 5% and q-value < 0.05 (Table A.3). PCA of the entire dataset of 2242 genes indicated that treatments as well as genotypes contributed almost equally to sample variance (Fig. 2A, left). As it can be seen in Fig. 2A, all Z (normoxia) samples were grouped relatively close to each other, indicating that transcriptome changes were minimal during normoxia conditions when compared to those observed under hypoxia (Y-samples). PC1, which accounts for 32.45% of variance, separated the samples according to whether they have undergone hypoxia stress or not (normoxia) with samples corresponding to longer stress exposure separating further from the rest of the samples in the PC1 axis (YA4 and YC4 further to the left than YA2 and YC2). PC2 (31.87% of variance) separated the short exposure samples (YA2 and YC2) from the rest indicating that there are important transcript differences affected transiently. Finally, PC3 (explaining 12% of variance) separated samples according to genotype (Fig. 2A, right).

Low O_2 conditions are known to induce the biosynthesis of ANPs in many plants (Sachs et al., 1980). Consistent with that, out of twenty described plant ANPs, 15 were found in the analysis of our dataset. Fig. A.2 shows the expression patterns of the 15 ANPs in Myrobolan 'P.2175' and 'Felinem' under waterlogging and normoxia conditions. Transcript levels for *alcohol dehydrogenase* (*Adh1*), *pyruvate decarboxylase* (*Pdc1*), *AlaAT* (PPN049F10) and *V-PPase* (PPN028B06) were induced in both *Prunus* rootstocks under hypoxia conditions. *Adh1* showed about a 2-fold induction from time 0 to 2 h in both genotypes, by 2 h *Pdc1* showed stronger induction in Myrobalan 'P.2175' than in 'Felinem', although this was transient and expression was clearly lower by 24 h (about 2.5-fold in both genotypes) (Table A.3). *AlaAT* was up-regulated in
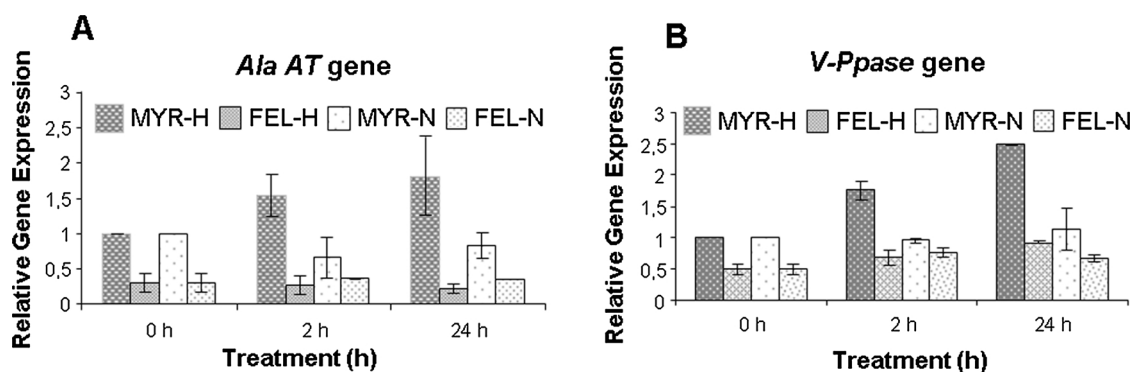
Fig. 1. Validation of induction hypoxia treatment. Real-time reverse-transcription polymerase chain reaction (RT-PCR) of A) *Alanine aminotransferase* (*AlaAT*) and B) *Vacuolar H+ pyrofosfatase* (*V-PPase*), selected because are hypoxia-induced genes. RNA samples were obtained from Myrobalan and almond × peach hybrid root harvested after 0, 2 and 24 h. The name of the gene or transcript model is shown in the upper part of the graph. Expression levels are relative to actin gene. An expression value of one is assigned to the 0 h sample. Data are means from two biological replicates, with error bars representing ± SD. H: hypoxia; N: normoxia; FEL: 'Felinem'; MYR: Myrobalan 'P.2175'.

Fig. 2. Global transcriptome analysis *Prunus* rootstocks subjected to normoxia and hypoxia treatments. A) Principal Component Analysis (PCA) of the global expression profile showing the most variation of each treatment condition (averaged from three replicates). In the left, the first principal component (PC1) is shown on x-axis, while the second principal component (PC2) is shown on y-axis. In the right, the PC1 is shown on x-axis and the third principal component (PC3) on y-axis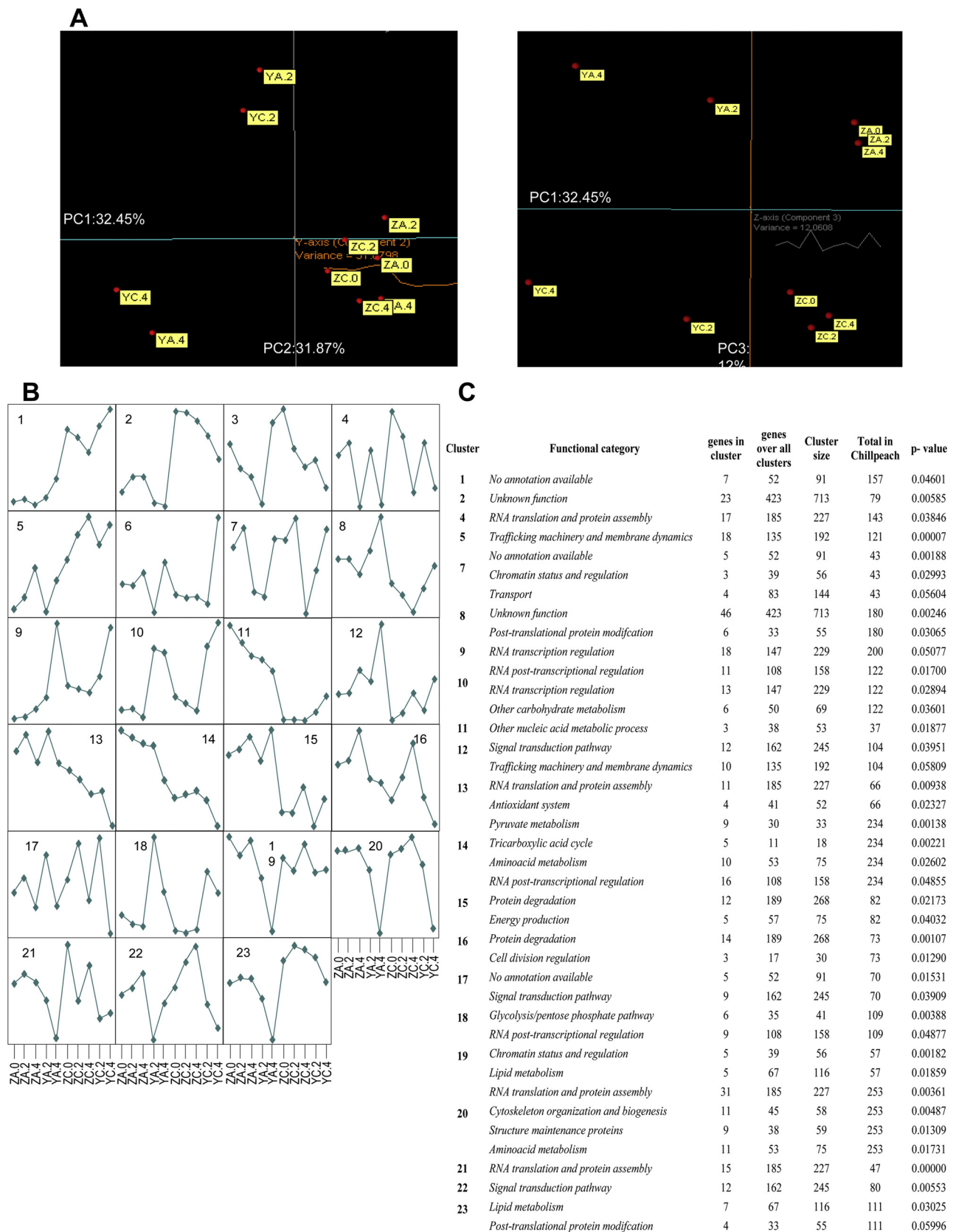. B) Clusters resulting from the unsupervised two-dimensional hierarchical clustering (Fig. A.1). Y-axis represents the normalized expression ratio (Log2 M) of three biological replicates in relation to a reference pool. The number of genes in each cluster is indicated. C) The functional categories overrepresented in each cluster (Fig. A.1) are shown as a table. Functional categories with Fisher test p-value < 0.05 and more than 3 genes are considered as enriching a given cluster. Clusters 3 and 6 are not enriched in any functional category. Y: hypoxia; Z: normoxia; A: 'Felinem'; C: Myrobalan 'P.2175'; 0: no treatment; 2: 2 h treatment; 4: 24 h treatment.

Cluster	Functional category	genes in cluster	genes over all clusters	Cluster size	Total in Chillpeach	p- value
1	No annotation available	7	52	91	157	0.04601
2	Unknown function	23	423	713	79	0.00585
4	RNA translation and protein assembly	17	185	227	143	0.03846
5	Trafficking machinery and membrane dynamics	18	135	192	121	0.00007
7	No annotation available	5	52	91	43	0.00188
	Chromatin status and regulation	3	39	56	43	0.02993
	Transport	4	83	144	43	0.05604
8	Unknown function	46	423	713	180	0.00246
	Post-translational protein modifcation	6	33	55	180	0.03065
9	RNA transcription regulation	18	147	229	200	0.05077
10	RNA post-transcriptional regulation	11	108	158	122	0.01700
	RNA transcription regulation	13	147	229	122	0.02894
	Other carbohydrate metabolism	6	50	69	122	0.03601
11	Other nucleic acid metabolic process	3	38	53	37	0.01877
12	Signal transduction pathway	12	162	245	104	0.03951
	Trafficking machinery and membrane dynamics	10	135	192	104	0.05809
13	RNA translation and protein assembly	11	185	227	66	0.00938
	Antioxidant system	4	41	52	66	0.02327
14	Pyruvate metabolism	9	30	33	234	0.00138
	Tricarboxylic acid cycle	5	11	18	234	0.00221
	Aminoacid metabolism	10	53	75	234	0.02602
	RNA post-transcriptional regulation	16	108	158	234	0.04855
15	Protein degradation	12	189	268	82	0.02173
	Energy production	5	57	75	82	0.04032
16	Protein degradation	14	189	268	73	0.00107
	Cell division regulation	3	17	30	73	0.01290
17	No annotation available	5	52	91	70	0.01531
	Signal transduction pathway	9	162	245	70	0.03909
18	Glycolysis/pentose phosphate pathway	6	35	41	109	0.00388
	RNA post-transcriptional regulation	9	108	158	109	0.04877
19	Chromatin status and regulation	5	39	56	57	0.00182
	Lipid metabolism	5	67	116	57	0.01859
20	RNA translation and protein assembly	31	185	227	253	0.00361
	Cytoskeleton organization and biogenesis	11	45	58	253	0.00487
	Structure maintenance proteins	9	38	59	253	0.01309
	Aminoacid metabolism	11	53	75	253	0.01731
21	RNA translation and protein assembly	15	185	227	47	0.00000
22	Signal transduction pathway	12	162	245	80	0.00553
23	Lipid metabolism	7	67	116	111	0.03025
	Post-translational protein modifcation	4	33	55	111	0.05996

Myrobalan 'P.2175′, but not in 'Felinem' (Fig. 1; Table A.3). These results indicated that the tissue samples used in this experiment: (i) underwent a typical waterlogging response; and (ii) Myrobalan showed a molecular response to hypoxia, which is consistent with being tolerant. Therefore, a detailed analysis of our microarray results should provide an expanded view of the waterlogging response and the mechanisms underlying the different tolerance observed by the two *Prunus* rootstocks.

The 2442 differentially expressed genes (Table A.3) were analysed in more detail by 2D − HCA clustering, followed by functional enrichment analysis of the 23 resulting clusters (Fig. 2B and C). Two large groups of clusters were formed, one enriched in metabolism related genes (clusters 3, 6, 10, 11, 13, 14, 15, 18, 19, 20 and 23) and the other enriched in genes associate to regulatory processes (Fig. 2C).

Functional enrichment indicated that the genes related to the functional categories *amino acid metabolism* (cluster 14), *antioxidant system* (cluster 13), *energy production* (cluster 15), *glycolysis/pentose phosphate pathway* (cluster 18), *pyruvate metabolism* (cluster 14) and *tricarboxylic acid cycle* (*TCA cycle*) (cluster 14) showed high levels of expression in the tolerant genotype Myrobalan 'P.2175′ (Fig. 2B and C; Table A.3). Furthermore, before hypoxia, the tolerant genotype Myrobalan 'P.2175′, had high levels of transcripts representing genes related to pyruvate (mainly the alanine fermentative pathway; Table A.3), *TCA cycle*, *amino acid metabolism* (cluster 14), *energy production* (cluster 15) and *antioxidant system* (cluster 13) (Fig. 2B and C; Table A.3). Furthermore, the expression levels of these genes remained higher in Myrobalan 'P.2175′ than in 'Felinem' during all treatments, although the genes from cluster 14 were repressed by 24 h, reflecting the important differential changes occurred at early stages in response to stress (Fig. 2B; Table A.3).

In contrast, genes in the *lipid metabolism* (clusters 19 and 23), *other carbohydrate metabolism* (cluster 10) and *sulfur metabolism* (cluster 6) classes showed the highest expression levels in the waterlogging-sensitive 'Felinem' (Fig. 2B and C). No functional enrichment was found for the genes associated to secondary metabolism in this analysis. This indicates that, although secondary metabolism is affected by waterlogging (see Table A.3), the early responses of *Prunus* rootstocks to waterlogging involve mainly a readjustment of primary and energy metabolism.

Most of the clusters in the second group (4, 7, 8, 10, 13, 14, 15, 16, 18, 19, 20 and 21) were rich in genes involved in gene expression regulation and signal transduction elements (Fig. 2C). Furthermore, during the early events of waterlogging, most of regulatory genes in the associated functional groups appear to participate in post-transcriptional processes. Thus, only one cluster is enriched in *RNA transcription regulation* (cluster 10), and the rest are enriched in genes related to *RNA post-transcriptional regulation*, basically *RNA biogenesis* and *splicing* (Tables 1 and A.3). Genes in this cluster, in addition to being induced by waterlogging in both genotypes, showed higher expression levels in the sensitive genotype 'Felinem'. Additionally, genes enriching clusters with high expression in 'Felinem' include processes other than transcriptional activation such as *chromatin-status and regulation* and *RNA translation and protein assembly* (cluster 19 and 4; Fig. 2B and C). But most striking is that genes highly expressed in the tolerant Myrobalan 'P.2175′ were rich in *RNA post-transcriptional regulation* (clusters 18 and 14), *RNA translation and protein assembly* (cluster 13), *post-translational protein modification* (cluster 8), *protein degradation* (clusters 15 and 16), *signal transduction pathway* (cluster 12) and *other nucleic acid metabolic process* (cluster 11) (Fig. 2B and C). This indicates that, at least, the tolerance of Myrobalan 'P.2175′ is probably associated to the adequate activation of post-transcriptional mechanisms.

3.3. Direct time-to-time comparisons revealed chronological events in the waterlogging response of Prunus rootstocks

A direct one-to-one comparison was made between the

transcriptomes of Myrobalan 'P.2175′ and 'Felinem' (Fig. 3; Table A.3) and the corresponding Venn diagrams (Fig. 3A) indicated how transcriptome differences between the two genotypes evolved with increasing hypoxia exposure. A total of 916 genes were differentially expressed between the two genotypes even before stress (ZA0 vs. ZC0; Fig. 3A), which indicates that the hypoxia response could be in part conditioned by differences in preformed mechanisms already existing in rootstocks before stress. Out of them, 517 genes were not hypoxia-responsive (NHG; non hypoxia-responsive genes) and 566 genes were differential between hypoxia treatments (Fig. 3A). In agreement with PCA results (Fig. 2A), our one-to-one analysis indicated that transcriptomes of our samples diverged with the time of hypoxia (Fig. 3A). Functional enrichment analysis of those genes differentially expressed before and during hypoxia revealed the *Prunus* temporal response program to waterlogging (Fig. 3B). Before hypoxia, the set of 434 genes highly expressed in the tolerant genotype Myrobalan 'P.2175′ was enriched for *glycolysis/pentose phosphate pathway*, *TCA cycle*, *pyruvate metabolism*, *other carbohydrate metabolism*, *antioxidant system*, *cofactor and vitamin metabolism* and *cell wall related* (Fig. 3B) gene functions. By 2 h into hypoxia, the functional categories *glycolysis/pentose phosphate pathway* and *TCA cycle* still enriched genes clusters highly expressed in Myrobalan 'P.2175′ and the *energy production* class was an important enriched category (Fig. 3B). By 24 h, *TCA cycle* and *energy production* still are the main functional categories enriching genes highly expressed in Myrobalan 'P.2175′, but other functional categories such as *cell wall related*, *trafficking machinery and membrane dynamics*, and *post-translational protein modification* also contribute with genes highly expressed in Myrobalan (Fig. 3B). This suggest that the tolerance of the genotype Myrobalan 'P.2175′ to waterlogging involves first a rapid metabolic adaptation (2–24 h) followed by the activation of genes that will be required later to modify the structure and anatomy of the root (24 h).

In contrast, genes that were highly expressed in 'Felinem' before hypoxia treatment and therefore part of the preformed program were enriched in *other carbohydrate metabolism*, *secondary metabolism*, *cytoskeleton organization and biogenesis* functions (Fig. 3B). Interestingly, *secondary metabolism*, *cytoskeleton organization and biogenesis* were, together with *signal transduction pathway*, the most prevalent functional categories in sensitive roots by 2 h into hypoxia treatment. No functional enrichment was observed for genes induced in 'Felinem' by 24 h. This may indicate that high levels of these genes may negatively affect the adaptation response of roots to waterlogging (Fig. 3B).

3.4. A PLS-DA analysis identifies gene expression biomarkers for waterlogging tolerance in Prunus

To gain a further insight into the expression changes caused by hypoxia / waterlogging in each of the two *Prunus* genotypes, a PLS-DA analysis, a supervised multivariate-regression technique involving a dummy variable for classification, was performed over the global dataset. The PLS-DA model allowed us to identifying which genes are important for each group separation. Two PLS-DA models were performed: a first model considering the two genotype / sensitiveness to hypoxia, no matter the treatment (Fig. 4A). The second model considered three groups: normoxia (independently of the genotype) and two hypoxia groups, corresponding to the response to hypoxia for each genotype (Fig. 4B). The results of the first model (MODEL1 PLS-component 2 components, $R^2X = 0.543$, $R^2Y = 0.993$, $Q^2 = 0.983$) produced clear separations between the genotypes irrespective of the normoxia or hypoxia conditions (Fig. 4A). The second model (MODEL2 5 components $R^2X = 0.754$, $R^2Y = 0.985$, $Q^2 = 0.909$, with the 3th, 4th and 5th components not adding much predictive value to the model), clearly separated samples normoxia and hypoxia; more effectively than genotypes (Fig. 4B). Only transcripts with a variable importance VIP-value > 2.5 were considered as contributing the most to the separation between the groups. In total 121 genes/transcripts were selected according to the VIP score from the two models: 77 genes from de first

Table 1
VIP genes.

Functional category	Process	Unigene annotation	Contig ID	Arab_AIG	Arabidopsis_thaliana_annotation	Arab Gene	Root global pattern	VIP score PLS-DA MODEL 1 (S vs. T)	PLS-DA MODEL 2 treatment & genotype
Genotype (26 genes)									
Antioxidant system	Gluthathione-glutaredoxin and thioredox redox homeostasis	Glutaredoxin	PPN048A04-T7_cs	AT5G63030	Glutaredoxin C1	GRXC1	1	2.67904	1.26028
		Putative peroxidase	PPN063G12-T7_cs	AT4G37530	peroxidase, putative		1	5.30967	2.9659
Cofactor and vitamin metabolism	Folate metabolism	OSJNBa0086B142 protein	CL5110Contig1	AT4G24380	expressed protein with DUF341		23	2.93251	2.36302
	Riboflavin metabolism	Riboflavin biosynthesis protein ribAB	PPN012A12-T7_cs	AT5G64300	GTP cyclohydrolase II	GCH	18	0.53324	3.48558
Cytoskeleton organization and biogenesis	Actin microfilament-actin monomer	Actin cytoplasmic 2	CL810Contig1	AT5G09810	actin 7/actin 2	ACT7	5	2.9118	1.54504
		Actin cytoplasmic 2	PPN047D11-T7_cs	AT5G09810	actin 7/actin 2	ACT7	1	3.68076	1.84529
	Microtubule-tubulin based motordynamics	Gb|AAB714791|	PPN062C07-T7_cs	AT5G66810	similar to unknown AAB714791		14	2.96206	1.42676
Energy production	Mitochondrial electron chain	2OG-Fe(II) oxygenase	PPN004E03-T7_cs	AT5G59540	oxidoreductase, 2OG-Fe(II) oxygenase family protein		11	4.5283	2.29954
Glycolysis/pentose phosphate pathway	Pay-off glycolytic phase	Nadp-dependent glyceraldehyde-3-phosphate dehydrogenase	PPN031F11-T7_cs	AT2G24270	aldehyde dehydrogenase 11 A3	ALDH11 A3	11	3.69909	2.11546
Nucleotide metabolism	de novo purine biosynthesis	SAICAR synthetase	CL1375Contig1	AT3G21110	SAICAR synthetase	PUR7	14	4.75676	2.31317
Other carbohydrate metabolism	Unknown sugar dehydrogenase	OSJNBa0081C0123 protein	CL1372Contig1	AT1G01800	short-chain dehydrogenase/reductase (SDR) family protein		1	8.19668	3.84036
Protein degradation	Protease inhibitor	Serine protease inhibitor-like protein	PPN055H03-T7_cs	AT4G01575	serine protease inhibitor, Kazal-type family protein		9	1.28024	2.63498
RNA transcription regulation	Ubiquitin degradation	Ubiquitin carboxyl-terminal hydrolase	CL635Contig1	AT5G06600	ubiquitin-specific protease 12	UBP12	1	6.00472	2.8064
	AP2/EREBP family	AP2/EREBP transcription factor ERF-2	CL1566Contig1	AT3G16770	ethylene response factor subfamily B-2	EBP	2	3.82867	2.05784
	AUX/IAA family	IAA16 protein	PP1009D02-T7_cs	AT1G04250	auxin-responsive protein	AXR3	10	0.613782	3.83349
	Jumonji-family	transcription factor	PPN055G03-T7_cs	AT1G08620	transcription factor jumonji (jmj) family	PKDM7D	9	0.773586	2.68822
Secondary metabolism	Flavonoid metabolism	Chalcone synthase 2	CL792Contig1	AT5G13930	chalcone synthase	TT4	23	2.9665	3.91076
	Polyamine metabolism/ ornithine cycle	Argininosuccinate lyase	PPN029D11-T7_cs	AT5G10920	argininosuccinate lyase		15	4.17534	2.03192
Signal transduction pathway	G-protein coupled receptor protein signalling pathway/ G-protein complex	F14O2322 protein	PPN070C02-T7_cs	AT1G71840	transducin family protein/WD-40 repeat family protein		5	3.25087	1.61524
	Phosphorylation cascades/ metabolic switch	AKIN beta3	CL1438Contig1	AT2G28060	protein kinase-related		1	3.18977	1.61784
		AKIN gamma	CL581Contig1	AT3G48530	SNF1-related protein kinase regulatory subunit gamma 1	KING1	10	2.29002	4.34029
Transport	ABC transporter-ATH family	Probable ABC-type transport protein	PPN033F03-T7_cs	AT3G47780	ABC transporter family protein	ABCA7	12	3.49895	3.11657
	Wax transport	White-brown complex homolog protein 15	PPN032C05-T7_cs	AT3G21090	ABC transporter family protein	ABCG15	11	2.87843	1.42774
Unknown function	Unknown chloroplast protein	Os09g0509400 protein	PPN052B04-T7_cs	AT5G41110	expressed protein		18	0.0688671	2.62667
	Unknown membrane protein	Similar to Arabidopsis clone: MJK13	PPN069D06-T7_cs	AT4G27450	similar to auxin down-regulated protein ARG10		10	0.575274	4.01074
	Unknown protein	Similar to Arabidopsis clone MQK4	CL1146Contig1	AT5G16550	expressed protein		9	1.01941	3.47506
Genotype and Treatment (84 genes)									
Amino acid metabolism	Alanine and Aspartate metabolism	Asparagine synthetase	CL283Contig1	AT3G47340	glutamine-dependent asparagine synthase 1	ASN1	20	1.58319	3.55445
		Alanine aminotransferase	PPN049F10-T7_cs	AT1G72330	alanine aminotransferase 2	ALAAT2	12	3.45768	2.7601
	Cyanoamino acid metabolism	Beta-cyanoalanine synthase 1	CL1105Contig1	AT3G61440	cysteine synthase C1	CYSC1	14	2.5124	2.01564

(continued on next page)

Table 1 (continued)

Functional category	Process	Contig ID	Unigene annotation	Arabidopsis thaliana annotation	Arab_AIG	Arab Gene	Root global pattern	VIP score PLS-DA MODEL 1 (S vs. T)	PLS-DA MODEL 2 treatment & genotype
Antioxidant system	Gluthathione-glutaredoxin and thioredoxin redox homeostasis	PP1006G04-T7_cs	Glutathione transferase	glutathione S-transferase TAU 19	AT1G78380	GSTU19	23	3.26173	2.87258
		PPN032B05-T7_cs	Glutathione transferase	glutathione S-transferase TAU 19	AT1G78380	GSTU19	14	3.82734	2.07541
	Oxidation to peroxide	CL487Contig1	Peroxidase 21 precursor	peroxidase 21	AT2G37130	PER21	5	4.01229	1.95489
	Peroxide detoxification	PPN059B02-T7_cs	Putative thioredoxin peroxidase 1	peroxiredoxin type 2	AT3G52960		4	2.85401	1.54583
	Regeneration of oxidized methionine	CL911Contig1	T4B215 protein	methionine sulfoxide reductase B 2	AT4G21860	MSRB2	1	3.39066	1.74028
Cell wall related	Cell wall biogenesis	PPN044B01-T7_cs	Gene induced upon wounding stress	vein patterning 1	AT4G24220	VEP1	11	3.1342	1.75993
	Glycan biosynthesis	CL157Contig1	GDP-mannose pyrophosphorylase	GDP-mannose pyrophosphorylase	AT2G39770	CYT1/GMP1	1	4.24883	3.16489
	Glycan degradation	CL205Contig1	Similarity to endo-1	Endo-1,31,4-beta-D-glucanase precur	AT3G23600		14	2.69054	1.38993
		CL392Contig1	Os05g0399100 protein	Endo-1,31,4-beta-D-glucanase precur	AT3G23600		14	2.68569	1.32716
		CL480Contig1	Similarity to endo-1	Endo-1,31,4-beta-D-glucanase precur	AT3G23600		14	2.8038	1.412
	Hemicellulosic polysaccharide biosynthesis	CL796Contig1	Alpha-16-xylosyltransferase	Alpha-16-xylosyltransferase	AT4G02500		5	2.78199	1.61167
Cofactor and vitamin metabolism	Pectin degradation	CL146Contig1	Polygalacturonase-inhibiting protein	polygalacturonase inhibiting protein 1	AT5G06860	1PGIP1	1	2.73911	1.38456
	Folate metabolism	PPN049C06-T7_cs	Protein At2g43840	UDP-glucoronosyl/UDP-glucosyl transferase family protein	AT2G43840	UGT74F1	23	3.99707	2.6528
Energy production	Photosynthetic machinery	PP1000F02-T7_cs	Cupredoxin	plastocyanin-like domain-containing protein	AT3G27200		1	3.37336	1.61913
Glycolysis/pentose phosphate pathway	Pay-off glycolytic phase	PPN021G06-T7_cs	Pyruvate kinase	pyruvate kinase, putative,	AT5G56350		10	0.58501	3.86392
Lipid metabolism	Phospholipid degradation	PPN065C11-T7_cs	CXE carboxylesterase	CXE carboxylesterase 12	AT3G48690	CXE12	18	0.959484	2.92084
	Wax biosynthesis	PPN032 A12-T7_cs	IMP dehydrogenase/GMP reductase	long-chain-fatty-acid-CoA ligase,	AT2G47240	LACS1	18	3.12462	4.10228
No annotation available	No annotation available	PP1006D03-T7_cs	no_annotation_available	no_annotation_available			1	5.90873	3.42145
		PPN063H12-T7_cs	no_annotation_available				1	3.83246	1.93614
Other carbohydrate metabolism	Trehalose biosynthesis	CL877Contig1	Trehalose-6-phosphate phosphatase	trehalose-6-phosphate phosphatase	AT5G65140	TPPJ	10	3.7685	4.08146
Other nucleic acid metabolic process	DNA repair and recombination	CL1248Contig1	Leucine rich repeat protein precursor	DNA-damage-repair/toleration protein precursor	AT3G12610	DRT100	2	3.48168	3.06259
Protein degradation	Adaptor for ubiquitination by Cul3-based ubiquitin ligase	CL730Contig1	F8K722 protein	BTB/POZ domain-containing protein	AT1G21780		18	0.13895	3.42129
	Protease	CL1010Contig1	Putative chloroplast nucleoid DNA-binding protein	aspartyl protease family protein,	AT2G17760		15	2.78039	1.67757
		CL928Contig1	Cysteine protease CP1	cysteine proteinase	AT1G47128	RD21 A	13	2.87213	1.81293
		PPN026C06-T7_cs	Similar Prunus persica cDNA clone PP_LEa0021C02f				1	3.88344	2.489
		PPN042F09-T7_cs	Tumour-related protein	trypsin and protease inhibitor family protein	AT1G17860		9	0.942412	4.69304
Pyruvate metabolism	Conversion of PEP to oxalacetate	CL1331Contig1	Pyruvate decarboxylase	pyruvate decarboxylase	AT5G01320	PDC1	18	0.231909	4.87347
		CL35Contig1	Pyruvate decarboxylase	pyruvate decarboxylase 1	AT4G33070	PDC2	18	0.219657	4.88751
	Glyoxal pathway	CL251Contig1	Hydroxyacylglutathione hydrolase cytoplasmic-like	hydroxyacylglutathione hydrolase	AT3G10850	GLY2GLX2-2	5	2.69617	1.61434
	Pyruvate fermentation to ethanol	CL654Contig1	Alcohol dehydrogenase	Alcohol dehydrogenase	AT1G77120	ADH1	10	1.0512	2.80122
RNA post-transcriptional regulation	RNA biogenesis and processing	PPN005H09-T7_cs	Poly(A) polymerase central domain putative	nuclear poly(A) polymerase	AT4G32850	nPAP	9	2.70177	2.05964
	Splicing	PPN054H01-T7_cs	TIR-NBS-LRR type R protein 7				5	4.49085	2.12548
RNA transcription regulation	b-ZIP family	CL1324Contig1	BZIP transcription factor bZIP41	bZIP transcription factor family protein	AT3G62420	BZIP53	1	2.9798	1.55962
	NAC-family	PPN014G09-T7_cs	Nam-like protein 10	no apical meristem (NAM) family protein ANAC081	AT5G08790	ATAF2	18	2.51379	2.11655

Table 1 (*continued*)

Functional category	Process	Contig ID	Unigene annotation	*Arabidopsis thaliana* annotation	Arab_AIG	Arab Gene	Root global pattern	VIP score	
								PLS-DA MODEL 1 (S vs. T)	PLS-DA MODEL 2 treatment & genotype
RNA translation and protein assembly	Regulation of protein biosynthesis	CL1170Contig1	Putative translation initiation factor IF-2	translation initiation factor IF-2	AT4G11160		11	3.98878	1.99834
		PPN047H03-T7_cs	Putative translation initiation factor IF-2 [Oryza sativa] T19E237				18	0.593288	3.26307
Secondary metabolism	rRNA binding	PPN065G07-T7_cs	Similar to Arabidopsis clone: MYH9	PAZ domain-containing protein	AT1G31280	AGO2	2	2.79225	2.72415
		PPN049B01-T7_cs		BolA-like family protein	AT5G09830		1	2.5653	1.28889
	Ethylene biosynthesis	CL235Contig1	1-aminocyclopropane-1-carboxylate synthase	alanine aminotransferase	AT1G72330	ALAAT2	12	3.6209	2.89871
	Flavonoid metabolism	CL649Contig1	Naringenin2-oxoglutarate 3-dioxygenase	naringenin 3-dioxygenase/flavanone 3-hydroxylase	AT3G51240	F3H	20	0.503438	2.60033
		PPN050F05-T7_cs	Chalcone isomerase	chalcone-flavanone isomerase	AT3G55120	TT5/CHI	20	1.86432	2.61906
	Nitrate assimilation	CL705Contig1	Non-photosynthetic ferredoxin precursor	Ferredoxin 3	AT2G27510	FD3	23	3.74435	2.49425
	Nitroalkane oxidation	CL1087Contig1	2-nitropropane dioxygenase-like protein	2-nitropropane dioxygenase family	AT5G64250		11	4.54125	2.24519
	Phenylpropanoid metabolism	CL362Contig1	Cinnamoyl-CoA reductase-like protein	cinnamoyl-CoA reductase-related	AT4G30470		20	0.215188	2.52359
		CL559Contig1	Cinnamyl-alcohol dehydrogenase 1	cinnamyl-alcohol dehydrogenase	AT4G34230	CAD5	23	2.57125	1.72143
	Salicylic metabolism / Salicylic conjugation	PPN012 A02-T7_cs	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase-like protein	IAA carboxylmethyltransferase 1	AT5G55250	IAMT1	5	3.45997	1.74317
	Steroid metabolism	PPN008A06-T7_cs	Cytochrome P450	steroid 22-alpha-hydroxylase	AT5G05690	CPD	1	2.96271	2.3342
	Sterol metabolism	CL1223Contig1	Delta-7-sterol-C5(6)-desaturase	delta 7-sterol-C5-desaturase	AT3G02580	STE1	18	1.72563	2.92433
		PPN063B12-T7_cs	Helix-turn-helix AraC type NAD-binding site Fumarate lyase	squalene epoxidase	AT4G37760	SQE3	10	0.131083	3.14522
	Terpene metabolism	PPN077B08-T7_cs	Putative Squalene monooxygenase	squalene monooxygenase	AT1G58440	XF1	10	0.845387	4.12733
		CL1466Contig1	Cycloartenol synthase	cycloartenol synthase	AT2G07050	1CAS1	5	2.71671	1.6219
		PPN007C05-T7_cs	Putative carbonyl reductase	short-chain dehydrogenase/reductase (SDR) family protein	AT3G61220	SDR1	5	2.62589	1.3109
	Unknown CYP450	PPN018G11-T7_cs	Cytochrome P450 monooxygenase CYP72 A26	cytochrome P450, putative	AT3G14650	CYP72 A11	8	2.96752	1.80176
	Unknown hydrolase	PPN016D04-T7_cs	Dreg-2 like protein	haloacid dehalogenase-like hydrolase family protein	AT5G44730		18	0.0575001	4.95122
	Unknown sugar-alcohol metabolism	CL495Contig1	Putativepod-specific dehydrogenase SAC25	short-chain dehydrogenase/reductase	AT5G02540		2	2.6819	1.60823
Signal transduction pathway	Calcium signalling / Calcium transducer	PPN050D05-T7_cs	Putative serine/threonine kinase	calmodulin, putative	AT1G12310		1	2.51778	1.20994
	G-protein coupled receptor protein signalling pathway / G-protein complex	CL101Contig1	Putative WD repeat domain 5B	transducin family protein/WD-40 repeat family protein	AT5G64730		2	3.06584	1.61239
	Phosphorylation cascades/ metabolic switch	PPN007E08-T7_cs	AKIN gamma	SNF1-related protein kinase regulatory subunit gamma 1	AT3G48530	KING1	10	1.88193	3.73545
		PPN009D07-T7_cs	AKIN gamma	SNF1-related protein kinase regulatory subunit gamma 1	AT3G48530	KING1	10	2.05667	4.03799
Structure maintenance proteins	Dehydrin	CL27Contig1	Type II SK2 dehydrin				1	3.51419	1.77477
Trafficking machinery and membrane dynamics	Nucleocytoplasmic transport	PPN052G09-T7_cs	Tic22	chloroplast inner membrane import protein	AT4G33350	Tic22-IV	23	2.87016	3.40212
Transport	Auxin transport	CL72Contig1	Gb|AAD329071	auxin efflux carrier family protein	AT2G17500	PILS5	11	4.69385	2.5017
	Carbohydrate transport	PPN051 A12-T7_cs	Major facilitator sugar transporter	hexose transporter	AT5G26340	MSS1	10	0.162699	3.58656
Tricarboxylic acid cycle	Isocitrate to? -ketoglutarate	PPN008F12-T7_cs	W25EPL23 M/W25EPL23M	NAD + isocitrate dehydrogenase	AT5G03290	IDH-V	14	2.62497	1.35039

Table 1 (continued)

| Functional category	Process	Contig ID	Unigene annotation	Arab_AIG	Arabidopsis_thaliana annotation	Arab Gene	Root global pattern	VIP score PLS-DA MODEL 1 (S vs. T)	PLS-DA MODEL 2 treatment & genotype
Unknown function	Unknown heme binding protein	PPN041F06-T7_cs	SOUL heme-binding protein-like	AT2G37970	SOUL heme-binding family protein	SOUL-1	11	2.6066	1.38954
	Unknown membrane protein	CL119Contig1	AI-induced protein	AT5G19140	auxin/aluminium-responsive protein	AILP1	9	1.14638	3.01132
		CL119Contig2	AI-induced protein	AT5G19140	auxin/aluminium-responsive protein	AILP1	9	0.607665	3.44079
		CL3395Contig1	UPI00000A82CD P0510F0919	AT5G11960	expressed protein		9	0.707866	2.99688
	Unknown plastidial protein	PPN042G09-T7_cs	AJ875782 Prunus persica fruit mesocarp plus epidermis 80 days after bloom Prunus persica cDNA clo	AT1G68680	expressed protein		9	0.62438	2.54127
	Unknown protein	CL175Contig1	Gb|AAF021421	AT3G17800	expressed protein		18	0.496557	3.26482
		CL179Contig1	Similar Prunus persica cDNA clone PP_1Ea0012C01f				2	4.84474	3.6658
		CL324Contig1	Putative senescence-associated protein SAG102	AT5G20700	similar to senescence-associated protein SAG102		10	2.22916	3.047
		PPN037A07-T7_cs	At1g68600/F24J5_14	AT2G17470	aluminium activated malate transporter 6	ALMT6	20	0.66658	2.73564
		PPN043G03-T7_cs	Unknown protein	AT1G25275	expressed protein		8	2.60639	1.88692
		PPN068C09-T7_cs	Similar to Arabidopsis clone MDC12	AT5G63220	expressed protein		14	3.50785	1.82194
		PPN069A06-T7_cs	AT5g16110/T21H19_30	AT3G02555	expressed protein		9	0.536094	2.81057
		PPN071E03-T7_cs	F21O36 protein	AT2G39650	expressed protein		10	1.08277	2.93193
	Unknown universal stress protein	CL463Contig1	Early nodulin ENOD18	AT3G53990	universal stress protein (USP) family		14	4.36513	2.17281
		PPN023C01-T7_cs	Early nodulin ENOD18	AT3G53990	universal stress protein (USP) family		8	3.45704	1.80344
	Unknown Zinc-finger protein	PPN007A01-T7_cs	UPI0000196CCB protein binding / zinc ion binding	AT2G27980	expressed protein		23	2.77711	1.6682
	Unknown Zinc finger RING-like	PP1001C04-T7_cs	Ring zinc finger protein	AT2G28840	ankyrin repeat family protein	XBAT31	18	0.372133	3.25855
Treatment (11 genes)									
Antioxidant system	Regeneration of oxidized methionine	CL238Contig1	Peptide methionine sulfoxide reductase	AT5G61640	peptide methionine sulfoxide reductase,	PMSR1	5	5.9048	2.84329
Cell wall related	Extracellular matrix protein biosynthesis	PP1003D07-T7_cs	AT3g28480/MFJ20_16	AT3G28480	similar to prolyl 4-hydroxylase, alpha subun		18	0.237958	2.51564
	Pectin degradation	PP1004B07-T7_cs	Pectinesterase	AT1G76160	multi-copper oxidase type I family protein,	sks5	9	1.27134	2.82676
Cofactor and vitamin metabolism	Porphyrin and chlorophyll metabolism	CL265Contig1	S-adenosyl-L-methionine-dependent uroporphyrinogen III methyltransferase	AT5G40850	urophorphyrin III methylase	UPM1	19	1.09332	3.67397
Glycolysis/pentose phosphate pathway	Glycolysis/pentose phosphate pathway by-pass	CL697Contig1	Rubisco activase	AT2G39730	ribulose bisphosphate carboxylase/oxygenase activase	RCA	18	0.599431	3.35256
Protein degradation	Peptidase	CL44Contig1	Serine carboxypeptidase	AT3G10410	serine carboxypeptidase III	scp149	1	2.51299	1.87925
RNA transcription regulation	NAC-family	PPN062G07-T7_cs	NAC family protein	AT1G01720	no apical meristem (NAM) family protein	ATAF1 ANAC002	18	0.465178	2.50118
Trafficking machinery and membrane dynamics	Protein targeting to vacuole	PP1004 A05-T7_cs	SRC2	AT1G09070	C2 domain-containing protein	SRC2	15	3.2879	2.08729
Unknown function	Unknown protein	CL1110Contig1	MTD1	AT5G21940	expressed protein		10	1.46169	5.02391
		PPN002B03-T7_cs	similar to F17O72 (Mouse-ear cress)				18	0.323318	6.28041
		PPN042C11-T7_cs	Similar Prunus persica cDNA clone Skin63F11				8	2.7944	2.20013
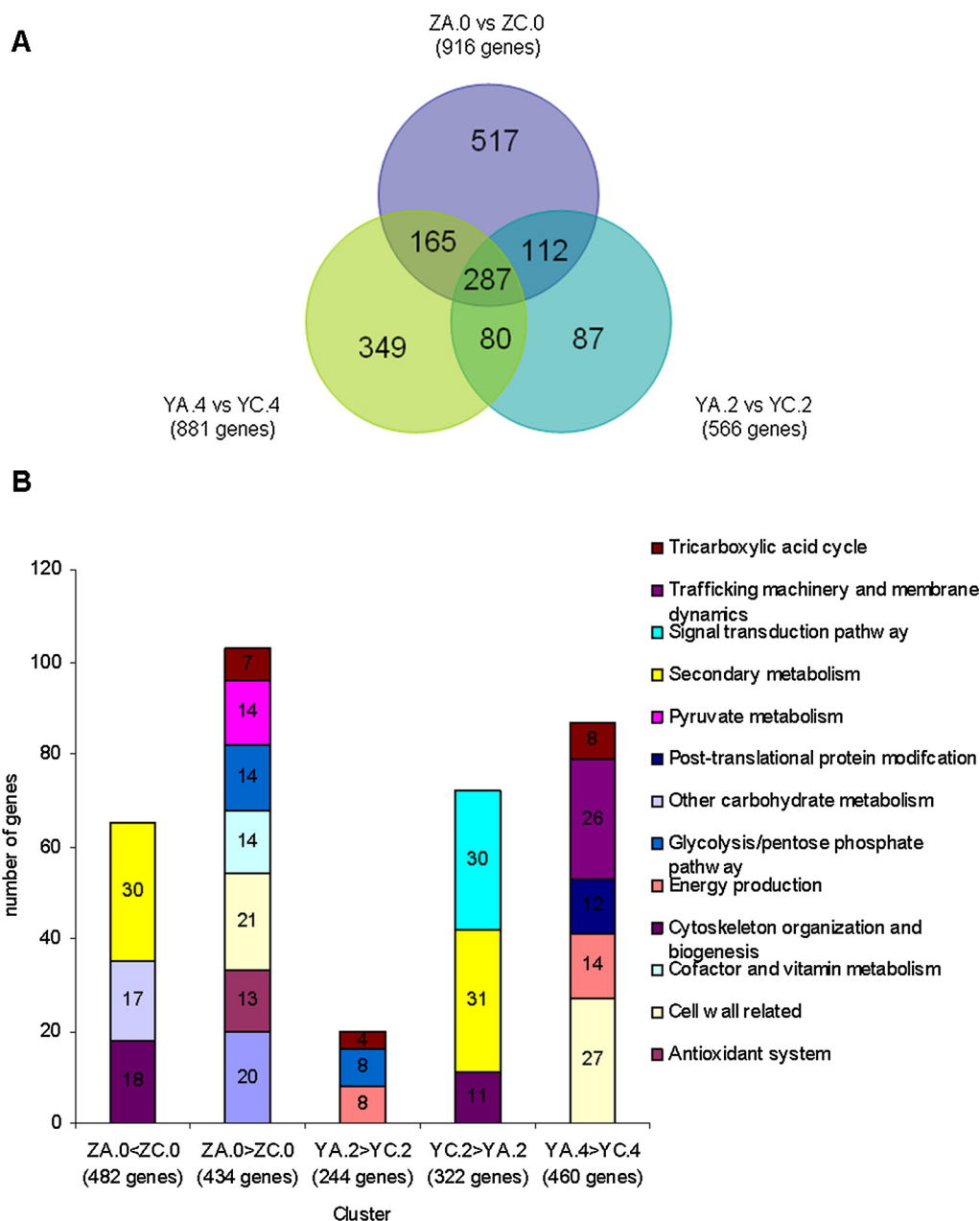
Fig. 3. Differential gene expression between Myrobalan 'P.2175' and 'Felinem' in normoxia and hypoxia conditions. A) Venn diagram depicting the differentially expressed genes (FDR < 0.05 and q-value < 0.05) between 'Felinem' and Myrobalan 'P.2175' at each time point. B) Functional categories enriching genes (p-value < 0.05) differentially expressed at each time between 'Felinem' and Myrobalan 'P.2175'. Y: hypoxia; Z: normoxia; A: 'Felinem'; C: Myrobalan 'P.2175'; 0: no treatment; 2: 2 h treatment; 4: 24 h treatment.

model and 63 genes from the second model (Tables 1 and A.3). A total of 19 genes with VIP score > 2.5 were shared between the two models and therefore their expression values differentiate both genotypes and stress responses (Table A.3).

The genes mostly contributing to the separation of samples in model 1, that is to the separation to genotypes (VIP > 4; Table 1) were: a gene encoding a short chain dehydrogenase (SDR; VIP = 8.19), probably related to carbohydrate metabolism, followed by a ubiquitin-specific protease 12 (UBP12; VIP = 6.00), a peptidethionine sulfoxide reductase 1 (PMSR1; VIP = 5.90), a peroxidase and GDP-mannose pyrophosphorylase/mannose-1-pyrophosphatase (CYT1; VIP = 4.24) all of them belonging to clusters of genes that were highly expressed in the sensitive 'Felinem' (clusters 1 and 5) and an auxin efflux carrier, (PILS5; VIP = 4.69) associated to the tolerant Myrobalan 'P.2175' and as well as part of cluster 11 (Fig. 2B; Table A.3).

In the second model, the genes mostly contributing to the separation between treatments (VIP > 4, Table 1) include an haloacid dehalogenase (VIP = 4.95) with similarity to an *Arabidopsis* gene that is up-regulated during hypoxia (Branco-Price et al., 2005), two pyruvate decarboxylase genes (PDC1-PDC2 VIP = 4.8) and a long-chain acyl-coA synthase 1 (LACS1; VIP = 4.10) all of them in cluster 18, which are highly induced by hypoxia in the tolerant Myrobalan 'P.2175' (Fig. 2B; Table A.3), and a squalene monoxygenase (XF1; VIP = 4.12), two SNF1-related protein kinase regulatory subunit gamma 1 (KING1; VIP = 4.34), a trehalose-6-phosphate phosphatase *J* (TPPJ; VIP = 4.08) all of them in cluster 10, corresponding to genes highly induced in the sensitive 'Felinem' (Fig. 2B; Table A.3). It is also remarkable that the ortholog of RAP2.3 an ethylene-response factor (ERF) described in *Arabidopsis* as a member of O_2 sensor family (Gibbs et al., 2011; Licausi et al., 2011a; Sasidharan and Mustroph, 2011) showed VIP scores of 3.8
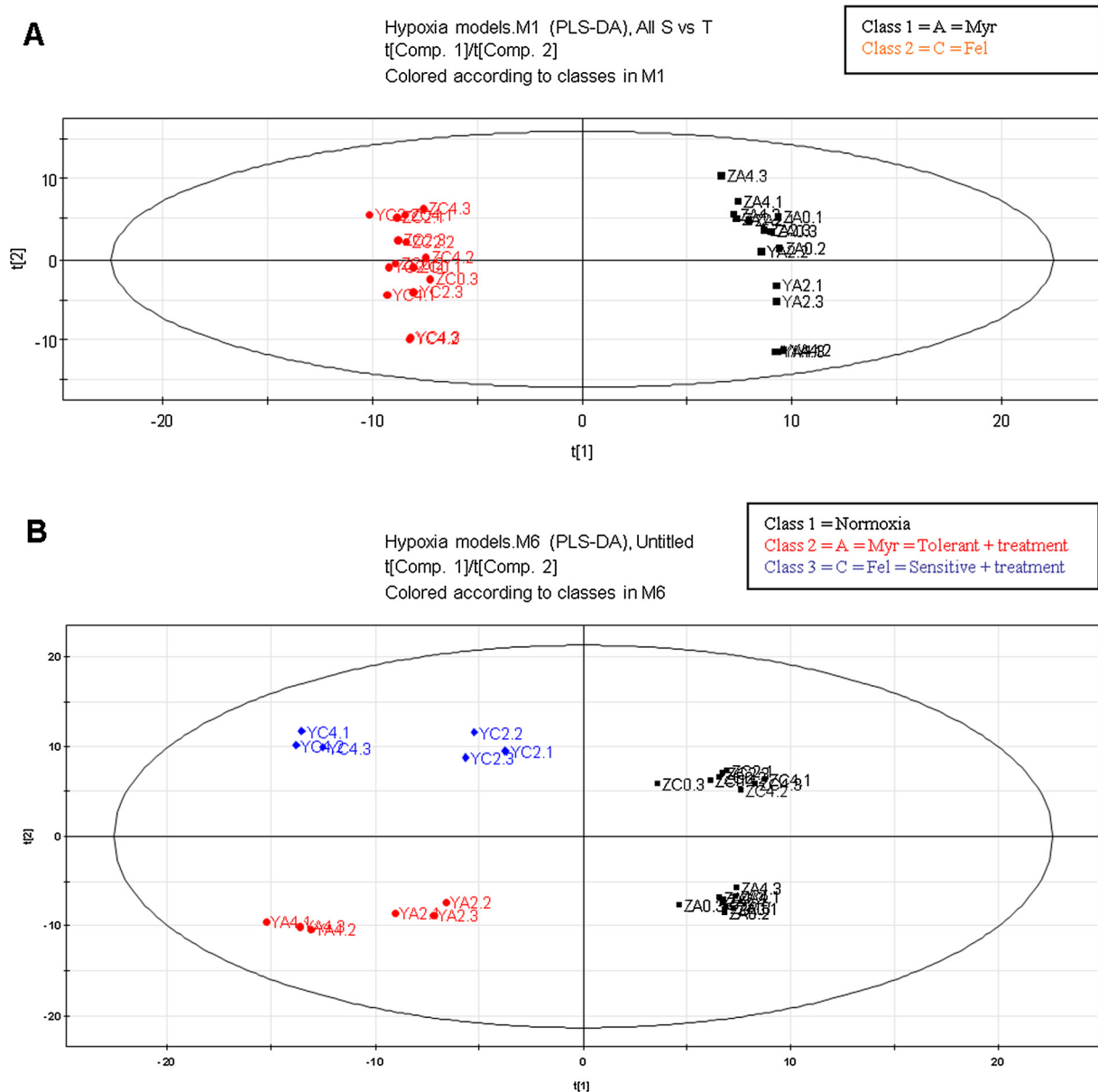
Fig. 4. Partial Least Squares Discriminant Analysis of differentially expressed genes. A) MODEL 1 considering two groups based in the genotype/sensitiveness to hypoxia, independent of the treatment. B) MODEL 2 considering three groups: normoxia (independently of the genotype) and two hypoxia groups, corresponding to the response to hypoxia for each genotype. Y: hypoxia; Z: normoxia; A: 'Felinem'; C: Myrobalan 'P.2175'; 0: no treatment; 2: 2 h treatment; 4: 24 h treatment.

and 2.0 in each of the corresponding models.

In addition, 121 genes selected by their high VIP values from the two models were classified by the factor mostly influencing its expression pattern, i.e. genotype (G), treatment (T) or their interaction (G × T) (Table A.3). These VIP genes fell into 16 of the 23 clusters of 2D − HCA (Fig. A.3). Out of them, clusters 1, 10 and 18 contained over 10% of all VIP genes each (Table A.4). Further, the analysis of functions associated to the VIP genes (Fig. A.3 and Table A.5) indicated that secondary metabolism (17 genes) or unknown function (17 genes) in G × T class accounted for around 30% of all VIP genes. The most abundant classes of genes whose main expression change is driven by genotype differences were related to *signalling, RNA transcription regulation, cytoskeleton organization* and genes with unknown function all accounting for around 10% of all VIP genes (Fig. A.3 and Table A.5). In the case of T class, the most abundant genes were related to *cell wall* and unknown function, accounting for about 4% of all VIP genes (Fig. A.3 and Table A.5).

3.5. Validation of microarray results by qRT-PCR

To increase the reliability on the results obtained from the gene expression array, the expression levels of 24 VIP genes identified in the PLS-DA analysis as potential biomarkers were evaluated by qRT-PCR using the same RNA samples than in the microarray (Tables A.1 and A.6). In addition, 11 genes reported by Arismendi et al. (2015) as hypoxia responsive were subjected to the same analysis. The expression profile of 19 VIP was validated (80%), with 17 of them having correlation coefficients higher than 0.75 between microarray and qRT-PCR results (Fig. 5A; Table A.6). A total of 8 genes out of the 11 genes from Arismendi et al. (2015) as hypoxia-responsive in peach roots, were validated in our experiment in our expression profiles (Fig. 5B; Table A.6). Taken together this indicates that our results are robust and can be interpreted with confidence.
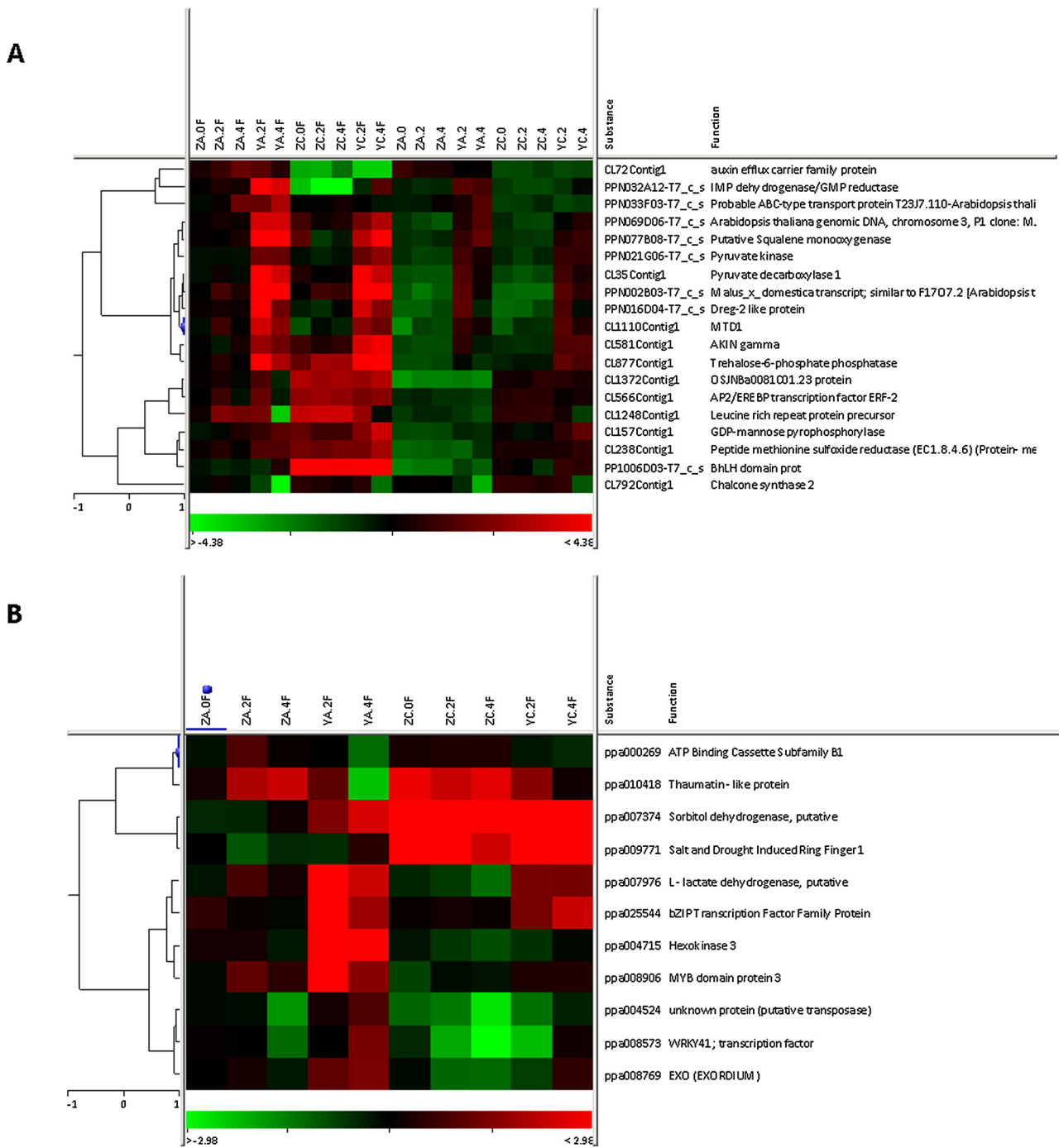
Fig. 5. Hierarchical cluster depicting the differential expression profiles of 30 hypoxia-associated genes (validated by qRT-PCR) in root tissues of flooding sensitive and tolerant genotypes. A) Comparison of the expression patterns of microarray and Fluidigm platform for 19 VIP genes showing a correlation higher than 0.75 between the two platforms. Samples labelled with F corresponded to those analysed by Fluidigm B) Expression patterns of 11 genes described previously as hypoxia-responsive genes and validated by qRT-PCR in 'Felinem' and Myrobalan 'P.2175' genotypes. Y: hypoxia; Z: normoxia; A: 'Felinem'; C: Myrobalan 'P.2175'; 0: no treatment; 2: 2 h treatment; 4: 24 h treatment.

3.6. Expression of candidate genes for oxygen sensing mechanism in Prunus during waterlogging

The *Prunus* genome was searched for homologs of the *Arabidopsis* genes encoding *ERF74/RAP2.12* (AT1G53910), two Acyl CoA binding proteins (ACBPs): *ACB1P1* (AT5G53470) and *ACBP2* (AT4G27780) genes, and *HCR1* (Hydraulic Conductivity Root 1 - AT3G24715). Then, these genes were classified as members of the hypoxia-sensing mechanism (Gibbs et al., 2015, 2011; Licausi et al., 2011a; Shahzad et al., 2016). Putative homologs for *ERF74/RAP2.12* (*Prupe.3G032300.3*),

ACB1P1 and *ACBP2* (*Prupe.2G314100*) and *HCR1* (*Prupe.8G206500*) were identified and named in our data as *ERF74/RAP2.12*, *ACBP1/2* and *HCR1*, respectively. Expression of these three *Prunus* genes was analysed by qRT-PCR using the same samples used in the microarray analysis. As it is shown in Fig. 6, the three genes presented a much higher and more rapid expression response in samples from tolerant genotype Myrobalan 'P.2175' (A) than in those of the sensitive 'Felinem' (C) or in the control (in which O_2 is also flushed with O_2 but at higher concentrations). In the case of the tolerant 'P.2175' (A) all three genes were very low expressed at time 0 and were quickly and
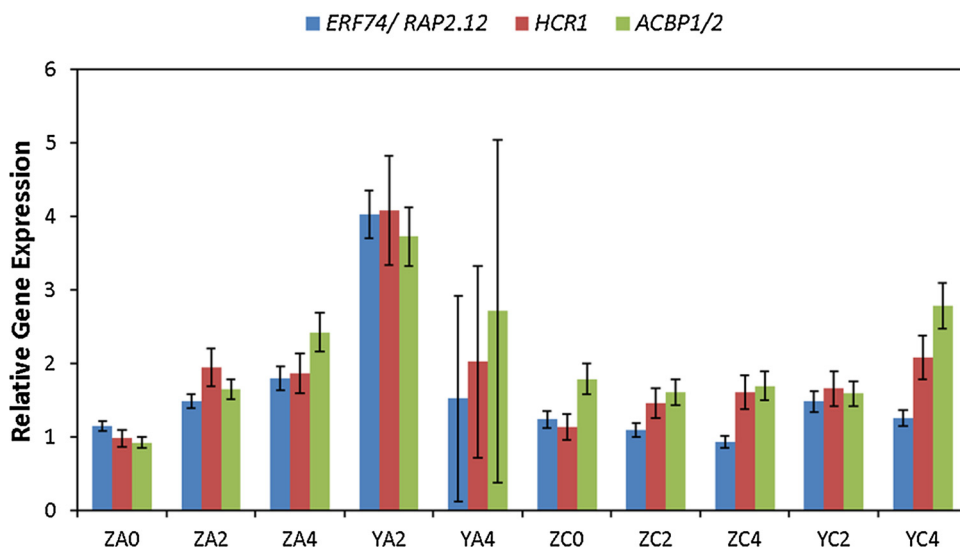
Fig. 6. mRNA expression profile of three selected oxygen sensing genes: *ERF74/RAP2.12*, *HCR1* and *ACBP1/2*, in roots of flooding tolerant and sensitive *Prunus* genotypes during short-term hypoxia in comparison with normoxia. Y: hypoxia; Z: normoxia; A: 'Felinem'; C: Myrobalan 'P.2175'; 0: no treatment; 2: 2 h treatment; 4: 24 h treatment.

transiently induced by 2 h, but this was not the case for 'Felinem' (C). In the sensitive rootstocks significant inductions (and different from the control) only occur by 24 h, only in *ACBP1/2* and *HCR1*. The *ERF74/RAP2.12* gene seems to be not affected by low O_2 conditions in our samples.

4. Discussion (shorten by 50%)

4.1. Metabolic changes associated to waterlogging in Prunus rootstocks

Our results indicate that early-mid time response of waterlogging in *Prunus* rootstock involve mainly a readjustment of primary and energy metabolism pathways, and this seems to precede structural changes, what is in agreement with results reported in previous studies(Narsai et al., 2011; Zou et al., 2013). Twelve of the genes in the specific metabolic classes that appear to be associated to the tolerance of Myrobalan 'P.2175' and two associated to the sensitivity of 'Felinem' were part of the set of significant genes revealed by PLS-DA multiclass analyses (Table 1). Those genes whose expression levels are influenced by both genotype and hypoxia (see Table 1). In the tolerant Myrobalan 'P.2175' roots, the expression of key genes involved in *fermentation pathways* and showing dramatic up-regulation as early as by 2 h in waterlogging were found to decline in expression by 24 h (clusters 18 in Figs. 2B and 3B). In contrast, *energy production* was up-regulated or had higher levels by 24 h in the tolerant Myrobalan 'P.2175' (cluster 12–15 in Figs. 2B and 3B). The decline in expression for these major *fermentation* and *glycolysis* genes indicates a decrease in metabolic flow for these pathways (Christianson et al., 2010; Kürsteiner et al., 2003; Rocha et al., 2010), or that the first steps of the shift towards an aerobic metabolism are completed by that time. In this sense, *AlaAT,* which is highly expressed in Myrobalan 'P.2175' roots even before treatments, was further up-regulated by 24 h (Fig. 1; Table A.3). *AlaAT* seems to play an important role in energy production during hypoxia, as it would prevent pyruvate accumulation while still producing ATP under the limiting oxygen availability (Rocha et al., 2010).

The crosstalk between carbon and amino acid metabolism reveals that amino acid metabolism performs two main roles: the regulation of cytoplasmic pH and the supply of energy through breakdown of the carbon skeleton (Zou et al., 2010). Several studies have pointed that *V-PPase* (highly expressed in Myrobalan 'P.2175' roots before and during hypoxia) has an important role in carbon-amino acid crosstalk during adaptation to anoxia (Agarwal and Grover, 2006), as well as to other stresses (Park et al., 2005) such as in the enhancement of H^+ extrusion to the apoplast produced by anoxic and consequently to the

alkalinisation of the cytoplasm. In alkaline media, PCD and ADH enzymes are activated (Drew, 1997), and it is known that the rates of alcoholic fermentation correlates with the tolerance to flooding in several plant species (Dolferus et al., 1997, 2000; Koizumi et al., 2011). Our results indicated that ADH expression levels, although up-regulated in both genotypes, were higher in sensitive 'Felinem' (Table A.3). We have previously showed that tolerant Myrobalan 'P.2175' efficiently reduced the toxic accumulation of acetaldehyde by controlling the levels of ADH (Amador et al., 2012), and therefore, the activation of ADH by pH in Myrobalan 'P.2175' may play an important role in hypoxia tolerance. Some of the genes, down-regulated in response to hypoxia in Myrobalan 'P.2175', are related with the storage of carbon as starch (Table A.3). This strategy appears to be an adaptive response of this genotype to hypoxia, as increased carbon for glycolysis/fermentation will inhibit its accumulation as starch (Gupta et al., 2009), but also limit carbon supply to other biosynthetic pathways like those leading to lipids, sulfur compounds, fenilpropanoids, flavonoids, mevalonate and sterols. In general, genes in these functional categories were expressed at higher levels in the sensitive 'Felinem' or even up-regulated in response to hypoxia as part of the adaptive response for alleviating the O_2 competition (Geigenberger, 2003; van Dongen et al., 2004).

4.2. Early events of waterlogging tolerance involve mainly genes involved in post-transcriptional regulation

Short hypoxia treatments resulted in a differential regulation of genes (Dennis et al., 2000) which, in the case of our experiments in *Prunus*, includes signalling elements and gene expression regulation classes. Most of genes in these functional categories appear associated to waterlogging tolerance and involved in regulatory processes downstream from transcription (Fig. 2C; Table A.3). *RNA post-transcriptional regulation* genes are clearly up-regulated by 2 h into hypoxia in both genotypes, but mostly (cluster 10 is an exception) with higher levels in the tolerant Myrobalan 'P.2175'. *Argonaute10* (*AGO10*) was found among the VIP genes, highly expressed in the tolerant Myrobalan 'P.2175' under normoxia conditions, but repressed by hypoxia in both genotypes (Table 1). Interestingly, *AGO2* has been described post-translational gene expression regulation and a key component in hypoxia response in humans (Wu et al., 2011). *SRO3* (*Similar to RCB one 3*) is probably negative regulated by *RCD1* (*Radical-Induced Cell Death1*), a gene described in stress-induced morphogenetic response (SIMR) (Teotia and Lamb, 2011) whose mutants have altered abiotic stress responses and ROS accumulation. In addition, the unknown protein MTD1 has been related with stress in root metabolism (VIP = 5.02)

([Table 1](#)) ([Curioni et al., 2000](#)).

Protein degradation plays a role in oxygen levels signalling ([Zou et al., 2013](#)) and the activation of post-translation mechanism provides a way to rapidly respond to stress, to fine-tune the strength of the response as well as to integrate multiple input signals ([Baena-González, 2010](#)). Myrolaban 'P.2175' appears to limit their energy demand and therefore, respiratory O_2 consumption. The low- O_2 quiescence strategy, is characterized by a general restriction in cellular metabolism and growth and displayed by species that regularly endure deep floods of short duration ([Bailey-Serres et al., 2012](#)) appears to be part of the strategy developed by Myrobalan 'P.2175' to cope with waterlogging stress.

In agreement with our results, a recent report using adult *Prunus* trees subjected to hypoxia indicated that the tolerant genotype specifically accumulated transcripts encoding enzymes of post-translational protein modifications ([Arismendi et al., 2015](#)). Among the 46 genes analysed in Fluidigm platform, 11 were among those described by these authors as involved in the fermentative pathway, glycolysis, anti-oxidant system and other cellular metabolic processes. Our results showed the same trend of expression in 8 genes. These small differences among the techniques (Fluidigm platform and qRT-PCR) might represent different levels of sensitivity. Therefore, with Fluidigm platform, we validated the microarray transcriptional analysis of two *Prunus* genotypes with contrasting response to hypoxia in short- and long-term steps.

4.3. Regulation of expression at DNA modification level and up-regulation of signalling elements are associated to the sensitivity to waterlogging

A number of signal transduction elements related to sugar, calcium, auxin and abscisic acid (ABA) signalling, such as KING1 and TPPJ in Cluster 10 ([Fig. 3](#)B; Table A.3), were expressed at higher levels before treatments and after 2 h in the sensitive genotype 'Felinem'. All the VIP genes involved in signal transduction pathway are found in cluster 1, 2, 5, 10 which are linked to sensitivity to waterlogging and therefore, low level of expression of these VIP genes could play an important role in tolerance response. Emerging data indicate that sugar derived signalling mechanism, including trehalose-6-phosphate phosphatase (TPPJ) and the SNF1-related protein kinase regulatory subunit gamma 1 (KING1) complex, also play important roles through modulating nutrient and energy signalling and metabolic processes ([Geigenberger, 2003](#)), especially under abiotic stresses when sugar availability is low ([Bailey-Serres et al., 2012](#); [Bailey-Serres and Voesenek, 2008](#); [Qi et al., 2012](#)). In addition, signal transduction plays a key role in activating genes related to the tolerance mechanism for survival during prolonged waterlogging ([Zou et al., 2010](#), [2013](#)). In waterlogged cotton, many core hypoxia-responsive gene mRNAs were up-regulated in both roots and shoots, whereas in waterlogged poplar, there was minimal effect on the shoot transcriptome ([Christianson et al., 2010](#); [Kreuzwieser et al., 2004](#)). In waterlogged *Arabidopsis*, systemic up-regulation of genes in the shoot was associated with ABA biosynthesis and response ([Hsu et al., 2011](#)), and even a shoot-specific response in *Arabidopsis* has been described ([Klecker et al., 2014](#)). Although we have focused our analysis to the roots and therefore, have no data for changes in the *Prunus* shoot transcriptome, we can hypothesized that our case is more similar to that other woody plants such a poplar and that adjustments of gene expression in response to low- O_2 regimes are influenced by O_2 level and/or energy homeostasis, cell type, and communication between stressed and unstressed organs ([Christianson et al., 2010](#)). Hypoxia-induced genes have been previously reported, including TFs and signal transduction components ([Bailey-Serres et al., 2012](#); [Bailey-Serres and Voesenek, 2008](#); [Baxter-Burrel et al., 2003](#); [Branco-Price et al., 2008](#)). Evidence have been presented that some of these elements could regulate hypoxia responses in several crops ([Licausi et al., 2010](#)) or even mutually controlled with phosphate starvation by post-translational mechanisms ([Klecker et al., 2014](#)). Regulation of hypoxia-induced

genes is controlled via simultaneous interaction of various combinations of TFs ([Licausi et al., 2011b](#)) with the participation of protein degradation in response to hypoxia ([Voesenek et al., 1993](#); [Zou et al., 2013](#)). In our case, changes in the expression of several TFs have been detected such as ethylene-responsive element binding protein (EBP) and auxin resistant 3 (AXR3), jumonji like (PKDM7D) (Table A.3). These hypoxia responsive TFs, belonging to the auxin response factor (ARF) and ERF families, has been described to accumulate when O_2 is scarce. These TFs have been shown to bind the "hypoxic core" promoters and activate the hypoxia-responsive genes ([Klecker et al., 2014](#); [Licausi et al., 2010](#)). The ortholog of RAP2.3 in *Arabidopsis* seems to be more related to plant defence responses than to hypoxia ([Sasidharan and Mustrophc, 2011](#)). We showed in this paper that three of the O_2 sensors, with a key role in hypoxia responses in *Arabidopsis* ([Gibbs et al., 2015](#); [Licausi et al., 2011a](#); [Xie et al., 2015](#); [Shahzad et al., 2016](#))were differentially expressed in our sensitive and tolerant rootstocks ([Fig. 6](#)) providing evidence for the first time of the participation of these genes in the differential response of *Prunus* to hypoxia conditions.

Very little information exists about chromatin structure and its dynamics in hypoxia. Chromatin could act as a primary O_2 sensor, with changes in histone and protein methylation giving rise to further structural changes in chromatin ([Melvin and Rocha, 2012](#)). We observe indications that this could happen in both genotypes in the genes in cluster 7, although none of the VIP genes fall into this functional category they are part of the late response in the tolerant Myrobalan 'P.2175' ([Table 1](#)). By 24 h of hypoxia, a different set of genes involved in cell structure and cell division emerged. So that the low-O_2 escape strategy, could cause structural changes among them a rapid elongation of underwater stems or leaves to enable photosynthetic tissue to outgrow shallow flood waters ([Gibberd et al., 2001](#); [Vidoz et al., 2013](#)). This strategy involves ethylene-mediated signal transduction mainly responsible for the ability to create more adventitious roots, as observed in the tolerant Myrobolan, as part of the late response strategy that could be related to the different ability to cope with waterlogging ([Voesenek and Sasidharan, 2013](#)). Myrobalan S.4 formed new roots, during flooding and their terminal tip maintaines a tissue organization and size similar to the roots developed under non-stress conditions ([Pistelli et al., 2012](#)).

5. Conclusion

In this work, a direct comparison of two *Prunus* genotypes differing in their waterlogging / hypoxia tolerance revealed a temporal program of events and provided molecular tools that we plan to use to study / classify different genotypes / conditions. We propose that the top VIP genes revealed by PLS-DA modelling of microarray expression data and those derived from literature mining and confirmed by Fluidigm in our samples can be used to develop markers for introducing waterlogging tolerance in *Prunus*.

Declarations of interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2018.06.004.

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