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Hypoxia responsive gene expression is mediated by various subsets of transcription factors and miRNAs that are determined by the actual oxygen availability

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Summary

- Reduced oxygen availability is not only associated with flooding, but occurs also during growth and development. It is largely unknown how hypoxia is perceived and what signaling cascade is involved in activating adaptive responses.
- We analysed the expression of over 1900 transcription factors (TFs) and 180 microRNA primary transcripts (pri-miRNAs) in *Arabidopsis* roots exposed to different hypoxic conditions by means of quantitative PCR. We also analysed the promoters of genes induced by hypoxia with respect to over-represented DNA elements that can act as potential TF binding sites and their *in vivo* interaction was verified.
- We identified various subsets of TFs that responded differentially through time and in an oxygen concentration-dependent manner. The regulatory potential of selected TFs and their predicted DNA binding elements was validated. Although the expression of pri-miRNAs was differentially regulated under hypoxia, only one corresponding mature miRNA changed accordingly. Putative target transcripts of the miRNAs were not significantly affected.
- Our results show that the regulation of hypoxia-induced genes is controlled via simultaneous interaction of various combinations of TFs. Under anoxic conditions, an additional set of TFs is induced. Regulation of gene expression via miRNAs appears to play a minor role during hypoxia.

Introduction

As ultimate electron acceptor of the oxidative phosphorylation reactions in mitochondria, oxygen represents an indispensable element for aerobic organisms, including plants. However, within plants, oxygen availability can vary strongly between tissues because of diffusional restrictions (Geigenberger, 2003, van Dongen *et al.*, 2003). Also, during organ development, the internal oxygen concentration can change considerably (Vigeolas *et al.*, 2003; Van Dongen *et al.*, 2004; Borisjuk *et al.*, 2007). Therefore, hypoxic conditions are common within plants even when oxygen is easily available from the environment. Moreover, severe hypoxic conditions or even anoxia can occur in plant tissues when the availability of environmental oxygen decreases, for example owing to reduced diffusion rates of gases in water during flooding or waterlogging (Armstrong, 1979).

Hypoxia not only represents a stress condition, but is also implicated as signal in the regulation of growth and development under optimal conditions. In human embryonic stem cells, for example, variations in oxygen levels are likely to play a role, by regulating pluripotency and proliferation (Forristal *et al.*, 2010). However, in plants, different cell types exhibit a conserved response to low oxygen levels at the molecular level (Mustroph *et al.*, 2010). This response includes the induction of a core set of genes after 30 min under hypoxia, whose expression is maintained for several hours (Klok *et al.*, 2002; van Dongen *et al.*, 2009). The increased transcript levels of these genes are further correlated with an enhanced association of the mRNAs with polysomes, suggesting that these transcripts are actively translated (Branco-Price *et al.*, 2008). Many of these genes that increase their expression when the oxygen availability reduces are required to maintain energy production via

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glycolysis, such as pyruvate decarboxylase (*PDC*), alcohol dehydrogenase (*ADH*; Ismond *et al.*, 2003) and alanine aminotransferase (*AlaAT*; Rocha *et al.*, 2010).

As for many other responses to environmental stimuli, a signaling cascade is likely to be required to transmit the hypoxic signal from its perception to the core anaerobic response and integrate it in the developmental program. As the mechanism by which oxygen is perceived in plants is still largely unknown (Licausi & Perata, 2009), hypoxia-responsive transcription factors (TFs) are a good starting point to investigate the regulation of the hypoxic response, as they represent the last regulatory element in the signal cascade. Furthermore, analysis of *cis*-acting DNA sequences over-represented in anaerobic gene promoters can be coupled to the isolation of *trans*-acting TFs, to identify master regulators in the transcriptional cascade.

Various studies have addressed the role of some hypoxia responsive TFs belonging to different protein classes, including the MYB (Myeloblastosis), NAC [NAM (No Apical Meristem), ATAF (Arabidopsis Transcription Activation Factor), CUC (Cup-shaped Cotyledons)], PHD (Plant Homeodomain) and ERF (Ethylene Responsive Factor) families (Hoeren *et al.*, 1998; Bond *et al.*, 2009; Christianson *et al.*, 2009; Licausi *et al.*, 2010). However, to date, no TF has been shown to act alone to trigger the induction of the core-set of anerobically induced genes. It therefore seems likely that a complex of transcriptional regulators is required to activate the molecular response to hypoxia. To understand the mechanisms and regulation of the molecular response to low oxygen, it is necessary to identify single members that comprise the TF complex first. Generally, TFs have very low transcript abundances, making proper detection a challenge. Although some changes in TF expression were identified via microarray approaches in the past, quantitative real-time PCR (qRT-PCR) is generally recognized to be better suited to identify differentially regulated TFs because of its higher sensitivity and accuracy (Czechowski *et al.*, 2002; Caldana *et al.*, 2007).

As TFs regulate the expression of other genes, including themselves or other TFs, reconstructing the hierarchy of their regulation can be a difficult task. Moreover, TFs constitute one of the widest functional classes of targets for small RNAs (Rhoades *et al.*, 2002). These noncoding transcripts are 20- to 24-nucleotide single-stranded RNAs, such as microRNAs (miRNAs) and *trans*-acting small interfering RNAs (ta-siRNAs) that promote degradation of the mRNAs to which they bind or hinder translation (Brodersen *et al.*, 2008; Lanet *et al.*, 2009). In plants, miRNAs have been shown to regulate development (Aukerman & Sakai, 2003; Emery *et al.*, 2003; Palatnik *et al.*, 2003) or the response to abiotic stresses (Sunkar & Zhu, 2004) including macronutrient limitation (Bari *et al.*, 2006; Chiou *et al.*, 2006; Pant *et al.*, 2008, 2009; Branscheid *et al.*, 2010; Huang *et al.*, 2010). Hypoxia-responsive miRNAs and ta-siRNAs were reported by Moldovan *et al.* (2009). These noncoding RNAs might also account for the variations in mRNA levels of the anaerobic genes, especially TFs.

In the present study, we report the comprehensive analysis and identification of upregulated and downregulated transcripts for TFs and pri-miRNAs in roots of Arabidopsis seedlings treated for 30 min and 2 h with different hypoxic conditions using qRT-PCR. An extensive correlation analysis was performed on the TF/miRNA data and available microarray analyses obtained from identically treated plant material. Several conserved DNA-binding elements were identified and subsequently validated to be involved in the regulation of the transcriptional response of *Arabidopsis* roots to low oxygen.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 seedlings were grown in a similar manner to that described in van Dongen *et al.* (2009), with a 12 h light (160 μE) and 12 h darkness cycle (day and night temperature was 22°C) on vertical plates with 10% agar dissolved in half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie BV, 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. Fifteen days after germination, seedlings were transferred to continuous darkness and a stream of premixed air containing different oxygen concentrations (as indicated in legends to Figs 3 and 5), 350 ppm CO_2, and N_2 (Air Liquide, Berlin, Germany) was blown over the plates. Each hypoxic treatment and the normoxic control treatments for all different time points were conducted similarly in darkness to abstract variation induced by variable factors other than the oxygen concentration, such as developmental variation or changes induced by the shift from light to dark. Roots were harvested in liquid nitrogen at the time-point indicated in the legends to Figs 3 and 5.

RNA extraction and cDNA synthesis

Total RNA was isolated from roots using TRIZOL reagent (Invitrogen), as described by Czechowski *et al.* (2002). Seventy-five micrograms of total RNA were subjected to a DNase treatment using a TURBO DNA free kit (Ambion), according to the manufacturer's instructions. Absence of genomic DNA contamination was tested by PCR, using primers designed on intron sequence of a control gene (At5g65080). cDNA was synthesized from 13 μg DNA-free RNA using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by real-time

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PCR amplification of control genes encoding *UBQ10* (*At4g05320*) and 1 kb distant regions on the *GAPDH* (*At1g13440)* gene. Only cDNA preparations that yielded similar C_T (cycle number at with the fluorescence signal crosses a fixed threshold of 0.2 units) values (± 3 cycles) for the GAPDH genes were used.

qRT-PCR for TF and pri-miRNA transcript quantification

Polymerase chain reactions were performed in an optical 384-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using SYBR Green to monitor dsDNA synthesis. Reactions contained 2.5 µl 2× FAST SYBR Green Master Mix reagent (Applied Biosystems), 13.0 ng cDNA and 100 nmol of each gene-specific primer in a final volume of 5 µl. The following standard thermal profile was used for all PCRs: 95°C for 2 min; 40 cycles of 95°C for 3 s and 60°C for 20 s. In order to compare data from different PCR runs or cDNA samples, C_T values for all TF genes were normalized to the average C_T value of the housekeeping genes *UBQ10* (*At4g05320*), *PP2A* (*At1g13320)*, *SAND Box Protein* (*At2g28390*) and *GAPDH* (*At1g13440*) (Czechowski *et al.*, 2005), obtaining a ΔC_T value for each gene tested in each sample. The TF relative gene expression between aerobic and anaerobic samples was obtained from the equation $(1 + E)^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ represents $\Delta C_{\text{Thypoxia}}$ minus $\Delta C_{\text{Tnormoxia}}$, and E is the PCR efficiency.

Quantification of mature miRNA by stem-loop quantitative PCR (SL-PCR)

The expression of mature miRNAs was assayed by SL-PCR and performed as described previously (Varkonyi-Gasic *et al.*, 2007). The stem–loop reverse transcription primers used have been previously described by Varkonyi-Gasic *et al.* (2007). The reverse transcription reactions were performed on 200 ng total RNA using Superscript III (Invitrogen) and carried out in a pulsed-reverse transcription reaction as described by Varkonyi-Gasic *et al.* (2007). Two cDNA reactions were performed, one containing the miRNA specific primer, and one for the housekeeping control U6, as in Moldovan *et al.* (2009). The reverse transcription product was amplified using a miRNA specific forward primer and a universal reverse primer on a Rotor-Gene 6000 (Invitrogen) in an optical 384-well plate with an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), using SYBR Green to monitor dsDNA synthesis. Reactions contained 5 µl 2× POWER SYBR Green Master Mix reagent (Applied Biosystems), 20 ng cDNA and 250 nmol of each gene-specific primer in a final volume of 5 µl. The following standard thermal profile was used for all PCRs: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 50 s. A final denaturation step (95°C for 15 min, 60°C for 15 min, 95°C for 15 min) was used to examine dissociation curves and test the presence of one single amplicon. Relative RNA levels were calculated using the $\Delta\Delta C_t$ method as described earlier.

Bioinformatic promoter analysis

Differentially expressed genes (DEG) (≥ 2 FC or ≤ −2 FC) from the van Dongen *et al.* (2009) dataset (Gene Expression Omnibus accession number: GSE11558; http://www.ncbi.nlm.nih.gov/geo/) were selected and split into four groups: induced after 30 min hypoxia (1% and 4% O_2), repressed after 30 min hypoxia, induced after 2 h hypoxia and repressed after 2 h hypoxia. Sequences of 1000 bp upstream from the transcription start site of the selected genes were downloaded from the Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/tools/bulk/index.jsp) using the Bulk Data Retrieval tool. Identification of over-represented hexameric and octameric motifs was carried out using the Motif Analysis (http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp) and by BioProspector Search tool (Liu *et al.*, 2001). Identification of known DNA elements bound by TFs in the promoter dataset was carried out using the web-based analysis tool PlantPAN (Chang *et al.*, 2008) and Pscan (Zambelli *et al.*, 2009), using default parameters.

Reporter transactivation assay in protoplasts

Coding sequences (CDSs) of At1g72360, At3g02550, At1g69570, At5g66980 and At4g29190 were amplified from cDNA of hypoxic Arabidopsis roots. *Renilla* luciferase CDS was amplified using pRL-null (Promega) as a template. The resulting amplicons were ligated into the pENTR/D-topo vector (Invitrogen) and subsequently recombined in p2GW7 (Karimi *et al.*, 2002), using the LR clonase enzyme (Invitrogen) to generate the 35S::TF test vector and the 35S::RLuc normalization vector. Arabidopsis plants stably expressing the firefly luciferase CDS under control of the full AtHB1 promoter were used to produce protoplasts according to the protocols described by Yoo *et al.* (2007). Protoplasts were transformed with the test and normalization vectors together using 5 µg plasmid DNA each, according to Yoo *et al.* (2007). Nonrecombined p2GW7 was used as a control. Dual luciferase reporter assay was performed using the kit provided by Promega and luminescence was read using a GloMax 20/20 Luminometer (Promega).

miRNA target gene prediction

Candidate miR target genes were determined using publicly available prediction algorithms, including miRU (Zhang,

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2005), the target search in WMD2 (Ossowski *et al.*, 2008) and the prediction tool in the UEA plant sRNA toolkit (Moxon *et al.*, 2008). The programs were used with their default settings.

Results

Hypoxic core genes share conserved motifs in their upstream regions

We analysed the genes that were selected for being differentially expressed in root tissues of Arabidopsis seedlings during hypoxia (van Dongen *et al.*, 2009) for the occurrence of overrepresented regulatory motifs. This dataset describes the changes occurring after 30 min, 2 h and 48 h at four different oxygen concentrations (1%, 4%, 8% and 21% O_2) in darkness. As most changes occurred within the first 2 h of the hypoxic treatments, we selected only those genes that showed at least a twofold change (positive or negative) under hypoxia (both 1% and 4% O_2) compared with the normoxic control (21% O_2) after 30 min and 2 h. Most of these genes were also induced at 8% O_2, although to a smaller extent. The resulting lists are shown in the Supporting Information Tables S1–S4. The overlap between the group of genes induced after 30 min (55 genes) and those upregulated after 2 h (52 genes) was high (45 genes). Conversely, out of 40 genes repressed after 30 min hypoxia only eight were still downregulated after 2 h.

We extracted the first 1000 nucleotides upstream of the annotated translation initiation codon (ATG) for each hypoxia-responsive gene (Tables S1–S4) and scanned them for over-represented DNA elements using various freely available analysis tools. Pscan (Zambelli *et al.*, 2009) and PlantPan (Chang *et al.*, 2008) were used to search for known motifs already reported to be bound by TFs and present in the Jaspar, Transfac and Place databases. Both tools coherently identified the binding site for the Arabidopsis Homeobox1 and Homeobox5 (AtHb5) TFs, being enriched in both 30 min and 2 h 'upregulated genes' datasets (Table S5). Pscan reported an enrichment of the homeobox elements AATAATT in the promoters of genes induced and repressed after 30 min, and the AGL3 elements in both upregulated and downregulated genes after 2 h (Table S5). Furthermore, the promoter sequences were analysed for the presence of motifs that were not annotated as regulatory elements before. For this purpose, the 'Motifsearch' tool from the TAIR website and the 'Bioprospector' tool were used. Among these newly identified motifs that were significantly ($P < 0.001$) over-represented in the set of hypoxia induced genes, six elements were present in both upregulated datasets (30 min and 2 h) (Fig. 1). Both prediction tools identified the sequences ATCTAT in the 30 min and 2 h datasets and TTGGAT in the 2 h dataset only (Fig. 1, Tables S6, S7). No common

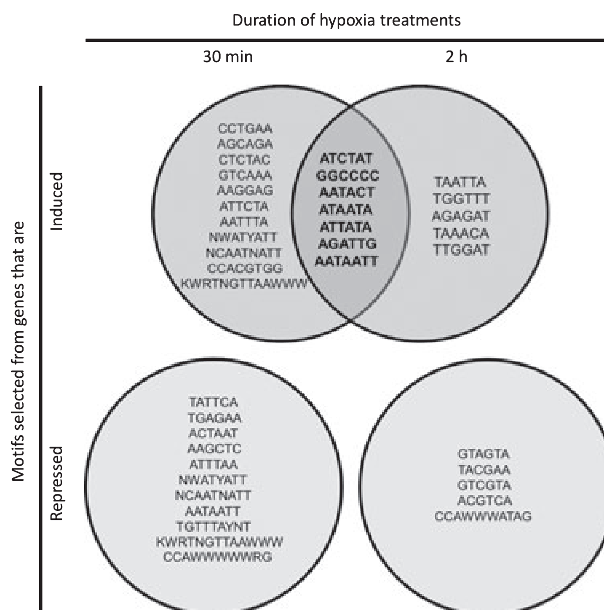

Fig. 1 Hexamer motifs overrepresented in hypoxia responsive promoters. Venn diagram depicting the number of DNA elements present in the 1 kb upstream region of induced (UP) and repressed (DOWN) genes after 30 min and 2 h hypoxia (1% and 4% O_2). A full overview with information about the statistical evaluation of the elements can be found in the Supporting Information, Tables S5–S8.

novel DNA element was identified in the downregulated datasets (Fig. 1 and Tables S8, S9).

Identification of TFs that are differentially expressed during hypoxia

In order to identify TFs that are differentially expressed by hypoxic conditions, we used a qRT-PCR platform (Czechowski *et al.*, 2002; Barrero *et al.*, 2009) that was successfully used in the past (Scheible *et al.*, 2004; Morcuende *et al.*, 2007; Osuna *et al.*, 2007). The current version of this tool allows the quantification of > 1900 Arabidopsis TF gene transcripts. The TF transcript profiling experiment was performed with the same set-up and conditions as described in van Dongen *et al.* (2009) for the microarray analysis to provide maximum comparability between the two datasets. Both the hypoxia-treated plants and the normoxic controls were kept in darkness in order to exclude changes induced by variable factors other than the oxygen concentration such as what could be induced by the shift from light to darkness.

The analysis revealed that mild hypoxic conditions (8% O_2) induced upregulation of a small number of TF genes after 30 min and 2 h from the hypoxia onset (7 and 11 genes, respectively) (Fig. 2). Lower oxygen concentrations increased the number of upregulated TF after 30 min hypoxia (29 and 24 genes under 4% and 1% O_2, respectively), with a further increase at 1% O_2 (81 genes). The

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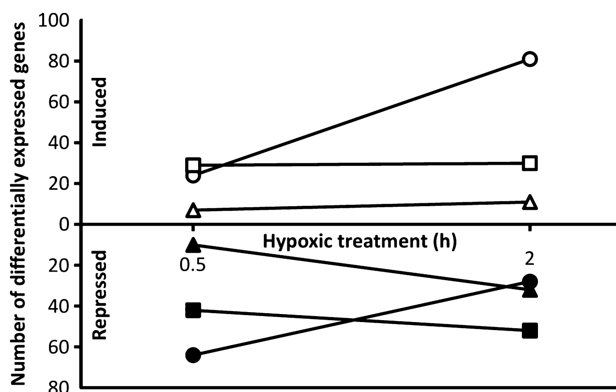

Fig. 2 Number of transcription factors (TFs) being upregulated or downregulated by hypoxia through time. Effect of a stepwise decrease in oxygen concentrations on the number of differentially expressed TFs. Arabidopsis seedlings were grown on vertical sterile plates and incubated at (open triangle) 8%, (open square) 4%, or (open circle) 1% (v : v) oxygen for 30 min and 2 h. The amount of genes is plotted that were at least two-times upregulated (open symbols) or downregulated (shaded symbols).

same trend was observed for downregulated genes, although after 2 h at 1% O_2 the number of downregulated genes was again reduced (Fig. 2). Among the induced TF genes, 21 were already induced after 30 min hypoxia and remained upregulated after 2 h and 10 showed increased transcript levels after 2 h only whereas 12 decreased their expression. Twenty-nine TF genes maintained reduced mRNA levels after 30 min and 2 h (Fig. 2). Eight TFs significantly increased their mRNA level after 30 min and were repressed to the aerobic levels or lower after 2 h hypoxia (Fig. 2).

The full list of differentially expressed TF genes is given in Fig. 3. Twelve TF genes were transiently induced by the hypoxic treatment (1% and 4% O_2) after 30 min and returned to levels comparable to the aerobic controls or lower after 2 h. By contrast, 17 TF transcripts progressively accumulated after 2 h treatment (Fig. 3a). Among the TFs that have been already characterized, members of the AP2/ERF-type family are the most commonly represented in the set of upregulated TFs (eight members), followed by Zinc-finger (six genes) and bHLH-type TFs (five genes) (Fig. 3b). Transcription factors belonging to the bHLH family are also highly represented among the downregulated genes (10 members), together with members from the bZIP and MYB families (eight members each).

Hypoxia responsive TFs belonging to the auxin response factor (ARF), DNA binding with One Finger (DOF), ERF and Homeobox families potentially bind the ‘hypoxic core’ promoters

To identify TFs that are supposed to play a role in the early induction of the hypoxic core genes, we searched the plant TF database PLANTTFDB (Guo *et al.*, 2008) for DNA elements specifically bound by the protein families to which the hypoxia responsive TFs belong. We also integrated the DNA elements characterized by previous studies but not included in PLANTTFDB. Subsequently, we carried out an extensive search for the presence of these DNA elements in the first 1000 base pairs upstream the transcriptional start site of the upregulated genes. Four protein families that included hypoxia-responsive TFs were able to bind DNA elements present in the promoter of at least half of the hypoxic responsive core genes. The ERF binding sites ATCTA (Welsch *et al.*, 2007) and ATATT (Kannangara *et al.*, 2007), and the DOF TF binding element CTTTT(T/A) were present at least once in all the promoters of the selected hypoxia-induced genes (upregulated after 30 min and 2 h) (Table S10). Also, the DNA motif bound by Homeobox proteins, AATAATT, is present in the promoter region of 44 hypoxia-induced genes (Table S10). Finally, the TGTCTC element recognized by the ARF TF family, was found in 24 promoters.

Hypoxia-responsive TFs can upregulate the expression of hypoxia-induced genes

To verify whether the identified TFs have indeed the potential to regulate the expression of hypoxia responsive genes, we assayed their *trans*-activation capacity on the promoters of two anaerobic genes, Alcohol Dehydrogenase (*ADH*) and Nonsymbiotic Hemoglobin1 (*HB1*). Five hypoxia-induced TFs were selected (*At4g29190;LBD41, At3g02550;HRE1, At1g72360; At1g69570; At5g66980*) from different TF families (Zinc Finger, LBD (Lateral Organ Boundary Domain), ERF, DOF, ARF) according to their predicted interaction with the *ADH* and *HB1* promoters (Fig. 4a). Mesophyll protoplasts were produced from transgenic Arabidopsis leaves expressing the firefly (*Photinus pyralis*) luciferase gene fused to the complete upstream sequence of the *At1g77120* (*ADH*, 905 nucleotides) and *At2g16060* (*HB1*, 369 nucleotides) as flanked by the start codon of either *ADH* or *HB1* and the 3′-untranslated region of the next first upstream gene on the chromosome. Protoplasts were then transiently transformed with vectors carrying the selected TFs under the control of the strong and constitutive 35S promoter of the Cauliflower Mosaic Virus (CaMV). The transformed protoplasts were incubated in the dark before the analysis of the reporter expression to exclude any putative effects of light on the selected hypoxic promoters.

In accordance with our *in silico* prediction, the selected TFs induced the expression of the reporter genes when the promoter contained the relevant binding element. The *HB1* promoter gave significant (≥ twofold) induction of the reporter gene when *LBD41*, *HRE1*, *At1g69570* and *At5g66980* were overexpressed in the protoplasts. The *ADH* promoter, instead, showed a more modest induction (≤ 1.5-fold) by overexpression of *HRE1*, *At4g29190* and *At5g66980*. Of the three TFs with at least one putative

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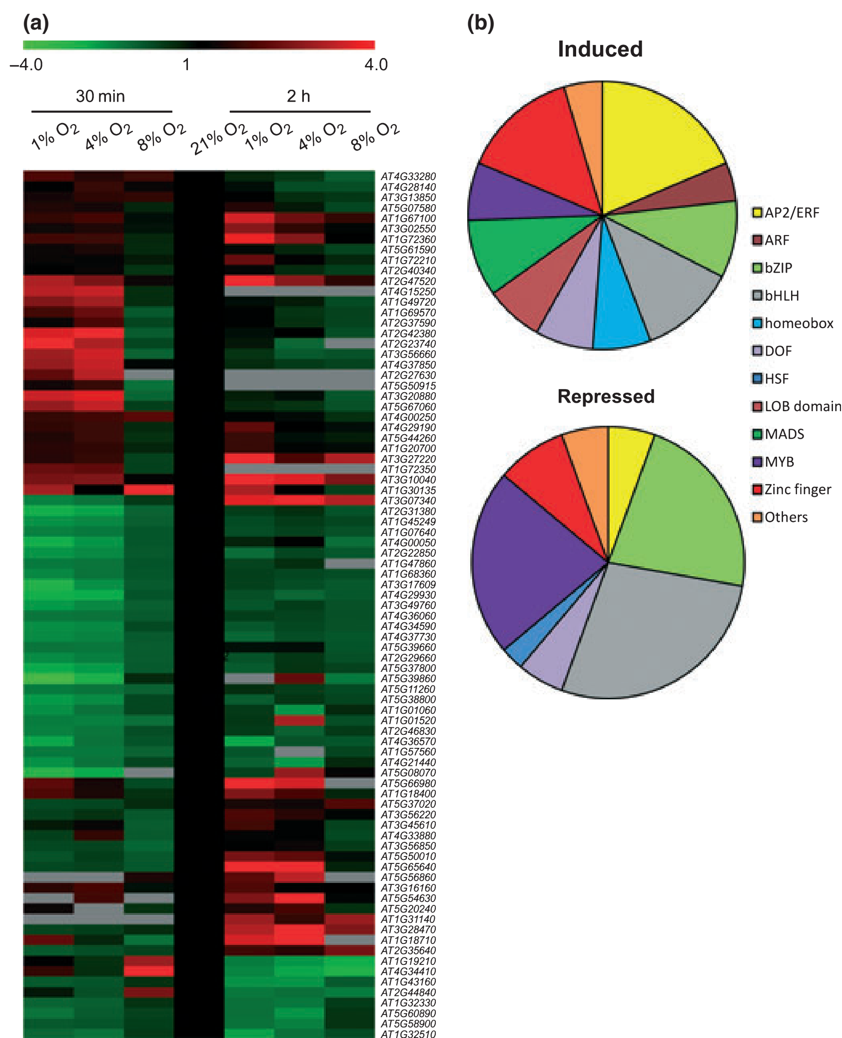

Fig. 3 Differentially expressed transcription factors (TFs) under different hypoxic conditions. (a) Heatmap showing the relative RNA level of microRNA primary transcripts (pri-miRNAs) significantly increased or decreased ($P \leq 0.05$) after 30 min or 2 h hypoxia (1%, 4%, or 8% (v/v) O_2) with respect to normoxia (21% (v/v) O_2). Each treatment was repeated twice and only TFs were selected that showed differential expression in at least two treatments. The fold-change is shown as log (2). (b) Pie-chart depicting the distribution of induced and repressed TF genes in protein families.

binding site in the two promoters, only HRE1 and At5g66980 were able to transactivate the expression of both reporter constructs, while At1g69570 only affected the luciferase expression in combination with the *HB1* promoter. In accordance with the prediction, At4g29190 only caused induction in combination with the *ADH* promoter, which contains a bipartite zinc finger binding site AATTAAANNNNNAATTA (Park *et al.* 2007). By contrast, overexpression of *LBD41* only led to transactivation when the reporter gene was fused to the *HB1* promoter, although a putative binding site for the LBD family (CGGCG) was identified only in the *ADH* promoter. Summarizing, it can be concluded that, with one exception, the transactivation assays confirmed the *in silico* prediction of the regulatory TF binding elements.

Identification of hypoxia-responsive pri-miRNAs

Moldovan *et al.* (2009) identified various mature miRNAs and their precursors being upregulated or downregulated after 5 h of near-anoxic (0.1% O_2) conditions. In order to test whether this regulation takes place after 30 min or 2 h hypoxia and at various oxygen concentrations, we used the pri-miRNA qRT-PCR platform recently reported by Pant *et al.* (2009). Only one pri-miRNA, miR159c, showed increased expression (logFC $\geq$ 2) in hypoxic conditions after 30 min, whereas 17 pri-miRNAs were downregulated (logFC $\leq$ −2) both at 1% and 4% O_2 concentrations (Fig. 5a). After 2 h the number of upregulated pri-miRNA increased to four, while the downregulated pri-miRNA decreased to eight (Fig. 5a). MiR159c is the most induced sequence after 30 min, although the increased expression does not last long, as after 2 h hypoxia it returned to the aerobic expression levels (Fig. 5b).

Specific miRNA biosynthesis takes place at the post-transcriptional level

A previous analysis of mature miRNA in Arabidopsis identified several sequences upregulated after 5 h hypoxia

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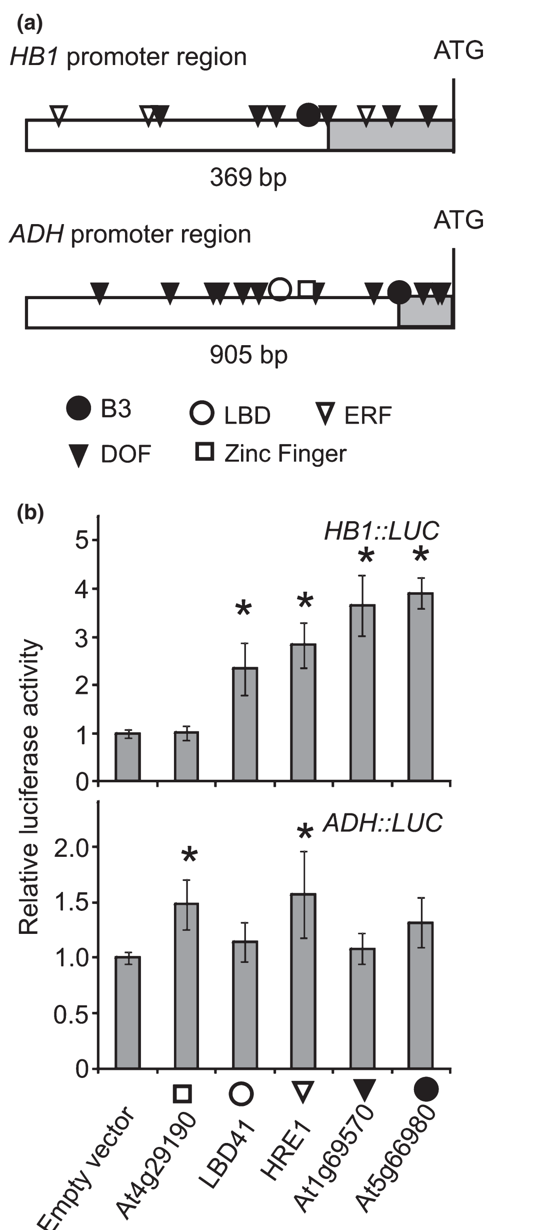

Fig. 4 Transactivation essay to test the ability of selected hypoxic transcription factors (TFs) to activate gene expression via interaction with promoter elements from *ADH* or *HB1 Arabidopsis* protoplast. (a) Schematic representation of the putative binding elements of the selected TF on the *ADH* and *HB1* promoters. The 5′ untranslated regions are the tinted part of the bars, the 5′ intergenic regions are indicated by the open part of the bars. (b) Regulation of *ADH* and *HB1* expression by transient expression of hypoxia-responsive TFs. Transgenic *Arabidopsis* plants stably transformed with the reporter constructs *ADH::fLuc* or *HB1::fLuc* were used to prepare mesophyll protoplasts. The protoplasts were simultaneously transfected with a P35S:TF effector construct and a P35S:rLUC construct for normalization of the signal. Averaged ($n \geq 6$) normalized fLuc/rLuc activities are plotted relative to the empty vector control. Error bars represent standard deviations. Asterisks indicate significant effects (one-way ANOVA test, $P < 0.05$).

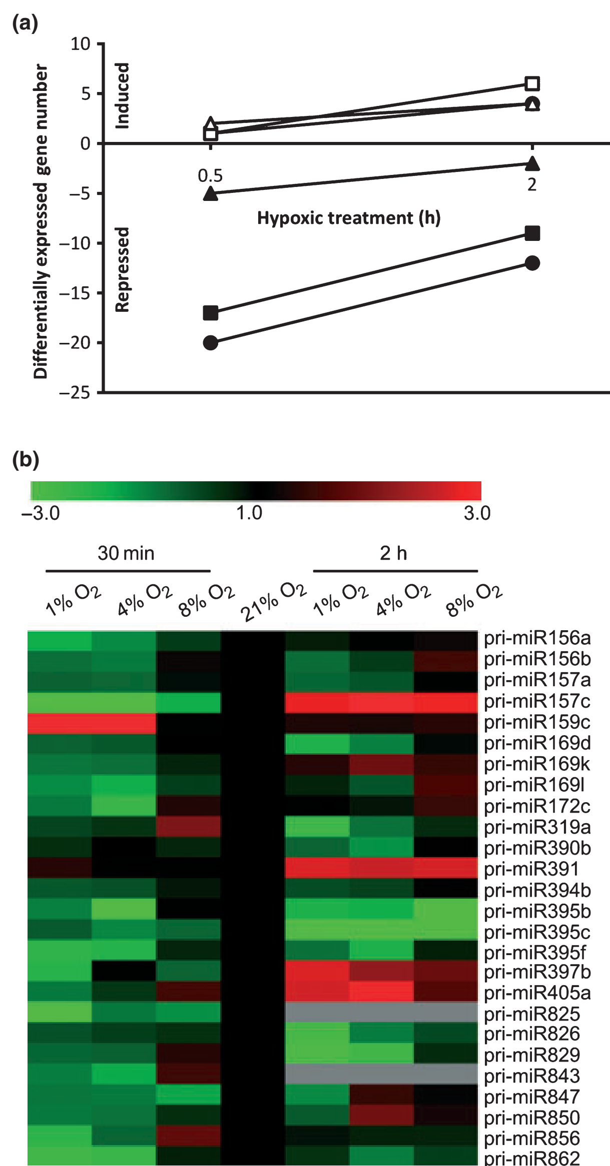

Fig. 5 Differentially expressed microRNAs (miRNA) under different hypoxic condition. (a) Effect of a stepwise decrease in oxygen concentrations on the number of differentially expressed microRNA primary transcripts (pri-miRNAs). Arabidopsis seedlings were grown on vertical sterile plates and incubated at different oxygen concentrations for 30 min and 2 h. Plotted here are the number of genes that were at least two-times up-regulated (open symbols) or down-regulated (shaded symbols) by either (open triangle) 8%, (open square) 4%, or (open circle) 1% (v/v) oxygen at the two different time intervals, as indicated on the horizontal axis. (b) Upregulated and downregulated pri-miRNA after 30 min or 2 h hypoxia. Heatmap showing the relative RNA level of pri-miRNAs significantly increased or decreased ($P \leq 0.05$) after 30 min or 2 h hypoxia (1%, 4%, or 8% (v : v) O_2) with respect to normoxia (21% (v : v) O_2). The fold-change is shown as log (2).

(Moldovan *et al.*, 2009). By contrast, in our analysis only one pri-miRNA was induced after 30 min hypoxia and three were induced after 2 h. Therefore, we tested the expression of the pri-miRNA reported by Moldovan *et al.* (2009)

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and those identified in the present study (pri-miR156g, pri-miR157a, pri-mi158a, pri-miR159a, pri-miR172a, pri-miR172b and pri-miR391) at a more detailed time resolution. we also analysed the level of mature miRNA in parallel. Nevertheless, we could only detect a constant increase of pri-miR391, as already observed in the complete pri-miRNA profiling (Fig. 6). Pri-miR157a and pri-miR172a showed a modest increase after 1 h but decreased again after 2 h and 4 h. Conversely, pri-miR156g remained extremely constant during the hypoxic treatment, while pri-miR158a, pri-miR159a and pri-miR172b were downregulated (Fig. 6).

We tested whether the mature miRNAs, produced from the pri-miRNAs previously analysed, were upregulated or downregulated under hypoxia. Mature miRNA391 showed a modest increase (> twofold) after 4 h, whereas the other mature miRNAs remained constant (miR156, miR157, miR158, miR159, miR172) (Fig. 6). In general, for all the miRNAs examined, changes at the pri-miRNA level did not result in the same magnitude of change in the mature miRNA pool.

Putative target transcripts of miRNAs that are differentially expressed under hypoxia do not change their abundance

miRNAs regulate gene expression by endonucleolytic cleavage of target mRNAs and they may also act through inhibition of translation (Bari *et al.*, 2006; Brodersen *et al.*, 2009; Pant *et al.*, 2009). Many of the known targets of miRNA are TF genes. Therefore, we searched for predicted or experimentally determined targets of the miRNA whose pri-miRNA was observed to be upregulated or downregulated under hypoxia (Table S11). The targets of the four upregulated miRNAs identified in this work included MYB TFs (pri-miR159c), protein kinases (pri-miR391), oxidoreductases (miR397) and squamosa promoter binding proteins (SBPs) (miR156e). Analysis of the TF dataset obtained in the present work and the previous whole-transcriptome profiling revealed that none of the targets of the induced pri-miRNA showed a significant downregulation at the transcriptional level within 2 h hypoxia. In a similar fashion, targets of downregulated miRNA did not show induction.

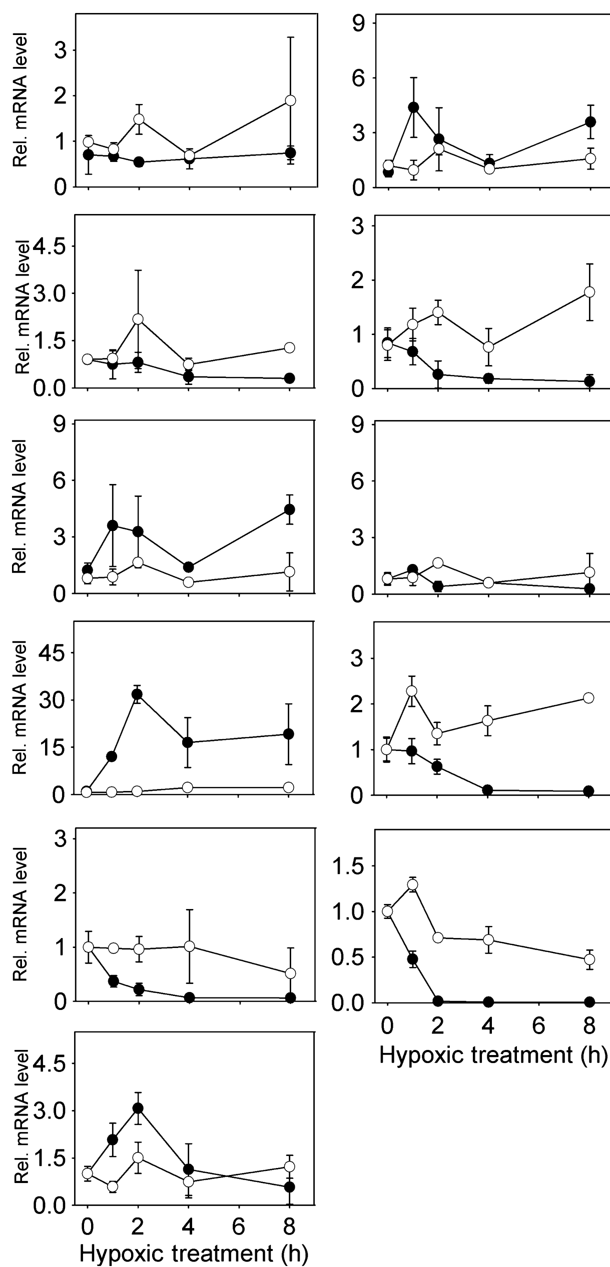

Fig. 6 Time-course analysis of the effect of hypoxia (1% O_2) on selected microRNA primary transcripts (pri-miRNAs) and their mature miRNA. Relative RNA levels of miRNAs precursors (*pri-miR156a*, *pri-miR157a*, *pri-miR158a*, *pri-miR159a*, *pri-miR72a*, *pri-miR172b* and *pri-miR391*, *pri-miR169d*, *pri-miR829*, *pri-mi395b*, *pri-miR397b*) and mature miRNAs (*miR156*, *miR157*, *miR158*, *miR159*, *miR172*, *miR391*, *miR169*, *miR829*, *miR395*, *miR397*) in roots of 15-d old seedling of Col-0 plants. Changes of the relative RNA levels were measured during an 8-h time-course by real-time quantitative PCR- calculated according the $2^{-\Delta\Delta CT}$ method and shown as fold-change values (1 = 0 h Hypoxia).

Discussion

Hypoxia-specific TFs identified by qRT-PCR

When plants are exposed to hypoxic conditions, they rapidly adapt their transcriptional program to cope with the developing stress conditions (van Dongen *et al.*, 2009). Most of the upregulated mRNAs are also loaded on polysomes to allow their translation (Branco-Price *et al.*, 2008). As TF proteins and small RNAs play a crucial role in the control of the transcriptional machinery, unveiling their targets and their regulation represent a milestone in the understanding of plant adaptation to oxygen shortage.

The qRT-PCR technique represents a sensitive assay to quantify changes in the abundance of TF and pri-miRNA

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transcripts, which are often expressed at levels that are undetectable by microarrays (Czechowski *et al.*, 2002; Scheible *et al.*, 2004; Morcuende *et al.*, 2007; Osuna *et al.*, 2007). Previous microarray analyses identified some TF genes upregulated by low oxygen conditions (Klok *et al.*, 2002; Loreti *et al.*, 2005; van Dongen *et al.*, 2009). Targeted analyses of specific cell population s, such as those performed by Mustroph *et al.* (2009), can circumvent the sensitivity limitation of the microarray technique. The signal for cell specific mRNA is increased by the enrichment derived by the immune-precipitation (Mustroph *et al.*, 2009). However, this study mainly addressed changes in the translatome and could not distinguish between changes at the transcript level or in the association of mRNA with polysomes. Furthermore, this technique still suffers of sensitivity limitation for mRNA whose amount is low in all cell types. For all these reasons, the qRT-PCR technique is considered to be optimally suited to address changes in the mRNA level of TFs and pri-miRNAs. Thirteen TF genes were upregulated and 33 TF-genes were downregulated after 30 min of either 1% (v/v) or 4% (v/v) oxygen compared with normoxia (21% v/v oxygen) (Fig. 2). After 2 h treatment, 49 TF transcripts were induced and 23 repressed by hypoxia (Fig. 2). A part of the differentially regulated TF genes found in the current study was not identified by previous microarray analyses. This may be because the corresponding probe set is not present on the chip or no significant change was detected as a result of the relatively low expression levels of TFs. Furthermore, it is possible that the discrepancies with previous studies are caused by the differences in experimental set-up: in particular, the upregulation of genes encoding bHLH and homeobox TFs has not been reported before. Interestingly, a set of 12 genes was transiently induced after 30 min hypoxia. Apart from HEC1 and ABF1 proteins, most of these TFs have not yet been characterized. They might be involved in the initiation of the hypoxic response. The low oxygen responsive genes *ADH* and *HB1* require protein synthesis, most likely of one or more TFs, to be induced under hypoxia (Licausi *et al.*, 2010). The rapid induction of these TF under hypoxia suggests that their regulation depends on release of a repressor or post-transcriptional activation of an activator. Within the group of TF genes induced by hypoxia (Fig. 3a), we also found some that were already reported as hypoxic genes and can be considered as markers of the hypoxic treatment, such as *Hypoxia Responsive ERF1* and *2* (*HRE1* and *HRE2*) (Licausi *et al.*, 2010), LOB (Lateral Organ Boundary) Domain *LBD41* and *At3g10040* (Fig. 3a).

Reactive oxygen species (ROS)-related TFs are induced by anoxia but not hypoxia

Not only did we discover new TFs or confirmed already characterized changes in TF expression upon hypoxia with our experiments, but there were also a few TFs that were not differentially expressed in our approach, although their hypoxia-induced regulation was reported in the literature previously. This is exemplified for heat shock factor *HSFA2*, and the zinc finger proteins *ZAT10* and *ZAT12*, which were not induced in our experiments even at 1% O_2, although these genes were reported to be induced under by low oxygen treatment by several other studies (Branco-Price *et al.*, 2005, 2008; Loreti *et al.*, 2005). HSFA2 is involved in the upregulation of several heat shock proteins (HSPs) and other oxidative stress-related proteins under anoxia (Banti *et al.*, 2008) and its over-expression improves anoxia and submergence tolerance in Arabidopsis (Banti *et al.*, 2010). Both ZAT10 and ZAT12 are also transcription factors that mediate the molecular response to ROS in plants (Davletova *et al.*, 2005; Mittler *et al.*, 2006). As our previous transcriptome analyses (van Dongen *et al.*, 2009; Licausi *et al.*, 2010) also did not show an increase in HFSA2 and its targets under constant hypoxia, it is likely that the upregulation of ROS-related TFs constitute a specific response to true anoxic conditions in the dark, whereas in our experimental system oxygen concentration never declined below 1%. Indeed, Loreti *et al.* (2005) used anoxic conditions and Branco-Price *et al.* (2005, 2008) flushed an O_2-free argon atmosphere in a sealed container, reaching complete anoxia after some hours. Apparently, the induction of hypoxic TF genes is (at least partly) regulated by a signaling pathway that is different from the regulation mechanism of anoxic responses.

At mild hypoxic conditions (8% O_2), the expression of fewer genes was affected compared with more extreme hypoxic conditions (1% v/v oxygen). Nevertheless, some TFs that are strongly induced by strict hypoxia also exhibit upregulation at 8% O_2. This is likely to be because the intensity of the hypoxic signal is inversely dependent on the oxygen levels within the cell. Considering the induction magnitude under mild and severe hypoxia, two groups of anaerobic genes could be distinguished: those that require more time to be induced, when treated with mild hypoxia (8% O_2) (such as *At3g10040*, *At3g02550* and *At1g67100*), and those that are not induced by the mild-hypoxic treatment (such as *At2g47520*, *At1g72210*, *At3g16060* and *At1g18400*). It is tempting to speculate that the sensing mechanism and signaling pathways that lead to the induction of these two groups of genes are different.

TFs induced by hypoxia bind overrepresented elements in the promoter of anaerobic genes

Individual members of a TF family are suggested to bind different oligonucleotide target sequences, although usually a consensus DNA element can be identified (Jin & Martin, 1999; Toledo-Ortiz *et al.*, 2003; Yang *et al.*, 2009). The promoter sequences of the core set genes were analysed for

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overrepresented regulatory sequence motifs. As expected, 5 bp sequences were found more frequently than 6- or 8-bp long ones. We identified several DNA elements that are known to be bound by some ERF, DOF and homeobox TFs (Table S9). The ERF-bound sequence ATATT (Kannangara *et al.*, 2007) is also homologous to part of the TATA-box, which constitutes a common feature of most genes transcribed by the RNA polymerase II (Smale & Kadonaga, 2003). However, the Motifsearch tool identified it as significantly overrepresented with respect to the genes present in the Arabidopsis genome, suggesting that A/T rich regions may have a further role under hypoxia unrelated to the assembly of the RNA polymerase II core complex, as enhancers of transcription.

We confirmed the over-representation of a GC motif (GCCCC) and a GT motif (TGGTTT) in the promoter of hypoxia-induced genes (Fig. 1 and Table S7) as already shown by Liu *et al.* (2005) and Mohanty *et al.* (2005). The GT motif has been proposed to be essential for AtMYB2 binding and hypoxia-induced *ADH1* expression (Hoeren *et al.*, 1998). The *MYB2* gene was not induced by hypoxia either in the microarray (Tables S1, S2) and the qRT-PCR (Table S12) dataset. It is possible that MYB2 is also part of the specific response to anoxia or to an anoxia-derived signal similar to that suggested for as HSFA2, ZAT10 and ZAT12.

Another DNA motif, ATCTA, already reported by Welsch *et al.* (2007), was also identified by Motiffinder and Bioprospector as significantly over-represented in the promoter of upregulated genes with respect to its average occurrence in the Arabidopsis genome. Moreover, this motif has already been reported as conserved in the promoter of anaerobic genes in different plant species (Mohanty *et al.*, 2005). The only TF reported until now to be able to bind this element is the ERF protein AtRAP2.2 (Welsch *et al.*, 2007). Interestingly, AtRAP2.2 was shown to be important for ethylene-mediated tolerance to hypoxia in Arabidopsis seedlings (Hinz *et al.*, 2010). RAP2.2 and its holomogs RAP2.12 and RAP2.3 do not respond to hypoxia or anoxia (Loreti *et al.*, 2005; van Dongen *et al.*, 2009; present study). Two other homologs of *RAP2.2*, *HRE1* and *HRE2*, were induced by hypoxia in our dataset (Table S2). These two genes are required to maintain the high expression of fermentative enzymes and indeed, HRE1 overexpression caused an increase in the upregulation of anaerobic genes under hypoxia (Licausi *et al.*, 2010). Interestingly, when HRE1 was transiently overexpressed in mesophyll protoplasts, it induced the expression of a reporter gene fused to the promoter of *ADH* or *HB1* genes, which both contain the ATCTA element (Fig. 4).

It is tempting to speculate that the bHLH TFs may also act as mediators in oxygen signaling, as oxygen sensors from animals (HIF; Wenger, 2002), and fungi (SRN1; Goldstein *et al.*, 2006) belong to this family. Our dataset revealed five bHLH genes being induced by hypoxia in our dataset, although only few genes in the 'hypoxia-induced' cluster (Table S10) contained the bHLH binding element in their promoters. This would rule out the bHLH proteins as regulators of the hypoxic response in Arabidopsis. However, bHLH are known to create homo-oligomer or hetero-oligomer complexes and therefore they may still be involved in the regulation of hypoxic genes, in association with other upregulated TFs. For example, some bHLH proteins have been shown to interact with members of the LBD protein family (Husbands *et al.*, 2007). Interestingly, two members of the LBD family were also upregulated by hypoxia in all the conditions tested in the current experiments (*LBD 40* and *LBD41*). Rubin *et al.* (2009) showed that three highly related *LBD* genes (*LBD37*, *LBD38* and *LBD39*) act as repressors of a larger subset of nitrogen-starvation induced genes. However, in our transactivation assay in protoplasts, the overexpression of *LBD41* caused an upregulation of the reporter gene controlled by the *HB1* promoter. Moreover, the data reported by Wang *et al.* (2003) showed that *HB1* is induced by nitrate and nitrite supplementation, together with *LBD37*, *LBD38* and *LBD39*, suggesting that LBDs may act as positive and negative regulators on different targets, perhaps through the interaction with members of the bHLH family. Analyses of the protein–protein interaction among the upregulated TFs will help in clarifying this aspect.

Pscan and Plantpan identified the binding site of *HOMEOBOX1* (*AtHB1*) and *AtHB5* genes as over-represented in the upregulated dataset. These specific TFs are not among the homeobox genes that exhibited upregulation (*AtWAX14*, *At4g33280* and *At4g02550*) by hypoxia in our dataset; however the general homeobox element AATAATT is present in the promoter of 44 anaerobic genes. Homeobox proteins are conserved among all eukaryotes and share basic structural similarity with the helix–loop–helix domains of some bacterial proteins. In plants, these TFs are involved in the regulation of meristem development, especially in stem cell niches (Scofield & Murray, 2006). Severe hypoxic stress, such as the one imposed by submergence, causes death of mature leaves and roots and reduces the size of new organs (Bailey-Serres & Voesenek, 2008). It is possible that the homeobox genes are involved in reprogramming the development of new roots and leaves to adapt to the environmental condition.

In rice, *in silico* analyses identified putative TF and DNA elements involved in the regulation of the molecular response to anaerobiosis also (Narsai *et al.*, 2009). Interestingly, only a little overlap was observed between the motifs over-represented in the promoters of hypoxia responsive genes in Arabidopsis and rice. For example, several elements containing the LBD binding site were over-represented in the promoter of anaerobic genes in rice (Narsai *et al.*, 2009), whereas the hypoxia responsive

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promoters do not contain such elements in Arabidopsis. This observation suggests that these two plant species possess different subsets of transcription factors and DNA binding elements to modulate their hypoxic response. Nevertheless, several orthologous members of specific families – including the ERF, LBD and trihelix TFs – exist that are coherently responding to hypoxia in several different species and therefore seem to constitute a core element in the response of plants to oxygen deprivation (Loreti *et al.*, 2005; Branco-Price *et al.*, 2005; Lashanti-Kudahettige *et al.*, 2007; Pasentsis *et al.*, 2007; Kreuzwieser *et al.*, 2009; Narsai *et al.*, 2009).

Hypoxia induces few pri-miRNAs although induction of their mature products was not always observed

Regulation of TF transcript levels may be by other TFs but also by the activity of miRNAs and ta-siRNAs (Shukla *et al.*, 2008). miRNAs have been shown to play important roles in the transduction of the hypoxic signal and the related development in animals (Crosby *et al.*, 2009). We used the qRT-PCR platform designed by Pant *et al.* (2009) to quantify changes in the expression of 177 Arabidopsis pri-miRNAs. We could identify only one pri-miRNA upregulated after 30 min hypoxia and four after 2 h hypoxia (Fig. 5). Instead, using high-throughput sequencing, Moldovan *et al.* (2009) identified 20 mature miRNA significantly upregulated after 5 h hypoxia (0.1% O_2). The only similarity between the two datasets is the upregulation of miR391 after 2 h hypoxia. For all the other miRNAs we could not observe an increase in the corresponding pri-miRNAs, as was reported by Moldovan *et al.* (2009) even when we analysed the expression in a detailed time-resolved manner (Fig. 6). Interestingly, the promoter of the gene coding for the pri-miR391 (*AT5G60408*) also contained the ATCTA elements that was identified in our *in silico* analysis.

The expression of pri-miRNAs that belong to the same family did not always reflect the levels of mature miRNAs, as observed for miR391, miR157a and miR172a (Fig. 6). Therefore, it is not surprising that we did not observe changes in the mRNA levels of the putative targets of the miRNAs whose precursor responded to hypoxia. However this cannot rule out the function of these mature miRNAs as translational inhibitors, as shown by Brodersen *et al.* (2008). Alternatively, the hypoxia-responsive miRNAs could also be acting in a tissue-specific manner and therefore the effect on their targets may only be observed in specific cell types. Little overlap was also observed between hypoxia-responsive miRNAs in Arabidopsis and submergence-responsive miRNA in maize roots (Zhang *et al.*, 2008;

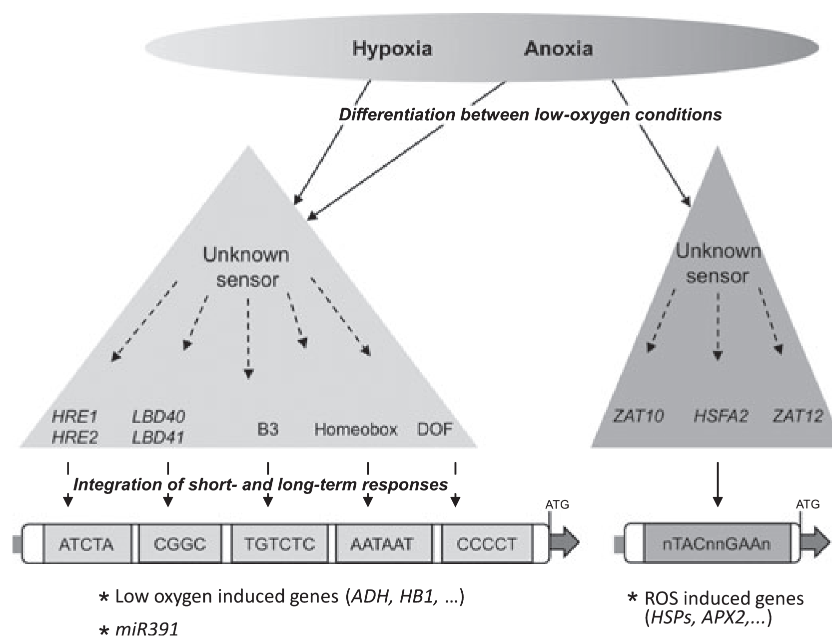

Fig. 7 Model of the genetic hypoxia signal transduction pathway in *Arabidopsis* roots. Oxygen deficiency is perceived by means of a yet unknown sensor, which triggers the upregulation of a set of hypoxia-responsive transcription factors (TFs) including members of the ERF (HRE1, HRE2), LBD (LBD40 and LBD41), B3 (At5g66980), homeobox (At3g33280) and DOF (At1g69570) families. Under conditions where the oxygen availability is reduced (hypoxia), the subtle regulation of hypoxia-induced effector genes such as *ADH* or *HB1* or specific miRNAs can be controlled via simultaneous interaction of various combinations of these TFs. Under anoxic conditions, an additional set of TFs is activated, including ZAT10, ZAT12 and HSFA2, that regulate the differential expression of genes that are only active when molecular oxygen is absolutely absent from the cell.

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Moldovan *et al.*, 2009), suggesting that the miRNA response under hypoxia is species-specific.

Molecular regulation of the low oxygen response in plants

With this study we provide an overview of expressional changes of TFs and miRNAs as transcriptional regulators under hypoxia. The results are summarized in a functional network depicted in Fig. 7. Different from animal systems, miRNAs do not seem to play a major role in the early phases of the regulation of hypoxic genes in plants. Few pri-miRNAs were significantly upregulated under hypoxia but this increase led to an increase of the corresponding mature miRNAs of miR391 only. Our experiments suggest a differentiation between hypoxia and anoxia-induced signals. Some TFs, including HSFs proteins, previously reported to be upregulated by anaerobiosis ($[O_2] \cong 0\%$ v/v oxygen), did not exhibit a significant increase in the hypoxic conditions tested in our study ($[O_2] \geq 1\%$ v/v oxygen), suggesting a differentiation of the signal to regulate the expression of various subsets of hypoxia- and anoxia-induced genes. Apart from the oxygen concentration itself, several related parameters can be involved in the transmission of the low-oxygen signal, such as the energy status (Zabalza *et al.*, 2009), the cellular pH (Dennis *et al.*, 2000), or the level of ROS and RNS (Blokhina & Fagerstedt, 2010). As all our hypoxic treatments, as well as the aerobic controls, were conducted in the darkness, we were able to separate the effect of the hypoxic treatment from that of other variables such as the shift from light to darkness shift (Hinz *et al.*, 2010). The reciprocal analysis of DNA motifs over-represented in the promoters of genes upregulated by hypoxia and of those elements bound by hypoxia-induced TF suggested that ERF, B3, homeobox, LBD and DOF proteins play a leading role in the regulation of the core set of hypoxic genes, including *miR391*. Regulation of these TFs appeared to occur in a time-dependent manner, with some TFs having their strongest induction in the very early (30 min) phase after the onset of hypoxia, and others being most strongly regulated after 2 h. Our analysis showed that most hypoxia-regulated genes posses various different TF binding elements in their promoter region. The time-resolved regulation of the expression of these genes during hypoxia is therefore suggested to result from the integration of TF-induced gene activation of various TFs binding to different promoter elements simultaneously.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 List of genes upregulated (≥ twofold) after 30 min hypoxia (1% and 4% O_2)

Table S2 List of genes upregulated after 2 h hypoxia (1% and 4% O_2)

Table S3 List of genes downregulated after 30 min hypoxia (1% and 4% O_2)

Table S4 List of genes downregulated after 2 h hypoxia (1% and 4% O_2)

Table S5 6-Mer over-represented motif in the promoter of genes in Table S1

Table S6 6-Mer over-represented motif in the promoter of genes in Table S2

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Table S7 6-Mer over-represented motif in the promoter of genes in Table S3

Table S8 6-Mer over-represented motif in the promoter of genes in Table S4

Table S9 Known DNA elements bound by transcription factors (TFs) in the promoter dataset (Tables S1–S4)

Table S10 DNA binding element of the differentially expressed transcription factors (TFs) under hypoxia and its occurrence in the promoter regions of low oxygen responsive genes (Tables S1, S2)

Table S11 Expression of the putative targets of differentially expressed microRNA primary transcripts (pri-miRNAs) in the TF dataset and previous microarray analyses

Table S12 Relative mRNA level of transcription factors (TFs) genes and microRNA primary transcripts (pri-miRNAs) in Arabidopsis roots under different hypoxic conditions

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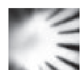

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