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ORIGINAL ARTICLE

A transcriptional analysis reveals an extensive range of genes responsible for increasing the tolerance of Carrizo citrange to oxygen deficiency

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Abstract

Little information is available on the *Citrus* genus and its relatives with regard to their ability to tolerate oxygen deficiency, establishing physiological and structural modifications. In order to gain insight into how citrus rootstocks respond to low-oxygen stress, a transcriptomic analysis (using a custom microarray) was performed on Carrizo citrange (CC) seedlings. These seedlings were transformed with OsMybleu transcription factor (TF), known for inducing tolerance to oxygen deficiency, and compared with CC wildtype. They were flushed for 24 h with N_2 and microarray, carrying out expressed sequence tags of *Citrus* and relatives isolated from the roots, was hybridized with RNA of roots before and after hypoxia treatment. The genes involved in fermentation, Krebs cycle, sugar metabolism, cell wall metabolism, hormones, and TFs all resulted significantly altered in response to hypoxia in both samples. Quantitative expression analysis was performed on 42 selected genes to validate microarray results. The outcome was that most of them were confirmed. The main results lead to the conclusion that CC is naturally tolerant to oxygen limitation. Transformed CC responded to hypoxia by activating the main genes which are known in other plants to be responsible for this type of tolerance such as pyruvate decarboxylase and alcohol dehydrogenase. Among TFs, several were also induced, such as an HDZipIII homologous to AtHB15, target of mir166, itself overexpressed exclusively in transformed CC under hypoxia compared with all other samples. The present manuscript represents one of the very few investigative works focused on hypoxia-responsive transcriptional networks in citrus.

Keywords Citrus · Hypoxia · Expression analysis · miRNA · Transcription factors

Introduction

Oxygen deficiency represents one of the most studied types of abiotic stress, considering that most plants cannot survive under prolonged waterlog. Oxygen is essential for roots to breathe (Reddy 2014). In scarcely drained soil or ones that have suffered short periods of flooding, an oxygen-deficit environment is created which causes a condition defined as hypoxia. Anoxia, on the other hand, refers to a total lack of oxygen which can be found in soils after prolonged periods of flooding. This condition is incompatible with the life of many plants. Sudden hypoxia is generally a rare condition in nature. What normally occurs is a gradual transition from normoxia to hypoxia and then to anoxia. In this way, the plants can gradually acclimate to a situation which, if abrupt, would be deadly (Drew 1997). Plants demonstrate a noteworthy variability in their capability to tolerate low oxygen stress. Wetland species such as *Oryza sativa* and *Rumex palustris* can survive in

Key message Transformed Carrizo citrange showed a higher tolerance to hypoxia, shifting the metabolism in favor of fermentation and inducing HDZipIII and mir166

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conditions of complete submergence. By contrast, dry-land species such as grains, legumes, and fruit crops such as grape, citrus, and apple can endure oxygen deprivation for short times (Fukao and Bailey-Serres 2004). The response of a plant to hypoxia is commonly divided into three phases (Dennis et al. 2000). From 0 to 4 h, we witness a rapid induction of signal transduction components. These signals are needed to activate the second stage (4–24 h), which consists in metabolic adaptation. The last stage, registered between 24 and 48 h, involves the formation of aerenchyma (gas-filled air spaces) in the roots. This is the result of adaptive mechanisms that increase a plant's survival under conditions of low soil oxygen, which is generally documented for some subtropical and tropical fruit trees (Dennis et al. 2000).

In order to lessen the damage caused by oxygen deprivation in plant cells, plants use various developmental, morphologic, and metabolic adaptations including the induction of three main fermentation pathways: ethanol, lactic acid, and alanine (Dennis et al. 2000; Hinz et al. 2010; Kennedy et al. 1992; Meguro et al. 2006). A set of transcription factors (TFs) play a crucial role in this stress response that in many cases is plant species-specific (Licausi and Perata 2009). TFs that belong to different protein families are upregulated in some species (*Arabidopsis, Triticum aestivum, O. sativa*) under anoxia, demonstrating themselves to be significantly involved in the tolerance to hypoxia (Katiyar et al. 2012; Licausi and Perata 2009). The Osmyb7 gene encoding for a transcriptional factor can be expressed in two different alternative transcripts (Mattana et al. 2007). The heterologous expression of the unspliced Osmyb7 (called Mybleu) in *Arabidopsis* leads to elongation of the primary roots and in the internodal region of the floral stem, together with an increase in tolerance for oxygen deficiency (Mattana et al. 2007).

Citrus is one of the most important woody plants worldwide, cultivated for its value as a fruit crop. It can suffer from oxygen deficiency, primarily due to flooding from excessive irrigation. Citrus rootstocks are generally classified as being sensitive to hypoxia, although limited studies have been reported on their responses to low oxygen environments. The tolerance of *Citrus* genus and its relatives to flooding and most abiotic stress greatly differ among genotypes (Syvertsen and Levy 2005). To date, flooding sensitivity studies have indicated that tolerance of citrus plants varies greatly with rootstock (Arbona et al. 2008; García-Sánchez et al. 2007; Hossain et al. 2009; Syvertsen and Levy 2005). The tolerance of Carrizo citrange (CC) to flooding has been demonstrated and documented in comparison to other rootstock genotypes such as sour orange, rough lemon, and Cleopatra mandarin (Arbona et al. 2008; Yelenosky et al. 1995). Recently, the role of Mybleu of *Arabidopsis* has turned out to also be crucial in transgenic lines of CC rootstock (Caruso et al. 2011). In the present study, CC wild type and genetically modified CC, for the presence of Mybleu, were subjected to hypoxia conditions, with the end goal of better understanding the transcriptional pattern responsible for the various aptitudes regarding oxygen deprivation.

Materials and methods

Plant material and hypoxia treatments

Three weeks old wild type (WT) and transgenic CC (TCC) in vitro plants were grown in a controlled growth chamber on MSC agar medium as described by Caruso et al. (Caruso et al. 2011). For the hypoxia and recovery experiments, the plants were flushed for 24 h at 5 l h^{-1} with 1% O_2/99% N_2 under sterile conditions.

Total RNA isolation

Root samples were collected after hypoxic treatments for the RNA extraction. Samples were frozen in liquid nitrogen and stored in aliquots at − 80 °C until needed. The roots from untreated WT and TCC of the same age and grown under the same conditions were used as controls. Total RNA was extracted from the roots according to Caruso et al. (Caruso et al. 2011). RNA quantity and quality were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and a Bioanalyzer Chip RNA 7500 series II (Agilent, Santa Clara, CA).

Microarray analysis, functional annotation, and expression analysis using quantitative reverse transcription polymerase chain reaction

The expression analysis was performed on a custom 90K Combimatrix microarray (CustomArray Inc., USA) as described by Licciardello et al. (Licciardello et al. 2013). Genes were considered significantly differentially expressed when the $\log_2$ fold change was ≥ 1 or ≤ 1 and the false discovery rate (FDR) was ≤ 0.05. The expression values of selected genes as analyzed by microarray hybridization were median normalized and represented using HeatMap (software MeV v4.9.0). Expression data are available on the NCBI GEO database (Edgar et al. 2002) with GEO number GSE86208.

All of the transcripts deriving from differential analysis (Table S1) were categorized according to their annotated function with respect to biological processes, based on the blast and GO term annotation using Blast2GO software (Götz et al. 2008). The biological processes included terms regarding metabolic processes, response to stimulus, and cellular and biological process. In order to have a better understanding of the functional involvement of each gene under hypoxia environmental conditions, differential genes were categorized using the most recent and available bibliographic literature and were classified as fermentation, Krebs cycle, γ-aminobutyric acid

(GABA) shunt, polyamine metabolism, sugar metabolism, hormones, cell wall metabolism, signal transduction, oxygen and nitrogen reactive species, transcription factors, secondary metabolism, chaperon, stress and defense response, processing, signaling, and chromatin modification.

A selection of differentially expressed genes (DEGs) was validated by quantitative reverse transcription polymerase chain reaction (qRT-PCR), conducted according to Licciardello et al. (Licciardello et al. 2013), on cDNA which was isolated from the roots. Primer sequences are listed in Table 1. Each gene was compared with the *Citrus sinensis* genome database (http://citrus.hzau.edu.cn/orange/) and the corresponding homologous was generally reported.

miRNA analysis

Unknown/unnamed proteins and non-annotated genes were blasted against the GreeNC database (all lncRNAs of all species) (Paytuvì et al. 2016). When blast reports indicated low confidence for lncRNA and findings for Pre-miRNA, we used the original nucleotide sequence to look for mature miRNA analysis using the miRBase, maintaining all default cutoff options (Griffiths-Jones and Grocock Russell 2006). The search for putative targets of selected miRNAs found was done using psRNATarget software (Dai and Zhao 2011).

Results

Identification of hypoxia-responsive genes through a microarray analysis with expressed sequence tags (ESTs) of citrus roots

In order to test if the expression of known genes was modulated by hypoxia, we prepared an EST microarray containing 7697 probes deriving from ESTs isolated under abiotic and biotic stress conditions from *Citrus* and related genera (retrieved from NCBI). CC wild type (WT) and genetically modified for the presence of Mybleu (TCC) were subjected to hypoxia conditions (WTA and TCCA). CC in vitro plant seedlings was uprooted and total RNA was extracted from the roots of three different plants for each sample (TCCA, TCC, WTA, WT), used as biological replicas.

The gene expression of WT and TCC were subjected to oxygen deficiency for 24 h and then compared with their relative control samples, which were maintained under normoxia conditions. In order to identify the changes in the expression patterns, four comparisons were performed: (1) WTA versus (vs) WT, (2) TCCA vs TCC, (3) TCCA vs WTA, (4) TCC vs WT. In the first three comparisons, the expression levels of 601 (8.1%) of the 7337 genes were altered by stress conditions. Figure 1 shows that many genes were altered in multiple comparisons, while others are unique and specific to single comparisons.

The comparison indicating the highest number of differentially expressed genes was WTA vs WT (96 upregulated, 285 downregulated, Table S1), followed by the comparison between TCCA and TCC, showing 98 upregulated genes and 113 downregulated ones (Table S2). Additionally, the comparison TCCA vs WTA only presented 3 overexpressed and 5 downregulated genes (Fig. 1; Table S3). Therefore, in addition to these 8 significantly different genes in TCCA vs WTA, we also considered 101 additional genes originating from previous, original, and single comparisons (TCCA vs TCC and WTA vs WT) for a total of 109 genes (Table S3).

Finally, non-differential genes were identified when comparing TCC vs WT data. This is because the only difference between these samples is due to the presence of the Mybleu gene (Caruso et al. 2011), that was not included in the microarray platform, because it is not specific to *Citrus* and related genera. The specific expression of Mybleu was only confirmed in TCC by qRT-PCR (Fig. S1).

Besides indicating the highest number of DEGs, the WTA vs WT comparison also showed the most significant fold change for both the upregulated (FC 4161 for id_1874) and downregulated genes (FC -4451 for id_7935) (Table S1).

Hypoxia-responsive genes belonging to various metabolic pathways

Differentially upregulated and downregulated genes were analyzed considering each comparison. Overall, 52 Probe IDs showed no annotation and 65 Probe IDs were annotated as predicted-hypothetical-proteins (Figs. 2, 3 and 4). The category indicated as "other" includes genes involved in a wide range of mechanisms and represents the most abundant category of differentially expressed genes among all of the comparisons (26% WTA vs WT, 25% TCCA vs TCC and 23% TCCA vs WTA). The WTA vs WT comparison was enhanced in five functional categories, unique and specific of this comparison, that are "chaperon," "chromatin modification," "GABA shunt," "signal transduction," and "signaling."

Metabolic pathways related to sugar, fermentation, Krebs cycle, GABA shunt, polyamine, and ethylene metabolisms are illustrated and outlined in Fig. 5.

Sugar metabolism

Plant cells react to low oxygen availability by shifting from aerobic to fermentative metabolism and require constant carbohydrate supplementation. One of the crucial adaptive mechanisms to oxygen deficiency consists in maintaining sufficient levels of fermentable sugar or accumulating more sugar or consuming carbohydrates more slowly (Sairam et al. 2009; Xia and Saglio 1992). Another alternative consists in

Table 1 Primer sequences used for quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Sweet orange gene locus	Annotation_probe ID	Sequence primer forward (5′-3′)	Sequence primer reverse (5′-3′)	Amplicon length (bp)
Cs3g17940	ADH_264	GGGGTCCACTGTTGCAATTT	GCGTATCCTTGCTCCTTCAG	70
Cs2g17920	XTHs_307	GAACCAAGGCCAATGACG	CCAATTAGTGCTCCTGCATGT	60
orage1.1 t00545	Mir166_349	CGTGGATAAGTTTCCATTCTGCTT	GAAACACTAGAAAAGAAAATGCAAGTTC	102
Cs8g05970	nitrite reductase_544	GCTCAAATGGCTTGGTTTGT	CCGTGTTTGCTCACTTGTTG	103
Cs5g20010	2OG_700	CTTCAAGAATGTTGCTGATGGT	CATCCAAGGTTTGCGTCCAA	73
Cs7g26660	Ap2_754	TCGAGAGCATGTGTGAAGGT	CCGGTCCGTTCATACACAAC	82
Cs6g11950	PAL_1789	CCTTAGCTTTTGCATCAATGGA	CACGTCTTTGCATACATTGATGAC	64
Cs5g04430	Nodulin_1864	CCAGTACCGGTGCCTTTGAT	AGTTCATGACGCCAGTGACA	97
Cs3g20830	ADH_1865	CCAGGAGACCATGTTCTTCCT	TGAGGAGGTCACACATATTGCT	92
Cs6g15060	GST_2079	TGCCCTTGATTCGAAGAGCT	GTAGACATGAAGGCTGGTGAACACA	112
Cs1g05500	Cell wall-related GT_2743	ACTACTTGGGGGGTAGTAGAT	TCAATTGCCAAGTTGGGAAG	59
Cs5g28410	nsHB1_2881	GCGGAGGCTTATGATCAACT	CCATGAAACAGCATCACGTT	97
Cs4g11200	Polyubiquitin 1 _3007	TCCAATTTACGAATCAATCGACAT	TGCTACCTTGAGCTTTTTGATTTTC	103
Cs3g25510	Nodulin_3280	CATGGTGGCCTTGCAGTTTG	ACAGGGAACACAAGCTTGCT	90
Cs6g15430	fructose 1,6 DP_3309	GGAACTTCGGTTTGAGTGGTT	CCATTAGGGGAAAGGGAAAA	103
Cs3g24700	PGI_3421	GACGCCAGTTGCGATTTCTAG	GGTTTAGTCCCGTGATACTTTTAGCA	72
Cs5g33470	SUS_3524	CCGTGATTTGGTGAAGTCTGTTC	CTTCCTTGAGTACCAGCAGCTTAAT	69
Cs2g05050	PDC_4457	CATTGACTCGAAGAGGCTCATTT	TGGAGAATTACCGCCGCATA	79
Cs7g07640	Chac_1270	GCATGCGGGATGATGACCCAACCAAC	AGAGCTGGCAAATGAAGTGAGG	92
Cs2g07920	HSP_4557	CATTGACTCGAAGAGGCTCATTT	TATGCGGCGGTAATTCTCCA	79
Cs8g10790	Transaldolase_4613	AAGAGAGGATGATGATGATCTGG	CATTTCGAAGCTTACGAATCCAGCT	70
Cs5g12420	Ep1-like_4818	GCCCCGCAATTAATTATCAAATA	TGAAAGCCCACATATCTATCTGTTG	75
Cs7g26180	Enolase_4834	CCATGCGTTCTCAATCTCAA	CAGAGTCCACGGTCAAACAA	102
Cs1g15640	Leucine zipper_4888	TGCTTGAGACGACCTTGGTT	CCTTCCGCCCATGATCATCA	72
Cs6g15060	Zinc finger_4913	ACGTCGTGTAAATCACGTCGTCAGG	ACCATCAAGCCTCGGGCATGACCA	59
orange1.1 t02887	Predicted_5356	GGGAATTGAAAAAGAGCAAAGAAA	TCTCACATGCCCGTTGTAGCT	66
orange1.1 t00323	MOQ_5344	TGCTCGAATGATATCACGCGGGGT	GATCCCTGGTTCAACAAGGATACTG	99
Cs6g19640	UDP-glucose transglucosidase_5565	TGTCGAAACAGGTCAAGGAA	TCCAGCTGGGTTAAGCTCAT	111
Cs7g12410	SAMDC_5828	GCTTGGGATGGGTGGTTCAA	TCCTCTTTCCAGCAGCACTT	96
Cs4g18250	R2R3 Myb_5620	ATTGCCCTCACCATGAGTCT	CAAGTGAAAGTGAAACGACAGG	106
Cs2g02500	ACO_5762	ACTCTATGCTGGGCTGAAGT	AGGACCCAGATTCACAATGG	85
Cs3g26100	Gibberellin-regulated protein 1_5834	CCTGGCCTGGCATGCA	GCAAAGGAACTGGTTATCCAAAA	59
Cs3g19060	Nitrate reductase_5947	GGCGCCTATTAAAGAAGTTGC	AAGAGACGCACATCGTGAGA	112
Cs2g12320	PK_6301	AAGTCTCCTCGGCCAACTC	TGCACTCTCACCACTAAGCA	93
Cs4g02260	SAMDC_6604	TCGGAACTGGCACGTTTACT	AAGCCGGTCATGCACATCTC	81
Cs7g12870	AP2_6823	GGTGTCGATAAGATTGGGTGC	GAGACAAGAGCTCCATTTCTCTAAG	82
Cs3g20810	ADH_6980	GCCTCGTTCTGACCTTCCTT	TCGGAGAATGGGACTGTATGG	93
Cs2g02540	PFK β subunity_7095	GTGCAAAGAGGTCCCTGCAAGC	CCGTGCATCTATCATGACATT	88
Cs9g04980	β amylase_7215	CAATTGGTCTGGCTACCTTGC	TTCAAAGCATGGAAGCAGAGT	87
Cs2g18290	UDP-GT 74E2_7508	CGGAATGCCCCCACTTG	AAGCCCAAATCCGTAAACGA	58
No similarity	bHLH101_7848	GAATCCAACTTCTGCTCTCTTAGG	CCAATGGACTATGAGCCAATTG	66
Cs3g03045	Predicted_7935	TTGGGAAAACCATTAGCGATTC	TCCCCACCCCTTACAACCA	68

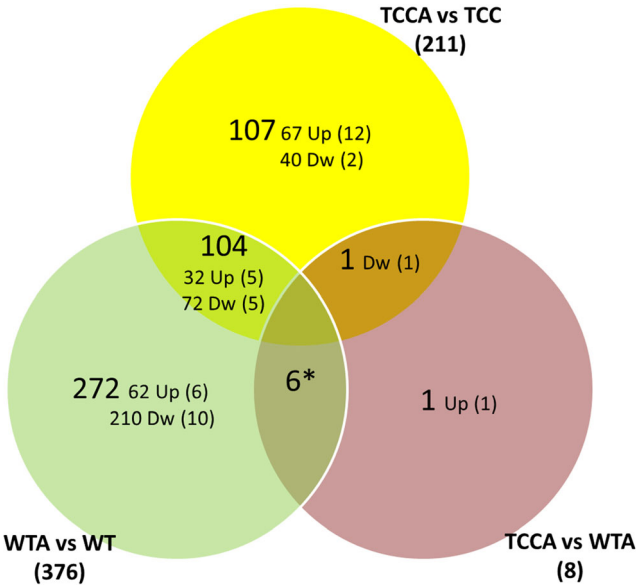


Fig. 1 Venn diagram showing differential gene expression for each comparison, specific and commonly shared. The upregulation (Up) and the downregulation (Dw) is also indicated. For each comparison and subset, the number of genes validated through real time PCR is showed in brackets. *means that the differential expression of six genes is not correspondent in WTA vs WT and in TCCA vs WTA

supplying hexoses for glycolysis by using a proficient starch mobilization via starch-degrading enzymes, including endo/exo-amylases, debranching enzymes, and starch phosphorylase (Magneschi and Perata 2009). In our experiment, the exo-amylases (corresponding to beta-amylase) resulted specifically upregulated in the TCCA vs TCC comparison (Table S2, id_2887 homologous to orange1.1t03470) and in WTA vs WT (Table S1, id_7215 homologous to Cs9g04980). The qRT-PCR performed on id_7215 confirmed the array data, showing more than four times the expression compared with the non-stressed sample (Figs. 5 and 6). This result brings us to hypothesize that hexoses, especially glucose, are primarily used to build starch molecules. Otherwise, the starch branching (id_1359) and the granule-bound starch synthase (id_1107) resulted in downregulation in both TCCA and WTA with respect to their relative not stressed samples (Table S3).

Sucrose degradation happens with the action of bidirectional sucrose synthase (SUS) and the unidirectional invertase (INV) pathways. By using SUS, the reaction releases fructose and UDP-glucose, which is then used by UDP-glucose pyrophosphorylase (UGPPase) and produces UTP and glucose-1P. In fact, these glycolytic reactions may use the PPi available instead of the ATP under oxygen deprivation, improving the net yield of ATP per mol of sucrose catabolized. In our experimental conditions, data from array indicate an overexpression of SUS (Table S1, id_3524) specifically in the WTA compared with the WT, and also confirmed by qRT-PCR (Figs. 5 and 6). Moreover, our results show a downregulation of UGPPase (Table S1, id_7142) as well as of G1P (Table S1, id_6667) specifically and exclusively in WTA. In addition, in the framework of genes involved in the biosynthesis of glycogen, we observed the downregulation of the UDP-glucose transglucosidase (Table S1, id_5565), whose expression was also confirmed by qRT-PCR (Figs. 5 and 6).

Among the genes involved in the glycolysis in our experiment, we found a gene coding for phosphoglucose isomerase (PGI, Table S3, id_3421) which was overexpressed in both the TCCA and WTA samples compared with their control samples, whose expression was also confirmed by qRT-PCR exclusively in transgenic CC (Figs. 5 and 6). In fact, the qRT-PCR demonstrated a downregulation in WTA instead of an upregulation that was reported by the array data. The phosphorylation of fructose-6P to fructose-1.6DP can be catalyzed by the unidirectional phosphofructokinase (PFK), which uses ATP as a phosphate group donor, or via the bidirectional PFK-PPi (PFP) that uses PPi. Microarray data highlighted a downregulation of PFP (Table S1, id_7095) which was also confirmed by qRT-PCR (Figs. 5 and 6). PFP is also known to be activated under anoxia in rice (Mertens et al. 1990; Mohanty et al. 1993). The expression of PFK is controversial in plants: in soybean (Nanjo et al. 2011) and in *Arabidopsis* (Mustroph et al. 2010), it is reported to be downregulated in low oxygen conditions, instead it is overexpressed in rice (Narsai et al. 2009) and poplar (Kreuzwieser et al. 2009).

We also found two different Probe ID coding for fructose 1.6 bisphosphate (DP) (id_2294 and id_3309). The first one resulted in down-expression of TCCA and WTA compared with the respective non-stressed controls (Table S3), the second one was upregulated in response to hypoxia in wild type CC seedlings (Table S1). qRT-PCR was performed on id_3309 confirming the data obtained after array hybridization (Figs. 5 and 6). Each Probe ID corresponds to a different gene in the sweet orange genome (Cs4g05700 and Cs6g15430), leading to think that the different response to chip hybridization could be due to the different genetic origin.

Among the genes involved in the transportation of glucose, we found a gene encoding for a sugar transporter (id_1266), and a gene encoding for transaldolase (id_4613), both highly expressed and exclusively retrieved in TCCA (Table S2). Nevertheless, the expression pattern of id_4613 had not been confirmed by qRT-PCR (Figs. 5 and 6).

Enolase represents a key enzyme involved in the metabolism of glucose which allows for limited energy production in the face of restricted oxygen supply and thus aids in survival when in this condition. In our case, enolase (id_4834) resulted in downregulation of WTA vs WT after microarray hybridization (Table S1), but its expression was not confirmed by the qRT-PCR experiment (Figs. 5 and 6).

The pyrophosphate (PPi) supply may be increased by a cyclic substrate coupled with pyruvate kinase (PK) in order

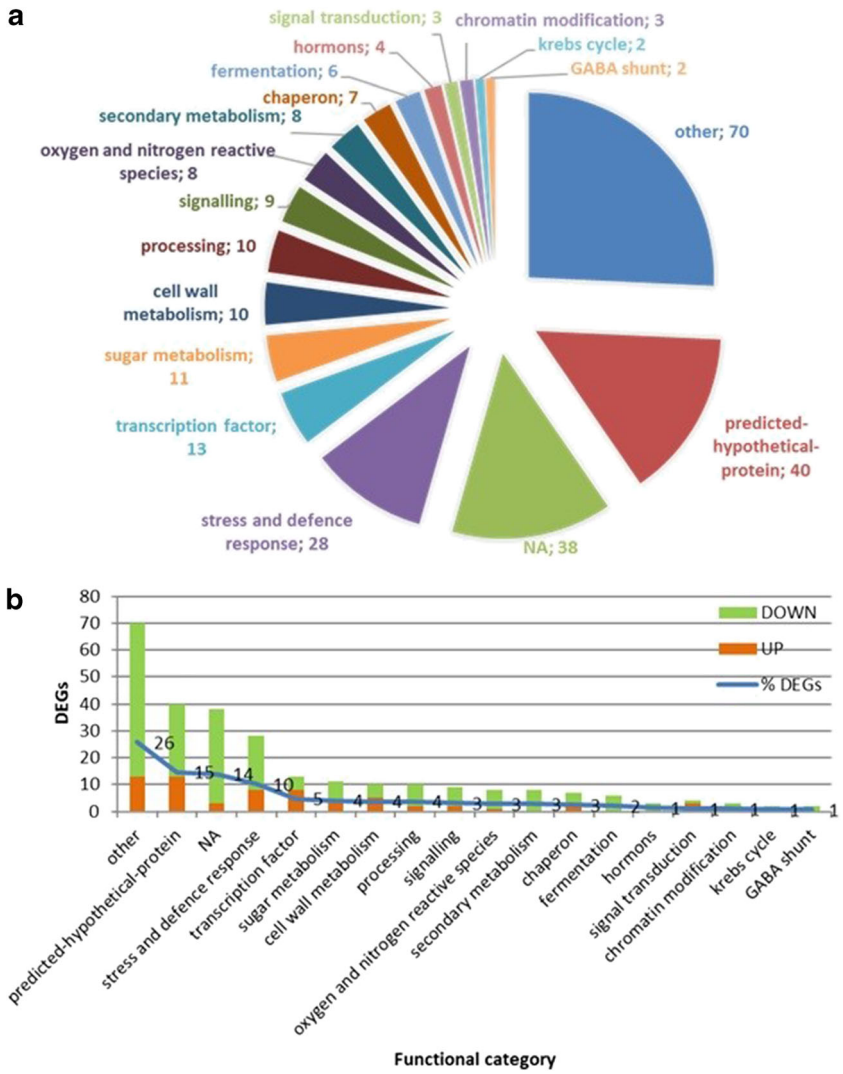


Fig. 2 Differential expressed genes (DEGs) pointed out in the comparison wild type Carrizo citrange under hypoxia conditions versus normoxia (WTA vs WT), ordered for functional categories, **a** using a pie chart and **b** a histogram. In this case, DEGs are separated in up (red bar) and downregulated (green bar), and the correspondent percentage of each category respect to the total is also indicated with a blue line

to accelerate glycolysis. The upregulation of PK (Table S3, id_6301) in both TCCA and WTA, also this confirmed by the qRT-PCR data (Figs. 5 and 6), can justify the ability to move the supply of carbohydrate and enzymes involved in the degradation of sugars in either the TCC or the WT by adopting the same exact strategy.

Fermentation, Krebs cycle, and GABAshunt

Fermentation consists of the production of substances and molecules alternative to respiration. Under oxygen deficiency, the end-product of glycolysis, which is the pyruvate, can proceed along two pathways: ethanolic and lactic fermentations. Generally, the activation of lactate dehydrogenase (LDH), reducing pyruvate to lactate, has been commonly observed in oxygen-deficit conditions (Banti et al. 2013). Regardless, the production of lactate is just transitory and produced only during the first hours of stress, then it is easily removed from the cell with the goal of preventing an excessive accumulation of this compound in the cytosol (Choi and Roberts 2007). Hypoxia-inducible Nodulin Intrinsic Protein is one of the main genes responsible for the lactate extrusion process. In our experiments, we found three different genes coding for nodulin-like proteins (id_7202 corresponding to Cs3g19460, id_3280 homologous to Cs3g25510, and id_4733 corresponding to Cs3g07150). Each of these is downregulated in the TCCA and WTA compared with their controls (Table S3), and one (id_1864, corresponding to Cs5g04430 in sweet orange genome) in WTA compared with WT (Table S1). The validation by the qRT-PCR was performed on id_3280 and id_1864 and correctly confirmed (Table 2), showing a lower expression in the non-stressed samples compared with the hypoxic one, for both genes. The downregulation of these genes could be explained by considering that the nodulin is activated during the initial hours of stress, but our experiment of oxygen deprivation was performed for 24 h and data were analyzed at the 24th hour.

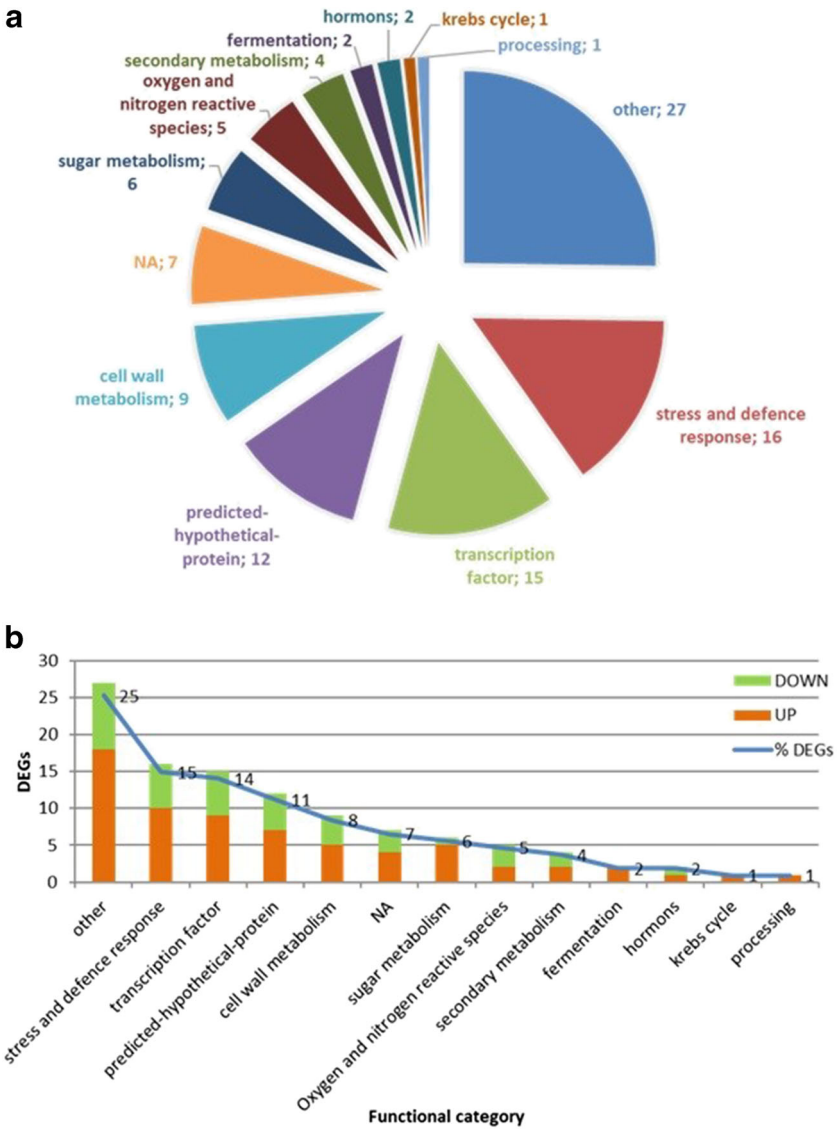


Fig. 3 Differential expressed genes (DEGs) pointed out in the comparison transformed Carrizo citrange under hypoxia conditions versus normoxia (TCCA vs TCC), ordered for functional categories, **a** using a pie chart and **b** a histogram. In this case, DEGs are separated in up (red bar) and downregulated (green bar), and the correspondent percentage of each category respect to the total is also indicated with a blue line

In addition to lactate metabolism, alternative pathways to aerobic respiration lead to ethanol, succinate, and GABA production, using pyruvate as a starting substrate, which is derived from sugar catabolism and glycolysis. Among the genes of ethanol fermentation, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) fulfill a key role in low oxygen tolerance. PDC (EC 4.1.1.1) catalyzes during the first step and it is responsible for the permanent conversion of pyruvate to acetaldehyde. ADH (EC 1.1.1.1) converts acetaldehyde to ethanol, generating at the same time, NAD^+. In our experiments, we found several Probe ID coding for ADH in the WTA vs WT comparison (Table S1, id_264) and in the TCCA vs TCC ones (Table S2, id_1865) in addition to one probe shared between TCCA and WTA (Table S3, id_6980). The qRT-PCR data (Figs. 5 and 6), confirmed the higher expression levels of id_1865 and id_6980 under hypoxia. Conversely, it further confirmed the downregulation of id_264 as revealed by the microarray data. The impressive high expression of ADH in the TCCA samples (more than 19 times for id_6980 and more than 5 times for id_1865 compared with the non-stressed transformed plants) could be associated to the concomitant high expression of PDC (id_4457, Figs. 5 and 6), which is consistent with the complementary roles of the encoded proteins in the conversion of pyruvate to ethanol during the ethanolic fermentation. In the WTA, we observed an ambiguous expression, up for id_6980 (also confirmed in the qRT-PCR, Figs. 5 and 6) and down for id_264 (confirmed as well by the qRT-PCR, Figs. 5 and 6). The apparently ambiguous behavior of ADH (in terms of high and low regulation) is not uniquely observed in citrus. In rice, Lasanthi-Kudahettige et al. (Lasanthi-Kudahettige et al. 2007) described that, among multiple isozymes of alcohol and

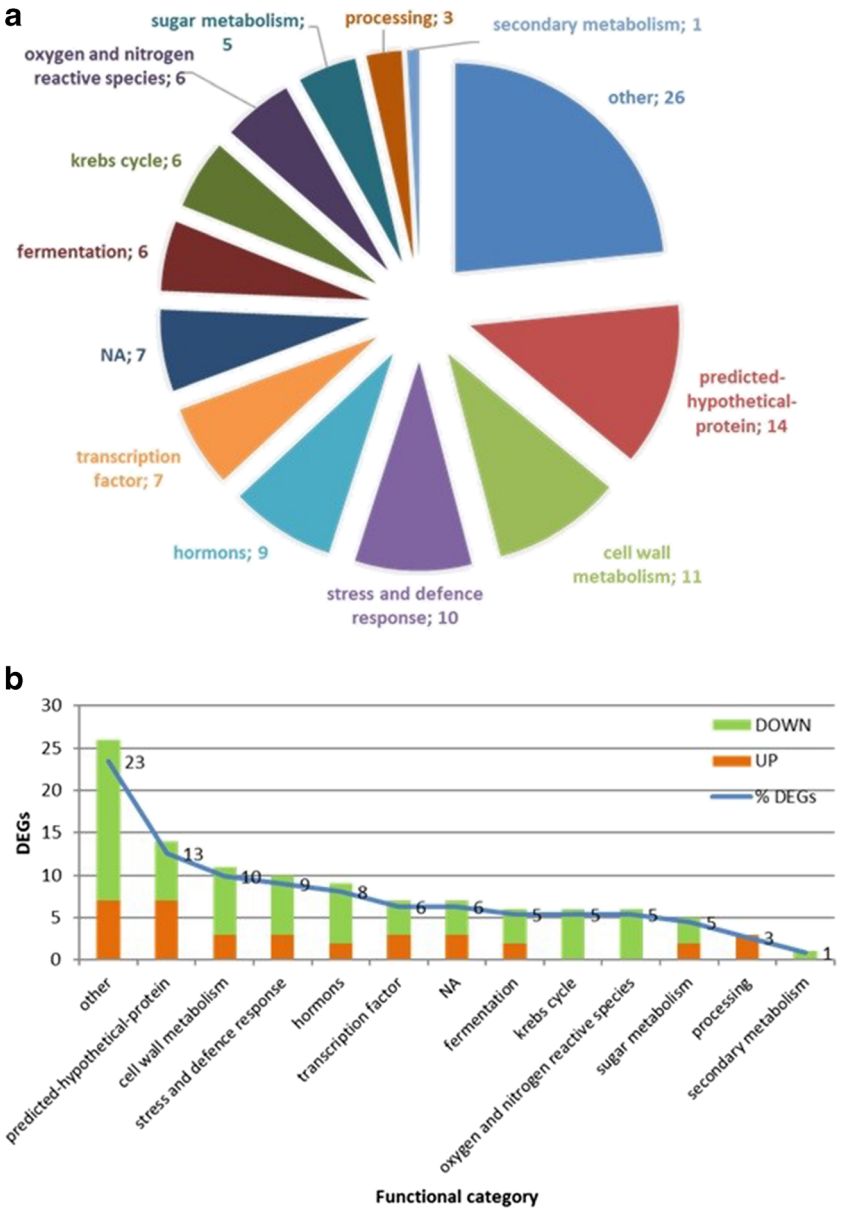


Fig. 4 Differential expressed genes (DEGs) pointed out in the comparison transformed and wild type Carrizo citrange both under hypoxia conditions (TCCA and WTA), ordered for functional categories, **a** using a pie chart and **b** a histogram. In this case, DEGs are separated in up (red bar) and downregulated (green bar), and the correspondent percentage of each category respect to the total is also indicated with a blue line

aldehyde dehydrogenases (ALDH), only a few of them (ADH1, ADH2, and ALDH2a) showed a radical increase in gene expression. In Arabidopsis, the overexpression of ADH1 is not correlated with the tolerance to flooding, while regular expression levels of ADH1 have been considered critical for the plant survival under low oxygen conditions (Ismond Kathleen et al. 2003). For these reasons, we cannot exclude that the ADH_id_264, as well as ALDH (Table S1, id_4314), correspond to different isoforms, probably not directly involved in the response to hypoxia. In fact, after the interrogation of the sweet orange genome database, we observed that id_ probes corresponded to different genes (id_264 = Cs3g17940, id_6980 = Cs3g20810, id_1865 = Cs3g20830). In fact, id_6980 and id_1865 are homologous to different isoforms of the same gene, justifying their similar expression trend.

In addition to ethanol, also alanine, GABA, and succinate have been suggested as additional products of anaerobic metabolism, belonging to the Krebs cycle and amino group transfer in the cytosolic steps. In our experiments, a gene coding for 2-oxoglutarate (2-OG, id_700) resulted in downregulation under hypoxia conditions in both TCCA and WTA samples, each one compared with non-stressed samples (Table S3). These data were also confirmed by qRT-PCR data (Fig. 5 and 6) showing a higher downregulation in both hypoxic samples compared with non-stressed. Similarly to 2-OG, glutamate decarboxylase (GAD, id_6831) is also down-expressed in WTA compared with a non-anoxic sample (Table S1), but no qRT-PCR was performed for this gene.

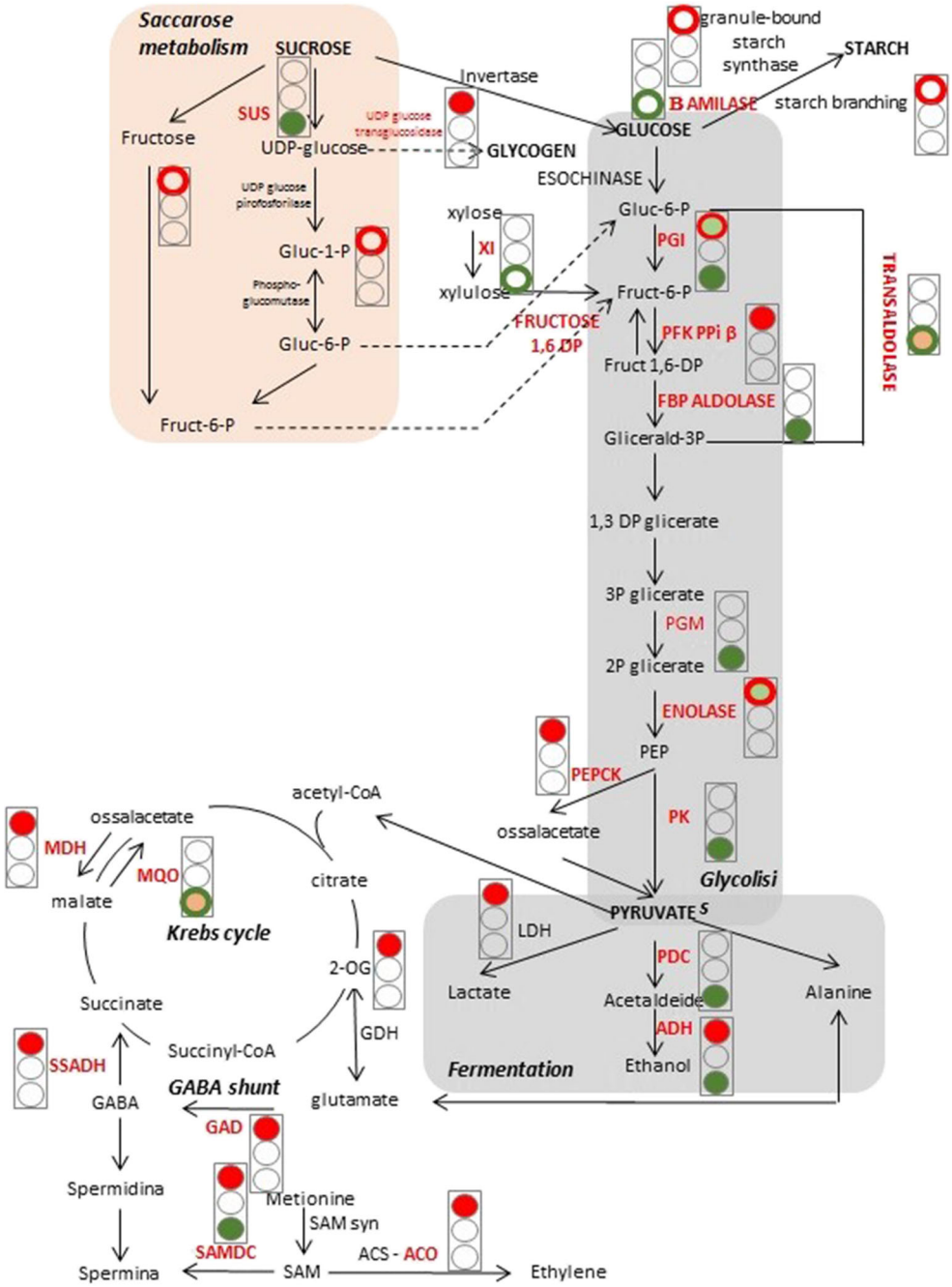
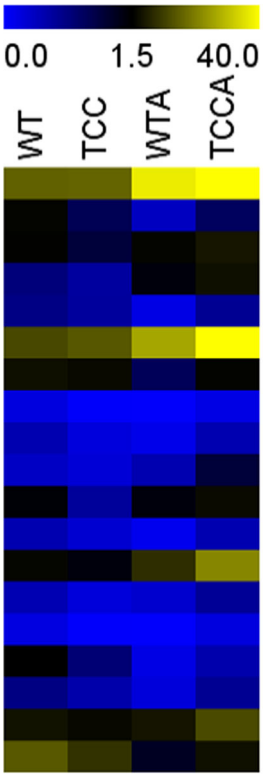


Fig. 5 Schematic view of genes coding for enzymes involved in sugar metabolism, fermentation, Krebs cycle, GABA shunt, polyamine, and ethylene metabolism. Enzymes, which expression resulted differential by microarray analysis, are indicated in red. Filled circle indicates the correct validation of array data by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and the color, green or red, indicates the modulation direction, upregulated and downregulated, respectively. Light green and light red mean that qRT-PCR did not confirm the expected modulation detected with the array. Empty circles mark genes that have been not analyzed by qRT-PCR

Focusing on the genes involved in the Krebs cycle, array data showed that the malate dehydrogenase (MDH) is downregulated in both TCCA and WTA (Table S3, id_6798 and id_6800 = Cs5g10730). Moreover, malate oxidoreductase (MQO, id_1338 and id_4769 = orange1.1 t00323.1) and succinate semialdehyde dehydrogenase (SSADM, id_2015 = orange1.1 t03248) also resulted in downregulation of WTA (Table S1). On the other hand, MQO resulted specifically and uniquely overexpressed in TCCA (Table S2, id_5344 = orange1.1 t00323.1). Nevertheless, the qRT-PCR which was performed to evaluate these data did not confirm the higher expression of MQO in TCCA compared with the control (Figs. 5 and 6). From a structural point of view, orange1.1 t00323.1 is made up of 19 exons. In our experiment, the first 9 exons of the total 19 are represented by id_5344, the last 6 exons include id_1338 and id_4769. In fact, although id_1338, id_4769, and id_5344 correspond to the same gene (orange1.1 t00323.1), probes spotted in the chip are different from each other, and qRT-PCR was performed exclusively only for id_5344 (Table 1).



Probe ID	Gene annotation
id_3524	SUS
id_5565	UDP_Gluc_Trans
id_7215	Beta_Amilase
id_3421	PGI
id_7095	PFK_PPI_Beta
id_3309	Fructose_1.6DP
id_2294	Fructose_1.6DP
id_4613	Transaldolase
id_4834	Enolase
id_6301	PK
id_4457	PDC
id_264	ADH
id_6980	ADH
id_1865	ADH
id_5344	MQO
id_700	2-OG
id_6604	SAMDC
id_5828	SAMDC
id_5762	ACO

Comparison	Foldchange Array	Foldchange qRT-PCR
WTA vs WT	1.356	1.932
WTA vs WT	-1.601	0.934
WTA vs WT	1.776	4.276
TCCA vs WTA	1.988 / 1.366	2.507 / 0.751
WTA vs WT	-1.884	0.836
WTA vs WT	1.492	2.396
TCCA vs WTA	-1.289 / -1.044	ND
TCCA vs TCC	2.664	0.787
WTA vs WT	-1.657	1.579
TCCA vs WTA	1.897 / 1.331	2.911 / 2.911
TCCA vs TCC	2.152	3.606
WTA vs WT	-1.681	0.495
TCCA vs WTA	3.47 / 2.941	19.108 / 7.801
TCCA vs TCC	1.777	5.165
TCCA vs WTA	-2.033 / -2.616	0.09 / 0.118
TCCA vs TCC	2.43	0.831
TCCA vs WTA	-1.224 / -2.616	0.219 / 0.093
WTA vs WT	-1.09	0.500
TCCA vs WTA	1.337 / 1.115	2.481 / 2.418

Fig. 6 Expression data related to the microarray and to the validation through quantitative reverse transcription polymerase chain reaction (qRT-PCR) relatively to the enzymes involved in sugar metabolism, fermentation, Krebs cycle, GABA shunt, polyamine and ethylene metabolism. Heat map reports the median expression of three biological replicates for each experimental condition as measured by microarray hybridization. For each gene comparison analyzed, fold changes of array and qRT-PCR analysis are reported. "ND" indicates that the gene was not evaluated in qRT-PCR. For the comparison, TCCA vs WTA two fold changes values are reported, that represent, respectively, the fold change of TCCA vs TCC and WTA vs WT.

Hormones

The category "hormones" is represented by all of the substances functioning as hormones, but do not necessarily have to be hormones themselves. In the framework of GABA shunt, there is a close link between glutamate (Glu) and polyamines (PA, including putrescine (Put), spermidine (Spd), and spermine (Spm)), and following, SAM decarboxylase (SAMDC - EC 4.1.1.50) is reported. The biosynthesis of Spd and Spm from Put is catalyzed by SAMDC. Gill and Tuteja (Gill and Tuteja 2010) described PAs as substances involved in regulating plant growth and also involved in the acquisition of tolerance to different abiotic stresses, including hypoxia. Furthermore, it was reported (Arbona et al. 2008) that polyamines accumulate in the leaves of citrus in response to flooding. In our experiments, we found a SAMDC, overexpressed in both anoxic samples of TCCA and WTA when compared with their respective control samples (Table S3, id_5828), whose higher expression result was also confirmed by the qRT-PCR experiment (Figs. 5 and 6). Conversely, SAMDC id_6604 (Table S1) resulted in downregulation of the WTA vs WT comparison, which was also confirmed by the qRT-PCR (Figs. 5 and 6). Both sequences correspond to two different genes, respectively Cs7g12410.3 and Cs4g02260.1, leading to hypothesize that they could correspond to different isoforms, probably involved in different functions.

Ethylene regulates the responses to adverse growth conditions, including hypoxia, by participating in several aspects of root formation, growth, and development (Ookawara et al. 2005). Ethylene is produced from methionine, through intermediates of S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylate (ACC) by SAM synthase, ACC synthase (ACS), and ACC oxidase (ACO). In several species under hypoxia conditions, cell death is mediated by ethylene as it causes an increase of expression of ACS and ACO expression (Van de Poe and Van Der Straeten 2014). However, under anoxic conditions, oxygen deficiency blocks the production of ethylene (Smith et al. 2012). In our experiment, both transformed and WT samples under hypoxic conditions (Table S3) shared the downregulation of ACO (id_5762) and ethylene-forming enzyme (id_2781). The down-expression of ACO was also confirmed by the qRT-PCR (Figs. 5 and 6), although our experiment was not conducted under conditions of anoxia, but rather hypoxia. Arbona reported (personal communication) a significant increase of

Table 2 Expression data related to array and quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments reported as fold change. Values in italics refer to not validated data

Gene name	Comparison	Fold change	
		Array	qRT-PCR
Nodulin	WTA vs WT (id_1864)	− 1.251	0.609
	TCCA vs WTA (id_3280)	− 1.579/− 1.431	0.122/0.118
UDP-GT 74E2	TCCA vs TCC (id_7508)	− 1.541	0.569
	TCCA vs WTA (id_7508)	− 1.206	0.991
Gibberellin-regulated protein 1	TCCA vs TCC (id_5834)	3.699	16.362
XTHs	WTA vs WT (id_307)	− 1.326	0.684
Cell wall-related GT	TCCA vs TCC (id_2743)	− 2.19	0.187
Nitrite reductase	TCCA vs WTA (id_544)	− 1.427/− 2.05	0.336/0.350
Nitrate reductase	WTA vs WT (id_5947)	*− 1.906*	*1*
nshb1	WTA vs WT (id_2881)	− 1.448	0.704
GST	TCCA vs TCC (id_2079)	*2.07*	*0.771*
HSP	WTA vs WT (id_4557)	1.222	4.391
Ap2	TCCA vs WTA (id_6823)	2.159/1.578	0.690/1.745
	WTA vs WT (id_754)	1.05	1.828
Zinc finger	TCCA vs TCC (id_4913)	2.068	1.637
BHLH101	TCCA vs TCC (id_7848)	2.757	4.989
Leucine zipper	TCCA vs TCC (id_4888)	− 1.139	0.259
R2R3 Myb	WTA vs WT (id_5620)	1.2	1.194
Chac	TCCA vs TCC (id_1270)	2.024	7.393
Ep1-like	TCCA vs TCC (id_4818)	2.591	4.349
PAL	TCCA vs TCC (id_1789)	2.737	4.477
Polyubiquitin 1	TCCA vs TCC (id_3007)	2.1	11.390
Predicted	WTA vs WT (id_7935)	− 4.451	0.112
	TCCA vs WTA (id_7935)	2.433	2.14
Predicted	TCCA vs TCC (id_5356)	2.83	5.973
Mir166	TCCA vs TCC (id_349)	2.252	2.221

ACO in the leaves of Cleopatra mandarin (and most likely ethylene as well), but no significant differences were found in the roots. It could be assumed that our experimental conditions simulated oxygen deficiency that was more similar to anoxia, rather than to hypoxia.

In the framework of molecules working as hormones, auxin is considered to have a strategic effect. Since the transcriptional profile of auxin signaling is very wide, the physiological responses are numerous. We found in particular that in TCCA and in WTA (Table S3), "auxin-induced proteins" (id_1909; id_5587) and "gibberellin receptor" (id_1133) as well as "dehydration-responsive protein rd22" (id_1707) and "ent-kaurenoic acid oxidase" (id_2174) resulted in downregulation. Conversely, "auxin associated-like protein" (id_2518) results down-expressed specifically and exclusively in terms of id_gene only in TCCA (Table S2). Moreover, the WTA (Table S1) is characterized by the down-expression of a SAUR family protein (id_2189), in addition to the auxin-induced protein (id_5576) and to the gibberellin receptor (id_4003) which resulted in down-expression, exclusively in WTA. A reduction of ABA levels in the roots is described in citrus, while an opposite behavior was observed in the leaves (Arbona et al. 2017). ABA depletion did not seem to be a side effect of O_2 depletion but rather a regulated and coordinated response involving UPD-glycosyl transferase, such as in our experiment, where the downregulation was also confirmed by qRT-PCR (Table 2). Even though the rd22 gene encodes an unknown function protein, it is reported that its transcriptional regulation is intensely associated with abiotic stresses and ABA treatment (Yamaguchi-Shinozaki and Shinozaki 1993). Interestingly, the biosynthesis of this last gene was described in hypoxic systemic responses (Hsu et al. 2011), confirming our findings of a higher expression in TCCA compared with WTA (Table S3).

Among the upregulated transcripts exclusively in TCCA, a strong expression of two gibberellins (GAs) regulated protein genes (Table S2, id_5834 and id_2982) were observed under oxygen deficiency. Both genes are the most highly expressed in terms of fold change in the microarray analysis. Moreover, the expression of the gibberellin-regulated protein (id_5834) was also validated using qRT-PCR, more than 16 times compared with the control thus not only confirming the

upregulation in the TCCA vs TCC (Table 2), but also the very high expression level (more than 10 times) in WTA compared with WT. It is known that gibberellins affect several physiological processes in the growth and development of rice and other species (Huerta et al. 2008). Interaction between GAs and other plant hormones under hypoxia and anoxia have been described (Armstrong and Drew 2002). These data confirm that TCCA developed an increase in the elongation of the roots, as previously reported (Caruso et al. 2011).

Cell wall metabolism and signal transduction

The involvement of genes responsible for cell wall modification is strategic during low oxygen conditions. The downregulation of xyloglucan endotransglycosylase/hydrolases (XTHs), exclusive in terms of Probe ID in WTA (Table S1, id_307), was also confirmed by the qRT-PCR data (Table 2), even though these data are contrasting compared with the very high expression level of XTHs id_4079 (FC 3081) highlighted by the microarray data in TCCA and in WTA (Table S3). These genes correspond to Cs2g17920 and Cs4g03200, respectively. We also observed a downregulation of alpha-expansin 4 in TCCA vs TCC (Table S2, id_4849), confirming what was previously reported. Most of the genes related to the development of the cell wall are downregulated under abiotic conditions affecting cell extensibility and reducing leaf growth and development (Devi et al. 2015). Other gene encoding proteins involved in the cell wall degradation, such as β-(1,4) glucanase (Table S3, id_2251), were downregulated under hypoxia conditions. Similarly, the array data exclusively in TCCA vs TCC indicated the downregulation of secondary cell wall–related glycosyltransferase family 8 (Table S2, id_2743), whose expression was also confirmed by qRT-PCR (Table 2). Overall, the downregulation of cell wall–related proteins was already emphasized in *Arabidopsis*, reflecting an attempt by the cells to cope with a reduced state of energy by conserving resources during the period of oxygen shortage (Liu et al. 2005). Moreover, some transcripts involved in the remodeling of the cell wall, such as pectin methylesterase (id_870) and glycoside hydrolase (id_2212) found in both TCCA and WTA (Table S3), as well as cell wall-associated hydrolase (id_833), COBRA-like protein 1 (id_4249), Endo−1,3;1,4-beta-D-glucanase (id_188, id_3823), pectin methylesterase (id_2936) specifically found in TCCA vs TCC (Table S2), and aquaporin (id_1640, id_2057, id_6185), invertase/pectin methylesterase inhibitor family protein (id_1663) specifically found in WTA vs WT (Table S1), resulted in overexpression under hypoxia conditions. The activation and, as a consequence, the upregulation of remodeling could be justified as a potential endeavor to enlarge and remodel the cell wall, particularly activate during oxygen deficiency.

Oxygen and nitrogen reactive species

One of the most controversial aspects related to the molecular response to low oxygen regards oxidative stress. The activation of ROS-regulated genes or those associated with ROS have been reported (Mittler et al. 2004). Similarly, the role of NO as a signaling molecule under low oxygen conditions is very probable. It was reported that nitrite (NO^{2-}) acts as an alternative electron acceptor and its reduction to NO, conversion to nitrate (NO^{3-}) by the non-symbiotic hemoglobin (nsHb1), and then reduction to NO^{2-} by nitrate reductase give rise to a cycle that produces a certain amount of ATP (Gupta et al. 2011; Igamberdiev et al. 2010). According to these data, we observed a downregulation of nitrite reductase (id_544) in transgenic and wildtype CC under hypoxia conditions (Table S3). These data were also confirmed through the qRT-PCR (Table 2). Similarly, we observed a specific down-expression of nitrate reductase (id_5947) and nsHb1 (id_2881) in WTA (Table S1), and these data were exclusively confirmed for nsHb1 (Table 2). A mechanism of hypoxia tolerance mediated by nsHb due to the O_2 affinity of nsHbs and NO detoxification was previously described (Perazzolli et al. 2006). According to this, we can hypothesize that the downregulation of nsHb1 in CC WT could be associated with a reduced tolerance of hypoxia, in comparison to transformed seedlings.

In our experiment, most of the genes involved in the ROS/RNS demonstrated a downregulation under hypoxia conditions (Table S1, S2, S3), with the exception of manganese superoxide dismutase (id_6945) and glutathione S transferase (GST, id_2079) specifically in TCCA vs TCC (Table S2). In particular, the upregulation of GST was also evaluated through qRT-PCR and it did not confirm array data (Table 2).

Previous studies have reported that heat shock proteins (HSPs) are expressed in plants subjected to high-temperature stress, as well as cold, salinity, water, osmotic, and oxidative stress (Wang et al. 2004). HSPs are the most diffused ROS-related proteins which are also induced by anaerobic conditions as a mechanism needed to activate a defense. HSPs' expression has been shown to be associated with the presence of hydrogen peroxide (H_2O_2) in different kingdoms (Vandenbroucke et al. 2008). In our experiment, we found an HSP specifically downregulated in TCCA vs TCC (Table S2, id_514) and other four (three downregulated and one upregulated) specifically in WTA vs WT (Table S1, id_3470; id_2906; id_4218; id_4557, respectively). The expression of id_4557 was validated and confirmed in qRT-PCR (Table 2).

Transcription factors

The regulation of hypoxia is under the intense control of transcription factors (TFs). Some of them, called "ethylene-

responsive element binding proteins" (EREBPs), regulate gene expression at the transcriptional level. In our experiments, a gene coding for Ap2 (Table S3, id_6823) was overexpressed in both TCCA and WTA, while id_754 resulted exclusively upregulated in WTA (Table S1). The qRT-PCR data confirmed the expression of Ap2 (id_6823) exclusively in the comparison WTA vs WT, as well as Ap2 (id_754, Table 2).

Among the genes specifically upregulated in TCCA vs TCC (Table S2), the most interesting is the zinc finger-like protein (id_4913) and transcription factor bHLH101 (id_7848). These data were also validated in qRT-PCR, confirming a consistent upregulation under hypoxia conditions compared with the control (Table 2). The induction of TFs, including zinc-finger types, has been reported in the roots of *Arabidopsis* under hypoxia conditions (Licausi et al. 2011). In addition, microarray data highlighted the overexpression of Ethylene-responsive transcription factor RAP2-3 (Table S2, id_3162), as previously reported (Gasch et al. 2016; Voesenek and Sasidharan 2013), although it was difficult to design more than one pair of primers to perform and validate the quantitative expression. Moreover, we validated the expression of the R2R3-Myb gene (id_5620, Table 2), whose upregulation was a confirmation of the array data. Among the downregulated genes, we focused our attention on the homeobox-leucine zipper protein ATHB-15 (Table S2, id_4888), whose expression was also confirmed by qRT-PCR (Table 2). The homeobox TFs participate in various circumstances of plant growth, where many genes of this family are reported to be downregulated (Chen et al. 2014).

The microarray data highlighted the induction of abscisic acid (ABA)-insensitive abi3-interacting protein 2 (Table S1, id_7613) as exclusively expressed in WTA vs WT. The expression of this gene is reported to be induced by auxin in the roots and, as a consequence, to regulate positively lateral root development (Duong et al. 2017).

Miscellaneous categories

In addition to the previous well-defined functional categories, we also reported data related to other functional categories.

The stress and defense response category resulted rich in DEGs in all the comparisons. The "cation transport protein chac" is shown to be enhanced during submergence as well as in stress-induced conditions by abscisic acid, salt, or drought in rice (Qi et al. 2005). In our oxygen deficiency conditions, the microarray experiments showed six "cation transport protein chac" genes as considerably upregulated, two overexpressed in both TCCA and WTA (Table S3, id_4102; id_4681), and four uniquely overexpressed in TCCA vs TCC (Table S2, id_1270; id_7251; id_5552; id_4524). We validated the overexpression of id_1270 through qRT-PCR, confirming the microarray results (Table 2).

Among the overexpressed genes, we focused on EP1 glycoprotein (id_4818), specifically overexpressed in TCCA vs TCC (Table S2). The qRT-PCR experiment confirmed and supported the high expression level of this gene (Table 2). References reported that EP1 may be involved in limiting water loss through the outer wall of the epidermal cells (Oliveira et al. 2015; Van Engelen et al. 1993). EP1 is included in the leucine-rich repeat receptor kinase (LRR-receptor kinase) family protein (Guerra-Guimarães et al. 2016), whose LRR domain is characterized by a versatile structure with a prominent role in plant defense (Kobe and Kajava 2001). It was also hypothesized that it could provide an early warning system and activate protective immune signaling in plants (Kobe and Kajava 2001).

The phenylalanine ammonia lyase (PAL), in addition to being involved in the biosynthesis of polyphenol in plants (Tanaka et al. 1989), is also affected by various biotic and abiotic conditions (Lee et al. 2003). In our findings, PAL resulted induced by hypoxia in TCCA vs TCC (Table S2, id_1789), and the higher expression was also confirmed through qRT-PCR (Table 2). In Argamasilla et al. (2014), phenolic acid levels increased in response to soil flooding. (Poly)Ubiquitin is involved in protein degradation. Diverse gene families in plants encode for ubiquitin ligases (Wang and Deng 2011). We found several genes coding for ubiquitin overexpressed in both TCCA and WTA (Table S3, id_1162, id_370 id_4167). Also, in WTA vs WT, three different genes coding for ubiquitin were shown (Table S1): id_3215 was overexpressed, while id_396 and id_5443 were downregulated. Moreover, the comparison of TCCA vs TCC is characterized by a unique and specific ubiquitin (Table S2, id_3007), which was overexpressed under hypoxia conditions. The data were also validated and confirmed by qRT-PCR, indicating a very high expression level which was more than 11 times compared with the non-stressed sample (Table 2). In our experiment, hypoxia induced the downregulation of several coding genes for cytochromes P450 (Table S3, id_1581, id_5188; Table S1, id_2757, id_1182, id_7442, id_551, id_6848, id_7340). Our results are in line with what was speculated by Lasanthi-Kudahettige et al. (Lasanthi-Kudahettige et al. 2007), according to whom oxygen deficit induces the downregulation of cytochromes P450 genes because it is needed in avoiding energy waste caused by the transcription of genes whose products require oxygen.

Lastly, we also thought it would be interesting to validate some predicted, unknown, and non-annotated genes because of their high expressions. We placed particular focused on id_7935, because it is shared between TCCA and WTA (Table S3) and because it represents one of the eight DEGs, whose expressions resulted very high in terms of fold change (Table S3). In fact, array

data highlighted a considerable downregulation in WTA compared with WT which was also confirmed by qRT-PCR and by the overexpression in TCCA compared with WTA (Table 2). Moreover, considering the high fold change, we also focused on id_5356 and id_349, both specifically overexpressed in TCCA vs TCC (Table S2). The higher expression result was also confirmed through qRT-PCR (Table 2).

Prediction of oxygen deficiency induced lncRNA, miRNAs, and their putative targets

Non-coding or non-annotated genes could serve as long non-coding RNA (lncRNA). Their behavior is reported to be involved in the response to biotic and abiotic stresses, including oxygen deficiency (Zhang et al. 2008). For this reason, genes categorized as "NA", "predicted, hypothetical, protein" and "TF" were further investigated to better understand their putative behavior as non-coding RNA (ncRNA). We used different databases to investigate if our unknown and putatively annotated sequences could match for lncRNA, and eventually for Pre-miRNA or miRNA. GreenC database highlighted that the only indicator for a Pre-miRNA (making a blast against lncRNA), and specifically for miRNA166, was the id_349. Interrogation of miRbase returned output highly promising in terms of *E* values and species belonging to the *Citrus* genus, supporting the probable realistic result. The search for the putative target of correspondent miRNA166, performed by interrogating psRNAtarget, predicted an HD-Zip.

Discussion

This is the first time in which a differential gene expression approach was used to investigate the genetic response of CC rootstock to low oxygen conditions. Few references are available to investigate the response to flooding and in general, oxygen deficiency in fruit trees, specifically in citrus. In fact, among fruit tree crops, this is the first time in which a citrus rootstock (Citrange) was used to illustrate the modification that genes undergo in low oxygen conditions. Scientists from various countries have attempted, not only to elucidate the mechanisms of plant damage and adaptation under conditions of oxygen deficiency but to also create plant organisms that are more tolerant to anaerobic stress (Caruso et al. 2011; Girhepuje and Shinde 2011; Liu et al. 2011). The availability of a microarray platform, specific to *Citrus* and its relatives, whose ESTs were exclusively isolated in the roots, could be considered a useful instrument to perform expression analysis of specific stresses that are mainly focused on the roots.

Hypoxia induces the activation of genes involved in the metabolism of sugar and alcoholic fermentation

Our findings confirm previous studies showing the activation of genes involved in the glycolytic and fermentative pathways under oxygen deficiency (Blokhina et al. 2003; Russell and Sachs 1991). This evidence led us to assume that the main genes involved in the metabolism of sugar and fermentation are expressed in both TCC and WT under hypoxia conditions suggesting that transformed plants are not that different from the wild type.

The immediate biochemical precursor to ethanol, that is acetaldehyde, is considerably more toxic to plant cells than ethanol and represents a key factor in plant cell death as a response to anaerobic root metabolism (Drew 1997; Vartapetian and Jackson 1997). The conversion of acetaldehyde to ethanol is catalyzed by the enzyme ADH. A significant amount of research on herbaceous plants has shown that increased ADH activity improves the plant's tolerance to anoxia. Although the importance of the fermentation pathway in hypoxia survival is known, the switch from respiration to fermentation in *Citrus* and relative *genera* appears to not be completely understood (Ismond Kathleen et al. 2003). Induction of ADH (in our case id_6980) showed an expression of more than 19 times, specifically in TCCA, representing a key step in the switch to anaerobic energy production. Therefore, these data support the idea that ADH is considered a marker of hypoxia or anoxia, switching to anaerobic metabolism mainly as a result of O_2 unavailability (Johnson et al. 1994).

Generally, under low oxygen conditions, plants reconfigure their metabolism, in order to maximize ATP production. Under anoxia conditions, evidence supports that lower glycolysis activation is used in order to avoid the dissipation of glucose and thus means that one of the main key mechanisms in response to oxygen deficiency is represented by the maintaining of sufficient levels of sugars (Sairam et al. 2009; Xia and Saglio 1992). Low oxygen conditions induce the expression of "anaerobic genes" (such as PDC and ADH), encoding enzymes involved in the glycolysis and ethanolic fermentation. Furthermore, our data support previous observations, according to which SUS (rather than INV) is reported to be the preferential pathway for sucrose degradation when oxygen is limited, because it partially preserves the energy of the glucose-fructose bond (Zeng et al. 1999). In this way, the availability of substrates for energy production, which uses glycolysis and fermentation, is guaranteed.

The quick activation of LDH, responsible for the reduction of pyruvate to lactate, has been frequently observed under low oxygen conditions (Banti et al. 2013). The production of lactate plays a minor role in low oxygen responses because lactate is generally produced only transiently during the first

hours of stress and is easily expelled from the cell, in order to prevent an excessive accumulation of this compound in the cytosol (Licausi and Perata 2009). Nodulin-like protein, responsible for the lactate extrusion process, is easily activated during the early first hours of stress. As we evaluated data after 24 h, the down-expression of these genes suggested that we were in a later phase of the activation of that mechanism.

The downregulation of 2OG, MDH, MQO, GAD, and SSADM suggested an inclination to specifically activating the fermentation pathway instead of the Krebs cycle and the GABA shunt. Moreover, SAMDC resulted in overexpression in response to hypoxia in both comparisons, suggesting the activation of the polyamine chain elongation pathway, leading to Spermidine and/or Spermine biosynthesis from putrescine or inducing to the ethylene biosynthesis via ACC synthase and ACC oxidase. To this respect, it could be speculated that the arginine-agmatine pathway is somehow blocked, as it could be deduced from the repression of arginine decarboxylase (id_7856, Table S3).

Cross-talk among ethylene, auxin, and gibberellin as a mechanism to control hypoxia tolerance in Carrizo rootstock

Hypoxia induces anatomical or morphological adaptations, including aerenchyma formation, in addition to increasing ethylene production. On the other hand, anoxia decreases ethylene formation due to the requirement of oxygen in the conversion of ACO to ethylene. Although ethylene has been shown to have an important role in flooding symptomatology of several herbaceous and some woody plants, more work is needed to clarify the role of ethylene in the responses of fruit trees. In fact, in addition to avocado (Schaffer 2006), no more references have currently been reported describing the modification of ethylene in roots of other fruit trees under oxygen deficiency. In our experimental conditions, a reduction of expression of ACO in TCCA compared with WTA was observed, contrary to what was widely reported. The role of ACO as a signal from the roots to shoots was well documented, for example, in waterlogged tomato (Bradford et al. 1982; Bradford and Yang 1980). The different behaviors related to tissue, developmental stages, stress, and plant species have to be extensively reported (Rudus et al. 2013).

The relationship between the induction of ubiquitin-dependent protein and ethylene in response to hypoxic conditions has been recently reported (Hsu et al. 2011). In the present manuscript, we reported a very high overexpression of polyubiquitin under hypoxia conditions, exclusively in TCCA. This also sustains the hypothesis provided by Mazzucotelli et al. (Mazzucotelli et al. 2006), according to whom, the involvement of ubiquitin is potentially regulated by hypoxia and anoxia. Moreover, massive protein degradation could support glutamate supply for non-cyclic TCA flux, as mentioned above.

Auxin is known to be involved in the growth for the distension of the cellular wall, of lateral roots, in the cellular division of apical root meristem, vessel ramification, and in the induction of ethylene synthesis. Under oxygen deficiency, the modulation of transcripts related to plant growth regulators (auxins and gibberellins) is known to be strongly suppressed (Armstrong and Drew 2002; Hsu et al. 2011). According to these findings, in our work, most of the genes differentially expressed specifically in TCCA, such as auxin and gibberellin regulators (auxin-induced protein and gibberellin receptor), have been reported to be downregulated, supporting previous evidence and also suggesting that the hypoxia conditions could alter the cell wall structure with consequent effects on root growth arrest and suppression of lateral root formation (Nanjo et al. 2011). These data lead to the hypothesis that the reduction of oxygen (strongly) limits the functionality of plant growth regulators. In contrast to our data, in Arabidopsis, the induction of several auxin-responsive genes by low-oxygen stress was reported (Liu et al. 2005). Otherwise, in agreement with our data, Loreti et al. (Loreti et al. 2005) found that some genes coding for auxin-responsive items resulted in repression by hypoxia and the addition of sucrose was reported to reduce the damaging effects of hypoxia. In addition to ACO, the simultaneous inhibition of "auxin induced" (id_1909, id_5587), "auxin associated-like protein" (id_2518), "SAUR family protein" (id_2189), and "auxin-induced protein" (id_5576) supports the hypothesis about a real ethylene-auxin cross-talk also for citrus, as previously reported by Hu et al. (Hu et al. 2017) and Abts et al. (Abts et al. 2017) in *Arabidopsis* and sugar beet. It, therefore, could be deduced that a close relationship exists between the activation of genes containing auxin and ethylene-responsive elements and the involvement of genes regulating the synthesis, transport, signaling, or response of hormones.

TFs network plays a key role in the response of Carrizo seedlings to hypoxia

Within the framework used to explore which mechanisms are modified in plants under oxygen-deficit conditions, hypoxia-responsive TFs represent primers for the investigation of the regulation of the hypoxic response (Licausi and Perata 2009). The main hypoxia-responsive TFs belong to the MYB, NAC, PHD (plant homeodomain), and ERF (ethylene responsive factor) families (Licausi et al. 2011).

Our data highlighted the differential expression of bHLH, Ap2, MYB, F-box/LRR-repeat protein, leucine-rich repeat-containing, bZIP, ERF, ZnF, and abi3 inducing to thinking that a potential involvement of transcription factors could be really hypothesized. AP2/ERF represents one of the largest groups of TFs in plants known to be induced by biotic and abiotic

stresses, including hypoxia and the stress-related hormones ethylene, jasmonic acid and ABA.

PDC along with TFs, miRNA166, and correspondent target HD-zip could be responsible for the higher tolerance of transformed Carrizo to hypoxia conditions

Data reported up to now supports that there are almost no significant differences between transformed and wild type CC in in vitro plants under hypoxia conditions. Nevertheless, there are some very interesting data uniquely differentially expressed in TCCA sample, bringing to the hypothesis that a higher putative tolerance could be due to the insertion of Mybleu.

A core-hypoxia-responsive gene was established in *Arabidopsis*, including PDC (PDC1 and PDC2) and ADH (Banti et al.2003). In our experiment, PDC is highly and exclusively expressed in TCCA. As previously reported in other plants, also in CC, the PDC transcription could also be considered the metabolic control point in the alcohol fermentation pathway, supporting its role as the control step in ethanol fermentation.

The deficit of oxygen prevents the utilization of pyruvate oxidative and induces an accumulation of glycolytic intermediates, as demonstrated by the overexpression of PGI and PK, shifting the metabolism in favor of glycolysis and alcohol fermentation, instead of other non-oxidative metabolisms. Pentoses are recycled into glycolytic intermediates through transketolase and transaldolase, generating a reversible link between the pentose phosphate pathway and glycolysis. These data fit well with the increased expression of transaldolase (id_4613) and sugar transporter ERD6 (id_1266) recorder in response to anoxic conditions exclusively in TCC.

Among TFs, TCCA is characterized by the high expression of the RAP2-3 gene (id_3162), supporting the knowledge that RAP2 represents a true molecular switch for the early response to hypoxia. While RAP2.12 mediates the quick activation of the low-oxygen response, HRE1/2 could instead be involved in the long-term acclimation of plants to prolonged oxygen deprivation. These data lead to hypothesize that transformed plants could have a major ability to tolerate low-oxygen levels at an earlier stage. Moreover, it was reported in *Arabidopsis* that AP2-18 and AP2-19 interact with class III HD-Zip such as PHAVOLUTA, PHABULOSA, REVOLUTA, and ATHB8. In our experiment among the downregulated genes, we found a gene that was homologous to Homeobox-leucine zipper protein ATHB-15 (id_4888), belonging to the group of PHABULOSA and REVOLUTA of *Arabidopsis*, corresponding to Cs1g15640 in the sweet orange genome. Among all the functions, Wu et al. (Wu et al. 2016) described that Cs1g15640 is also involved in root elongation. As previously described, also in CC rootstock, we found a reduced expression of genes involved in the cell wall modification and auxin metabolism, in this way reducing both the unnecessary lengthening of roots and the unnecessary use of energy. Therefore, the overlapping of the down-expression data of the homologous gene AtHB15 supports the hypotheses previously formulated. Moreover, *ATHB8/14/15* (Cs4g19310, Cs2g09770, Cs1g15640) are reported to function as targets of csi- miR166d and are involved in the vascular development and auxin signaling (Baima et al. 2014; Ohashi-Ito and Fukuda 2003). It is known that ATHB-15 is the target of the mir166 (Singh et al. 2017). Among unknown genes, we also found (exclusively in TCCA) a gene (id_349) whose investigation on miRNA and correspondent target database resulted to be homologous to mir166. MicroRNAs (miRNAs), 20–24 nucleotides in length, are a class of single-stranded and small regulatory RNA molecules, regulating the expression of target genes mainly at post-transcriptional levels. Thus, miRNAs mediate most of plant cellular and metabolic processes via regulating post-transcriptional gene silencing (Jones-Rhoades et al. 2006; Reinhart et al. 2002). MiR166s are highly conserved in plants (Xie et al. 2012) as well as the target HD-Zip III family genes, such as REVOLUTA, PHABULOSA, PHAVOLUTA, CORONA, and ATHB8. To date, miR166s act on their targets by controlling various developmental processes negatively (Li et al. 2017). This means that also in our case, the overexpression of mir166, interacting with ATBH15, induces its degradation and as a consequence, the lengthening of the roots, thus also confirming data related to auxin and all of the genes involved in the root and cell wall modifications.

Concluding remarks

Very few papers on fruit trees, and even less on *Citrus* and its relatives, are available on the evaluation of transcriptional modifications under oxygen deficiency conditions. Even though microarray is no longer considered one of the latest, widest, and highly performing approaches to investigating differently expressed genes, the use of a Combimatrix platform, as well as investigating genes involved in the citrus iron chlorosis (Licciardello et al. 2013), helped in the identification of strategically involved genes under hypoxia conditions. These findings highlight that CC rootstock globally reconfigures its molecular machinery under hypoxic stress by selectively synthesizing the necessary coding transcripts for enzymes that produce and conserve energy. The principal and most interesting data supported the fact that CC tolerate hypoxia conditions, with or without the insertion of Mybleu gene. Nevertheless, transformed CC seedlings showed a better aptitude to tolerating hypoxia conditions thanks to the activation of typical fermentative products, such as ADH and

especially PDC. The identification of an HD-Zip TF, working as a target of mir166, never reported before, represent the main discovery in the present paper. We suppose that further investigation is required to better identify a network of genes to be used for new biotechnology approaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Data archiving statement Microarray expression data are available at NCBI GEO database (Edgar et al. 2002) with GEO number GSE86208.

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