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ORIGINAL RESEARCH

Expression Profile Analysis of Hypoxia Responses in *Arabidopsis* Roots and Shoots

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Abstract Physiological and molecular adaptation mechanisms enable plants to improve their survival under harsh conditions, including low oxygen levels caused by flooding. When *Arabidopsis* was exposed to hypoxia, we observed hyponastic response, shoot elongation, leaf chlorosis, and inhibited growth. To understand this response, we used a specialized complementary DNA microarray from our laboratory to examine the time-dependent profiles of gene expression in *Arabidopsis* roots and shoots. From this, we identified 282 hypoxia-responsive genes. These included novel genes for a zinc finger protein, WRKY family transcription factor, and glycosyl hydrolase as well as those previously identified as hypoxia-related genes including alcohol dehydrogenase (*ADH*), pyruvate decarboxylase (*PDC*), and phosphofructokinase. Cluster analysis of these profiles suggested that the hypoxia response occurs in two distinctive phases: early and late. The early response to imposed stress (hours 1, 3, and 8) includes increased expression of fermentation-related genes and transcription factors, such as by members of the C_2H_2 zinc finger family and WRKY family. The late response (hours 24 and 72) involves the down-regulation of genes that function in secondary metabolic pathways and up-regulation of transcription factors that are mostly related to the ethylene-responsive element binding protein family. Mutants of *Arabidopsis* defective in sucrose synthase1 (*SUS1*), the At1g05060 gene (with unknown function), *ADH*, and the *WRKY33* were more sensitive to hypoxic stress, evidence of the importance of these genes in that response. The genes presented here allow us to deepen our understanding of the mechanism for this stress response and, eventually, will aid in the development of more flood-tolerant crops.

Keywords *Arabidopsis thaliana* · cDNA microarray · Flooding · Hypoxia response · Hypoxic stress

Plants are routinely exposed to environmental stresses such as low temperatures, heat, drought, high salinity, and flooding, all of which can affect their development. Among these, flooding is one of the most severe factors to reduce crop productivity. Oxygen is diffused about 10,000 times more slowly in water than in air, so plants suffer greatly and immediately initiate hypoxic stress responses when they inhabit water-logged soil (Armstrong 1979).

The most obvious changes after flooding are leaf chlorosis, necrosis, defoliation, cessation of growth, and premature plant death (Huynh et al. 2005). Expression levels of several plant growth regulators and other signaling molecules can also be interrupted (Dat et al. 2004). These flood-induced alterations in physiology and morphology are apparently due to enhanced ethylene production, which then causes shoot extension, the development of aerenchyma,

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and initiation of adventitious roots (Vartapetian and Jackson 1997). In plants, ethylene is a prime signal required for adventitious root growth in deepwater rice (*Oryza sativa*) and aerenchyma formation in maize (*Zea mays*) roots (Voesenek et al. 1993; Lorbiecke and Sauter 1999).

Plants respond to hypoxia metabolically as well as morphologically. For the former, sucrose catabolism, glycolysis, and the fermentation pathway are important for energy conservation (Sachs et al. 1980; Kennedy et al. 1992). Fermentation pathways to ethanol and lactate are necessary for the re-oxidation and recycling of NADH when O_2 is less available. Such responses have been shown in several species, including maize, rice, barnyardgrass (*Echinocloa crusgalli*), dock (*Rumex palustris*), and *Arabidopsis thaliana* (Perata and Alpi 1993; Dolferus et al. 1997, 2003; Ellis et al. 1999; Visser et al. 2003). The plant response to hypoxia also requires increased expression of genes that encode for transcription factors, signal transduction components, non-symbiotic hemoglobin, ethylene biosynthesis, cell wall loosening, and nitrogen metabolism (Voesenek et al. 1993; Mattana et al. 1994; Saab and Sachs 1996; Visser et al. 1996; Baxter-Burrell et al. 2002; Dordas et al. 2003, 2004; Liu et al. 2005). Transcriptome analysis of *A. thaliana* has revealed some hypoxia-related genes (Klok et al. 2002; Branco-Price et al. 2005; Liu et al. 2005; Loreti et al. 2005). Nevertheless, despite having an intensive impact on the various adaptations to hypoxia in plants, the molecular understanding of this response remains limited. Therefore, to gain more comprehensive insight, we used a specialized complementary DNA (cDNA) microarray developed in our laboratory to examine gene expression profiles for *Arabidopsis* under hypoxic stress.

Materials and Methods

Plant Materials and Growth

A. thaliana ecotype Columbia (Col-0) was used as our wild-type (WT) control. We obtained transfer DNA (T-DNA) insertional mutant lines of *SUS1* (SALK_014303), the At1g05060 gene with an unknown function (SALK_034347), *ADH* (SALK_066824), and *WRKY33* (SALK_064436) from the *Arabidopsis* Biological Resource Center at The Ohio State University (Columbus, OH, USA; Supplemental Fig. 1). Seeds of all genotypes were surface sterilized in 0.05% Tween-20 and washed five times with sterile water. The imbibed seeds were incubated under darkness at 4°C for 3 days. Afterward, they were germinated in one-half strength Murashige and Skoog media (Duchefa, Haarlem, The Netherlands) containing 1% phytoagar and were cultured at 23°C under continuous light (approximately 100 μmol m^{-2} s^{-1}) in an environmentally controlled growth chamber.

Treatments with Low Oxygen and Submergence

For low-oxygen treatment, plants were grown on plates for 2 weeks in an environmentally controlled growth chamber at 23°C and under continuous light (approximately 100 μmol m^{-2} s^{-1}). The plates were then transferred to a darkened vacuum chamber at 23°C. Gas mixtures (5% O_2/95% N_2 or 0.1% O_2/99.9% N_2) were flushed into this air-tight chamber to retain low-O_2 conditions. The oxygen concentration in the chamber was monitored by an XP-3180 oxygen meter (Cosmos, Osaka, Japan).

For the submergence treatment, plants were grown in soil for 3 weeks in an environmentally controlled growth chamber at 23°C under continuous light (approximately 100 μmol m^{-2} s^{-1}). They were then entirely submerged in a tank of 25-cm-deep water and held for 7 days at 23°C under continuous light (approximately 100 μmol m^{-2} s^{-1}).

Measurement of Chlorophyll Content

Chlorophyll was extracted from untreated (control) and hypoxia-treated plants by boiling the shoots in 95% ethanol at 80°C. The concentration of total chlorophyll per fresh weight of the shoots was calculated as described by Lichtenthaler (1987): Total chlorophyll (mg g^{-1}FW) = $[5.24\ (OD_{664}) + 22.24\ (OD_{648})] \times 8.1/FW$, where, OD is the optical density at 664 or 648 nm and FW is the fresh weight of a shoot sample.

RNA Isolation and Purification

Total RNA was isolated with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Hypoxia-treated root (or shoot) samples were homogenized in the presence of liquid nitrogen and were lysed in buffer containing guanidine isothiocyanate. The lysed samples were placed in the RNeasy column and washed with an ethanol-containing buffer. Total RNA was eluted with RNase-free water. For ethanol precipitation, 1 mL of 95% ethanol was added, and the total RNA sample was held at −80°C for 20 min. After centrifuging at 12,000×*g* for 15 min at 4°C, the entire RNA pellet was washed with 1 mL of 70% ethanol and centrifuged at 12,000×*g* for 5 min at 4°C. The pellet was then dissolved in RNase-free water. The concentration and purity of this isolated total RNA were determined by measuring absorbances at 260 and 280 nm.

Semi-quantitative RT-PCR

Total RNA (2 μg) was extracted from roots exposed for 0, 1, 3, 8, 24, or 72 h to 5% O_2/95% N_2. After DNase treatment, the RNAs were reverse transcribed with Superscript®II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The target cDNA was PCR-amplified in a 20-μl reaction volume containing 2 μl of 10× PCR buffer, 25 mM of each dNTP, 1 μl of cDNA, and 1 U of *Taq* DNA polymerase (Takara, Shiga, Japan), plus 8 pmol of the appropriate primer sets (see Supplemental Table 1). PCR conditions included 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Microarray Hybridization and Image Acquisition

Total RNA (5 μg) was reverse transcribed using a reverse transcription (RT) primer tagged with either a Cy3- or Cy5-3DNA capture sequence from Array 350 MPX Expression Array Detection Kits (Genisphere, Hatfield, PA, USA). The synthesized, tagged cDNAs were then fluorescently labeled with Cy3- or Cy5-3DNA based on the sequence complementary to the 3DNA capture reagent, which contained an average of 375 fluorescent dyes. The labeled cDNA was hybridized on a version-3-specialized cDNA microarray, which included additionally 110 flooding stress-related cDNA probes (Klok et al. 2002) on 1,482 cDNA probes of a version-2-specialized cDNA microarray as described by Eom et al. (2006). The hybridization and washing procedures were performed according to the Genisphere technical protocol. After washing, the slides were immediately scanned with ArrayWoRx (Applied Precision, Issaquah, WA, USA). To maximize the camera's dynamic range without saturation and to normalize the two channels for signal intensity, we adjusted the exposure setting so that the intensity level of the brightest spot on a slide was 80–90%. Experiments were performed with two replicates, which generally used the same material except for the dye labels, which were reversed between duplicate samples for each microarray.

Analysis of cDNA Microarray Data

Intensity values were quantified from the pairs of TIFF image files from each channel, using ImaGene software (Version 5.6; BioDiscovery, Los Angeles, CA, USA). Analyses were conducted with a GeneSight software package (Version 4.1; BioDiscovery, Los Angeles, CA, USA). For each slide, the local background was subtracted from the signal intensity, and the minimum intensity was raised to 20 via a "floor" function. The mean intensity for each element was normalized by the locally weighted scatterplot smoothing method in this GeneSight software.

Results

Effects of Hypoxic Stress on *Arabidopsis* Growth and Development

We investigated the hypoxia-related changes in *Arabidopsis* under treatment with a low-oxygen atmosphere or complete submergence. In one set of experiments, 2-week-old plants initially grown under normal conditions were then placed in a darkened incubator maintained with 5% O_2/95% N_2 for 1–72 h. Although their survival was not affected by this, several morphological changes were observed in the first 8 h, including shoot elongation, hyponastic responses, and leaf chlorosis (Fig. 1a). For the second set of experiments, plants were completely submerged in water for 7 days, leading to even more dramatic alterations both morphological and developmental. The most obvious was elongation of the petiole (Fig. 1b), which was enhanced by about 50% compared with our untreated control. However, widening and lengthening of the leaves themselves was slightly inhibited by this treatment. Bolting frequency was also affected, being decreased by 60% while that of the untreated plants was at 100% (Fig. 1c).

Comparison of Hypoxic Stress Responses between Roots and Shoots

Hypoxia responses differ between roots and shoots. In the former tissue type, genes involved in the fermentation pathway are highly expressed, while those in the latter are not (Dolferus et al. 1994; Ellis et al. 1999). Our RT-PCR results also confirmed that two fermentation-related genes (*ADH* and *PDC1*) were differently expressed in the shoots and roots (Fig. 2). In the roots, induction of both was detectable within 1 h under hypoxic conditions, then lasting up to 24 h, whereas their induction was barely detectable in the shoots.

For a more complete analysis of the differential expression patterns in roots and shoots under hypoxia, we used a specialized cDNA microarray to compare their levels of transcripts after 0, 3, and 72 h of stress. The expression profiles of previously identified hypoxia-induced genes also were examined for tissue-specific responses (Table 1). Ethylene-related genes, such as ethylene-responsive element binding protein (*EREBP*) and ACC oxidase (*ACO*), were more highly expressed in the shoots. By contrast, transcripts of fermentation-related genes (*ADH*, *PDC1*, and *PDC2*), as well as carbohydrate metabolism-related genes (*SUS1*; phosphofructokinase; glutamate decarboxylase 1, *GAD1*; and glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*) were more abundant in the roots. In general, these well-known hypoxia-induced genes were more highly expressed in the roots than in the shoots.

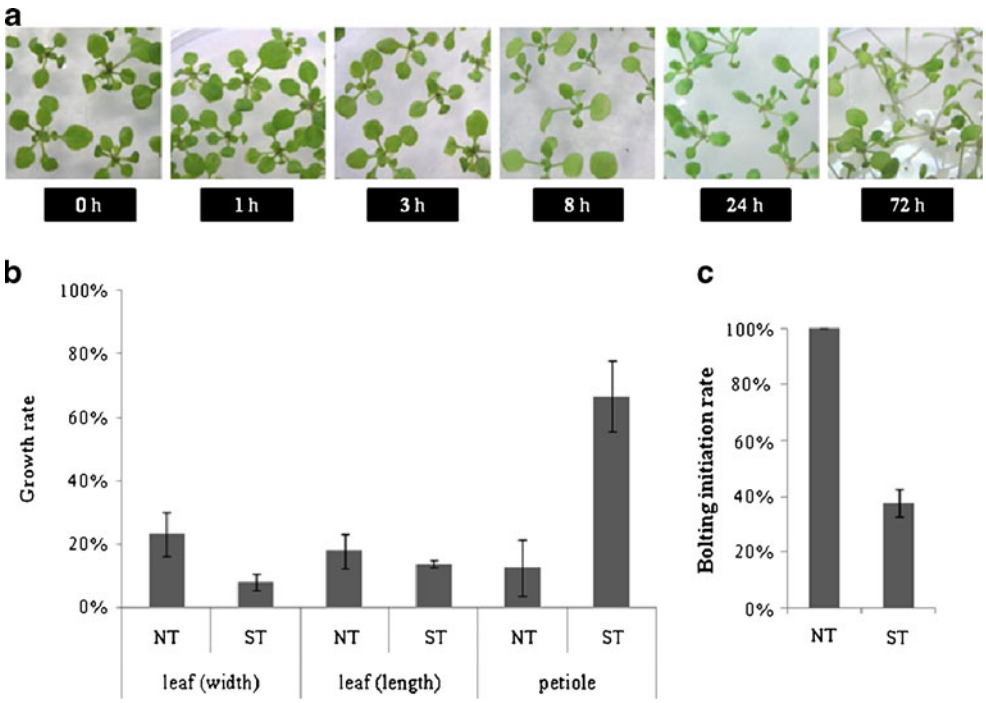

Fig. 1 The morphological and developmental changes of *Arabidopsis* under hypoxic stress. **a** Effects of hypoxic stress on morphology. Two-week-old plants were exposed to hypoxia (5% O_2/95% N_2) for 0, 1, 3, 8, 24, and 72 h in vacuum chamber at 23°C in the dark. Photographs were immediately taken after hypoxic treatment. **b** Morphological change of leaf and petiole by submergence. Three-week-old plants were submerged for 7 days. After the submergence, the leaf width, leaf length, and petiole length of plants were measured for comparison with untreated ones. Growth rate (%)=[change of length (or width)/initial length (or width)]×100. Experiments were repeated three times, and more than ten plants per set were tested in each experimental condition. **c** Bolting frequency under submerged condition. Bolting initiation rate (%)=(number of bolted plants/total number of plants)×100. Experiments were duplicated, and more than ten plants per set were tested in each experimental condition. *NT* non treatment, *ST* submergence treatment

Gene expression was compared between tissues under normal and hypoxic conditions, and those genes were annotated based on the functional classification of MapMan ontology (Thimm et al. 2004; Fig. 3). The 22 functional categories could be divided into three groups based on prominent tissue-specific responses that occurred late in the experimental period (hour 72). Such a response was evident for 13 out of 22 categories (groups A and B) that were predominantly represented by either of the tissue types. In group A of prominent root-specific response under hypoxia stress, expres-

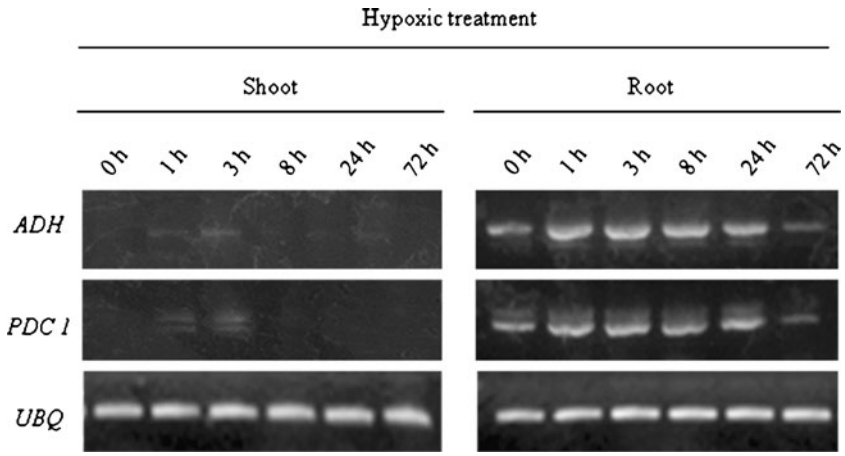

Fig. 2 RT-PCR analysis of expression *ADH* and *PDC* under hypoxic condition. Two-week-old plants were exposed to hypoxic stress (5% O_2/95% N_2) for 0, 1, 3, 24, and 72 h. The isolated total RNA (2 μg) in roots or shoots was reverse transcribed and then used for the PCR analysis. The transcripts for the target genes (*ADH* and *PDC1*) were detected using specific primers (Supplement Table 1). The *UBQ10* was used as a control

Table 1 The expression profiles of previously identified hypoxia-induced genes in roots and shoots

Functional group	Gene ID	Locus ID	Description	Relative expression value (log_2; shoots/roots)		
				HT00	HT03	HT72
Hormone metabolism ethylene	LRU30	At3g16770	Ethylene responsive element binding protein	0.82	1.25*	1.44*
	PT62	At1g05010	ACC oxidase (*ACO*)	0.77	1.58*	1.12*
Fermentation	L48_1	At1g77120	Alcohol dehydrogenase (*ADH*)	−0.61*	−1.71**	−0.35
	F40	At4g33070	pyruvate decarboxylase 1 (*PDC1*)	−4.77**	−4.75**	−3.12*
	PT79	At5g54960	Pyruvate decarboxylase 2 (*PDC2*)	−1.71*	−2.15*	−0.5
Carbohydrate metabolism	F29	At5g10830	Sucrose synthase/sucrose-UDP glucosyltransferase (SUS1)	−3.78*	−4.71*	−2.1
	F66	At2g31390	Phosphofructokinase	−4.46*	−3.95*	−2.75
Amino acid metabolism	F80	At5g17330	Glutamate decarboxylase 1(*GAD1*)	−4.85*	−4.81*	−4.47*
Glycolysis	PT34	At3g04120	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	−2.08	−2.3*	−1.95*

Expression values of each gene were represented comparative ratio of transcript levels in shoots per those in roots. The positive value of genes indicates high expression in shoots, and the negative value of genes indicates high expression in roots

HT hypoxic treatment

*$p<0.05$; **$p<0.005$

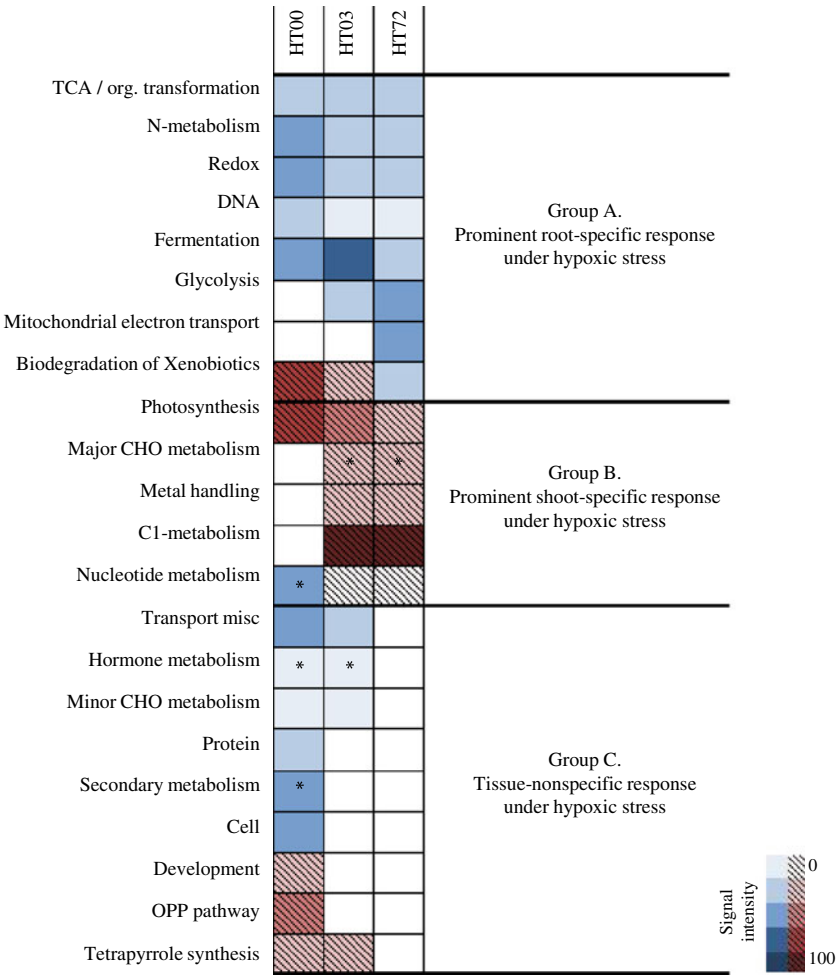

Fig. 3 Functional classification of differentially expressed genes in shoots and roots. The differentially expressed genes in roots or shoots of untreated and hypoxia-treated plant are annotated based on the functional classification of MapMan ontology. The 22 functional categories are grouped based on their tissue-specific responses that occurred late in the experimental period (hour 72). The *blue colored boxes* indicate the up-regulated functional categories in roots. The *red colored* and vertical hatched boxes indicate the up-regulated functional categories in shoots relatively, and *asterisk in box* indicates slightly up-regulated functional categories in other tissue as well as in each tissue. The down- and up-regulated level of functional category is represented different shades of color as shown in *color bar on the bottom right*

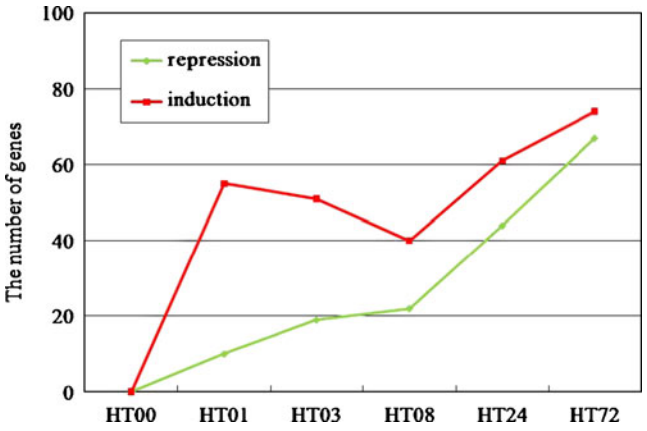

Fig. 4 The number of differentially expressed genes under hypoxic stress. The total number of differentially transcribed genes (more than 1.8-fold) at the six time points was plotted

sion of genes belonging to the functional categories of TCA/organic acid transformation, N-metabolism, redox, and DNA were slightly decreased in roots by hypoxic stress. However, prolonged treatment led to increased expression by genes involved with fermentation, glycolysis, mitochondrial electron transport, and the biodegradation of xenobiotics. That last category, in particular, was associated with up-regulation in the shoots both under normal conditions and at the early time point of stress (3 h) as well as in the roots at the later stages. Group B included genes more strongly expressed in the shoots than in the roots during hypoxia. For shoots, expression of photosynthesis-related genes was decreased while that of genes functioning in major CHO metabolism, metal handling, C1 metabolism, and nucleotide metabolism was increased. Finally, genes from group C showed no tissue-specific responses to prolonged stress. For example, under normal conditions, those in the categories of transport miscellaneous, hormone metabolism, minor CHO metabolism, proteins, secondary metabolism, and cell growth were expressed more in the roots, while those for development, the OPP pathway, and tetrapyrrole synthesis were more highly expressed in the shoots. When hypoxia was imposed, however, genes in all of these categories were similarly expressed in both roots and shoots. Overall, these results demonstrate that two tissue types differ in essential responses to hypoxic stress.

Gene Expression Profiles on Hypoxic Stress

Because responses to hypoxia varied between tissues and survival relied more upon the status of the roots, we examined changes in the transcriptome during 1, 3, 8, 24,

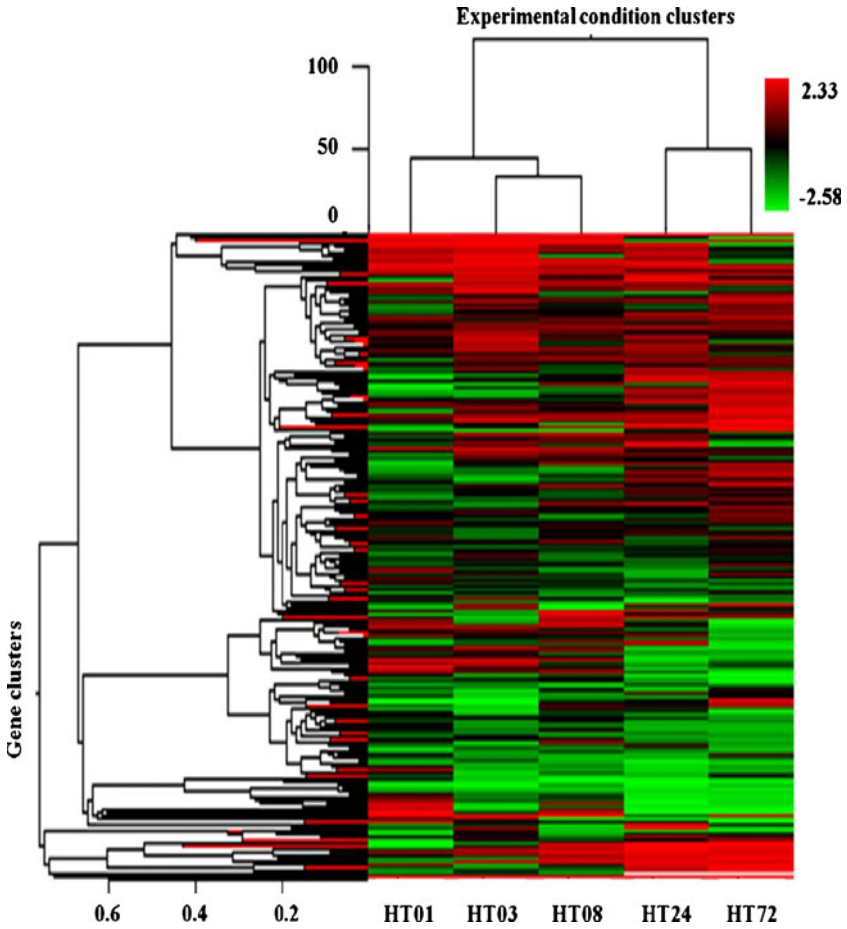

Fig. 5 Hierarchical cluster analysis of gene expression pattern under hypoxic stress. Only 282 hypoxia-responsive genes during hypoxia acclimation were clustered by Euclidean distance. Logarithmic scales indicating the color assigned to each fold change are shown to the right of cluster

and 72 h of stress. Our cDNA microarrays showed a fold change of at least 1.8 for 282 genes expressed in root samples (Supplemental Table 2), with the total number rising as treatment was prolonged (Fig. 4). Expression was confirmed by RT-PCR for five genes previously identified as hypoxia-regulated, i.e., *ADH*, *PDC1*, *PDC2*, *SUS1*, and *ETR2* (Supplemental Fig. 2).

Hierarchical clustering of these 282 genes revealed that their responses could be divided into two distinctive stages: "early response," within 8 h of stress imposition, and "late response," corresponding to 24 or 72 h (Fig. 5). Using two-dimensional self-organizing map (2D-SOM) analysis, we further categorized them into six clusters according to their expression profiles (Fig. 6). The characteristics of those clusters were described based on MapMan ontology, and the five most highly induced or repressed genes within each cluster were listed in Table 2. Cluster 1 included genes for which expression drastically declined within the first hour and then remained repressed for up to 72 h. Genes in that cluster were mostly related to secondary metabolic pathways and stresses. For clusters 2, 4, and 5, expression was typified by undulating changes under hypoxic stress, and covered primarily photosynthesis, proteins, and miscellaneous genes associated with stress, respectively. Cluster 3 included genes for which expression increased steadily in response to hypoxia, being directly or indirectly involved in the regulation of transcription. Finally, cluster 6 contained genes that showed initially rapid increases in expression before slowly decreasing as the stress continued. It included genes related to transcription factors as well as fermentation.

The Effect of Knocking-Out Candidate Genes on Sensitivity to Hypoxic Stress

To address whether the hypoxia-responsive genes identified by our microarray analysis indeed play a role in hypoxia sensitivity, we selected four genes, such as *SUS1* (At5g20830), a At1g05060 gene with unknown function, *ADH* (At1g77120), and *WRKY33* (At2g38470). Using T-DNA insertion mutant lines confirmed homozygous (Supplemental Fig. 1B), we examined the effects of knocking-out genes.

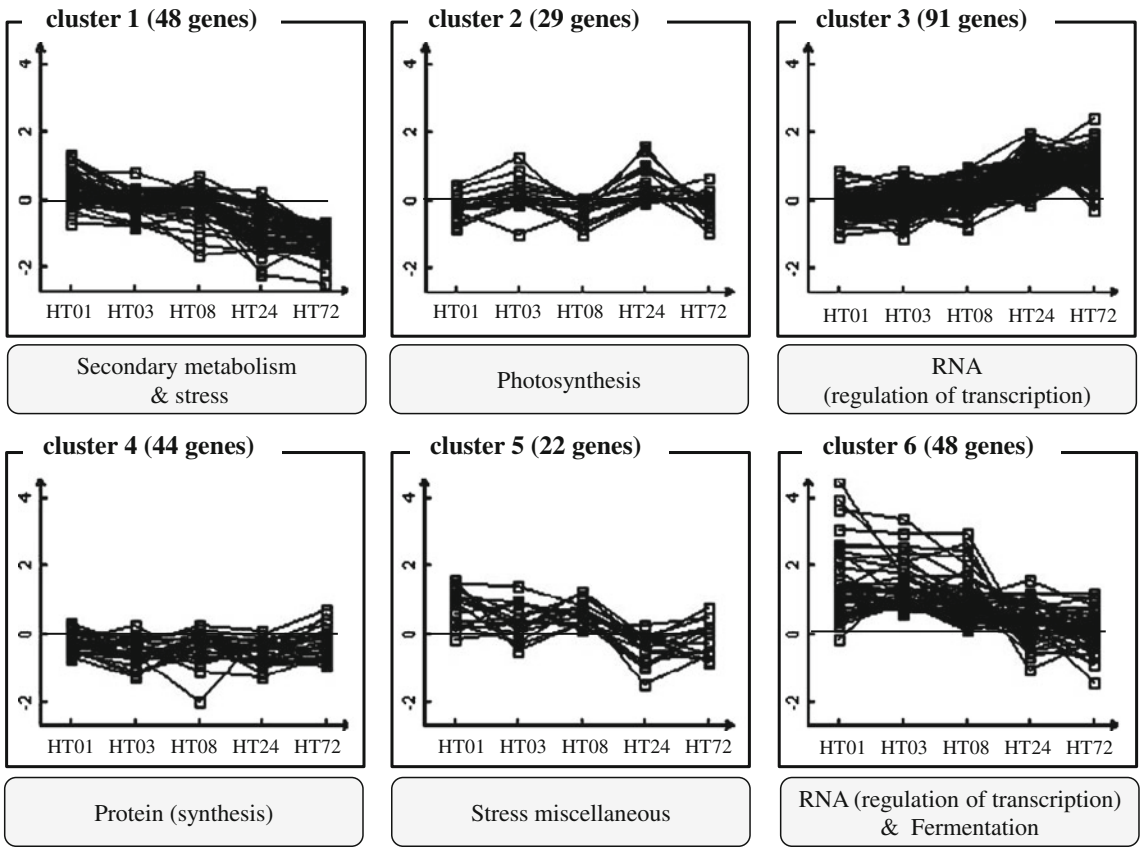

Fig. 6 The 2D-SOM cluster of 282 differentially expressed genes. The hypoxia-responsive genes were categorized into six clusters according to their expression profiles during the time course of the hypoxic treatment. The functional terms in a box at the bottom of clusters indicate characteristics of genes included in each cluster. The characteristics of those clusters were described based on MapMan ontology

Table 2 The five most differentially expressed genes within six clusters

Gene ID	Locus ID	Description	Expression level (log_2)				
			HT01	HT03	HT08	HT24	HT72
Cluster 1							
G10	At4g22880	Putative leucoanthocyanidin dioxygenase (LDOX) mRNA	0.93	−0.19	0.31	−2.31*	−2.56
LIU4	At5g13930	Chalcone synthase (naringenin chalcone synthase; testa 4 protein; MAC12.14)	0.63	−0.29	−0.19	−1.61	−1.22
LIU78	At5g05270	Chalcone-flavanone isomerase family protein	0.17	−0.19	−0.08	−0.73	−0.84
S18	At5g15970	Cold-regulated protein COR6.6 (stress-induced protein KIN2)	1.07	0.10	−0.20	−1.39	−1.63
G3	At5g52310	lti140 mRNA for a 140 kDa cold acclimation related polypetide	0.06	0.14	−0.76	−0.73	−1.26
Cluster 2							
2945_1	At1g29920	CAB2; chlorophyll binding	−0.34	−0.02	−0.62	1.50*	−0.66
3579	At1g29910	Chlorophyll a/b binding protein (F1N18.5) mRNA	−0.38	−0.03	−0.65	1.70	−0.34*
LRU22	At3g08940	Lhcb4.2 protein (Lhcb4.2) mRNA, complete cds	−0.45	0.53	0.17	0.90	0.24
L54-1	At2g21620	mRNA for RD2 protein	0.26**	0.81	−0.06	0.35	0.04
PT105	At2g14610	PIP; aminopeptidase/catalytic/hydrolase/prolyl aminopeptidase	−0.64	−0.38	−0.63	1.78	−0.33
Cluster 3							
3499	At3g16770	mRNA for ethylene-responsive element binding protein	−0.37	0.19*	0.21	1.00	1.34
PT28	At3g16770	AP2 domain-containing protein	−0.61	0.16	0.47	1.41	1.91
F30	At3g14230	AP2 domain-containing protein RAP2.2 (RAP2.2)	−0.27	−0.13	0.21	0.60	0.89
F96	At3g26720	Glycosyl hydrolase family 38 protein	−0.55	−0.26**	0.21	1.35*	1.27
LRU64	At4g02520	Atpm24.1 mRNA for glutathione S transferase	−0.62	−0.05	−0.14	0.57	1.43
Cluster 4							
S24	At2g34480	60S ribosomal protein L18A (RPL18aB) mRNA	0.00	−0.29	0.10	−0.17	−0.80
S30	At3g46740	Chloroplast import-associated channel protein homolog mRNA	−0.29	0.22	−0.90	−0.40*	−0.24
S31	At3g53020	60S ribosomal protein L24 (RPL24B) mRNA	0.14	−0.26	0.19	−0.22	−0.98
S5	At1g77940	60S ribosomal protein L30 (RPL30B)mRNA	−0.11	−0.13**	−0.09	−0.29*	−0.80
2898_1	At1g07920	Elongation factor 1 alpha (EF 1 alpha) mRNA	−0.24	0.00	−0.87	−0.03	−0.25
Cluster 5							
3623	At5g13690	Alpha N acetylglucosaminidase (At5g13690) mRNA, complete cds	1.01	0.39	0.15	−0.51	0.06
2923	At4g31500	mRNA for cytochrome Q450 monooxygenase	0.00	0.00	1.98	−0.03	0.00
F97	At4g20830	Reticuline oxidase-like protein	1.26	0.55	0.70	−0.58	−0.63
L95-1	At1g79920	Putative heat shock protein (F19K16.12) mRNA	0.45	0.82	0.19	0.36	−0.11
AA042289	At2g46520	Cellular apoptosis susceptibility protein (importin alpha re exporter)	−0.25	−0.04	1.21	−0.12	0.39
Cluster 6							
L48-1	At1g77120	Alcohol dehydrogenase (ADH)	1.26	2.18*	0.88	0.79	−0.72
F40	At4g33070	Pyruvate decarboxylase1(PDC1)	3.61*	3.35	1.99	0.35	−1.53**
PT79	At5g54960	Pyruvate decarboxylase1(PDC2)	1.86	1.77	0.39	0.28	−0.95
3356_1	At1g27730	mRNA for ZAT10 protein	2.12	2.32*	2.25	−0.16	0.49
F101	At2g38470	WRKY family transcription factor	1.40	1.13*	1.30	−0.43	0.15

The five most highly expressed genes are listed with both description of genes and expression level. The complete list of differentially expressed genes in six clusters is shown in the Supplemental Table 2

HT hypoxic treatment

*$p<0.05$; **$p<0.005$

Under normal conditions, the phenotype of those knockout mutants was similar to WT plants. However, when hypoxic stress was imposed, leaf chlorosis was more obvious in the former (Fig. 7a). Whereas the knockout mutants lost ~40% of their chlorophyll, the WT lost only ~20% (Fig. 7b). This hypoxia-sensitive response by the mutants was also manifested as a decrease in biomass to 56–72% of normal (control) values compared with 88% retention by the stressed WT plants (Fig. 7c). Taken together, our results strongly indicated that these genes

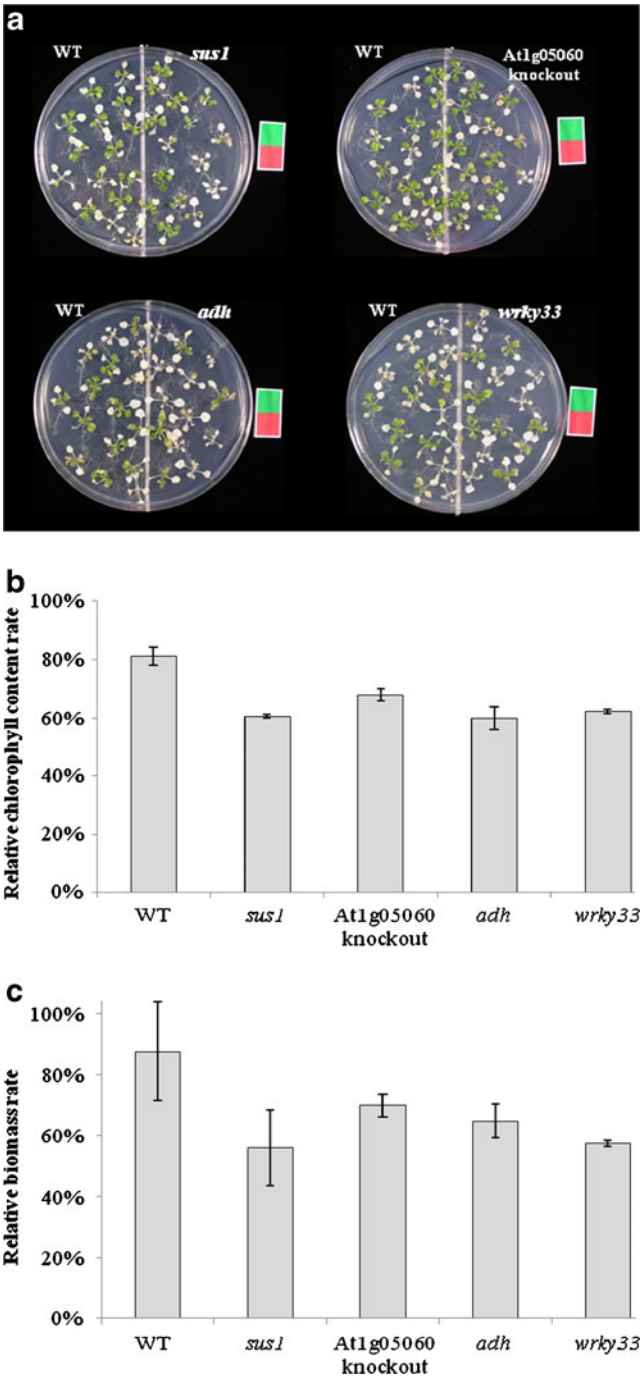

Fig. 7 Effect of hypoxic stress on growth in wild type and T-DNA insertion mutant lines. Two-week-old wild type (WT) and four knockout mutants (*sus1*, At1g05060 gene with an unknown function, *adh* and *wrky33*) were exposed to hypoxia treatment (0.1% O_2/99.9% N_2) for 5 days. **a** Phenotype comparison of WT and knockout mutants in hypoxic treatment. Photographs were taken 2 days after hypoxic treatment. **b** Total chlorophyll content change of WT and knockout mutants after hypoxic stress. Relative chlorophyll content rate (%)=(total chlorophyll content of hypoxia-treated plants/those of untreated plants)×100. **c** Biomass change of WT and knockout mutants after hypoxic stress. Relative biomass change (%)=(biomass of hypoxia-treated plants/that of untreated plants)×100. Experiments were repeated three times, and more than 15 plants per a plate were tested in each experimental condition

play an important role in conferring hypoxia tolerance in *Arabidopsis*.

Discussion

To better understand how plants respond to hypoxia, we first investigated the growth and development of relatively flooding-sensitive *A. thaliana* under stress conditions. When submerged in water or placed in an environment with a low-oxygen concentration, their growth was inhibited, and various morphological changes, e.g., hyponastic responses, shoot elongation, and leaf chlorosis, were very evident. Those alterations in morphology have also been reported as a mechanism for overcoming the effect of complete submergence with wetland rice and *Rumex* sp. (Kende et al. 1998; Almeida et al. 2003; Voesenek et al. 2004).

Plant stress responses are complex processes involving thousands of genes. Therefore, we examined expression profiles for *Arabidopsis* using cDNA specialized micro-array. By comparing transcriptional differences between roots and shoots, we found that well-known anaerobic genes related to fermentation and many previously identified hypoxia-induced genes (Dolferus et al. 1997; Ismond et al. 2003) were expressed more abundantly in the roots. In contrast, genes related to ethylene metabolism were more highly expressed in the shoots. Geigenberger (2003) has shown that, under hypoxia, roots respond by up-regulating metabolite-related genes in order to obtain more ATP. By comparison, under the same stress, shoots will up-regulate genes related to morphological adaptations, including those for ethylene because the accumulation of ethylene is the primary signal for promoting shoot elongation (Musgrave et al. 1972; Kende et al. 1998; Voesenek et al. 2003).

Because hypoxia is more damaging to the root system, we conducted a time trial to follow changes in the root transcriptome and found 282 hypoxia-responsive genes. This also enabled us to cluster genes and identify sets of transcription factors and signal transduction components that could play a role in regulating that response. These genes included AP2/EREBP transcription factors, and members of the WRKY and C_2H_2 zinc finger families, as well as previously identified hypoxia-induced *EREBP*, *ADH*, *PDC*, *SUS1*, and phosphofructokinase (Hoeren et al. 1988; Rowland et al. 1989; Salanoubat and Belliard 1989; Ricard et al. 1991; Dolferus et al. 1997; Porterfield et al. 1997; Ismond et al. 2003; Kürsteiner et al. 2003; Kato-Noguchi 2004). Transcription factors of the C_2H_2 zinc finger and WRKY were induced at the early stages, while those of the EREBP family were induced later. The fact that each stage was associated with a different family of transcription factors suggests that distinctive regulatory pathways are functioning at various times throughout the stress period.

Finally, we examined the role that knockouts play in the hypoxia response. For example, the importance of ethanol fermentation during anaerobic stress has been demonstrated in several species by studying *adh* null mutants, for which survival is lower compared with the WT under hypoxia (Ismond et al. 2003; and in this study). This is because production of ethanol and lactic acid via fermentation is a mechanism by which organisms maintain glycolysis to sustain short-term cell viability (Ismond et al. 2003; Mancuso and Marras 2006). The substrates needed to feed those pathways are also increased by enhancing the flux of glycolysis. Here, knocking-out *SUS1* limited substrate availability, thereby substantially diminishing the tolerance of *Arabidopsis* to anaerobic stress. The same was noted with our *wrky33* mutant. *Arabidopsis* contains 72 WRKY genes that can be divided into three groups and several subgroups according to their WRKY domains and inferred phylogeny (Eulgem et al. 2000). The role of *WRKY33* has already been characterized in pathogen responses (Zheng et al. 2006; Lippok et al. 2007), mediation of NaCl stress (Jiang and Deyholos 2006), and regulation of *WRKY33* activity through a MAPK cascade (Andreasson et al. 2005). However, our research is the first to show that *WRKY33* plays a significant role under low-oxygen stress. In separate experiments, we also determined that expression of the At1g05060 gene was up-regulated upon hypoxia (Supplemental Fig. 3) and that its knockout plants were sensitive to that stress. Using the motif analysis program (http://bioinformatics.Psb.ugent.be/webtools/plantcare/html/) to study its putative promoter region, we found that it contained response sequences for drought, low temperature, abscisic acid, methyl jasmonate, gibberellic acid, and salicylic acid (Supplemental Table 3A). Sequences for putative response elements to heat, low temperatures, wounding, ABA, and SA were also identified in the promoter region of *WRKY33* (Supplemental Table 3B). Interestingly, the promoter regions for both contained an anaerobic-responsive element that is known to be required for anaerobic induction (Walker et al. 1987). This suggests that these genes are involved in responses to general stresses, but especially hypoxia.

In summary, we utilized microarray analysis to find a large number of genes not previously described as hypoxia regulated. We confirmed that some of those genes indeed confer hypoxia tolerance in plants. Together with information from the other independent studies referred to here, our current results from gene expression profiling and functional analysis of key responsive genes provide a more comprehensive understanding of the flooding response in plants. Furthermore, the outcome of this research will aid in developing flooding-resistant or flooding-tolerant crop plants.

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