Global Transcription Profiling Reveals Comprehensive Insights into Hypoxic Response in Arabidopsis[1][w]

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Plants have evolved adaptation mechanisms to sense oxygen deficiency in their environments and make coordinated physiological and structural adjustments to enhance their hypoxic tolerance. To gain insight into how plants respond to low-oxygen stress, gene expression profiling using whole-genome DNA amplicon microarrays was carried out at seven time points over 24 h, in wild-type and transgenic P_{SAG12}:*ipt* Arabidopsis (*Arabidopsis thaliana*) plants under normoxic and hypoxic conditions. Transcript levels of genes involved in glycolysis and fermentation pathways, ethylene synthesis and perception, calcium signaling, nitrogen utilization, trehalose metabolism, and alkaloid synthesis were significantly altered in response to oxygen limitation. Analysis based on gene ontology assignments suggested a significant down-regulation of genes whose functions are associated with cell walls, nucleosome structures, water channels, and ion transporters and a significant up-regulation of genes involved in transcriptional regulation, protein kinase activity, and auxin responses under conditions of oxygen shortage. Promoter analysis on a cluster of up-regulated genes revealed a significant overrepresentation of the AtMYB2-binding motif (GT motif), a sugar response element-like motif, and a G-box-related sequence, and also identified several putative anaerobic response elements. Finally, quantitative real-time polymerase chain reactions using 29 selected genes independently verified the microarray results. This study represents one of the most comprehensive analyses conducted to date investigating hypoxia-responsive transcriptional networks in plants.

Hypoxia occurs when internal oxygen levels fall below their ambient concentration. Poor drainage in soil after transient flooding or irrigation is a common cause of hypoxia, as plants or their roots are under water. Metabolically active cells such as those in the root apical zone are particularly liable to hypoxia because of their higher O_2 demand and fewer intercellular spaces to conduct O_2 by gaseous diffusion (Drew, 1997). The deleterious effects associated with hypoxia, such as a decrease in cellular energy charge, a drop in cytoplasmic pH, and accumulation of toxic end products from anaerobic respiration and reactive oxygen species (ROS) during recovery, are responsible for the slowed growth and reduced yield of many agriculturally important crops in the event of flooding (Subbaiah and Sachs, 2003). Plants have developed adaptation mechanisms at both the physiological and structural levels to enhance their ability to survive prolonged hypoxic stress.

Exposure of maize (*Zea mays*) seedling primary roots to hypoxia has been shown to selectively induce the synthesis of approximately 20 proteins known as anaerobic polypeptides (ANPs), most of which are enzymes involved in sugar metabolism, glycolysis, and fermentation pathways (Sachs et al., 1980). Additional proteins whose rates of synthesis were altered during hypoxic acclimation have been identified from maize root tips using a proteomics approach (Chang et al., 2000). Gene expression is also altered under conditions of limited oxygen supply, and anaerobic response elements along with their binding factors have been identified (Olive et al., 1991; Dolferus et al., 1994; Hoeren et al., 1998; Klok et al., 2002; Paul et al., 2004). Besides those encoding ANPs, additional hypoxia-induced genes identified include transcription factors (de Vetten and Ferl, 1995; Hoeren et al., 1998), signal transduction components (Baxter-Burrell et al., 2002; Dordas et al., 2003), nonsymbiotic hemoglobin (Dordas et al., 2004), as well as those involved in ethylene biosynthesis (Olson et al., 1995; Vriezen et al., 1999; Nie et al., 2002), nitrogen metabolism (Mattana et al., 1994), and cell wall loosening (Saab and Sachs, 1996). Calcium is an essential messenger for the induction of hypoxia response (Subbaiah et al., 1994; Sedbrook et al., 1996), while ethylene plays an important role in alcohol dehydrogenase (ADH) induction during later stages of hypoxia and in long-term structural adaptation mechanisms, such as formation of aerenchyma and adventitious roots in certain species

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(He et al., 1996; Lorbiecke and Sauter, 1999; Drew et al., 2000; Peng et al., 2001). Despite several attempts to engineer plants with improved flooding tolerance using *adh1*, *pdc1*, *myb2*, *GLB1*, P_{SAG12}:*ipt* genes (Zhang et al., 2000; Dolferus et al., 2003; Dordas et al., 2003; Ismond et al., 2003), and microarray studies on subgenome scales using hairy roots (Klok et al., 2002) and shoots (Paul et al., 2004), the regulation of anaerobically induced genes and the molecular basis of the adaptation to low-oxygen conditions have not been fully characterized.

To gain a comprehensive insight into how plants respond to hypoxia at a genome level, we carried out the gene expression profiling at seven time points during a 24-h period following hypoxic treatment using a whole-genome microarray representing nearly all the nuclear, plastid, and mitochondrial genes encoded in Arabidopsis (*Arabidopsis thaliana*). We identified sets of positively and negatively significantly expressed genes (induced and repressed, respectively) in response to hypoxia whose expression shows distinct temporal profiles. Our analyses based on gene ontology (GO; http://www.geneontology.org) terms represented by these responsive genes suggest that hypoxia affects a broad spectrum of functional categories. Promoter analysis of the genes exhibiting similar response profiles expanded the list of genes that potentially are regulated by AtMYB2 and identified some novel putative regulatory elements, suggesting a cross talk between hypoxic response and other signaling pathways.

RESULTS

Validation of the System

Wild-type and flooding-tolerant transgenic P_{SAG12}:*ipt* Arabidopsis plants (Zhang et al., 2000) were grown in liquid media for 13 d and then treated, respectively, with air of ambient composition (21% oxygen, 79% nitrogen, 0.03% carbon dioxide) and of low-oxygen concentration (3% oxygen, 97% nitrogen, 0.03% carbon dioxide) to assess the changes in gene expression in response to hypoxia. At 13 to 14 d old, the plants consisted of two pairs of true leaves and had the primary root lengths of approximately 50 to approximately 60 mm. Both wild-type and transgenic plants appeared green and healthy with no visible signs of distress before and after the treatment. Plant biomass in each flask was 13.11 ± 4.00 g (average ± SD) of fresh weight. We applied low-oxygen stress to plants grown in liquid media primarily to simulate submergence stress and ease the collection of whole plants, as compared with collecting roots in soil-grown or agar-grown plants, and to facilitate a uniform treatment of all plants. To validate the system, we first measured the transcript levels of *adh1*, a molecular marker known to be induced by hypoxia in wild-type plants, during a 24-h period following the gas treatment. The quantitative real-time PCR (qRT-PCR) results (Fig. 1) showed that low oxygen significantly induced the expression of *adh1* starting as early as 30 min after the gas was switched on, whereas the pretreatment control and posttreatment controls kept in ambient air for up to 48 h showed little change in *adh1* expression, demonstrating that the system is appropriate to study the low-oxygen response. All the subsequent treatments from which samples were collected for microarray analysis were performed following the same protocol (at the same rate of gas flow, using plants grown under identical controlled conditions).

Identification of Differentially Expressed Genes

To compare gene expression between samples subjected to normoxia and hypoxia, we used a pooled reference design in which individual experimental mRNA samples were hybridized to a common reference created by pooling an equal amount of mRNA from every test sample. Significance analysis of microarrays (SAM; Tusher et al., 2001) was used to identify genes with significant differences in expression between normoxic and hypoxic samples. SAM uses a modified *t* test to assess the significance of the expression level of each gene on the array, taking into account both its absolute level as well as the SD of the replicates; SAM then uses permutations to estimate the fraction of positive genes identified simply by chance, the false discovery rate (FDR). SAM was run at a median FDR of 0% to identify a set of positively significant genes (PSGs; hypoxia-induced genes) and a set of negatively significant genes (NSGs; hypoxia-repressed genes) at each time point for wild-type and transgenic plants, respectively (Fig. 2). There are 1,266 significant genes

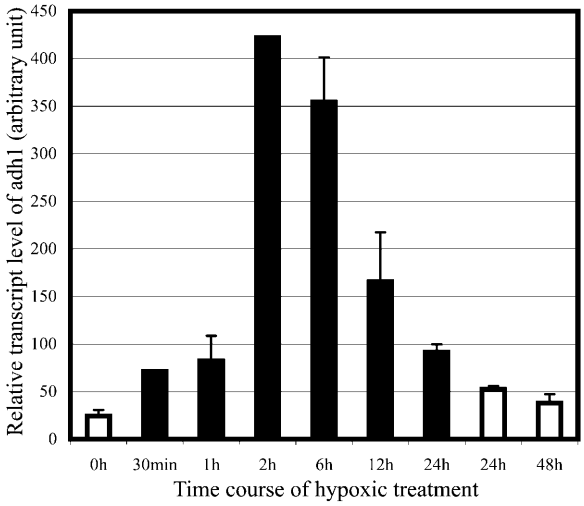

Figure 1. Induced expression of *adh1* gene in wild-type plants in response to low oxygen. White bars represent the level of *adh1* transcript measured using qRT-PCR in plants exposed to air of ambient composition, whereas black bars designate the level of *adh1* transcript in samples treated with 3% oxygen for the duration of time indicated on the graph.

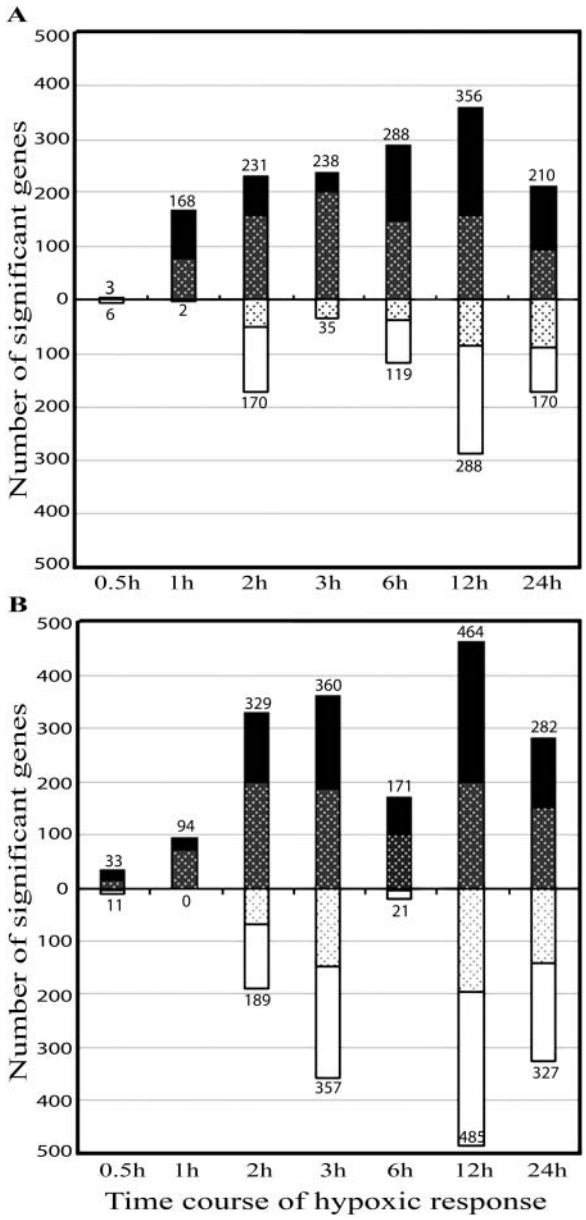

Figure 2. Differentially expressed genes identified using SAM. The number of PSGs and NSGs for wild-type (A) and transgenic P_{SAG12}:*ipt* plants (B) are represented with black bars above and white bars below the *x* axis, respectively, for the time points indicated. The hashed area within each bar designates the number of significant genes within the category whose expression changed by at least 2-fold in response to the low-oxygen treatment.

identified in wild-type plants representing a union of PSGs and NSGs across all the hypoxic time points and 1,732 significant genes in transgenic plants. The union of these two datasets contains 2,085 genes, of which 972 genes have greater than 2-fold, 224 genes have greater than 3-fold, and 105 genes have greater than 4-fold change in expression for at least one time point. The entire list of significant genes is provided in Supplemental Table I. As shown in Figure 2, a number of PSGs were identified starting at 1 h after treatment, whereas NSGs were not identified until 2 h after the hypoxic stress, suggesting that up-regulated genes responded sooner than down-regulated genes to oxygen deficiency.

Temporal Patterns of Expression of Differentially Expressed Genes

Transgenic P_{SAG12}:*ipt* Arabidopsis plants are capable of self-regulating cytokinin production under environmental stresses that trigger onset of premature senescence and have been shown to produce more biomass; accumulate higher levels of cytokinin, Suc, and chlorophyll; and perform better in poststress recovery relative to wild-type plants under conditions of flooding (Zhang et al., 2000). One of our original goals was to analyze the molecular mechanisms underlying the improved flooding tolerance in P_{SAG12}:*ipt* plants. To assess the differences of hypoxic response between wild-type and P_{SAG12}:*ipt* plants, we carried out a two-factor ANOVA analysis using the 2,085 differentially expressed genes, with one factor set as time and the second factor as genotype; only two genes were identified as significantly different between the two genotypes at $P \leq 0.01$ (17 genes were different with $P \leq 0.05$). We also carried out a two-class paired SAM to compare the difference in gene expression between wild-type and transgenic plants across all seven time points, but only five of the 2,085 genes were identified as significantly different between the two genotypes with a median FDR of 38%, suggesting that only three of the five were truly significant. These results suggest that there is a high degree of congruency between the gene expression profiles of the wild type and the transgenic line in response to the 24-h low-oxygen treatment, even though a different number of significant genes was identified at different time points between the two genotypes. Consequently, we focused our analyses on the 2,085 differentially expressed genes and treated the two genotypes as if, for this analysis, they represented biological replicates. While it is known that these genotypes produce different phenotypes over the long term, our data clearly show concordance of early gene transcriptional response to hypoxic stress.

The highly similar gene expression profiles obtained for wild-type and transgenic plants also prompted us to look into whether promoter P_{SAG12} was activated under our experimental conditions. In both wild-type and transgenic plants, *SAG12* gene showed a decreased expression in response to low-oxygen stress, whereas the transcripts of the *ipt* gene were undetectable within the 24-h period of hypoxic treatment as measured using qRT-PCR (data not shown). The results suggest that the low-oxygen treatment for 24 h under our experimental conditions was not sufficient to activate promoter P_{SAG12}, consistent with previous observations that the SAG12 gene was not significantly induced until 3 d after flooding treatment (L.N. Huynh and T. VanToai, unpublished data) and β-glucuronidase (GUS) activity

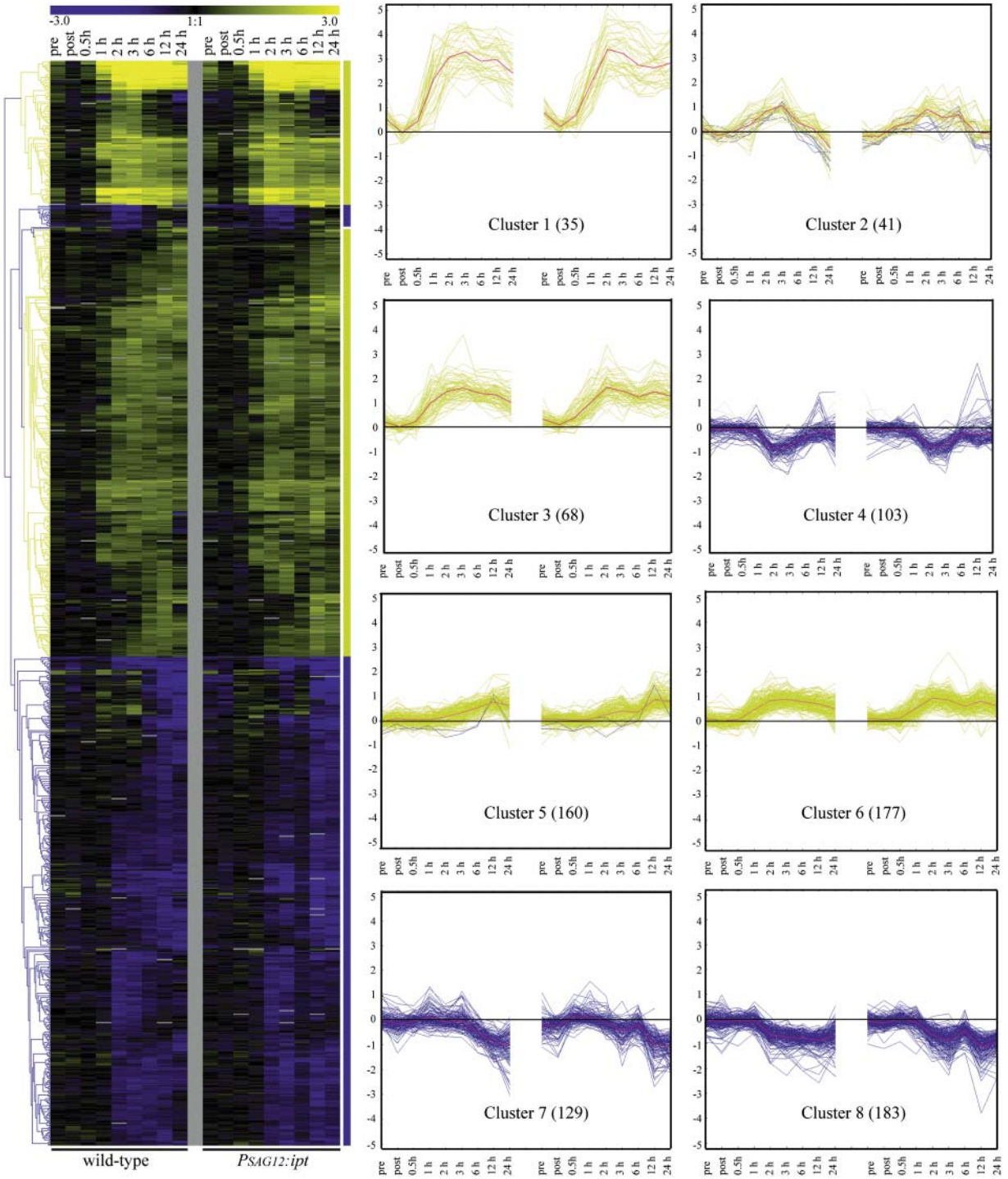

Figure 3. Representation of expression changes for differentially expressed genes. Temporal patterns of expression in wild-type and transgenic P_{SAG12}:*ipt* plants during the time course of hypoxic stress (pretreatment, posttreatment, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, and 24 h) were visualized using average HLC (left) and KMC (right), both with a Euclidian distance metric. The color of each element in the left section represents the expression level of each gene as $\log_2$(hypoxia/normoxia), with blue representing down-regulation and yellow representing up-regulation. The color of individual lines in the right section represents temporal profiles in each of the eight clusters corresponding to the gene groups highlighted in the hierarchical clustering dendrogram; the pink lines represent the average expression of each group.

was not detected in P_{SAG12}:*GUS* plants until the eighth day of waterlogging (Zhang et al., 2000).

A total of 896 genes (see Supplemental Table II) representing the overlap between the two lists of differentially expressed genes in wild-type and P_{SAG12}:*ipt* plants were subjected to average linkage hierarchical clustering (HCL) and *k*-means clustering (KMC) analysis with a Euclidian distance metric (Fig. 3). The majority of the significant genes exhibited either consistent up- or down-regulation in response to anaerobic stress, with only a few exceptions exhibiting a pattern of an up-regulation followed by a down-regulation or the reverse. Using KMC, these 896 genes were grouped into eight clusters according to their expression profiles during the time course of hypoxic treatment. The grouping by KMC is consistent with the clustering profile based on HCL, as genes in up-regulated clusters (labeled in yellow) and genes in down-regulated clusters (labeled in blue) in HCL generally fall into the up- and down-regulated groups, respectively, in KMC (Fig. 3). Cluster 1 (35 genes) showed the strongest and most sustained induction, starting at 1 h and lasting through the 24-h period, with the peak average $\log_2$(ratio) of the cluster being greater than 3 (corresponding to 8-fold induction). Within this cluster are a number of genes encoding ANPs, including two pyruvate decarboxylases (PDCs), ADH1, two Suc synthases, 1-aminocyclopropane-1-carboxylate (ACC) oxidase, and nonsymbiotic hemoglobin 1 (GLB1). Expression profiles of clusters 3 (68 genes) and 6 (177 genes) were similar to that of cluster 1, except they showed only moderate and weak induction, with the peak average $\log_2$(ratio) at 1 to approximately 2 (2–4-fold) and close to 1 (2-fold induction), respectively. There was a transient induction in cluster 2 (41 genes), with transcript levels peaking at 3 h and returning to base line as stress progressed. Cluster 5 (160 genes) exhibited a slow and steady increase in transcript levels starting 2 h after the beginning of low-oxygen stress. Genes in clusters 4 (103 genes), 7 (129 genes), and 8 (183 genes) showed an early, late, and sustained decrease in transcript levels, respectively, during anaerobiosis. A complete list of genes in different clusters along with their expression levels is provided in Supplemental Table III.

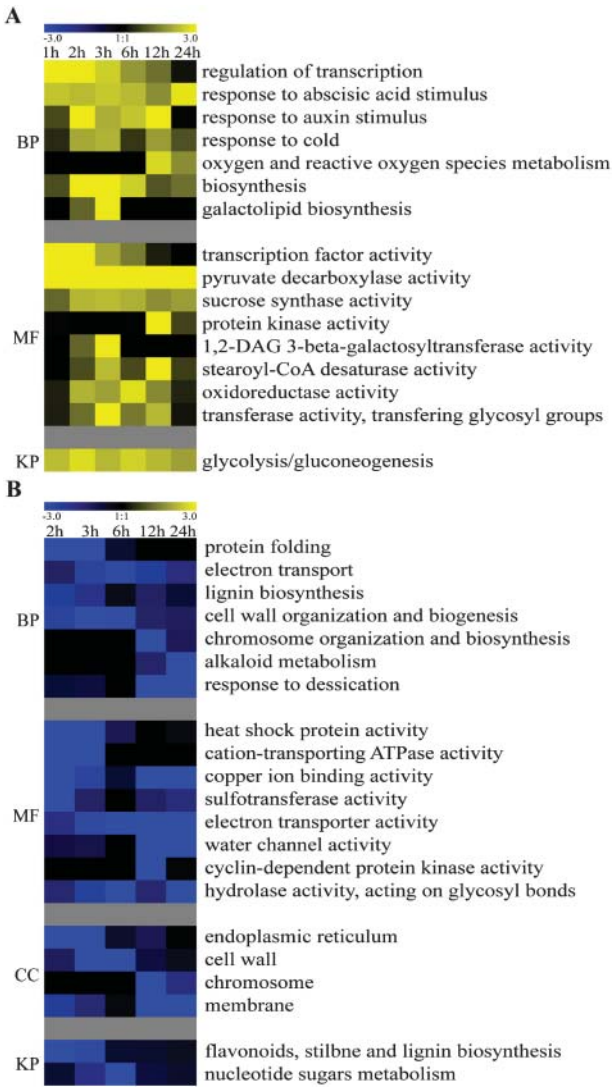

Figure 4. Hypoxia-induced and suppressed functional classes and pathways. Selective GO terms and KEGG pathways overrepresented in positively (A) or negatively (B) significantly expressed gene classes identified by EASE are presented as heat maps. Here, minus $\log_{10}$ transformed *P* values $[-\log_{10}(P)]$ and $\log_{10}$ transformed *P* values $[\log_{10}(P)]$ were presented in A and B, respectively, for up- and down-regulated genes. BP, Biological process; MF, molecular function; CC, cellular component; KP, KEGG pathway.

Hypoxia-Perturbed Pathways and Functional Categories

We used Expression Analysis Systematic Explorer (EASE; Hosack et al., 2003) to investigate higher level functional perturbations in response to hypoxia. EASE uses a Fisher Exact Test to identify functional classes and metabolic pathways occurring in a selected gene set relative to the representation on the array as a whole. Functional assignments are defined by GO terms (http://www.geneontology.org/), which provide broad functional classifications for genes and gene products representing their corresponding biological processes, molecular function, and cellular localization; pathway assignments were derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.ad.jp/kegg). EASE allowed us to sift through lists of hundreds of genes to extract functional information, which would not be possible by simply examining genes individually from the lists. EASE analysis was run for lists of PSGs and NSGs at each time point, except at 1 h for NSGs because there were only 11 genes identified as negatively significantly expressed at this time point. The selected functional categories and pathways with $P < 0.001$ are presented in Figure 4.

A wide spectrum of physiological processes was affected by the low-oxygen stress, as evidenced by an overrepresentation of the corresponding GO terms in

the PSG and NSG subsets. Besides the expected GO terms associated with glycolysis and fermentation pathways, processes such as response to auxin and response to abscisic acid (ABA) stimulus were significantly enriched in the induced gene datasets (Fig. 4A), suggesting a possible role for auxin and ABA in the hypoxia-induced signaling transduction. Overrepresented GO terms in the NSG datasets provided new insights into hypoxia-repressed processes and functions, such as a down-regulation in cell wall biosynthesis, secondary metabolism, nucleosome assembly, electron transport, and water channel activity (Fig. 4B), reflecting an attempt by the cells to cope with a reduced energy state by conserving resources during the period of oxygen shortage.

The temporal patterns of overrepresented GO terms suggest a cascade of responses following the hypoxic stress; for example, an induction of oxygen binding and transcription factor activity preceded an up-regulation of protein kinase activity in the PSG datasets (Fig. 4A), whereas a suppression of lignin biosynthesis and heat shock protein activity was followed by a down-regulation of alkaloid metabolism, nucleosome assembly, and water channel activity (Fig. 4B). GO terms identified as being significantly overrepresented were consistent among the molecular function, biological process, and cellular component classes and agreed with KEGG pathway analysis; for example, an enrichment of glycolysis (process) and PDC activity (function) indicated an up-regulation of a particular KEGG pathway (glycolysis/glycogenesis), and an overrepresentation of lignin biosynthesis (process) and cell wall (component) suggested a down-regulation of flavonoids and lignin biosynthesis (pathway). The complete lists of significant GO terms and pathways, along with the associated *P* values, are provided in Supplemental Table IV. Below, we characterize selected significant GO terms and pathways identified by EASE and also examine the significance of representative genes exhibiting the greatest alteration in expression in response to hypoxia.

Transcription Factors and Protein Kinases

Transcription factors and protein kinases are important components of signal amplification and transduction networks conveying diverse signals to specific responses. EASE analysis indicated that transcription factor activity and protein kinase activity were overrepresented during the early and late phases of anaerobiosis, respectively (Fig. 4A). To gain further insight into the regulation of anaerobic adaptation, we identified 64 genes with GO assignments of either transcription factor activity or protein kinase activity from the entire set of differentially expressed genes (896 genes as shown in Fig. 3) and then performed average HCL using a Euclidian distance metric (Supplemental Fig. 1). Among the five transcription factors that were most strongly induced by hypoxia was ANR1, a MADS-box protein known to be essential for lateral root development in response to nitrate supply (Zhang and Forde, 1998), suggesting a possible connection between hypoxia response and nitrogen metabolism. A closer examination of the differentially expressed genes also found an induction of other genes involved in nitrogen assimilation and utilization, such as nitrate reductase (At1g77760, NR1) and Asp aminotransferase (At5g19550, Asp-2). Genes encoding nitrate reductase have been identified previously as hypoxia inducible (Mattana et al., 1994; Klok et al., 2002).

The transcription factors spanned several major families, including the AP2-domain, basic helix-loop-helix, zinc-finger, myb, Leu-zipper, and WRKY family proteins. The wide distribution to multiple families and the different expression profiles suggest they may be involved in the control of different processes and/or different phases of responses. Consistent with results from EASE analysis, most protein kinases identified were grouped into a cluster with a profile of late up-regulation; several of these belong to Leu-rich-repeat protein kinase family, and members of this family have been linked to diverse biotic and abiotic stress responses (Dievart and Clark, 2004). One cluster of genes including several transcription factors and two protein kinases showed reduced expression, suggesting the genes may be negative regulators of the low-oxygen response.

Increased Sugar Flux to Glycolysis and Fermentation Pathways

Oxygen deficiency induces a rapid metabolic shift from aerobic to anaerobic respiration for limited ATP production via glycolysis and for NAD^+ regeneration via fermentation at the expense of low-efficiency utilization of substrate reserves (Dennis et al., 2000). Consistent with results from previous expression profiling and proteomic studies (Chang et al., 2000; Klok et al., 2002; Paul et al., 2004), we observed that genes involved in glycolysis and fermentation pathways are among those most strongly induced by hypoxia. There are multiple steps in this central carbon metabolic pathway in which one or more genes encoding the corresponding enzymes are significantly induced (Supplemental Fig. 2). The increased expression for the majority of these genes has been verified by qRT-PCR (Table I). In several steps, including those catalyzed by Suc synthase, Fru kinase, and PDC, more than one gene encoding the same enzyme showed increased expression. As an energy-saving strategy, Suc synthase was selectively induced over invertase for breaking down Suc, since the breakdown of a molecule of Suc by Suc synthase and UDP-Glc pyrophosphorylase requires only one molecule of pyrophosphate as compared with two molecules of ATP consumed by invertase and hexokinase (Zeng et al., 1999).

Trehalose is a disaccharide ubiquitously present in plants (Leyman et al., 2001). Recent evidence has

Table I. *Differentially expressed genes validated by qRT-PCR*

The values listed in the four columns on the right are the $\log_2$(hypoxia/normoxia) values measured by microarrays and qRT-PCR for wild-type (WT) and transgenic P_{SAG12}:*ipt (T5) plants.*

AGI Locus	Common Name	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Microarray		Real-Time PCR	
				WT	T5	WT	T5
At1g77120	Alcohol dehydrogenase	GAATCGCTGGTGCTTCTAGG	CTCAGCGATCACCTGTTGAA	3.88	3.88	3.13	2.82
At3g43190	Suc synthase, putative	ACAAACTCAACGGGCAATTC	AAAGAGCAGGCTGCACAAAT	4.08	3.78	3.30	2.93
At5g20830	Suc synthase	CGCCGTTACCTTGAAATGTT	CTTCAAACACCGGAACCACT	3.19	3.24	2.03	1.99
At4g33070	Pyruvate decarboxylase 1 (PDC1)	GGTGGTCCTAAGTTGCGTGT	CTGCTCCCCAATAAGTTCCA	4.33	4.48	3.59	3.65
At4g17260	Lactate dehydrogenase (LDH1)	TGGTGGTGATGTTTTCCTCA	CTGCAGCTTCTCAGCCTCTT	1.80	2.09	0.10	1.55
At2g31390	Putative fructokinase	TCCTTTGTCGGTGCTCTTCT	CTGAGGGAAGAGCTGGA-ATG	1.87	1.57	1.18	0.90
At4g26520	Fru-bisphosphate aldolase	GCACAGTCCCACCAGCTATT	CCGCCAAAAGAGAAAGT-GAG	0.89	0.76	0.54	−0.58
At4g32840	Phosphofructokinase family protein	CGATCTCCCCACTTATCCAA	GGCCTGCACGTCTAAAATGT	2.02	1.38	1.18	0.77
At2g36580	Putative pyruvate kinase	CTGTTGTCATTCCCCGACTT	CATGGGGAAAAGACCTCTGA	1.11	1.03	0.83	0.76
At1g72330	Putative Ala aminotransferase	AGAGCCGAAGGAGCAAT-GTA	TTGCAGTAGAACGCATCTGG	0.68	−0.03	0.00	−0.14
At5g51460	Trehalose-6-P phosphatase (AtTPPA)	CCGATGATCGATAAGGTGCT	TGTGCAACCAATGTCCAGTT	1.04	1.16	1.08	0.91
At5g65140	Trehalose-6-P phosphatase, putative	TTCGGTCGGTGGTAAAGAAC	TCAAGAGCCTTGCCTTTGTT	1.95	1.87	1.17	1.41
At4g24040	Trehalase-like protein	AAACCGTGTTTTGGAACGAG	TAGACGCAAAGACGTTGGTG	1.37	0.85	0.65	0.66
At1g76640	Putative calmodulin	GGCTGTGTTTGCTTACATGG	CCTCGGCTTCTTCATCAGAC	1.78	1.52	0.81	−0.46
At1g76650	Putative calmodulin	CATGGATGCAAACAGAGACG	ACAGCAGCTACGGCTTCTTC	2.87	2.65	3.41	3.36
At2g19590	ACC oxidase	CTCAGCAAGACGATGGATGA	TTGGACCAGAAAAGGCATTC	2.76	2.68	1.88	1.85
At3g23150	Ethylene receptor ETR2	TTGTGCTACTGCGATTACGC	CAACTTCACGACCAAGCTCA	1.10	0.60	0.42	−0.15
At3g11930	Ethylene-responsive protein, putative	GTGGAGAAGCAGCCAGA-GAC	CCATTGGAGAGCGTAGAAGC	1.90	1.48	0.84	0.86
At2g34390	Major intrinsic family protein (NIP2;1)	TTACTGTCTCGGCCACCTCT	CCAAGGTTGATCCGATGACT	4.55	4.31	4.66	3.88
At5g60660	Major intrinsic family protein (PIP2;4)	CCGATGGCTACAACAAAGGT	CACGTGAGAGTCACGAGCAT	−1.21	−1.57	−1.52	−1.25
At5g47450	Major intrinsic family protein (TIP2;3)	GCAGCTGATCCAAAGAAAGG	ACCTTGCTGGATTCATGGAG	−2.32	−2.43	−2.99	−2.18
At3g02550	Lateral organ boundary domain protein (LBD41)	AAGCTTGTGGGAGGATTGTG	TGACCGGTTCTCCTTTCATC	3.10	2.90	1.51	1.70
At2g14210	MADS-box protein (ANR1)	GAGAGATGGGGAGAGGG-AAG	TTCTGCATCACAAAGGATCG	3.04	2.40	2.87	2.68
At2g16060	Nonsymbiotic hemoglobin protein (GLB1)	AACACTTTGAGGTGGCCAAG	CAGCTTTAATGGCAGCAACA	3.37	3.19	2.53	2.61
At2g17850	Putative senescence-associated rhodanese protein	CCAAACCGATCATCTCATCC	TGACCCAAGCAATGTAACCA	4.28	4.58	3.81	3.83
At2g39510	Nodulin MtN21 family protein	GCCTTCAATCCATTGAGCAT	TTGCTTTTTCCCCACAAAAC	3.56	3.69	2.22	2.07
At2g47460	Putative MYB family transcription factor	TGATGGGGAGTTGCATAACA	ACTCCACCGATGGACAAGAC	1.07	1.20	0.60	0.48
At2g47520	Putative AP2 domain transcription factor	GCCAAACTGAATTTCCCAAA	CATCAGGTCCTCCGATAAGC	3.18	2.83	2.61	2.63
At5g54470	CONSTANS B-box zinc-finger family protein	CTGAATCTTGGCCCAACTGT	GCTCCGTCGTTACAACCAAT	1.75	1.29	1.42	1.43

established that the initial step of its synthesis is essential for embryo development in Arabidopsis (Eastmond et al., 2002), and the level of its precursor, trehalose-6-P (T6P), plays an important role in controlling sugar influx into glycolysis in yeast (Thevelein and Hohmann, 1995) and may also play a similar role in plants (Eastmond and Graham, 2003). Three genes (At5g51460, At1g78090, and At5g65140) encoding T6P phosphatase and one gene (At4g24040) encoding trehalase showed increased expression under

oxygen shortage, whereas a gene (At2g18700) encoding T6P synthase exhibited a decreased expression at 6, 12, and 24 h after the stress (Supplemental Figs. 2 and 3B). These changes in gene expression may result in a potential decrease in the level of T6P, which in turn might alleviate its inhibition on glycolysis and facilitate the increased sugar flux to anaerobic respiration.

Signal Transduction Networks Mediating Anaerobic Responses

Transient elevation and/or oscillation in the concentration of cytosolic free calcium are involved in signal transduction pathways in response to various developmental cues and environmental conditions (Sanders et al., 2002). Evidence from studies using Ca^{2+} antagonists and channel blockers has implicated calcium as an essential messenger for establishing ion homeostasis and triggering downstream signaling pathways following oxygen deprivation (Subbaiah et al., 1994; Sedbrook et al., 1996), but how the Ca^{2+} signal is deciphered by various Ca^{2+}-sensing proteins is not clear. We observed an induced expression of multiple genes encoding proteins whose activity is regulated by Ca^{2+} or that are capable of altering Ca^{2+} concentration by modulating its release from internal storage sites. Supplemental Figure 3A shows the expression profiles for some of these, including Ca^{2+}-binding proteins such as EF-hand-containing proteins (At1g76650, At2g33380, and At5g54130), C2 domain-containing protein (At3g61720) and calmodulin (At1g76640), calcineurin B-like protein-interacting protein kinases (At3g17510 and At5g25110), and phosphotidylinositol 3,4-kinase (At1g26270). The expression of these genes increased as early as 1 h after the start of hypoxic stress and gradually returned to basal levels as the stress continued, consistent with their roles as mediators of calcium signal transduction and alteration of target gene expression. This result suggests a complex calcium sensing and regulatory network is involved in low-oxygen response.

Ethylene has long been recognized as a signaling molecule for low-oxygen response, especially for such long-term adaptations as aerenchyma formation and epinastic growth (He et al., 1996; Vriezen et al., 1999; Drew et al., 2000; Peng et al., 2001), and limited evidence also points to a possible role for ABA (de Bruxelles et al., 1996; Ellis et al., 1999), but little information is available to suggest an involvement of auxin in hypoxic adaptation. The transcript levels of genes involved in ethylene synthesis (At2g19590 [ACC oxidase] and At5g65800 [ACC synthase]), perception (At3g23150 [receptor ETR2]), and target gene regulation (At5g07580 [ethylene-responsive element-binding protein]) significantly increased in response to hypoxia (Supplemental Fig. 3C). Several auxin-responsive genes (At3g23030 [IAA2], At5g19140, and At1g19840) were induced while a gene encoding an auxin carrier protein (At2g17500) was suppressed by low-oxygen stress (Supplemental Fig. 3D). The up-regulation of ABA-responsive genes was supported by both EASE and the analysis of individual differentially expressed genes, which also suggest the involvement of multiple plant hormones in the transcriptional regulation of response to hypoxia.

A number of adverse environmental conditions promote the generation of ROS. ROS can serve as potential signaling molecules to mediate diverse biotic and abiotic stress responses, although the excess accumulation of ROS is detrimental to the cells (Laloi et al., 2004). Hypoxia-induced increase of *ADH* mRNA is associated with an increase of hydrogen peroxide levels mediated by the activation of a Rop (RHO of plants) G-protein and a diphenylene iodonium-sensitive calcium-dependent NADPH oxidase (Baxter-Burrell et al., 2002). We identified three genes encoding respiratory burst oxidase protein (Rboh)/NADPH oxidase that were significantly induced by low-oxygen stress; specifically, At5g07390 (RbohA) and At4g25090 (putative Rboh) showed a late induction while At5g47910 (RbohD) exhibited an early and sustained induction 1 h after the start of the hypoxic treatment, supporting that ROS may play a role in mediating anaerobic responses.

Major Intrinsic Proteins and Lateral Organ Boundaries Proteins

The identification of more than 2,000 genes whose expression was significantly altered by a 24-h low-oxygen treatment is indicative of the complexity of the anaerobic adaptation process. The analysis of the dataset confirms our current knowledge of hypoxic response, yet also identified a collection of genes either with unknown functions or involved in processes that had not previously been identified as involved in the anaerobic response. Here, we present two representative examples.

Major intrinsic proteins (MIPs) are membrane channels involved in transport of water (aquaporins or water channels), uncharged solutes, gases, and micronutrients (Tyerman et al., 2002). The 35 MIPs in Arabidopsis are classified into four major groups: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), and small basic intrinsic proteins (Johanson et al., 2001). A drop in cytosolic pH associated with low-oxygen stress has been demonstrated to inhibit channel activity of PIP proteins, leading to a decrease in root permeability (Tournaire-Roux et al., 2003). EASE analysis indicates an overrepresentation of water channel activities in suppressed gene sets (Fig. 4B), and multiple genes in the PIP and TIP subgroups indeed showed reduced expression; however, a gene encoding NIP2;1 (At2g34390) was significantly induced in response to hypoxia (Supplemental Fig. 3E). At2g29870 is annotated as a pseudogene because it

potentially encodes a protein (NIP2;1ps) that is 96% identical to NIP2;1 but with an N-terminal truncation (Quigley et al., 2002), which suggests the almost identical expression profiles of these two genes may be due to cross-hybridization. While the decreased expression of MIPs in response to hypoxia correlates with a flooding-associated decrease in the permeability of water channels, the significance of a selective induction of one particular MIP gene (NIP2;1), belonging to a subgroup that is involved in transport of other small metabolites besides water in bacteria (Tyerman et al., 2002), remains to be investigated.

Lateral Organ Boundaries (LOB) proteins are a family of recently identified plant-specific proteins that share a previously uncharacterized domain called LOB (Shuai et al., 2002). There are a total of 43 genes encoding LOB domain-containing proteins (LBDs) in Arabidopsis, and these are primarily expressed in the base of lateral organs and thought to play a role in boundary establishment or communication between the meristem and initiating of lateral shoots or roots (Lin et al., 2003). We identified several genes in this family whose expression was significantly altered by low-oxygen stress, including induction of LBD4, LBD40, and LBD41 and suppression of LBD37 and LBD39 (Supplemental Fig. 3F). All five of these are expressed in roots, and one, LBD40, showed a root-specific expression as demonstrated previously using RT-PCR (Shuai et al., 2002). Flooding induces adventitious root formation in species such as maize and rice (*Oryza sativa*; Drew et al., 2000; Mergemann and Sauter, 2000), but whether oxygen shortage induces the development of adventitious roots in Arabidopsis and whether these LBD proteins play a role in the root initiating process await further experimental investigation.

Coexpressed Genes Share Common Regulatory Elements

The promoters of coexpressed genes are likely to share common regulatory motifs and are potentially regulated by a common set of transcription factors, and, therefore, identification of cis-regulatory elements in the promoter regions provides a means of uncovering new mechanisms of transcriptional regulation networks underlying hypoxic response. Two anaerobic response elements (AREs), GT motif (5′-TGGTTT-3′) and GC motif (5′-GCCCC-3′), were first characterized in the promoters of the aldolase and *adh1* genes in maize (Walker et al., 1987); mutation of these elements results in a loss of response to hypoxia (Olive et al., 1990). The Arabidopsis *ADH1* promoter contains sequence motifs similar to the maize AREs (Dolferus et al., 1994) but with the GT motif in the reverse orientation relative to that in maize (5′-AAACCA-3′). The GT motif, also present in the promoters of many other ANPs, is essential for AtMYB2 binding and hypoxia-induced *adh1* expression (Hoeren et al., 1998). Transcriptional repression in response to hypoxia is not as well studied as induction, and neither cis-regulatory elements nor transcription factors have been reported for the repression of gene expression by hypoxia. We used an enumerative algorithm, SIFT (Hudson and Quail, 2003), which counts the frequency of exactly matched short nucleotide sequences in the selected subset of promoters relative to the occurrences of the same sequence in the entire promoter dataset, to search for 5- to 10-mer motifs that are overrepresented in the 500-bp promoter regions of the selected subsets compared with all the genes analyzed on the array. Because genes in clusters 1, 3, and 6 (Fig. 3) show similar expression profile and only differ in the magnitude of the induction, the analysis was run with 237 genes from these three clusters whose expression was increased by at least 2-fold at any time point. A list of selected promoter motifs and their corresponding sequence variants that are enriched significantly in the selected subset are provided in Figure 5. The most significant hit is the GT motif, AAACCA, which is present in 142 of the 237 genes (60%, $P \leq 5.17 \times 10^{-10}$), suggesting that AtMYB2 potentially regulates a significant subset of hypoxia-induced transcripts. The next best hit from the set is TATGGAAAA ($P \leq 4.46 \times 10^{-7}$), the last eight nucleotides of which exactly match part of a sugar-responsive element (SURE) sequence (Grierson et al.,

Motif	*Sequence*	*P-value*	*Binding*
GT-motif	AAAACCA	5.17×10^{-10}	MYB2[a]
	AAACCAACG	1.51×10^{-6}	
	AAAACCAT	9.43×10^{-6}	
	AAACCAACGG	1.63×10^{-5}	
	AAACCAGC	1.93×10^{-5}	
	AACCAACGGC	2.73×10^{-5}	
	AAACCAACG	3.01×10^{-5}	
	AAACCA	3.65×10^{-5}	
	AACCAACG	4.90×10^{-5}	
	AAACCAGCC	5.87×10^{-5}	
	AAACCAA	5.96×10^{-5}	
	AACCAACGG	7.15×10^{-5}	
	CAAAACCA	7.16×10^{-5}	
SURE-a-like	TATGGAAAA	4.46×10^{-7}	WRKY[b]
	TATGGAAA	9.98×10^{-6}	
	TATGGAAAAA	6.60×10^{-5}	
G-box-like	CCACG	4.95×10^{-6}	bZIP[c]
pARE-1	CCCATAG	5.29×10^{-6}	Unknown
	CCATAG	4.91×10^{-5}	
	CCCATAGA	9.16×10^{-5}	
pARE-2	GAGGAATA	2.69×10^{-5}	Unknown
pARE-3	AATGGTA	2.82×10^{-5}	Unknown
	AATGGT	3.73×10^{-5}	

Figure 5. Sequence motifs overrepresented in hypoxia-induced promoters. Each sequence was individually identified as significantly enriched in the selected subset. Sequences representing the same motif are aligned in subgroups. The *P* value was determined using the binomial distribution (Hudson and Quail, 2003) representing the likelihood of the observed occurrences in a randomly selected set of promoters. Footnotes: a, Hoeren et al. (1998); b, Sun et al. (2003); and c, Guiltinan et al. (1990), Hong et al. (1995).

1994). SURE elements have been demonstrated to bind to a WRKY family transcription factor in barley (*Hordeum vulgare*), and mutations in the elements led to a reduction in their binding to the factor and to the plant nuclear extracts (Sun et al., 2003), suggesting a potential connection between sugar signaling and anaerobic adaptation. Another significantly overrepresented motif, CCACG ($P \leq 4.95 \times 10^{-6}$), is related to G-box1 sequence (5′-CCACGTGG-3′) of Arabidopsis *adh1* promoter (Dolferus et al., 1994). G-box (5′-CACGTG-3′) also is present in the promoters responsive to plant hormones such as auxin and ABA and to environmental conditions such as light and low temperature (Siberil et al., 2001). The overrepresentation of the G-box-related motif in the hypoxia-induced gene promoters suggests a potential cross talk among different signaling transduction pathways. Several sequence motifs identified as overrepresented significantly in the dataset have not been characterized previously, and we refer to these sequences as putative AREs. Only sequence elements selected from those having an overrepresentation *P* value (Hudson and Quail, 2003) $P < 10^{-5}$ are shown. The complete list of genes along with the overrepresented motifs is provided in the supplemental material. The same analysis performed with other clusters either resulted in motifs that did not meet the significance cutoff or returned the most significant motifs as basal promoter elements, such as the TATA box and some simple sequence repeats such as CACACA; these are not presented here.

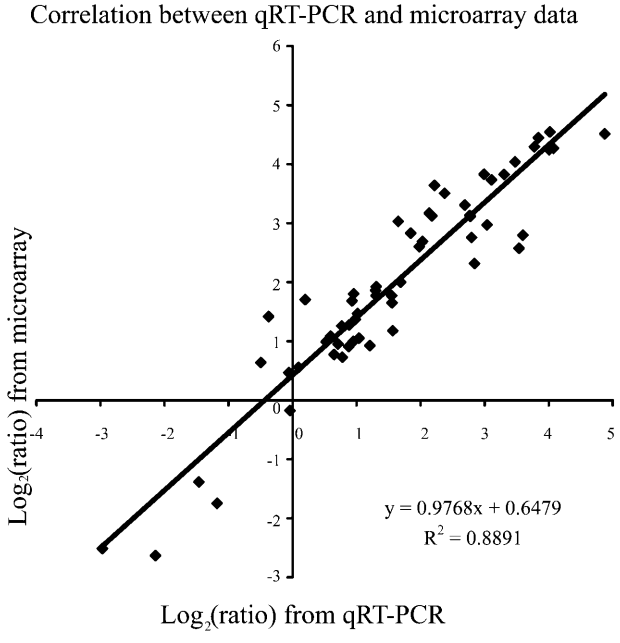

Figure 6. Correlation of gene expression values measured by microarrays and qRT-PCR. Changes in gene expression for 29 genes represented as $\log_2$(hypoxia/normoxia) derived from qRT-PCR and microarray hybridizations were compared. Each gene had two pairs of values, which are also listed in Table I.

Quantitative Real-Time PCR Verification of Microarray Results

For an independent verification of expression profiling data, a selected set of 29 significantly expressed genes identified in the microarray results were tested with qRT-PCR at selected time points using the same RNA samples that were used for the microarray analysis. Rather than generally validating genes across a range of representative expression levels, we selected genes with a focus on those involved in glycolysis, fermentation pathways, trehalose metabolism, and calcium and ethylene signaling, as well as transcription factors. The genes tested and their expression values derived from microarray and qRT-PCR are listed in Table I. As shown in Figure 6, there is a strong correlation between measurements of gene expression by microarrays and by qRT-PCR, with correlation coefficient $r^2 = 0.89$ ($n = 58$; $r = 0.94$). Twenty-five genes showing an induction or a repression in the microarray analysis were confirmed for both wild-type and P_{SAG12}:*ipt* plants by qRT-PCR. Three genes (At4g26520, At1g76640, and At3g23150) exhibiting an induction for both genotypes in the microarray analysis showed an up-regulation only in the wild type by qRT-PCR. Only in one case (At1g72330) did the qRT-PCR results show no change in gene expression while microarray analysis showed a weak induction for wild-type plants alone at the selected time point. The differences might be explained by a potential cross-hybridization among related probes in the microarray experiment; however, we have previously reported similar patterns of correlation for cDNA arrays and qRT-PCR, with the greatest discrepancies occurring for genes exhibiting small changes and expressed at low levels (Larkin et al., 2004). This may simply represent the limitations of sensitivity of detection using these techniques.

DISCUSSION

We used whole-genome amplicon microarrays representing nearly all gene content in Arabidopsis to investigate the low-oxygen stress response at multiple time points during a 24-h period. We applied several approaches to achieve the goal of reliably identifying sets of genes that are significantly differentially expressed in response to hypoxia. First, we used repeated measurements, from both biological replicates and dye-reversal hybridizations, to compensate for biological and technical variations. A strong correlation between data obtained from wild-type and from transgenic P_{SAG12}:*ipt* plants added another level of confidence (even though we did not achieve our initial aim for including transgenic plants in the design). Second, we inferred the change in gene expression using a parallel comparison between a pair of samples respectively subjected to hypoxia and normoxia at the same time point, rather than

comparing every time point back to time 0, to minimize the nonspecific effects potentially due to developmental and nutritional factors that may have changed during the 24-h period. Third, we used SAM with the median FDR set to 0% to identify differentially expressed genes. The quality of the resulting datasets is evident in several ways. First, there was a strong correlation in measurements between the dye-reversal pairs and between biological replicates. Second, there was little change in gene expression in either the pre- or the posttreatment controls. This is also true for the 30-min time point, where plant cells may not have been exposed to a low-oxygen environment long enough to mount defense responses. This further suggests that low-oxygen stress was applied to plants in a relatively stable state in which there was little variation in gene expression between replicates prior to the treatment, producing reproducible responses. Third, the level of gene expression at time points separated by short intervals, such as at 2 and 3 h, exhibited very consistent patterns of response. Fourth, we identified in our datasets many of those genes reportedly previously to be hypoxia responsive. Finally, the expression profiles of a select set of 29 genes were verified by qRT-PCR.

Falling internal oxygen levels trigger a variety of adaptive responses, including a rapid reduction of energy consumption, a shift to alternative pathways for energy generation and more efficient utilization, an establishment of systems to cope with accumulation of toxic products and ROS, as well as long-term morphological changes to help plants better survive the prolonged stress (Geigenberger, 2003). Consistent with the complexity of the low-oxygen response, we were able to identify a total of 2,085 genes whose expression was significantly altered in at least one genotype, of which 896 genes showed significant changes in expression in both genotypes, whereas the majority of the genes that did not overlap had relatively low expression levels. Of the 210 differentially expressed genes identified in a previous microarray study using hairy roots (Klok et al., 2002), 68 overlapped with our dataset. The overlapped genes include those involved in sugar utilization (notably Suc synthase, fructokinase, GAPC, PDC1, PDC2, ADH1, and LDH1), nitrogen metabolism (NR1), hormone actions (ETR2, IAA2, and gibberellin-regulated protein 4), and oxidative stress response (RbohD). The maximal induction level for some genes was significantly higher in their analysis (Klok et al., 2002), possibly because these genes are preferentially or exclusively expressed in the roots. We detected little change in gene expression at the 0.5-h time point, whereas they observed a distinct transcriptional response at 0.5 h compared with later time points. While the substantially larger set of significant genes in our study may be explained by the larger number of target genes analyzed on the arrays (26,777 amplicons versus 3,500 cDNAs), two fundamental differences may account for the differences between these analyses. First, we used whole plants rather than the cultured hairy roots used by Klok et al. (2002). Second, we administered the low-oxygen treatment in the light, whereas Klok et al. applied the stress in the dark. Given the greater diversity of tissues profiled, as well as the differences in basal gene expression in light and dark where many physiological processes are different, one would expect to identify different sets of hypoxia-responsive genes in these experiments. There is evidence to show that shoots and roots have different mechanisms to cope with low-oxygen stress and that plants respond differently to oxygen deficiency under dark and light conditions (Ellis et al., 1999; Mohanty and Ong, 2003), as oxygen generated from photosynthesis in the light may be used internally to mitigate the stress from oxygen shortage. When we compared our dataset with another microarray study using Arabidopsis 8K Affymetrix GeneChip arrays (Santa Clara, CA) to investigate the differential response of Arabidopsis to hypobaria and hypoxia, genes responsive to hypoxia were primarily overlapped in the glycolysis and fermentative pathways (such as Suc synthase, fructokinase, and *pdc1*), possibly because only shoot samples were used for their analysis such that genes exclusively expressed in roots were missed (Paul et al., 2004).

Genetic manipulation of individual transcription factors has successfully been used to engineer plants with improved tolerance to various environmental conditions (Jaglo-Ottosen et al., 1998; Tamminen et al., 2001). AtMYB2 is the only transcription factor that has been experimentally shown to regulate anaerobic response (Hoeren et al., 1998), even though the diverse processes perturbed by hypoxia (Fig. 4) and the differential expression of a suite of transcription factors (Supplemental Fig. 1) imply the existence of a complex transcriptional regulation network. We identified the GT motif as the most significantly overrepresented element in the promoters of hypoxia-induced genes, suggesting AtMYB2 may potentially regulate a substantial subset of hypoxia-responsive genes, consistent with the lethal phenotype observed for transgenic plants constitutively overexpressing the AtMYB2 gene (Dennis et al., 2000). Promoter analysis also identified other enriched regulatory elements, representing potential putative anaerobic response elements for different families of transcription factors (Fig. 5). Further characterization of hypoxia-responsive transcription factors using promoter-reporter constructs combined with elucidation of regulatory motifs using DNA-binding assays and yeast one-hybrid systems will, on one hand, yield a better understanding of hypoxia regulatory networks and, on the other hand, provide insights for choosing candidates to express in an inducible and tissue-specific manner to achieve the goal of improved flooding tolerance in plants.

As an important adaptation to flooded conditions, plants respond to low-oxygen concentrations by forming adventitious roots at the base of shoots capable of

longitudinal oxygen transport (Drew et al., 2000). Calcium, auxin, and ethylene are important signaling components for their development, and ethylene is essential for the subsequent formation of aerenchyma in adventitious and existing roots. Expression profiling in pine has revealed a complex transcriptional network involved in adventitious root formation, and mutants defective in different phases of its development have been isolated from Arabidopsis (Konishi and Sugiyama, 2003; Brinker et al., 2004). We speculate that oxygen deficiency might stimulate the formation of adventitious roots in Arabidopsis, based on several pieces of evidence derived from this study. First, a suite of genes encoding signaling components necessary for the development of adventitious roots was induced in response to hypoxia, including those involved in auxin signaling, ethylene synthesis and perception, and Ca^{2+} sensing. Second, as an essential MADS-box transcription factor involved in the root initiation process (Zhang and Forde, 1998), ANR1 showed a strong induction in response to low-oxygen stress. Third, members of the MtN21 nodulin gene family were significantly induced, especially At2g39510 (see Table I) and At3g53210; a homolog of this family in loblolly pine (*Pinus taeda*) is specifically induced by auxin prior to adventitious root formation only in tissues that are competent in forming lateral or adventitious roots (Busov et al., 2004). In addition, a significant alteration in gene expression of LBD proteins may also imply the initiation of new organs, such as adventitious roots. Whether the anaerobic response simply happens to share these regulatory components with root initiation or these changes in gene expression in response to hypoxia are associated with the development of adventitious roots needs further investigation by monitoring the morphological changes following a prolonged low-oxygen treatment.

Through systematically exploring sets of differentially expressed genes for higher-level biological themes based on GO and pathway assignments, as well as examining individual genes exhibiting the strongest altered response to hypoxia, we confirmed much of what is currently know of low-oxygen stress responses and, more importantly, obtained new insights into processes that were not previously associated with anaerobic response. This study represents a comprehensive gene expression profiling experiment conducted to investigate hypoxia-perturbed transcriptional networks in plants. The information provides a basis for formulating working models and establishing testable hypotheses to identify the underlying low-oxygen sensing mechanisms, to elucidate the signal transduction pathways, and to further characterize hypoxia-responsive genes. Together with information from independent approaches such as proteomics, metabolite profiles, and the utilization of reverse genetics resources, insights from gene expression profiling will lead to a better understanding of low-oxygen stress response and aid the effort to develop flooding-tolerant crops.

MATERIALS AND METHODS

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor. All expression data were collected in compliance with the MIAME standards (Brazma et al., 2001; Ball et al., 2002, 2004) and are available through ArrayExpress with accession IDs E-TIGR-20, E-TIGR-21, E-TIGR-22, and E-TIGR-23. Software used in this analysis, with the exception of SIFT, was developed at The Institute for Genomic Research (TIGR) and is available with source code under the open-source Artistic license through http://www.tm4.org.

Plant Materials and Hypoxic Treatment

About 100 Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) seeds, wild-type or transgenic P_{SAG12}:*ipt* (Zhang et al., 2000), were surface sterilized and stratified at 4°C overnight. The seeds were then sown into 200 mL of liquid medium containing Gamborg's B5 basal salt supplemented with vitamins (Sigma, St. Louis) and 2% Suc, and the seedlings were grown under continuous light of 90 μE m^{-2} s^{-2} intensity at 23°C for 13 d, with constant shaking at 100 rpm. Hypoxic and normoxic treatments were carried out under the same conditions by feeding flasks containing plant seedlings with low-oxygen gas composed of 3% O_2:0.03% [^{13}C]CO_2:97% N_2 and ambient gas composed of 21% O_2:0.03% [^{13}C]CO_2:79% N_2 (Sigma-Isotek, St. Louis), respectively. At 30 min, 1, 2, 3, 6, 12, and 24 h after gas treatment, whole plants from randomly selected flasks, one wild type and one transgenic, were collected and snap frozen in liquid nitrogen. Two flasks each, both wild-type and transgenic plants, were also collected immediately before and immediately after the treatment to serve as pre- and posttreatment controls. Both hypoxic and normoxic treatment runs were repeated to provide biologically replicated samples for both wild-type and transgenic plants. In total, 87 plant samples in four sets of 22 each from the two hypoxic and the two normoxic runs (in one of the normoxic runs, one of the posttreatment control samples for transgenic plants was lost) were collected. Using a reference design and dye-reversal replication, 174 hybridizations were performed in this analysis.

RNA Isolation

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), and poly(A) RNA was subsequently isolated from total RNA using Dynabeads oligo$(dT)_{25}$ (Dynal Biotech, Lake Success, NY) and further purified using RNeasy mini kit (Qiagen, Valencia, CA), following manufacturers' specifications.

Microarray Fabrication, Probe Labeling, and Hybridization

Arabidopsis genomic DNA amplicon microarrays were constructed as described previously (Kim et al., 2003). In brief, based on TIGR Arabidopsis genome release 2.0, genomic DNA fragments covering the predicted 3′ end of the 26,777 protein-encoding nuclear, plastid, or mitochondrial genes were amplified by PCR. The PCR amplicons were purified and then resuspended in 50% dimethyl sulfoxide and printed onto UltraGAPS aminosaline-coated slides (Corning, Corning, NY) using an Intelligent Automation System arrayer (Cambridge, MA). After printing, the spotted DNA was cross-linked to the slide surface by UV irradiation at an integrated intensity of 120 mJ cm^{-2} using a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA), and slides were stored in a desiccated chamber until use. Functional annotations for the arrayed elements are from TIGR Arabidopsis genome release 5.0 (http://www.tigr.org/tdb/e2k1/ath1/).

Probe labeling and hybridization protocols were described previously (Kim et al., 2003) and are also available in detail at http://atarrays.tigr.org. Briefly, starting with 1 μg of poly(A)-enriched mRNA, single-stranded cDNAs were synthesized during reverse transcription reaction using random hexamer primers (Invitrogen) in the presence of aminoallyl-dUTP (Sigma). Following the removal of unincorporated aminoallyl-dUTP and dNTPs using Microcon YM-30 columns (Millipore, Bedford, MA), the reaction products were conjugated to either Cy3 or Cy5 NHS-ester fluorescent dye (Amersham-Pharmacia, Piscataway, NJ). The Cy3- and Cy5-labeled probes were further

purified using a Qiaquick PCR purification kit (Qiagen), combined as an appropriate pair, and lyophilized.

Slides were prehybridized in 1% bovine serum albumin in 5× SSC, 0.1% SDS for 45 min at 42°C, followed by several washes in water and isopropanol, and then dried by centrifugation. The labeled probes were resuspended in hybridization buffer containing 50% formamide, 5× SSC, 0.1% SDS, and 0.2 μg/μL salmon sperm DNA and hybridized to the microarray slide at 42°C for 16 to 20 h in a sealed, humidified chamber. Following hybridization, slides were sequentially washed once in 2× SSC and 0.1% SDS for 4 min at 42°C, once in 0.1× SSC and 0.1% SDS for 4 min at room temperature, and twice in 0.1× SSC for 4 min at room temperature, and then dried by centrifugation. Slides were scanned using an Axon 4000B microarray scanner (Axon Instruments, Union City, CA), and data were saved as two independent 16-bit TIFF files corresponding to the Cy3 and Cy5 channels, respectively.

Experimental Design, Data Acquisition and Normalization, and Statistical Analysis

Gene expression levels were measured using a reference design for microarray analysis in which each test sample was hybridized to a common reference created by pooling an equal amount of poly(A) RNA from every test sample. At each time point, the change in gene expression in response to hypoxia for each array element was derived from eight data sources (16 for pre- and posttreatment controls): two indirect (hypoxic versus reference and normoxic versus reference) comparisons, two (four for pre- and posttreatment controls) biologically replicated samples, and each performed using dye-reversal labeling. The $\log_2$(ratio) of comparisons between hypoxic and normoxic samples at each time point, T_i, were inferred for each gene using the relationship:

$$\log_2\left(\frac{hypoxia}{normoxia}\right)_{T_i} = \log_2\left(\frac{hypoxia}{reference}\right)_{T_i} - \log_2\left(\frac{normoxia}{reference}\right)_{T_i},$$

where T_i = pretreatment, 30 min, 1 h, 2 h, 3 h, 6 h, 12 h, 24 h, and posttreatment.

The raw intensity data were extracted from the two TIFF images using TIGR Spotfinder, one of the four modules implemented in TIGR TM4 software suite (http://www.tigr.org/software/tm4; Saeed et al., 2003), and data points were not considered for further analysis if a spot was flagged during data acquisition as saturated or nondetectable at either channel, or if greater than 50% of the pixels within the spot were less than the median plus one SD of background intensity. For the remaining spots, the raw signal intensity was reported as the mean spot intensity minus the mean background intensity. To compensate for nonlinearity of intensity distributions, difference in probe labeling efficiency, and variation in printing pins, raw data were normalized using a locally weighted scatterplot smoothing regression algorithm (Cleveland, 1979) implemented in MIDAS (Saeed et al., 2003) with smoothing parameter set at 0.33, followed by a variance regularization algorithm with Cy3 signals selected as reference. Only those array elements with valid data in at least three of the four (six out of the eight for pre- and posttreatment controls) hybridizations were considered for further analyses; approximately 20,000 genes were included in the analyses.

To identify differentially expressed genes in response to hypoxia, a two-class unpaired SAM algorithm (Tusher et al., 2001), as implemented in TIGR MeV (Saeed et al., 2003), was applied to normalized data to compare four $\log_2$(ratios) from group A (hypoxia versus reference) to four $\log_2$(ratios) from group B (normoxia versus reference) at a full permutation (70 for $n = 8$), with the median FDR set at 0% and missing values imputed using k-nearest neighbors with $k = 10$. Genes identified as being significantly expressed in both wild-type and transgenic plants in at least one time point were further analyzed using average HLC and KMC algorithms with a Euclidean distance metric as implemented in TIGR MeV (Saeed et al., 2003).

To assess the differences in hypoxic response between wild-type and transgenic plants, two-factor ANOVA (time as factor 1 and genotype as factor 2) and two-class paired SAM (pair-wise comparison between wild-type and transgenic plants across seven hypoxic time points), both implemented in TIGR Mev (Saeed et al., 2003), were applied to a dataset consisting of 2,085 differentially expressed genes.

For EASE (Hosack et al., 2003) analysis, we identified genes that were differentially expressed in both genotypes relative to normoxic controls at each time point, by applying two-class unpaired SAM to normalized data to compare eight $\log_2$(ratios) from group A (hypoxia versus reference) to eight $\log_2$(ratios) from group B (normoxia versus reference) with the number of permutations set at 500 and the median FDR set at 0%. To discover higher-level biological themes from the resulting list of positively (hypoxia-induced) and negatively (hypoxia-suppressed) significant genes, EASE was performed to find significantly overrepresented functional classes in the selected subset relative to all the genes analyzed on the microarray, with the statistical significance (EASE score) estimated as a Fisher Exact score corrected for the number of hits in the subset representing a particular functional class. In addition to analyzing GO (http://www.geneontology.org) terms for biological process, cellular component, and molecular function, EASE also was employed to identify significantly overrepresented metabolic pathways using information from KEGG (http://www.genome.ad.jp/kegg), in which a gene is mapped to a particular metabolic pathway by the enzyme commission (EC) number of its encoded enzyme.

To identify the conserved motifs in the promoters of a set of genes that were clustered together based on their expression profiles, SIFT, an enumerative approach-based algorithm (Hudson and Quail, 2003), was used to compare the promoter sequences of the selected cluster to those of all the genes analyzed on the microarray. The genomic sequences were retrieved from the TIGR ftp site (ftp://ftp.tigr.org/pub/data/a_*thaliana*/ath1/), and both DNA strands were searched for exactly matched 5- through 10-mer motifs in the potentially coregulated set of "promoters," referred to in this study as 500-bp sequences upstream (5′ end) of the annotated translation start site.

Quantitative Real-Time PCR Analysis

Total RNA was purified using RNeasy MinElute cleanup kit following the treatment with RNase-free DNase I (Qiagen) and then used as template to synthesize single-stranded cDNAs using the Taqman reverse transcription kit (Applied Biosystems, Foster City, CA). PCRs were then performed in an optical 384-well plate with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems), which monitors the incorporation of the fluorescent dye SYBR Green into PCR product in real time during the dsDNA synthesis and computes for each reaction the threshold cycle (C_T), defined as the PCR cycle at which exponential growth of PCR products begins. Standard curves were derived from reactions with universal 18S ribosomal RNA primers (Ambion, Austin, TX) and a dilution series of cDNA templates. The relative quantity of the transcript assayed in each RNA sample was determined by normalizing to the standard curve and calculated as an arithmetic mean of the duplicated reactions. The presence of a single amplicon in each PCR reaction was confirmed by dissociation curves.

The expression levels of 29 genes selected from a set of differentially expressed genes identified in the microarray analysis and two genes (*SAG12* and *ipt*) not present on the microarray were analyzed by real-time PCR. The list of genes along with their forward and reverse primers designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) is provided in Table I. The expression level in response to hypoxia for each gene was calculated as the ratio of the average expression level from the two hypoxic duplicates over that from the two normoxic duplicates, and then $\log_2$-transformed to facilitate comparison with the microarray results. The hypoxic time points analyzed were 2 h for At4g32840, 6 h for At1g72330, 12 h for At4g26520 and At5g60660, 24 h for At5g47450 and At5g65140, and 3 h for the remaining 23 genes.

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