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Transcript and metabolite profiling of the adaptive response to mild decreases in oxygen concentration in the roots of arabidopsis plants

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• *Background and Aims* Oxygen can fall to low concentrations within plant tissues, either because of environmental factors that decrease the external oxygen concentration or because the movement of oxygen through the plant tissues cannot keep pace with the rate of oxygen consumption. Recent studies document that plants can decrease their oxygen consumption in response to relatively small changes in oxygen concentrations to avoid internal anoxia. The molecular mechanisms underlying this response have not been identified yet. The aim of this study was to use transcript and metabolite profiling to investigate the genomic response of arabidopsis roots to a mild decrease in oxygen concentrations.
• *Methods* Arabidopsis seedlings were grown on vertical agar plates at 21, 8, 4 and 1 % (v/v) external oxygen for 0·5, 2 and 48 h. Roots were analysed for changes in transcript levels using Affymetrix whole genome DNA microarrays, and for changes in metabolite levels using routine GC-MS based metabolite profiling. Root extension rates were monitored in parallel to investigate adaptive changes in growth.
• *Key Results* The results show that root growth was inhibited and transcript and metabolite profiles were significantly altered in response to a moderate decrease in oxygen concentrations. Low oxygen leads to a preferential up-regulation of genes that might be important to trigger adaptive responses in the plant. A small but highly specific set of genes is induced very early in response to a moderate decrease in oxygen concentrations. Genes that were down-regulated mainly encoded proteins involved in energy-consuming processes. In line with this, root extension growth was significantly decreased which will ultimately save ATP and decrease oxygen consumption. This was accompanied by a differential regulation of metabolite levels at short- and long-term incubation at low oxygen.
• *Conclusions* The results show that there are adaptive changes in root extension involving large-scale reprogramming of gene expression and metabolism when oxygen concentration is decreased in a very narrow range.

Key words: *Arabidopsis thaliana*, microarray, transcriptomics, metabolomics, low oxygen, root growth.

INTRODUCTION

Unlike animals, plants never evolved active distribution systems to deliver oxygen to their internal tissues. As a consequence, oxygen can fall to relatively low concentrations in many plant tissues – especially those which either exhibit high metabolic activity or tissues that lack large intercellular air spaces (aerenchyma) to improve oxygen transport driven by diffusion gradients (Colmer, 2003; Armstrong and Armstrong, 2005). These problems are evident even within well-oxygenated surroundings (21 % v/v external oxygen), however, problems are exacerbated when external oxygen is reduced due to flooding or water-logging of the rhizosphere (Jackson, 2002; Dat *et al.*, 2004; Pederson *et al.*, 2006; Voesenek *et al.*, 2006). Under anoxic conditions, cytochrome oxidase activity becomes oxygen limited and consequently ATP has to be produced by fermentation. This represents a severe stress, since the efficiency of ATP formation is sharply reduced in combination with a falling cytosolic pH and an accumulation of the toxic products of fermentation (Drew, 1997; Geigenberger, 2003; Bailey-Serres and Chang, 2005). Moreover, re-entry of oxygen into highly reduced anoxic tissue leads to harmful oxygen radicals and toxic oxidation products (Crawford and Braendle, 1996; Biemelt *et al.*, 1998). Within short-time frames, these deleterious effects lead to strongly negative growth responses and ultimately to the death of many plants.

Much research attention in the past has focused on the molecular response of plants to such severe stress conditions. Proteomic analyses in many plant species revealed the induction of a specific set of anaerobic proteins involved in carbohydrate utilization, glycolysis and fermentation (Sachs *et al.*, 1980; Chang *et al.*, 2000). With recent progress in genome sequencing and the subsequent availability of microarrays for many species, transcript profiles of the hypoxic response were analysed across a broad range of species including arabidopsis (Klok *et al.*, 2002; Paul *et al.*, 2004; Branco-Price *et al.*, 2005; Gonzali *et al.*, 2005; Liu *et al.*, 2005; Loreti *et al.*, 2005), rice (Lasanthi-Kudahettige *et al.*, 2007) and citrus (Pasentsis *et al.*,

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2007) and many diverse tissues including roots (Klok *et al.*, 2002), fruits (Pasentsis *et al.*, 2007) and germinating seeds (Lasanthi-Kudahettige *et al.*, 2007). Transcripts that were found to be differentially expressed upon oxygen deprivation stress encoded not only the known anaerobic proteins, but also proteins involved in other processes including amino acid metabolism, oxidative stress responses, detoxification programmes and the regulation of gene expression and signalling. In addition to transcriptional regulation (Walker *et al.*, 1987; Dolferus *et al.*, 1994), translational mechanisms were recently found to contribute to the control of expression of these genes (Branco-Price *et al.*, 2005). However, comprehensive analysis of metabolite-mediated post-translational control mechanisms which could play a role in this process have, as yet, not been attempted.

In the majority of the studies mentioned above, plants were subjected to very low external oxygen concentrations leading to (near) anoxic conditions within the tissues. A drawback of that approach is that it is hard to attribute changes in gene expression to the change of the oxygen concentration itself, or that the changes in gene expression are induced by the various severe side-effects as induced by the anoxic treatment, like the acidification of the cytosol or a change in the cellular energy status. Less attention has been paid to analyse the response of plants to relatively small changes in oxygen concentrations, such as those occurring under physiological conditions within many plant tissues such as seeds, tubers, fruits and roots. Under physiological conditions the prevailing environment generally affords conditions ranging between 1 % and 21 % (v/v) oxygen. To date, studies were mainly performed in potato tubers (Geigenberger *et al.*, 2000; Bologa *et al.*, 2003), developing seeds (Gibon *et al.*, 2002; Rolletscheck *et al.*, 2002, 2003; Vigeolas *et al.*, 2003; van Dongen *et al.*, 2004) and phloem tissue (van Dongen *et al.*, 2003), wherein metabolic fluxes and selected metabolites were analysed in response to small changes in the prevailing levels of internal oxygen concentrations. The consensus of the results from these studies revealed that a decrease in oxygen concentration leads to adaptive changes in metabolism in order to decrease oxygen consumption, involving a rapid inhibition in respiration, a fall in the energy charge and an overall depression of many ATP-consuming biosynthetic activities (Geigenberger, 2003). However, the molecular mechanisms underlying this response remain unknown – most likely due to a lack of molecular studies aimed at analysing the response of plants to changes in oxygen concentration within a narrow range.

In order to gain a comprehensive insight into the genomic response of plants to mild decreases in oxygen concentration, expression profiling was carried out in roots of arabidopsis seedlings grown on vertical agar plates at 21, 8, 4 and 1 % (v/v) external oxygen for 0·5, 2 and 48 h, using Affymetrix whole genome DNA microarrays. In addition, the sampled material was also used for GC-MS-based metabolite profiling. In parallel to these analyses, root extension was monitored to investigate adaptive changes in growth under these conditions. Results from these studies revealed that relatively small changes in the oxygen concentration lead to adaptive changes in root extension rates, which are correlated with the induction of a small set of low-oxygen-specific genes. The entire dataset resulting from this study is discussed in the context of current models for oxygen signalling in plants.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana ecotype col2 seedlings were grown in a 12 h light (intensity 160 μE)/12 h dark cycle (day and night temperature was 22 °C) on vertical plates with 15 % agar dissolved in half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 1 % sucrose. The roots of the plants were growing on top of the agar, thus avoiding restricted oxygen diffusion towards the roots by the agar medium. Ten days after germination, seedlings were transferred to continuous darkness and a stream of premixed air containing different oxygen concentrations (as indicated in the text), 350 ppm CO_2, and N_2 (Air Liquide, Berlin, Germany) was blown over the plates. Oxygen concentrations within the plates were regularly controlled to ensure precise regulation of the actual oxygen concentration around the roots. Roots were harvested in liquid nitrogen after 0·5, 2 or 48 h of each oxygen treatment.

Analysis of root extension rates

Root extension rates were determined on seedlings growing on vertical agar plates as described above. After the plates with seedlings were transferred to the dark, the actual root length was marked on the plate with a marker pencil. After 2 d of incubation at the various oxygen concentrations as indicated in the text, the root extension was measured.

Transcript profiling

RNA was isolated using Trizol (Invitrogen) and further processed according to Kolbe *et al.* (2006). Chip hybridizations of at least two independent biological replicates for each treatment were performed at the Deutsches Ressourcenzentrum für Genomforschung (Berlin) using protocols recommended by Affymetrix. Probe signal intensities were processed with the Affymetrix MicroArray Suite software package (MAS 5·0) and the resulting CEL files were imported for data quality control in the Bioconductor software package in R (Gentleman *et al.*, 2004). Normalization of the raw data and estimation of signal intensities was done using the Robust Multichip Average (RMA) methodology (Bolstad *et al.*, 2003). Average expression values and their *P*-values were calculated using the affylmGUI package in R (Smyth, 2004, 2005) using the Benjamini & Hochberg adjustment method. The MapMan visualization tool (Thimm *et al.*, 2004; Usadel *et al.*, 2005) was used for functional categorization of the expressed genes. The Wilcoxon Rank Sum test with Benjamini–Hochberg *P*-value correction was used to test whether the observed combined expression values of a functional bin differ from the expression changes as observed for the collection of genes from all other bins.

Raw data of the transcript profiles can be found in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/) with the Series record number GSE11558.

Metabolite profiling

Soluble metabolites were extracted and analysed as described in detail by Erban *et al.* (2007) and Lisec *et al.* (2006). In short, homogenization of the tissue was performed in liquid nitrogen. Metabolites were extracted from the tissue with methanol and water. Polar metabolites were separated using chloroform purification. The polar phase was dried by vacuum centrifugation (Concentrator 5301, Eppendorf, Hamburg, Germany) and chemically derivatized using a methoxyamination and silylation reagent. Subsequently, the samples were analysed by GC-TOF-MS. Metabolite fingerprinting was performed after baseline correction (ChromaTOF software version 1·00, Pegasus driver 1·61, LECO, St Joseph, MI, USA). Peak heights of the mass (m/z) fragments were normalized using the amount of the sample fresh weight and an internal standard (ribitol). Anotation was manually supervised using the TagFinder visualizations of TimeGroups (Anotation) and Clusters (minimum three correlating fragments as unique masses for quantitication) with maximum of 5 % of time deviation between expected RI from library and measurement (Luedemann *et al.*, 2008). Statistical analysis (two-way ANOVA) of the data was done using the MeV (Multi Experiment Viewer) software (Saeed *et al.*, 2003). Independent component analysis (ICA) was performed according to Scholz *et al.* (2004).

RESULTS

Effect of a stepwise decrease in oxygen concentrations on root extension rates

In order to investigate the effect of low oxygen on root growth, arabidopsis seedlings were grown on vertical agar plates for 1 week at 20 °C, before they were transferred to 0, 1, 4, 8, 12 and 21 % (v/v) oxygen in the dark. After 48 h, root extension growth was measured. The data in Fig. 1 show that deceasing oxygen from 21 % to 8 % (v/v) already leads to a significant reduction in root extension growth by 25 %. Root extension rates decreased progressively by 45, 70 and 100 % when oxygen is further decreased to 4, 1 and 0 % (v/v), respectively. These results show that root extension growth decreases as the result of a progressive reduction in the oxygen concentration. This growth reduction is seen as part of the energy-saving adaptive response of plants to hypoxia.

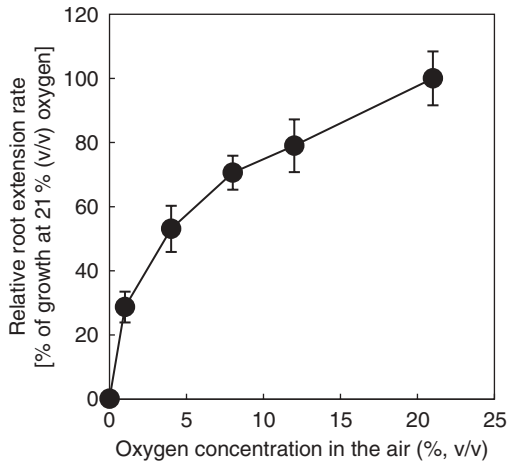


FIG. 1. Effect of a stepwise decrease in oxygen concentrations on extension growth of arabidopsis roots. Arabidopsis seedlings were grown for 10 d on vertical sterile plates. Root growth was measured during a 2-d period of incubation at different oxygen concentrations. Values are expressed as a percentage of growth in normal air [21 % (v/v) oxygen] and are the mean (± s.e.) of at least 30 seedlings.

Effect of a stepwise decrease in oxygen concentrations on whole-genome transcript profiles

In order to investigate the effect of low oxygen on whole-genome transcript profiles, samples were taken from roots of seedlings subjected to 1, 4, 8 and 21 % (v/v) oxygen for 0·5, 2 and 48 h within a parallel experiment. Figure 2 shows the number of differentially expressed genes, when referred to 21 % (v/v) oxygen as respective control. Only those genes were considered of which the mean expression level of the biological replicates changed at least two times. This analysis indicates a global tendency of more genes being up-regulated than down-regulated in response to low oxygen, indicating reprogramming of gene expression to induce genes that are important for adaptation rather than an inhibition of gene expression upon hypoxia. The number of up-regulated genes increased with time and with the extent of the decrease in oxygen concentration, reaching a maximum of approx. 300 genes at 1 % oxygen (v/v) after 48 h. Inspection of the number of down-regulated genes showed a similar tendency, but at a lower level, reaching a maximum of approx. 180 genes.

Changes in gene expression were categorized on the basis of the functions of the protein which they encode using the MapMan software. Table 1 shows a selection of functional bins in which the genes are significantly different expressed

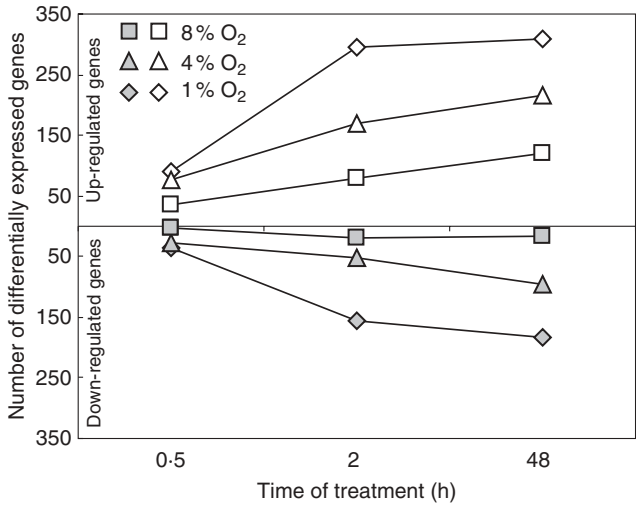


FIG. 2. Effect of a stepwise decrease in oxygen concentrations on the number of differentially expressed genes. Arabidopsis seedlings were grown on vertical sterile plates and incubated at different oxygen concentrations for various time periods. Plotted are the number of genes that were at least two-times up-regulated (open symbols) or down-regulated (shaded symbols) by either 8 %, 4 %, or 1 % (v/v) oxygen at the three different time intervals as indicated on the horizontal axis ($P < 0·05$).

TABLE 1. *Transcript profiling in roots of arabidopsis seedlings subjected to 1, 4 or 8 % (v/v) oxygen for 0·5, 2 and 48 h: statistical evaluation of the behaviour of transcript levels of genes within a functional category (bin) as compared with all the other remaining bins*

			48 h			2 h			0·5 h		
Bin	Name	Elements	1 %	4 %	8 %	1 %	4 %	8 %	1 %	4 %	8 %
34	Transport	570	**0·000**	**0·000**	0·249	**0·000**	0·766	**0·000**	**0·000**	**0·000**	0·328
30	Signalling	700	0·099	**0·000**	0·571	**0·000**	**0·001**	**0·000**	**0·001**	**0·000**	0·081
9	Oxidative phosphorylation	85	**0·013**	**0·002**	0·493	**0·048**	0·077	0·088	**0·021**	0·312	**0·000**
10	Cell wall	268	0·589	**0·000**	**0·000**	**0·013**	0·691	**0·000**	**0·025**	0·749	0·685
27	RNA	1528	0·939	**0·000**	**0·008**	0·976	**0·000**	0·960	0·770	**0·039**	**0·000**
11	Lipid metabolism	269	**0·001**	0·075	0·239	**0·014**	**0·000**	0·148	0·131	**0·013**	0·587
16	Secondary metabolism	230	**0·048**	0·064	0·933	**0·002**	**0·000**	0·676	**0·003**	0·444	0·479
21	Redox regulation	137	0·899	**0·027**	0·994	0·301	**0·002**	**0·033**	0·394	0·166	**0·006**
29	Protein	2086	**0·000**	**0·002**	0·474	0·571	0·443	**0·002**	0·553	0·954	0·401
31	Cell	421	0·096	0·386	0·676	**0·022**	0·124	0·109	0·101	**0·005**	**0·015**
28	DNA	317	0·308	0·219	0·246	**0·013**	0·068	0·329	**0·001**	0·249	**0·002**
23	Nucleotide metabolism	123	0·154	**0·005**	**0·031**	0·634	0·161	0·931	0·526	0·969	0·829
33	Development	303	0·242	**0·000**	0·106	0·134	0·934	0·123	0·209	0·175	**0·037**
17	Hormone metabolism	303	0·065	0·118	0·772	0·113	0·911	**0·002**	0·588	0·831	0·477
4	Glycolysis	54	0·340	0·105	0·151	0·980	0·849	0·173	0·996	0·811	**0·008**
13	Amino acid metabolism	233	0·362	0·237	0·479	0·439	**0·026**	0·665	0·269	0·234	0·809
2	Major CHO metabolism	69	0·384	0·361	0·630	0·155	0·934	0·926	0·226	0·181	**0·000**
14	S-assimilation	12	0·872	0·689	0·922	0·411	0·081	0·682	0·457	0·731	**0·029**

Genes that belong to a bin that has a *P*-value below 0·05 (in bold) are likely to be co-regulated. Categorization of genes and subsequent statistic analyses were performed with the MapMan software, using the Wilcoxon Rank Sum test with Benjamini–Hochberg correction. See also Fig. 4 for a visualization of some selected functional bins.

as compared with all other remaining genes on the microarray, independent of whether the change is an increase or a decrease. Overall, the most significantly differentially expressed category contains genes encoding transport proteins, revealing that the combined response of transcripts from genes assigned to this functional category is highly significantly different compared with the response of all other expressed genes on the array. As the responses of transcripts for genes assigned to the bins, signalling, respiration, cell walls, RNA metabolism, lipid metabolism, secondary metabolism and redox-regulation also had *P*-values $<0{\cdot}05$ in at least one of the oxygen concentrations in each time interval, it can be assumed that the expression of genes assigned to these bins is co-ordinately regulated by low oxygen. Figure 3 provides an overview of the direction of changes in transcript levels in selected bins. These data reveal that low oxygen leads to a preferential decrease in transcripts for genes encoding proteins associated with ATP-consuming processes such as transport, signalling, lipid-metabolism, secondary metabolism and redox-regulation, but a preferential increase in transcripts for genes encoding proteins associated with ATP-generating processes such as respiration.

In a more stringent selection procedure to find genes that are specifically induced by oxygen, only significant 2-fold changes ($P < 0{\cdot}05$) were considered. The mean expression values of all these genes are given in Files 1–4 in Supplementary Information. Figure 4 shows a Venn diagram of the selected genes that were up-regulated at 1, 4 and 8 % (v/v) oxygen during the three respective time intervals. After 0·5 h, seven genes were commonly induced at all three oxygen concentrations, encoding a wound-response family protein, an LOB domain protein 41, a kelch repeat-containing protein and alcohol dehydrogenase, as well as three proteins of unknown function. The same seven genes were also induced at all three oxygen concentrations after 2 h, together with five additional genes, encoding sucrose synthase, pyruvate decarboxylase, non-symbiotic haemoglobin 1 and two proteins of unknown function. After 48 h, only one of these genes remained up-regulated at all three oxygen concentrations, the gene encoding non-symbiotic haemoglobin-1. The expression levels of the core set of genes that are expressed at all oxygen concentration are shown in Table 2. The extent of up-regulation of these genes at 8 % oxygen varies between the different time intervals, being high at 0·5 h and 2 h, and decreasing at 48 h, indicating short- and long-term responses to low oxygen.

These data reveal that a small but highly specific set of genes is up-regulated very early in response to a moderate decrease in oxygen concentrations from 21 % to 8 % (v/v) oxygen. At all different time intervals, those genes that were induced at 8 % (v/v) oxygen were also induced when the oxygen concentration decreased further to 4 % and 1 % (v/v) oxygen, respectively. This indicates that the induction is likely to be a specific result of the change in oxygen rather than being induced by any of the anoxia-induced side-effects.

Effect of a stepwise decrease in oxygen concentrations on metabolite profiles

As indicated above, decreased oxygen concentrations led to specific alterations in transcript profiles which showed different responses at different time intervals. To investigate whether this is paralleled by global changes in metabolite levels, metabolite profiles were analysed using GC-MS.

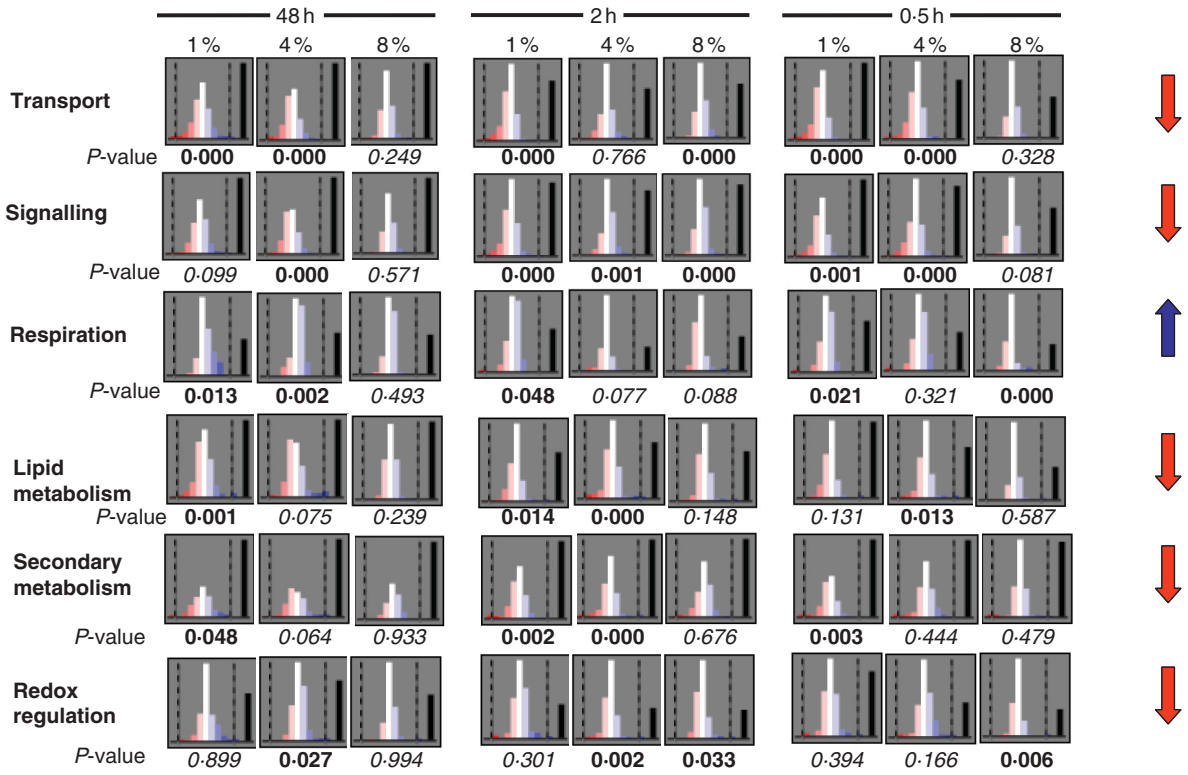
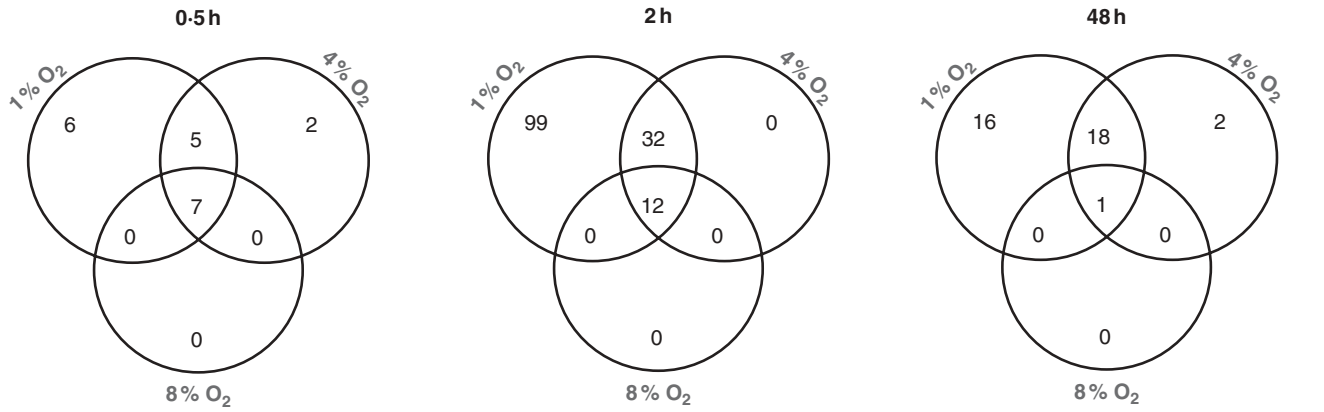


FIG. 3. Frequency distribution of low-oxygen induced changes in transcript levels of genes categorized in functional bins that divert significantly from the mean change in expression of the genes from all other bins. Functional categories are selected that show differential expression of genes in at least one of the oxygen treatments per time period. A significant change in expression is characterized here by a *P*-value below 0·05. These *P*-values are depicted below each panel; *P*-values $<0{\cdot}05$ are in bold, *P*-values $>0{\cdot}05$ are in italics. Statistical analysis is as described in Table 1. Red colours indicate down-regulated genes, and blue colours indicate up-regulated genes. The white bar in the middle represents the relative number of genes that are not changed by the oxygen treatment that is indicated. The black bar indicates the relative number of genes within the category that were not expressed. The arrow at the right side indicates a predominant up- (blue arrow) or down-regulation (red arrow) of the genes that belong to the respective bin. Incubation of arabidopsis roots with different oxygen concentrations was performed exactly as described in Fig. 2.

Independent component analysis of the metabolome data (for a review on this method and its interpretation, see Steinfath *et al.*, 2008) grouped the various oxygen treatments within each time point together (Fig. 5). Apparently, the variance between the samples taken at different time points is larger than the effect of the oxygen treatment. However, within each time-group, a slight separation of the various oxygen concentrations can be observed. Tag-intensities (defined as the intensities of mass (m/z) fragments within a specified time-window; for a detailed definition see Luedemann *et al.*, 2008) from the various experimental conditions (different oxygen concentrations and time) were examined by a two-way ANOVA to select metabolite fragments that showed a significant change in at least one of the experimental conditions. Hierarchical clustering analysis based on Eucledian distance of the metabolite fragment tag intensities revealed differential regulation of metabolite levels at short- and long-term incubation at low oxygen concentrations. The relative tag intensities that were grouped within a same cluster are visualized in a heat map (Fig. 6). It must be noted that the size of each group is not indicative for the total number of metabolites with similar changes. Because the selection of metabolite fragments was done from all available tag-intensities in a sample without any attempt to annotate the fragments to a known metabolite, it is to be expected that some co-regulated fragments are derived from the same metabolite. Therefore, the groups that are selected by the hierarchical clustering analysis just indicate different tendencies of metabolite changes as induced by the various low-oxygen treatments. Group A (Fig. 6A) shows an increase in tag-intensities only after long-term incubation (48 h), while group C (Fig. 6C) shows an increase only at short-term incubation (0·5 h and 2 h), but not at long-term (48 h). In group B (Fig. 6B), tag-intensity values showed an initial increase very early after exposure to low oxygen concentrations (0·5 h), which was not present at the subsequent time intervals (2 h and 48 h).

From the approx. 40 metabolites that were routinely identified in the chromatograms, 15 metabolites were selected that were significantly changed in response to at least one of the low oxygen concentrations (Fig. 7). These metabolites included minor sugars such as galactinol, myo-inositol and trehalose, amino acids such as alanine, proline, glutamate, γ-amino butyrate (GABA) and cycloserine, phosphorylated intermediates such as glucose-6-P and glycerol-3-P and organic acids such as galactonic acid, glutarate, threonic acid and nicotinic acid. Most of these metabolites showed different responses to low oxygen after different time intervals. The minor sugars galactinol and myo-inositol increased in response to 4 % and 1 % oxygen incubation after 48 h (Fig. 7A and B),



AGI code	Gene annotation
At4g10270	wound-responsive family protein
At3g10040	expressed protein
At4g33560	expressed protein
At3g02550	LOB domain protein 41
At3g27220	kelch repeat-containing protein
At5g15120	expressed protein
At1g77120	alcohol dehydrogenase

AGI code	Gene annotation
At1g77120	alcohol dehydrogenase
At3g27220	kelch repeat-containing protein
At4g33560	expressed protein
At5g62520	ADP-ribosyltransferase
At5g15120	expressed protein
At4g24110	expressed protein
At3g43190	sucrose synthase, putative
At4g10270	wound-responsive family protein
At3g02550	LOB domain protein 41
At3g10040	expressed protein
At2g16060	non-symbiotic hemoglobin 1
At4g33070	pyruvate decarboxylase, putative

AGI code	Gene annotation
At2g16060	non-symbiotic hemoglobin 1

FIG. 4. Identification of a specific set of genes that is up-regulated in response to low oxygen. Genes were selected that were significantly ($P < 0{\cdot}05$) and at least two times up-regulated at 1, 4 and 8 % (v/v) oxygen during the three time intervals. (A) For each time point, a Venn diagram shows the co-ordinate expression of genes at the various oxygen concentrations tested. In the table (B), the genes that are induced at all three oxygen concentrations are listed. An overview of the expression values of these genes can be found in Table 2. The expression values of all other genes are listed in File 1a–c in Supplementary Information.

whereas trehalose increased in response to 1 % oxygen after 0·5 h (Fig. 7C). In response to low oxygen incubation, the amino acids alanine (Fig. 7D), proline (Fig. 7E), glutamate (Fig. 7H) and GABA (Fig. 7F) decreased at 0·5 h and 2 h, while they increased at 48 h. By contrast, the phosphorylated intermediates glucose-6-P (Fig. 7I) and glycerol-3-P (Fig. 7J) increased in response to low oxygen at 48 h but showed a tendency to decrease upon low oxygen treatment at 0·5 h. Conversely, the organic acids, galactonic acid (Fig. 7K), glutarate (Fig. 7L), threonic acid (Fig. 7M) and nicotinic acid (Fig. 7 N) showed an increase at 4 % oxygen after 48 h but a tendency to decrease at low oxygen concentrations after 2 h and 0·5 h.

Visualization of changes in the levels of metabolites within the context of primary metabolic pathways (glycolysis, TCA-cycle and amino acid metabolism) as induced by the

TABLE 2. *Transcript profiling in roots of arabidopsis seedlings subjected to 1, 4 or 8 % (v/v) oxygen for 0·5, 2 and 48 h: selection of genes that are already up-regulated at 8 % oxygen for the three different time periods*

	0·5 h			2 h			48 h			
AGI (Tigr5)	8 %	4 %	1 %	8 %	4 %	1 %	8 %	4 %	1 %	Gene annotation
At4g10270	**4·090**	**5·933**	**6·053**	**4·704**	**6·210**	**6·553**	2·905	**6·063**	**5·218**	Wound-responsive family protein
At3g27220	**3·073**	**3·558**	**3·612**	**2·730**	**4·078**	**5·123**	1·101	**2·617**	**2·141**	Kelch repeat-containing protein
At4g33560	**2·928**	**4·331**	**4·093**	**3·554**	**5·372**	**6·238**	1·141	**4·753**	**4·086**	Expressed protein
At5g15120	**2·613**	**3·021**	**2·696**	**2·800**	**3·756**	**4·559**	1·739	**3·129**	**2·787**	Expressed protein
At1g77120	**2·283**	**2·664**	**2·323**	**3·836**	**5·312**	**5·561**	1·746	**3·652**	**4·192**	Alcohol dehydrogenase
At3g02550	**2·988**	**3·658**	**3·977**	**2·945**	**4·010**	**4·682**	0·830	**2·386**	1·545	LOB domain protein 41
At3g10040	**3·961**	**5·015**	**5·159**	**3·370**	**5·211**	**5·855**	1·270	2·839	1·949	Expressed protein
At4g24110	2·069	**3·823**	**3·922**	**2·557**	**4·420**	**5·637**	0·976	**3·275**	**2·806**	Expressed protein
At5g62520	1·541	**3·167**	**3·823**	**1·876**	**3·417**	**5·075**	−0·021	**1·752**	**1·677**	ADP-ribosyltransferase
At4g33070	2·640	**2·886**	**2·765**	**2·851**	**4·076**	**4·359**	1·016	**3·273**	**3·550**	Pyruvate decarboxylase, putative
At3g43190	2·144	2·672	**2·062**	**3·666**	**5·162**	**5·605**	2·171	**4·223**	**4·613**	Sucrose synthase, putative
At2g16060	1·783	**2·537**	**2·288**	**2·652**	**3·890**	**4·156**	**2·603**	**4·261**	**3·918**	Non-symbiotic haemoglobin 1

Expression levels ($\log_2$ values) that are significantly different from the 21 % control ($P < 0{\cdot}05$) are indicated in bold.

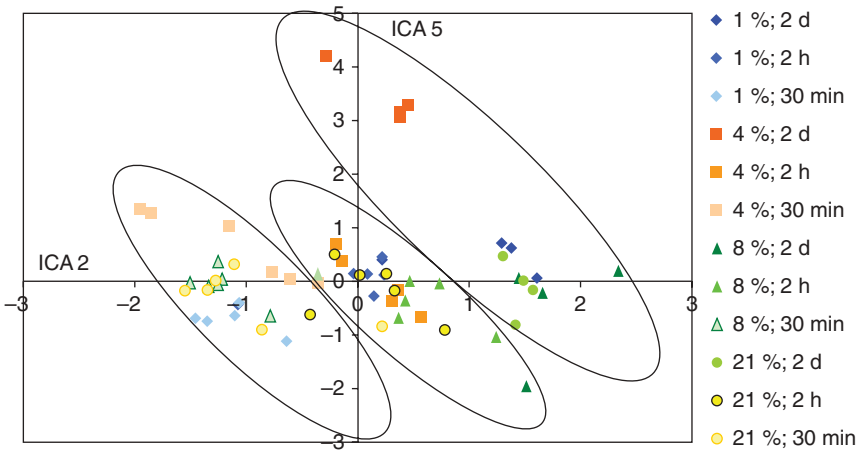


FIG. 5. Independent component analysis of GC-MS profiles from arabidopsis roots treated with different oxygen concentrations and time periods as indicated in the key. Plotting of the second and fifth component of ICA against each other shows grouping of samples taken at the same time interval (marked by the circles). Also within each group, a slight separation of the various oxygen concentrations can be discerned. Incubation of arabidopsis roots with different oxygen concentrations was performed exactly as described in Fig. 2.

low-oxygen treatments clearly reveals general trends in metabolite levels within the pathway (as an example, the 48-h time point is shown in Figure 8; the other pathways are given in File 5 in Supplementary Information). When oxygen is decreased to 4 %, there is a general tendency for an increase in the levels of the intermediates both of sucrose degradation and the TCA cycle and in the levels of most amino acids. Metabolic flux analysis (Geigenberger *et al.*, 2000) revealed an inhibition of carbon metabolism when oxygen is decreased to 12–4 % as compared with normoxia (21 %). The general increase in primary metabolite levels could therefore result from a depression in metabolic activity. When the oxygen further decreased to 1 %, the levels of most of the metabolites decreased again, indicating that the inhibition of metabolism has been partly released. This change correlates with the strong increase in glycolytic carbon flux under anoxic conditions due to induction of fermentative metabolism (Geigenberger *et al.*, 2000).

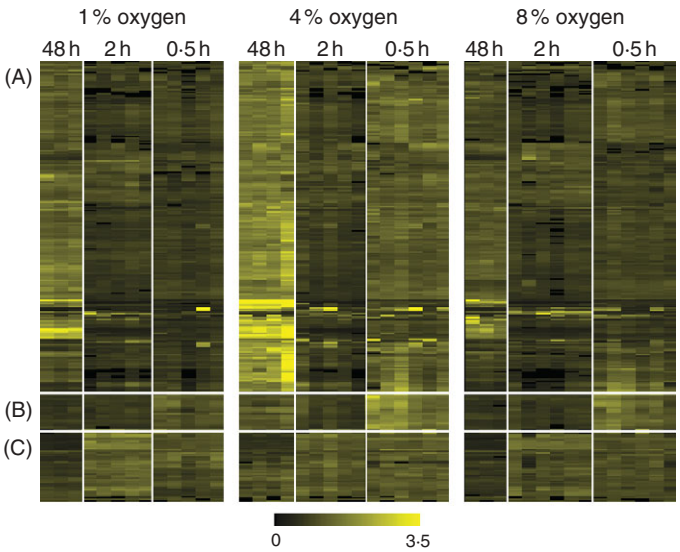


FIG. 6. Visualization of taq-intensities by a heat map shows differential regulation of metabolite levels at short- and long-term incubation at low oxygen concentrations. Shown are those mass fragments that were significantly changed in at least one of the treatments, independent of whether they could be assigned to a known metabolite or not. (A) Cluster A showing increase of metabolites after 48 h; (B) cluster B showing increase of metabolites after 0·5 h; (C) cluster C showing increase of metabolites after 0·5 h and 2 h at low oxygen. The intensity of the colour represents the relative intensity of the respective m/z value after normalization of the data from the various experiments.

DISCUSSION

Microarrays have been used as a powerful tool to explore responses of the plant transcriptome to anaerobic conditions (Gonzali *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007). Studies performed so far focused mainly on plants, or excised plant tissues, incubated under strict anoxic conditions (see Loreti *et al.*, 2005; Lasanthi-Kudahettige *et al.*, 2007) or under very low concentrations of external oxygen in liquid media which will inevitably lead to (near-)anoxic conditions inside the tissues under investigation (see Klok *et al.*, 2002; Liu *et al.*, 2005). Anoxia has many deleterious effects and represents an extreme stress situation for the plant (see Introduction). It is therefore difficult to dissect specific responses to low oxygen from general stress responses from the data obtained in earlier experiments. In the present study, the response of arabidopsis roots to a stepwise decrease in external oxygen concentrations from 21 to 8, 4 and 1 % was investigated. This represents a moderate decrease in oxygen reaching values that are comparable to the oxygen concentrations measured in a variety of plant tissues under physiological conditions (see Porterfield *et al.*, 1999; Geigenberger *et al.*, 2000; Rolletschek *et al.*, 2002, 2003; van Dongen *et al.*, 2003, 2004; Geigenberger, 2003; Vigeolas *et al.*, 2003). Moreover, arabidopsis seedlings were grown on vertical plates with the roots growing on the surface of the agar and being therefore directly exposed to the external gas-phase of which the oxygen concentration could be precisely

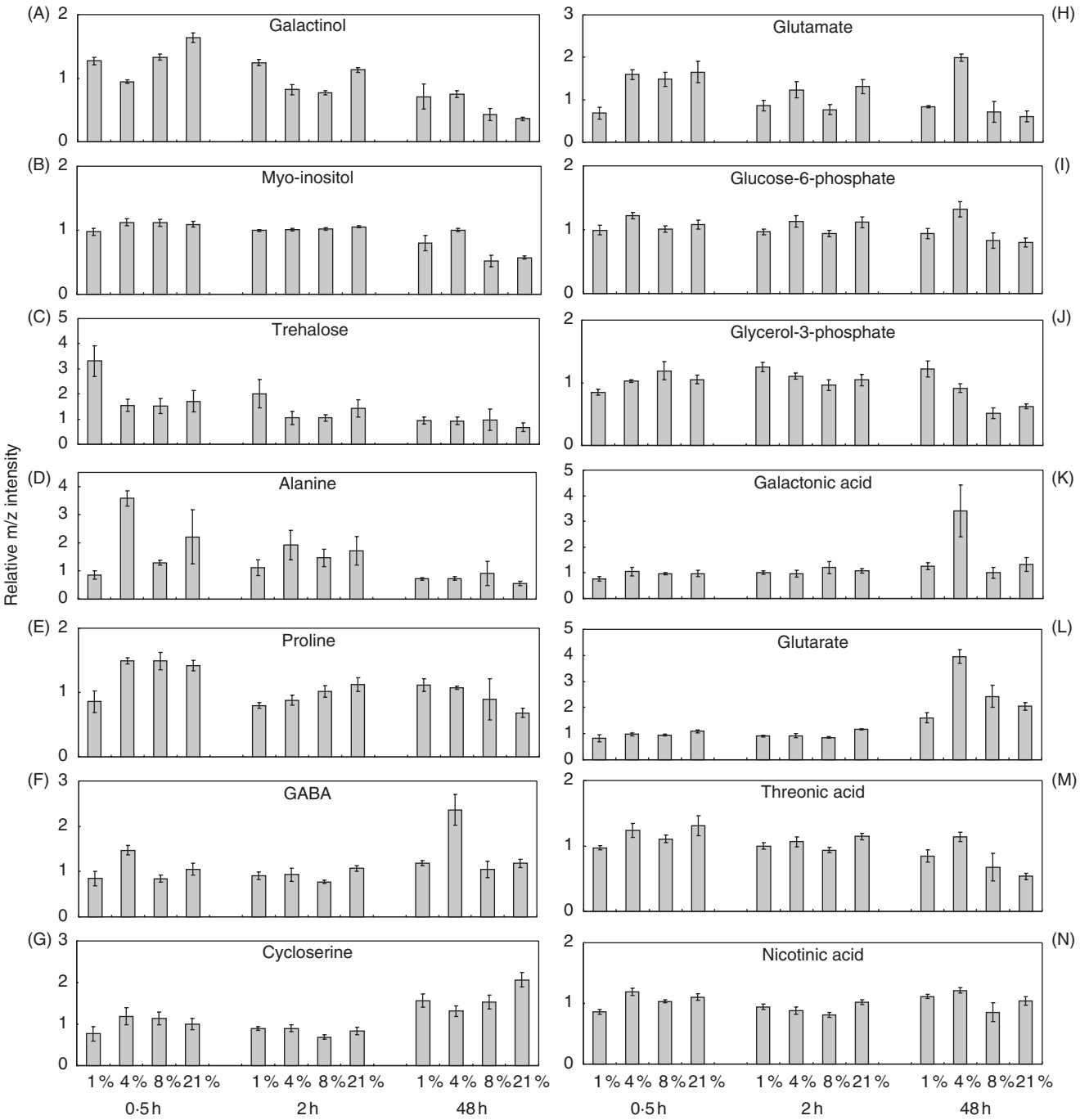


FIG. 7. Changes in the levels of selected annotated metabolites that changed significantly in response to low oxygen in at least one of the treatments. Data are the mean ± s.e. from three to six biological replicates. A significance test was performed using a two-way ANOVA ($P < 0{\cdot}01$).

determined. The present results show that transcript and metabolite profiles were significantly altered already in response to a moderate decrease in oxygen concentration, indicating large-scale reprogramming of gene expression and metabolism under these conditions. These changes were accompanied by an adaptive inhibition of root extension.

Figure 2 shows that a moderate decrease in oxygen leads to a preferential up-regulation of genes. At 8 % oxygen an approx. 5-fold greater proportion of genes were up-regulated (40–120 genes) than down-regulated (2–15 genes). With decreasing oxygen, the ratio of up-regulated to down-regulated genes decreased to 1·6 at 1 % (v/v) of oxygen. Results from previous studies revealed that under anoxic conditions, more genes were down-regulated than up-regulated. In the study by Loreti *et al.* (2005), the number of genes induced by anoxia was 629, whilst 1063 genes were down-regulated. It seems likely that genes which are induced under low oxygen conditions (as in the present

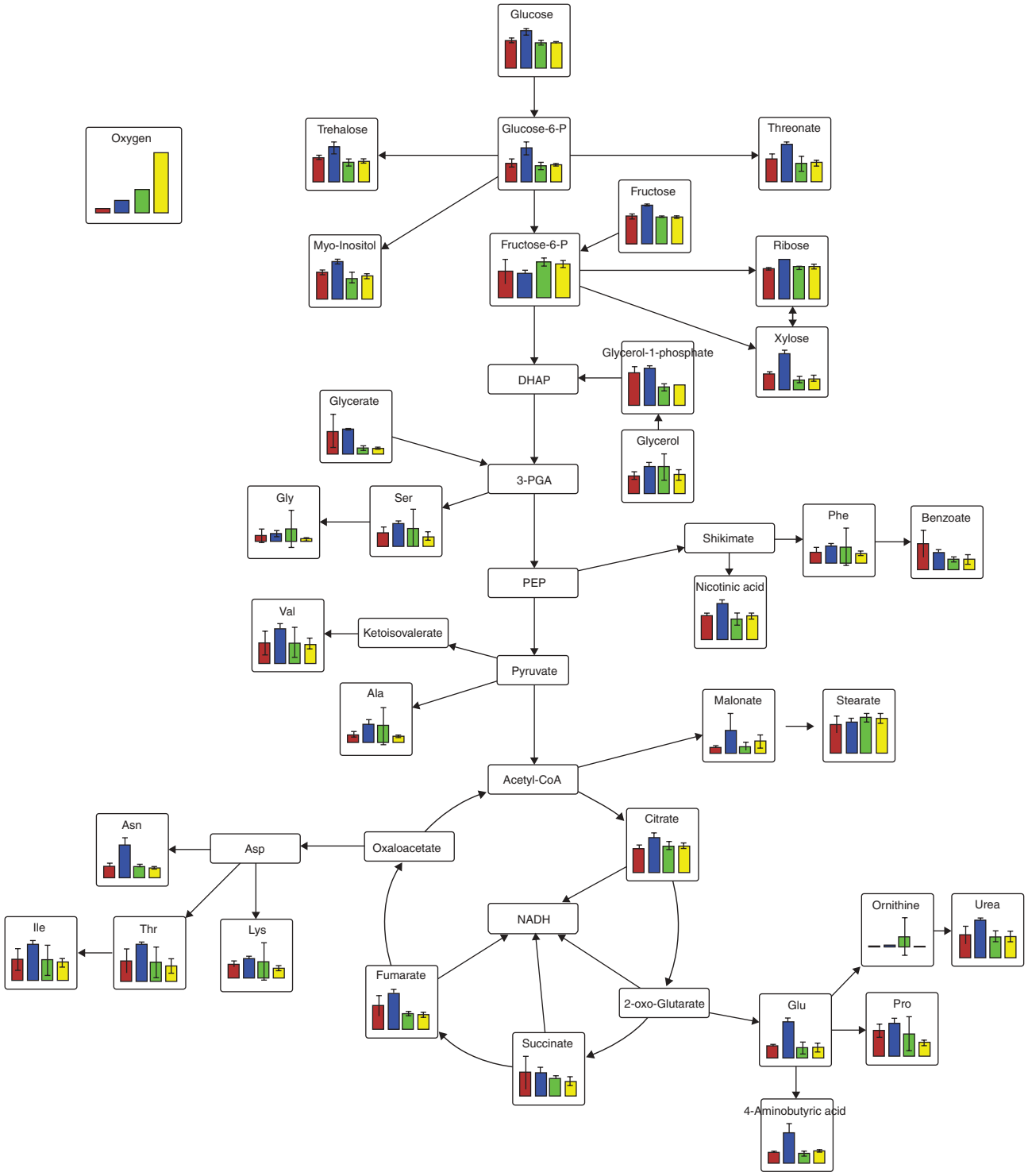


FIG. 8. Low-oxygen-induced changes in the levels of metabolites within metabolic pathways. Metabolite levels in roots after 48-h treatment at low oxygen were analysed using GC-MS and visualized in their adequate pathways using the VANTED software (Junker *et al.*, 2006). The bars represent the following conditions: 21 % oxygen = yellow; 8 % oxygen = green; 4 % oxygen = blue; 1 % oxygen = red. DHAP, dihydroxyacetone phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate. Amino acids are abbreviated with their common three-letter code. Data are the mean ± s.e. ($n = 3–6$).

study) will be important to trigger adaptive responses in the plant, those that are repressed are most probably indicative of strategies to conserve ATP such as the down-regulation of metabolic processes (Fig. 3).

As mentioned above, reprogramming of gene expression is already evident at 8 % (v/v) oxygen. Figure 4 shows a selection of genes that are already induced in response to a moderate decrease in oxygen concentration to 8 %. The same genes

are also induced when external oxygen is decreasing more strongly to 4 % and 1 %. Many of the genes described here have been previously documented to be also induced by anoxia, including the genes coding known anaerobic proteins such as alcohol dehydrogenase, pyruvate decarboxylase and sucrose synthase, or other proteins such as a kelch-repeat containing protein, a LOB domain 41 protein, a non-symbiotic haemoglobin class 1 protein and four proteins with unknown function (see Gonzali *et al.*, 2005; Liu *et al.*, 2005; Loretti *et al.*, 2005). This selection of genes can therefore be regarded as oxygen responsive. Some of them are very likely to be involved in adaptive responses to decreases in oxygen concentrations (like alcohol dehydrogenase, pyruvate decarboxylase and sucrose synthase) and others might probably be involved in oxygen signalling. Reverse genetic approaches and mutant studies are underway to probe the exact functionality of these genes and to test their candidacy for the improvement of low oxygen tolerance.

In parallel to the changes described above, statistical analysis of the data also reveals that low oxygen leads to a preferential decrease in transcripts for genes encoding for proteins involved in transport, signalling, lipid metabolism, secondary metabolism and redox-regulation (Fig. 3). Especially, transport, lipid metabolism and secondary metabolism represent processes with a relatively high energy demand (Riewe *et al.*, 2008). A global depression of ATP-consuming processes represents a powerful strategy to save oxygen since it allows oxygen consumption to be decreased (Geigenberger *et al.*, 2000; Bologa *et al.*, 2003; Geigenberger, 2003). A reduction in oxygen from 21 % to 8 % (v/v) has been reported to lead to an adaptive depression in metabolism in various tissues such as stems, seeds, tubers, roots and fruits (see Solomos, 1982; Geigenberger, 2003) This involves a global decrease in many ATP-dependent biosynthetic processes, such as lipid synthesis, protein synthesis and phenylpropanoid synthesis (Geigenberger, 2003).

The inhibition of root growth also provides additional evidence that biosynthetic processes have been inhibited. The present data show that already a decrease from 21 % to 8 % oxygen leads to a significant reduction in root extension growth (Fig. 1). Root extension growth decreased progressively with decreasing oxygen, providing evidence that it is linked to the availability of oxygen. This represents an adaptive response, since a decrease in growth will ultimately save ATP and decrease oxygen consumption. In agreement with this postulate are recent observations that tomato plants deficient in the expression of the genes encoding aconitase, succinyl CoA ligase, fumarase or malate dehydrogenase (see Sweetlove *et al.*, 2007), exhibited both altered energy metabolism and root growth.

In addition to changes in gene expression, there is also a large-scale reprogramming of metabolism under the low oxygen conditions. Evaluation of significant changes in metabolite levels, independently of the identification of these compounds shows differential regulation of metabolite levels at short- and long-term incubation at low oxygen (Fig. 6). Analysis of changes in metabolites that could be identified revealed an increase of the level of many metabolites that were already been found to be induced at low oxygen in earlier studies, such as the amino acids alanine, proline and GABA and the phosphorylated esters glucose-6-P and glycerol-3-P (see Geigenberger *et al.*, 2000; Gibon *et al.*, 2002, van Dongen *et al.*, 2003; Vigeolas *et al.*, 2003). However, there were also changes in the levels of minor sugars and various organic acids that have not previously been reported to be oxygen responsive. Figure 8 reveals that there is a tendency of metabolite levels to be increased when oxygen is reduced from 21 % to 4 %, while metabolites dropped again when oxygen is further reduced to 1 % (v/v). A similar increase in metabolite levels upon a moderate decrease in oxygen concentration has been reported in previous studies to be accompanied by the general depression of biosynthetic activities and growth (for a review, see Geigenberger, 2003). The drop in metabolite levels when oxygen is further decreased is most likely attributable to increased fluxes of sucrose degradation and glycolysis when fermentative processes are induced.

In conclusion, the results of this study show that there is an adaptive decrease in root extension involving large-scale reprogramming of gene expression and metabolism in response to a mild decrease in oxygen concentration. A small but highly specific set of oxygen-responsive genes is induced very early in response to a moderate decrease in oxygen concentrations. Some of these genes are very likely to be involved in oxygen signalling and adaptation. Reverse genetics studies are in progress to test the functionality of these genes to mediate low oxygen responses. More studies will be needed to dissect the underlying oxygen-sensing systems and regulatory components.

SUPPLEMENTARY INFORMATION

Supplementary information is available online at www.aob.oxfordjournals.org/ and consists of the following files.

File 1: list of genes that are significantly up-regulated within 0·5 h under 1, 4 or 8 % oxygen, respectively ($P < 0{\cdot}05$). In addition, the expression levels (as signal-log ratio) of the selected genes as measured for the other treatments are given. Expression values with a *P*-value $>0{\cdot}05$ are written in italics.

File 2: list of genes that are significantly up-regulated within 2 h under 1, 4 or 8 % oxygen, respectively ($P < 0{\cdot}05$). Also, the expression levels (signal-log ratios) of the selected genes as measured for the other treatments are given. Expression values with a *P*-value $>0{\cdot}05$ are written in italics.

File 3: list of genes that are significantly up-regulated within 48 h under 1, 4 or 8 % oxygen, respectively ($P < 0{\cdot}05$). Also, the expression levels (signal log ratios) of the selected genes as measured for the other treatments are given. Expression values with a *P*-value $>0{\cdot}05$ are written in italics.

File 4: list of all genes on the micro-array with their signal-log ratios and corresponding *P*-values as determined after 0·5 h (a), 2 h (b) and 48 h (c) under 1, 4 or 8 % oxygen, respectively. The raw data are available from the GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/) with the Series record number GSE11558.

File 5: low-oxygen induced changes in the levels of metabolites within metabolic pathways. Metabolite levels in roots after 0·5 and 2 h treatment at low oxygen were analysed

using GC-MS and visualized in their adequate pathways using the VANTED software (Junker *et al.*, 2006).

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