

Reactive Oxygen Species-Driven Transcription in *Arabidopsis* under Oxygen Deprivation^{1[W]}

Chiara Pucciariello, Sandro Parlanti, Valeria Banti, Giacomo Novi, and Pierdomenico Perata*

PlantLab, Institute of Life Sciences, Scuola Superiore Sant'Anna, 56127 Pisa, Italy

Reactive oxygen species (ROS) play an important role as triggers of gene expression during biotic and abiotic stresses, among which is low oxygen (O_2). Previous studies have shown that ROS regulation under low O_2 is driven by a RHO-like GTPase that allows tight control of hydrogen peroxide (H_2O_2) production. H_2O_2 is thought to regulate the expression of heat shock proteins, in a mechanism that is common to both O_2 deprivation and to heat stress. In this work, we used publicly available *Arabidopsis* (*Arabidopsis thaliana*) microarray datasets related to ROS and O_2 deprivation to define transcriptome convergence pattern. Our results show that although *Arabidopsis* response to anoxic and hypoxic treatments share a common core of genes related to the anaerobic metabolism, they differ in terms of ROS-related gene response. We propose that H_2O_2 production under O_2 deprivation is a trait present in a very early phase of anoxia, and that ROS are needed for the regulation of a set of genes belonging to the heat shock protein and ROS-mediated groups. This mechanism, likely not regulated via the N-end rule pathway for O_2 sensing, is probably mediated by a NADPH oxidase and it is involved in plant tolerance to the stress.

Oxygen (O_2) deprivation in plants occurs frequently, due either to natural flooding events in flood-prone areas (Bailey-Serres et al., 2010) or to the slow diffusion of O_2 in bulky organs (Geigenberger et al., 2000; van Dongen et al., 2003). Energy production is tightly regulated in the absence of O_2 . The respiratory metabolism switches from aerobic to anaerobic, to sustain ATP generation and guarantee survival (for review, see Geigenberger, 2003; Sachs and Vartapetian, 2007; Colmer and Voesenek, 2009). The way that plant morphology and physiology allows plants to withstand O_2 deprivation has been a subject of study for many years (for review, see Voesenek et al., 2006).

Recently, considerable progress has been made toward the comprehension of the molecular mechanisms governing these traits and which are responsible for plant sensitivity/tolerance to low- O_2 stress (Xu et al., 2006; Hattori et al., 2009; Lee et al., 2009; Licausi, 2011). Moreover, very recently a direct homeostatic low- O_2 sensor has been identified in plants (Gibbs et al., 2011; Licausi et al., 2011). Ethylene-responsive factors (ERFs) of group VII, among which HYPOXIA RESPONSIVE1 (HRE1) and HRE2 and RELATED TO AP2 12 (RAP2.12), have been shown to be substrate of the N-rule path-

way, where the N-terminal Met-Cys motif is subjected to targeted ubiquitin-dependent protein degradation under normoxic condition, possibly through the oxidation of the tertiary destabilizing residue Cys (Gibbs et al., 2011; Licausi et al., 2011). The stabilization of the N-terminal motif under low O_2 leads to increased plant survival, through the control of the expression of hypoxic core genes (Gibbs et al., 2011). Whether Cys (in)stability depends directly on O_2 or cellular changes associated to its availability, such as cytosolic pH or reactive oxygen species (ROS) balance, is still unclear (Gibbs et al., 2011).

ROS production has been suggested to be a component of low- O_2 signaling. Baxter-Burrell et al. (2002) proved that the activation of a RHO-like small G protein of plant (ROP) under low O_2 induces hydrogen peroxide (H_2O_2) accumulation via an NADPH oxidase mechanism that was shown to be required for ALCOHOL DEHYDROGENASE (ADH) expression and activity, thus tolerance. The ROP family modulates signaling cascades associated with a variety of mechanism in plants and eukaryotic kingdoms in general (for review, see Yang and Fu, 2007). It seems that tolerance to O_2 deprivation also requires ROP activation and deactivation and that their activity be controlled by negative feedback regulation that in turn requires a ROP GTPase activating protein, also regulated by H_2O_2 (Baxter-Burrell et al., 2002). The mechanism that drives the ROP rheostat activation under low O_2 is currently not known, but it should involve a novel mechanism because RAC1, the counterpart of ROP, regulates NADPH oxidase through binding to the p47^{phox} regulatory subunit that is absent in plants (Gu et al., 2004).

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* Corresponding author; e-mail pierdomenico.perata@sssup.it.

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H₂O₂ production is observed under both anoxia and heat stress (Banti et al., 2010), suggesting that it can be involved in the induction of heat shock transcription factors (HSFs) and heat shock proteins (HSPs) found to be induced under these stresses (Banti et al., 2010). Indeed, among the families of conserved H₂O₂-responsive proteins across kingdoms, DNAJ-type HSPs and small HSP proteins were identified (Vandenbroucke et al., 2008). Moreover, a cross-kingdoms comparison of transcriptome regulation under low O₂ indicates a general increase of HSP transcripts (Mustroph et al., 2010). HSFs have been proposed to be specific H₂O₂ sensors in plants (Miller and Mittler, 2006) and Arabidopsis (*Arabidopsis thaliana*) seedlings that overexpress *HsfA2* are markedly more tolerant to anoxia (Banti et al., 2010).

Overall, these results indicate that the response to anoxia and to heat often overlaps, and it seems that ROS production may occur upstream of the signaling pathway required for tolerance. HSFs have not been proposed as directly regulated by the N-end rule pathway, suggesting ROS as possible actors in a side mechanism. However, ROS production due to general oxidative stress generated by low-O₂-dependent secondary alterations cannot be excluded.

To verify whether a subset of hypoxic/anoxic transcripts are in fact regulated by ROS, we examined some Arabidopsis Affymetrix genome arrays related to both O₂ deprivation and ROS-producing experiments that are available in the literature. Our results demonstrate that a certain overlap exists between genes induced by ROS and genes induced by anoxia. Here we show that genes induced by both ROS and anoxia include some encoding HSPs and ROS-related transcription factors (TFs) and we propose that the expression of those genes under anoxia is regulated by a ROS-dependent way that requires the activation of an NADPH oxidase involved in tolerance.

RESULTS AND DISCUSSION

The Low-O₂ Core Transcriptome in Arabidopsis Plants

Transcript profiling datasets related to O₂ deprivation (Table I) and ROS-related experiments (Table II) were gathered and analyzed. From cluster analysis of the datasets, it became evident that shoots and roots respond differently to ROS (Supplemental Fig. S1) and low-O₂ conditions (Supplemental Fig. S2), as previously observed (Ellis et al., 1999; Mustroph et al., 2009; Lee et al., 2011), and that the seedlings' response was more similar to the shoot one. Therefore roots and seedlings/shoots datasets were analyzed separately.

The microarray datasets related to O₂ deprivation were investigated first. The majority of the experiments were performed using the Columbia (Col) ecotype at growth stage 1.0 (Boyes et al., 2001), under dark conditions. Treatment in the dark should eliminate the

possibility that O₂ be produced by photosynthesis (Mustroph et al., 2006; Colmer and Pedersen, 2008).

A core of genes up-regulated under anoxia and hypoxia was defined for both seedlings and shoots, by querying the whole microarray datasets at the arbitrary threshold of $\log_2 \geq 1$ ($P < 0.05$). For experiments containing several time points (i.e. Lee et al., 2011), we considered the regulation to be significant when at least one of the time points analyzed was significantly up-regulated.

The regulation of anaerobic core genes was also investigated in *35S::HRE1* and *35S::HRE2* Arabidopsis transgenic plants under hypoxia. The *HRE1* gene is an ERF acting as positive regulator of a set of anaerobic genes under hypoxia (Licausi et al., 2010). Overexpression of *HRE1* results in an increased anoxia tolerance (Licausi et al., 2010). Moreover, we investigated whether low-O₂ up-regulated transcripts were ectopically accumulated in the N-end rule mutants *prt6* and *ate1/2* and thus directly regulated by the N-end rule pathway (Gibbs et al., 2011; Licausi et al., 2011). These mutants lack some of the recognition steps required for proteasomal degradation under normoxia, thus they constitutively express hypoxic core genes related to the anaerobic metabolism such as *ADH1*, *PYRUVATE DECARBOXYLASE1* (*PDC1*), and *SUC SYNTHASE4* (*SUS4*; Gibbs et al., 2011).

Six genes were found that constitute a core of transcripts significantly up-regulated under all the anoxic experiments but not under hypoxia (Fig. 1A). These genes encoded mostly HSPs (Fig. 1B), most of which are regulated by heat stress and that have been indicated to be *HsfA2* targets (Nishizawa et al., 2006). HSPs were previously indicated to be part of the genes in which expression in dependence of low O₂ is conserved among different kingdoms (Mustroph et al., 2010). Their expression has been observed since very short anoxia treatment (e.g. 2 h in seedlings; Mustroph et al., 2010), indicating a fast mechanism of response. Moreover, HSP response is suggested to be H₂O₂ specific (Vandenbroucke et al., 2008). Interestingly, none of these genes was up-regulated in the HRE mutants subjected to hypoxia (Fig. 1B), neither were ectopically accumulated in N-end rule mutants *prt6* and *ate1/2* (Gibbs et al., 2011). Thus, the expression of these HSPs is likely not directly regulated by this mechanism. A single mitochondrial HSP (*HSP23.5-M*, *At5g51440*) is accumulated under normoxia in the *ate1/2* mutant only (Gibbs et al., 2011). Secondary signaling molecules to monitor O₂ availability might relay on calcium flux, energy charge, and ROS balance. These three parameters seem to be interrelated, thus suggesting the presence of downstream events that could converge (Bailey-Serres and Chang, 2005). Emerging evidence suggests that ROS-mediated activation of plasma membrane calcium (Ca²⁺) channels is involved in plant signal transduction related to both biotic and abiotic stress and development events (for review, see Lecourieux et al., 2006). Previous results demonstrated the involvement of NADPH-oxidase-

Table I. Overview of the O₂ deprivation Affymetrix Arabidopsis microarrays studies used for the analysis

A 6h¹, 6 h of anoxia treatment without or with 90 mM Suc (s) added before the treatment (Loreti et al., 2005); A 6h², 6 h of anoxia treatment (Banti et al., 2010); A 12h, 12 h of anoxia treatment, total and polysomal (p) RNA (Branco-Price et al., 2005); H 4h, 4 h of hypoxia treatment at 1% O₂ (Licausi et al., 2010); S 7/24h, 7 and 24 h of submergence treatment (Lee et al., 2011); Hyp, 0.5, 2 and 48 h at 1%, 4%, and 8% O₂ (van Dongen et al., 2009); GS, growth stage (Boyes et al., 2001).

Sample Description	GS	Growth	Treatment	Accession No.	Arrays	Replicates	Reference
Col <i>glabra</i> seedlings	1.0	Petri dishes, MS liquid medium	6 h anoxia ± 90 mM Suc, dark conditions	GSE2133 GSE3704	8	2	A 6h ¹ A 6h ^{1s}
Col-0 seedlings	1.0	Petri dishes, MS liquid medium (90 mM Suc)	6 h anoxia, dark conditions	GSE16222	4	2	A 6h ²
Landsberg <i>erecta</i> seedlings	1.02	Vertical plates, MS solid medium (1% Suc)	12 h O ₂ -free argon atmosphere, dim light	GSE2218	4	1	A 12h A 12h ^p
Col-0 seedlings	1.02	Petri dishes, MS liquid medium (1% Suc)	4 h at 1% O ₂ , dark conditions	GSE17099	4	2	H 4h
Col-0 roots and shoots	1.09	Pot, soil	7 to 24 h submersion, dark conditions	GSE24077	16	2	S 7h S 24h
Col2 roots	1.02	Vertical plates, MS solid medium (1% Suc)	0.5, 2 h and 48 h at 1%, 4%, and 8% O ₂ , dark conditions	GSE11558	28	2	Hyp

dependent H₂O₂ production under O₂ deprivation response (Baxter-Burrell et al., 2002). Moreover, transient changes in Ca²⁺ during low O₂ was found to be involved in ADH regulation (Sedbrook et al., 1996; Subbaiah et al., 1994a, 1994b). A connection between NADPH-oxidase-dependent ROS production and Ca²⁺ oscillation has been suggested in Arabidopsis, where Ca²⁺ likely binds to the EF hands of the N-terminal region of NADPH oxidase and, together with phosphorylation, promotes ROS production (Ogasawara et al., 2008; Takeda et al., 2008; Suzuki et al., 2011). Whether and how this mechanism could in part overlap with N-end rule pathway is still unknown.

Five transcripts were found to be consistently up-regulated by hypoxia. These same genes were also significantly up-regulated in some anoxic microarrays but not necessarily in all (Fig. 1B). This was not true the other way around. Although we found genes that were consistently up-regulated in anoxia, they were not significantly up-regulated in any of the hypoxic microarray experiments. This suggests that the response to anoxia contains all elements of the response to

hypoxia but that anoxia also contains additional elements (Fig. 1B), as was previously proposed by Licausi (2011). Among the set of five hypoxic genes, we found *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE1* (*At2g19590*), a gene that plays a key role in ethylene biosynthesis and requires O₂ for its catalytic activity. Ethylene increases within minutes in plants subjected to soil waterlogging or complete submergence due to entrapment in submerged organs and reduced catabolism (Kawase, 1972, 1978; Könings and Jackson, 1979; Geisler-Lee et al., 2010).

A specific group of eight genes that are up-regulated after both anoxia and hypoxia were also identified, they include *PDC1* (*At4g33070*), *ADH1* (*At1g77120*), and *SUS4* (*At3g43190*; Fig. 1B). Suc synthase catalyzes the conversion of Suc to UDP-Glc and Fru that will be used in glycolysis (Ricard et al., 1998). *ADH1* and *PDC1* are involved in catalyzing reactions involved in ethanol fermentation, which occurs in response to O₂ deprivation to ensure the regeneration of NAD⁺ for glycolysis with concomitant production of ATP, in the absence of mitochondrial respiration (Kürsteiner et al.,

Table II. Overview of the ROS-related Affymetrix Arabidopsis microarrays studies used for the analysis

flu 0.5/1/2h, *flu* mutant shift from dark to light (op den Camp et al., 2003); AOX1a-AS, AOX1a-AS mutant (Umbach et al., 2005); MV 1/6/12h, MV 1, 6, 12 h treatment (Dr. Kirch, NASCArray repository); O₃, 1 h of O₃ fumigation at 200 nL L⁻¹ (Dr. Shirras, NASCArray repository); H₂O₂, 1 h of H₂O₂ treatment at 20 mM (Dr. Mittler, NASCArray repository); GS, growth stage (Boyes et al., 2001).

Sample Description	GS	Treatment	ROS	Accession No.	Arrays	Replicates	Reference
<i>flu</i> mutant in Landsberg <i>erecta</i> background leaves	1.02	Dark to light 0.5, 1 h, 2 h	¹ O ₂ plast	GSE10876	6	1	<i>flu</i> 0.5/1/2h
AOX1a-AS mutant in Col background leaves	1.09	–	O ₂ ⁻ mit	GSE2406	4	2	AOX1a-AS
Col-0 root and shoot	1.04	10 μM MV 0.5, 1, 3, 6, 12, 24 h	O ₂ ⁻ mit/chl	NASCARRAYS-143	44	2	MV 1/6/12h
Seedlings	1.02	1 h O ₃ 200 nL L ⁻¹	O ₂ ⁻ , H ₂ O ₂	NASCARRAYS-26	6	3	O ₃
Seedlings	1.0	20 mM H ₂ O ₂ 1 h	H ₂ O ₂	NASCARRAYS-338	6	3	H ₂ O ₂

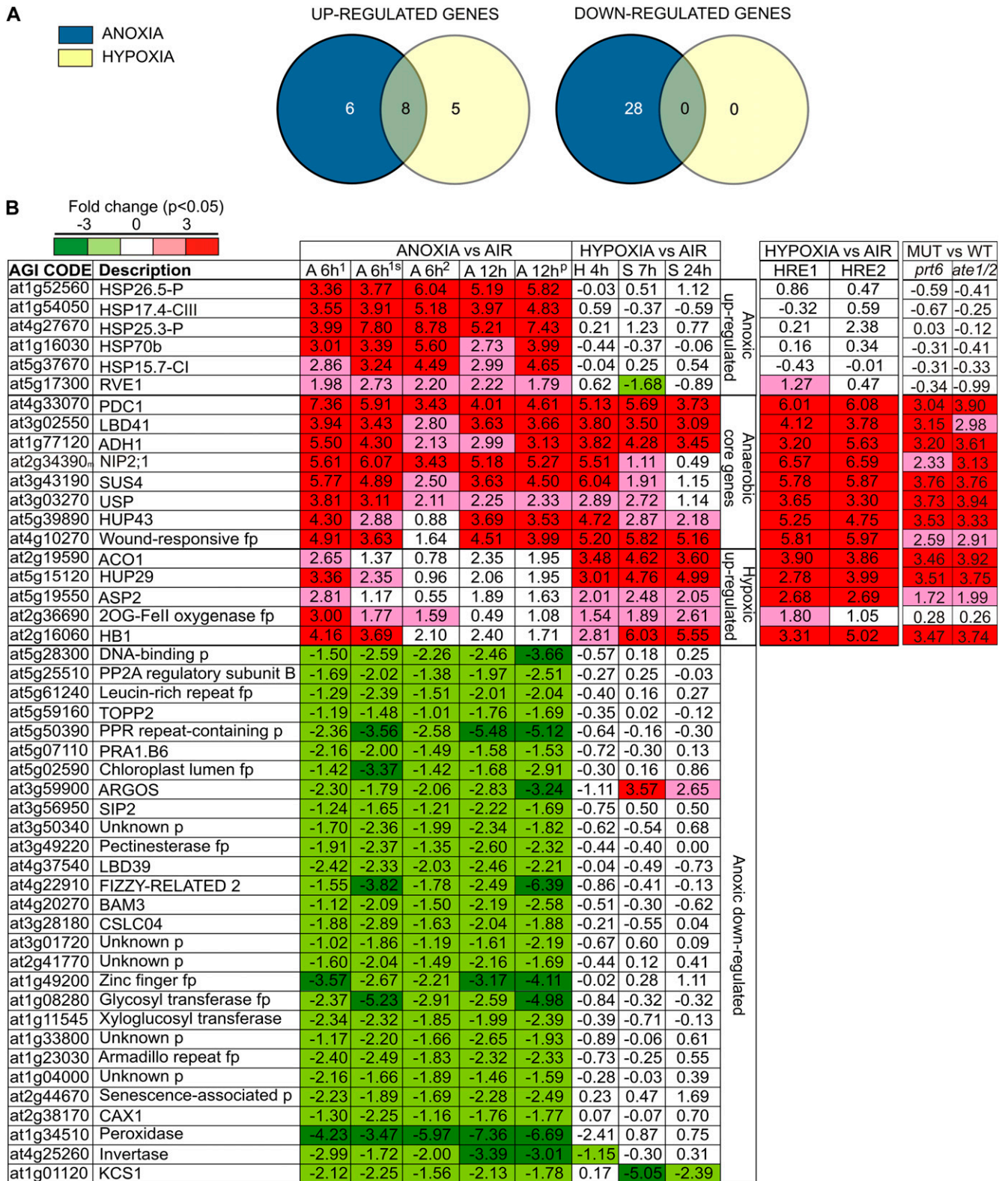


Figure 1. Genes regulated by anoxia, hypoxia, and O₂ deprivation in the seedlings/shoots of Arabidopsis microarray datasets. The core genes were obtained selecting significantly regulated genes ($-1 > \log_2 > 1$, $P < 0.05$) in all the experiments and in at least one time point in the experiments with several time points. A, Venn diagram showing significantly regulated genes under anoxia, hypoxia, and the overlap between anoxia and hypoxia. B, Heat map showing the expression profile of anoxia, O₂ deprivation, and hypoxia-regulated genes. Value represents log₂, and color indicates significant change ($P < 0.05$). A 6h¹, 6 h of

2003). These genes are regulated by low O₂, independently of the degree of O₂ deprivation, and they encode enzymes required for the anaerobic respiration. Hypoxia-responsive genes (i.e. *ADH1* and *SUS4*) induction is augmented in *HRE1* overexpressor under anoxia (Licausi et al., 2010) and in *35S::Δ13RAP2.12* plants in air, where the first 13 amino acid residues were deleted, resulting in loss of recognition for degradation under normoxia (Licausi et al., 2011). *HRE1* and *RAP2.12* are members of the group VII of the ERFs Arabidopsis family, composed by five members (Nakano et al., 2006). They share homology to the rice (*Oryza sativa*) ERF SUBMERGENCES and SNORKELS, found to be necessary for the activation of the quiescence strategy in lowland rice (Xu et al., 2006) and escape strategy in deepwater rice (Hattori et al., 2009), respectively. Arabidopsis ERFs of group VII are activated under low O₂ to support gene expression for acclimation strategy (Gibbs et al., 2011; Licausi et al., 2011). Through the presence of the initiating motif MetCys at the N-terminal site, these ERFs are degraded through ubiquitination under normoxia, by the N-end-rule mediated removal of Met and oxidation of the Cys residue (Gibbs et al., 2011; Licausi et al., 2011). Under low O₂, they are stabilized and activated and move to the nucleus to trigger anaerobic gene expression (Licausi et al., 2011). As previously described, anaerobic genes are also activated ectopically in N-end-rule mutants *prt6* and *ate1/2* (Fig. 1B; Gibbs et al., 2011).

Two other genes were also part of the group up-regulated by both anoxia and hypoxia, namely the LATERAL ORGAN BOUNDARIES DOMAIN CONTAINING PROTEIN41 (*LBD41*; At3g02550), and a UNIVERSAL STRESS PROTEIN (*USP*; At3g03270). *LBD41* is member of the LBD gene family that encodes plant-specific TFs (Husbands et al., 2007). *LBD* genes are suggested to be involved in developmental processes, including leaf polarity establishment (Lin et al., 2003; Xu et al., 2003) and lateral root formation (Inukai et al., 2005; Liu et al., 2005; Okushima et al., 2007). Recent results also suggest some LBDs be involved in auxin signaling (Lin et al., 2003; Inukai et al., 2005; Taramino et al., 2007; Mangeon et al., 2011). The USP protein family has been identified and studied in bacteria, but its biochemical role is not yet fully understood. These proteins are believed to play a function in response to a plethora of stresses, including carbon starvation, O₂ deprivation, nitrate, phosphate, and sulfate starvation, oxidative stress, and heat shock (Nachin et al., 2008). In plants, they have been classified but not deeply stud-

ied (Kerk et al., 2003). Indeed, an USP was found to be induced in rice under submergence and to be positively regulated by ethylene, suggesting a role in adaptation to this stress (Sauter et al., 2002).

We have identified a set of genes that are down-regulated by anoxia but not hypoxia (Fig. 1, A and B). Many of them belong to the cell wall functional class, suggesting a mechanism of general repression of growth under severe O₂ deprivation, whereas others belong to the protein posttranslational modification functional class. Intriguing was the behavior of the AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (*At3g59900*), which, while down-regulated under anoxia, was up-regulated in some hypoxic experiments (Fig. 1B). This gene is induced by auxin and controls lateral organ size (Hu et al., 2003).

The available root datasets were screened for significantly regulated genes (Supplemental Fig. S3A). The root material from the submergence experiment of Lee et al. (2011) was defined as anoxic since the measurement of the O₂ partial pressure demonstrated its absence. The specific group of genes that are up-regulated after both anoxia and hypoxia were defined to be those significantly regulated in at least five conditions out of the nine analyzed by van Dongen et al. (2009), and one condition out of the two analyzed by Lee et al. (2011); anoxic the genes significantly regulated in both the conditions analyzed by Lee et al. (2011) and none or one from van Dongen et al. (2009), and hypoxic the genes regulated in at least five conditions out of the nine analyzed by van Dongen et al. (2009) and never regulated under the conditions of Lee et al. (2011). Also for root experiments a core of genes up-regulated under both anoxia and hypoxia was defined. Among these genes, six were in common with the up-regulated genes of the seedlings/shoots group and some of them encode for the enzymes required for the anaerobic respiratory pathway (e.g. *ADH1* and *SUS4*).

In roots, like in seedlings/shoots, some genes were found to be up-regulated more specifically under anoxic conditions (Supplemental Fig. S3A). None of them was in common with the corresponding category of the seedlings/shoots group. In particular, the group of HSPs found in seedlings/shoots was not present in the genes up-regulated under anoxia in roots. Checking each single root array, it was evident that none of them showed a positive regulation of these HSP genes (Supplemental Table S1). However, previous results showed the induction of HSPs under low O₂ in roots (Mustroph et al., 2010) with an enrichment in the phloem companion cells (Mustroph et al., 2009).

Figure 1. (Continued.)

anoxia treatment without or with 90 mM Suc (s) added before the treatment (Loreti et al., 2005); A 6h², 6 h of anoxia treatment (Banti et al., 2010); A 12h, 12 h of anoxia treatment, total and polysomal (p) RNA (Branco-Price et al., 2005); H 4h, 4 h of hypoxia treatment at 1% O₂ (Licausi et al., 2010); S 7/24h, 7 and 24 h of submergence treatment (Lee et al., 2011); *HRE1/2*, *HRE1* and *HRE2* overexpressing plants under 4 h of hypoxia treatment at 1% O₂ (Licausi et al., 2010); *prt6* and *ate1/2*, N-end rule mutants under air compared with the wild type (Gibbs et al., 2011); M, multiple hit; fp, family protein; P, protein.

No genes were found to be significantly down-regulated in roots under hypoxia, after our analysis of data (five conditions out of nine, $\log_2 \geq 1$, $P < 0.05$; Supplemental Fig. S3A), in line with the seedlings/shoots core genes dataset (Fig. 1B). However, genes were down-regulated in single experiments as previously reported (van Dongen et al., 2009) but none of them was present in all the time sets following our criteria. van Dongen et al. (2009) reported a low tendency of genes to be down-regulated, suggesting a preferential reprogramming toward adaptation rather than inhibition. Down-regulation of gene expression under low O_2 could be a side effect of low- O_2 treatment that does not depend on a direct and conserved modulation of gene expression, as it happens instead for up-regulated genes (van Dongen et al., 2009).

Questioning the organ-specific response of gene regulation, only six genes were found to be in common between roots and seedlings/shoots (Supplemental Fig. S3B; Supplemental Table S1). Ten genes regulated by O_2 deprivation conditions in seedlings/shoots were also found to be regulated in some of the root experiments (Supplemental Fig. S3B; Supplemental Table S1). The contrary was observed for seven genes. Thus, a number of genes both up- and down-regulated were organ related. These genes are interesting because they could define an organ-specific response to O_2 limitation. Indeed, transcriptome adjustment has been suggested to be organ specific (Ellis et al., 1999; Mustroph et al., 2009; Lee et al., 2011). Because of the absence of a direct ROS signature in the genes commonly regulated in roots, we did not report data about subsequent analysis.

Convergence in the Gene Transcripts Regulated by ROS and O_2 Deprivation

Some authors have proposed the involvement of ROS-driven signaling under O_2 deprivation (Banti et al., 2010) to be mediated by NADPH oxidase (Baxter-Burrell et al., 2002). This idea might seem counterintuitive because O_2 is required for ROS production. However, O_2 deprivation at the cellular level occurs later than deficiency in the environment, because internal O_2 concentration depends also on the resistance to O_2 diffusion through tissue (Gupta et al., 2009). Cytochrome c oxidase catalyzes the reduction of O_2 to water with high affinity for O_2 [$K_m(O_2)$ approximately $0.1 \mu M$; Gupta et al., 2009] along the mitochondrial electron transport chain (Cooper, 2002), whereas the oxidative burst that produces ROS via the cell-membrane-located NADPH oxidase occurs in the apoplast (Torres and Dangl, 2005). It is tempting to speculate that this oxidative burst occurs prior to the use of O_2 by cytochrome c oxidase inside the cell. Recent findings report the involvement of mitochondria in oxidative burst after anoxia where ROS production is likely to occur at the mitochondrial electron transport chain (Chang et al., 2012).

Should ROS be part of the mechanism(s) behind gene regulation under hypoxia or anoxia, we would expect to find a convergence in gene regulation when comparing ROS and low- O_2 -related datasets. The ROS-related datasets used for our analysis, all of which were performed at growth stage 1.0 (Boyes et al., 2001), are described in Table II. A very detailed analysis on the specificity of ROS signaling was previously published by Gadjev et al. (2006). We instead filtered both the ROS and the O_2 deprivation (anoxia and hypoxia) datasets related to seedlings/shoots, and selected genes that were regulated ($\log_2 \geq 1$ and $\log_2 \leq -1$, $P < 0.05$) in at least one experiment. The genes identified with these criteria were 1,762 (data not shown). We searched this group of genes to identify those found both in O_2 deprivation and ROS signaling, identifying 431 genes (Fig. 2A). One hundred and ninety seven of them were commonly up-regulated (Supplemental Fig. S4) and 90 commonly down-regulated (Supplemental Fig. S5). Among the functional classes that were coregulated, S-assimilation, fermentation, N-metabolism, and stress were overrepresented and some of them consisted of up-regulated genes only (Fig. 2B). However, only the stress cluster contained a large number of genes (Fig. 2B). A large number of co-down-regulated genes belonging to the overrepresented cell wall cluster was also found (Fig. 2B).

Among the 197 genes up-regulated in both O_2 deprivation and ROS datasets, the hierarchical average linkage clustering identified a functional group composed of 22 genes related to heat stress (Fig. 3), including several HSPs but also heat shock TFs (i.e. *HsfA2*, *HsfA7A*, and *HsfB2A*). This group included genes induced both under anoxia and some of the ROS-related experiments. Hsfs are encoded by a large gene family and act by binding to a highly conserved heat shock element in the promoter region of many target genes related to defense (Mittler and Zilinskas, 1992; Storozhenko et al., 1998; Davletova et al., 2005a). Several reports suggest the existence of a direct link between heat shock and oxidative stresses, as they show that Hsfs are activated during various environmental stimuli (Li et al., 2005; Nishizawa et al., 2006; Banti et al., 2008, 2010). Hsfs were also proposed to be ROS sensors in plants (Miller and Mittler, 2006), as suggested for *Drosophila* and mammals (Zhong et al., 1998; Ahn and Thiele, 2003). Hierarchical average linkage clustering associated the heat-related genes to H_2O_2 and O_2^- -producing experiments as well as to the anoxic (but not hypoxic) ones (Fig. 3), a result that is consistent with the seedlings/shoots core gene dataset for hypoxia, showing no significantly up-regulated HSP genes (Fig. 1B).

The expression of HSP gene orthologs was investigated in the available microarrays of rice plants tolerant and sensitive to low O_2 (Kottapalli et al., 2007; Mustroph et al., 2010). Indeed, some HSP orthologous genes showed an up-regulation in the tolerant varieties only (Supplemental Table S2). In particular, *Os03g14180* likely orthologous to *HSP25.3-P* of Arab-

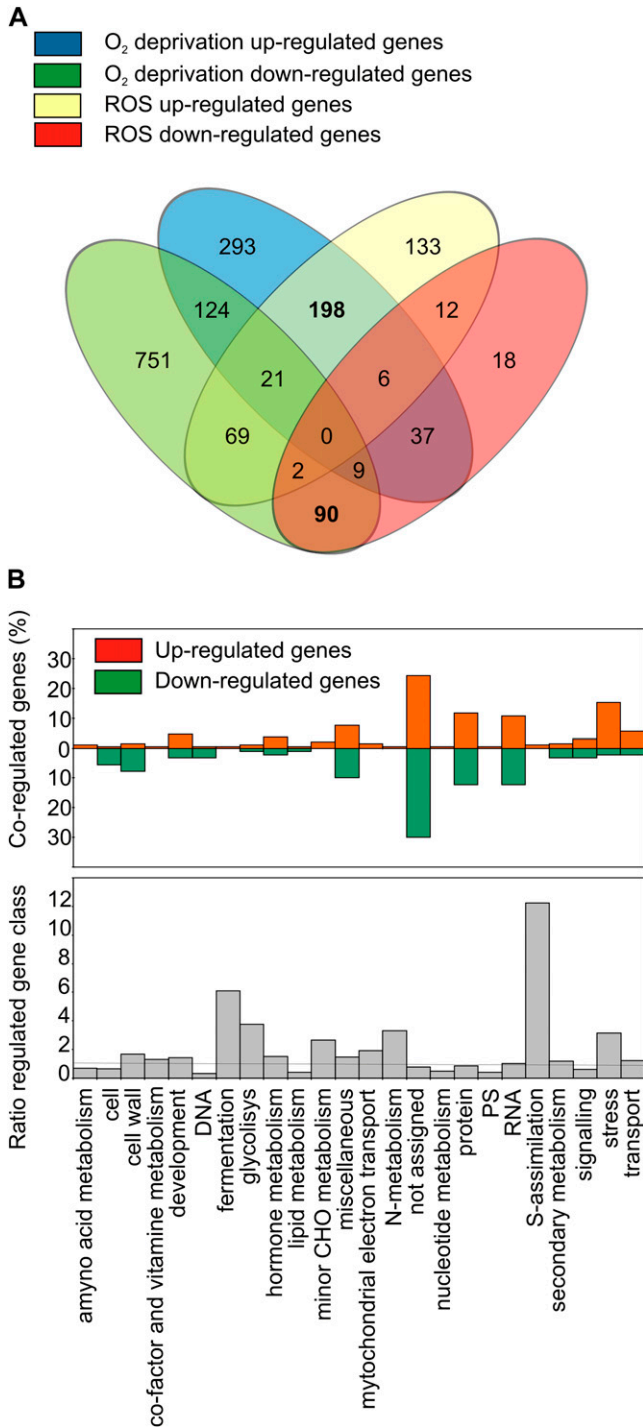


Figure 2. Genes coregulated in O₂ deprivation and ROS-related seedlings/shoots microarray experiments. A, Venn diagram showing the overlap of up- and down-regulated genes under O₂ deprivation and/or ROS-related experiments, considering at least one significantly regulated transcript for each condition. In bold, up and down coregulated transcripts. B, Percentage of up- and down-regulated functional gene categories under O₂ deprivation and ROS-related microarray experiments, and ratio of coregulated class percentage divided by the relative percentage of gene classes represented in the microarray. The line indicates ratio = 1, all the classes above this value are overrepresented in the O₂ deprivation and ROS-related coregulated gene classes.

idopsis, was induced in tolerant rice under submergence and was also present in the anoxia up-regulated genes category defined in Figure 1B. Indeed, conserved induction of HSPs under low O₂ has been found in several plant species analyzed before (Mustroph et al., 2010). The activation of the HSP way could be a successful strategy under low O₂. This is demonstrated by the anoxia tolerance of the *HsfA2* overexpressor plants that, although not showing up-regulation of classical anaerobic genes, shows increased transcription and translation of HSPs (Banti et al., 2010). Up-regulation of HSP genes in rice-tolerant plants strengthens this hypothesis. However, it appears that this way is not fully executed in *Arabidopsis* under anoxia (Banti et al., 2010). This might be related to the energy need of the de novo protein synthesis, that likely is dampened under the energy shortage occurring under anoxia. In this context, some of the HSPs found to be up-regulated under anoxia (Fig. 1B) are part of the small HSP class that likely act in an ATP-independent way (Sun et al., 2002).

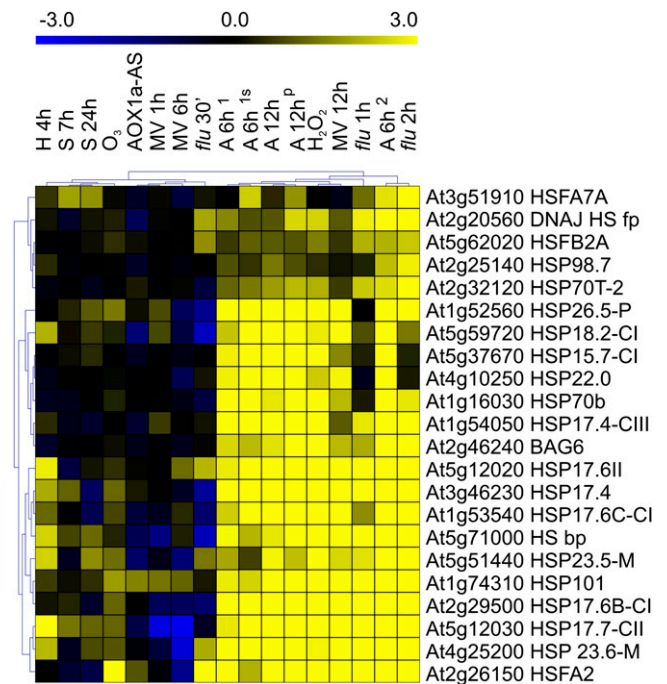


Figure 3. Hierarchical average linkage clustering of heat-stress-related genes regulated in seedlings/shoots in both O₂ deprivation and ROS-related experiments. H 4h, 4 h of hypoxia treatment at 1% O₂ (Licausi et al., 2010); S 7/24h, 7 and 24 h of submergence treatment (Lee et al., 2011); O₃, 1 h of O₃ fumigation at 200 nL L⁻¹ (Dr. Shirras, NASCArray repository); AOX1a-AS, AOX1a-AS mutant (Umbach et al., 2005); MV 1/6/12h, MV treatment (Dr. Kirch, NASCArray repository); *flu* 0.5 (30') / 1/2h, *flu* mutant shift from dark to light (op den Camp et al., 2003); A 6h¹, 6 h of anoxia treatment without or with 90 mM Suc (s) added before the treatment (Loreti et al., 2005); A 12h, 12 h of anoxia treatment, total and polysomal (p) RNA (Branco-Price et al., 2005); H₂O₂, 1 h of H₂O₂ treatment at 20 mM (Dr. Mittler, NASCArray repository); A 6h², 6 h of anoxia treatment (Banti et al., 2010); bp, binding protein; fp, family protein.

The overlap between heat stress and anoxia was already described by Banti et al. (2010), who proposed H_2O_2 as the signaling element linking anoxia and heat stress. *HSPs* are induced by anoxic stress in several species, suggesting a conservation of this mechanism across different kingdoms (Vandenbroucke et al., 2008; Mustroph et al., 2010). Anoxia, differently from hypoxia, could harbor a more complex mechanism of response/adaptation rather than a simple switch to fermentative metabolism, as suggested by Licausi (2011) and we suggested that induction of heat-related genes is mostly related to anoxia (Fig. 3).

Suc feeding drastically enhances both the induction of *HSPs* and anoxia tolerance in *Arabidopsis* seedlings (Loreti et al., 2005). Suc triggers higher *HSP* expression, especially when Suc is added to the growth medium some days prior to the anoxic treatment (Supplemental Fig. S6). Transcript convergence in processes involving sugar, ROS, and scavengers suggests that sugar modulation of gene expression is likely related to oxidative stress control (Couée et al., 2006). Suc feeding enhances transcription of *HSPs* (Loreti et al., 2005) that, acting as chaperones, may prevent aggregation, denaturation, misfolding, and degradation of proteins important for plant survival. Indeed, exogenous sugar strongly enhances low- O_2 tolerance in a variety of plant species (Vartapetian and Andreeva, 1986; Perata et al., 1992; Germain et al., 1997; Loreti et al., 2005).

AtRbohD Helps Mediate ROS Signaling in Seedlings/Shoots under Anoxia

Anoxia triggers an oxidative burst in which H_2O_2 plays a role (Baxter-Burrell et al., 2002; Banti et al., 2010). Transient, early H_2O_2 accumulation was observed in *Arabidopsis* seedlings under anoxia but not hypoxia (Fig. 4A). *HsfA2* and *HSP22* were up-regulated by H_2O_2 (Fig. 4B) and the induction of these genes under anoxia but not hypoxia is suggestive of a direct role of the H_2O_2 produced under anoxia as a trigger for the early expression of these genes (Fig. 3).

ROS generated by NADPH oxidase regulate plant development as well as biotic and abiotic stress responses (Torres and Dangl, 2005). The NADPH oxidase gene family is composed of 10 *Rboh* genes (Torres et al., 1998; Dangl and Jones, 2001) encoding plasma membrane proteins that display a cytosolic extension with two Ca^{2+} -binding EF hands, likely responsible for their regulation by Ca^{2+} (Keller et al., 1998). NADPH oxidase rapidly reduces apoplastic O_2 to O_2^- as a primary product that then may be further converted to H_2O_2 (Neill et al., 2002). ROS generated via NADPH oxidase might represent the link in common stress signaling (Miller et al., 2009). Previous data suggest that different *Rboh* genes are active in different cellular contexts. While *AtrbohC* has a function in root-hair development (Foreman et al., 2003), *AtrbohD* and *AtrbohF* are required in response to plant pathogens (Torres et al., 2002) and abscisic acid signaling (Kwak et al., 2003). The survey of *Rboh* gene expression in the

microarray datasets, allowed us to identify *RbohD* as significantly up-regulated in some of the low- O_2 experiments (Fig. 5A). *RbohD* is also positively regulated in some ROS-related microarrays (Fig. 5A). Interestingly, the *rbohD* mutant, previously shown to display reduced O_2^- production and H_2O_2 accumulation during defense responses (Torres et al., 2002), displayed reduced survival under anoxia (Fig. 5B). We also screened the list of genes commonly up-regulated in some ROS and low- O_2 experiments for TF specifically related to ROS signaling (Gadjev et al., 2006). Hierarchical clustering linkage of these genes showed association of gene induction with anaerobic experiments (Fig. 6A). A survey of expression of some of these gene under anoxia, revealed a lower induction of *HsfA2* and *ZAT12* in the insertional *rbohD* mutant when compared with the wild type (Fig. 6B), suggesting a role for this NADPH oxidase in inducing these genes under anoxia, and their partial requirement for survival. *HsfA2* has previously been shown to be fast up-regulated after 1 h under anoxia (Banti et al., 2010). The regulation of some other genes like *ZAT6* and *ADH1* was instead mostly not affected by the lack of

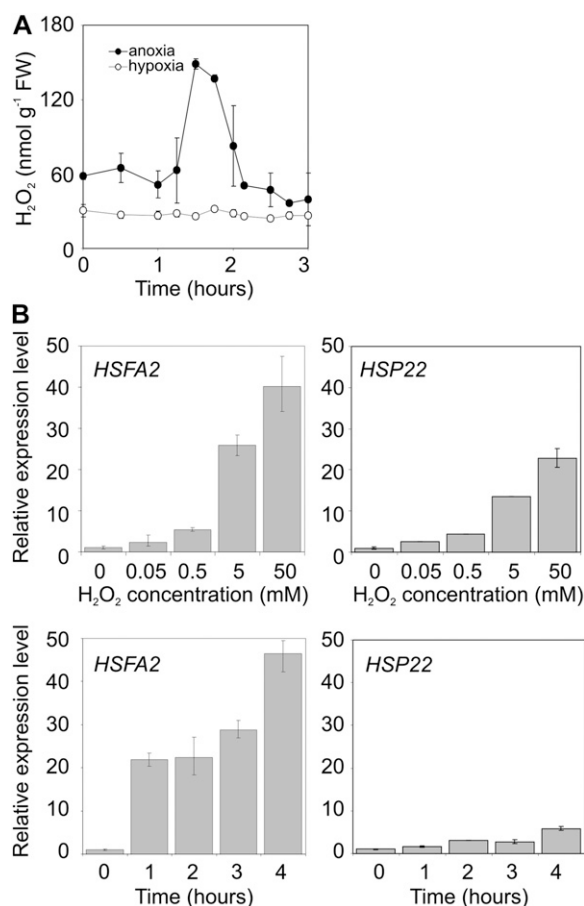


Figure 4. ROS-related experiments under anoxia. A, H_2O_2 production under anoxia and hypoxia. B, Regulation of *HsfA2* and *HSP22* gene expression at different exogenous H_2O_2 concentration and in a time course up to 4 h (H_2O_2 5 mM).

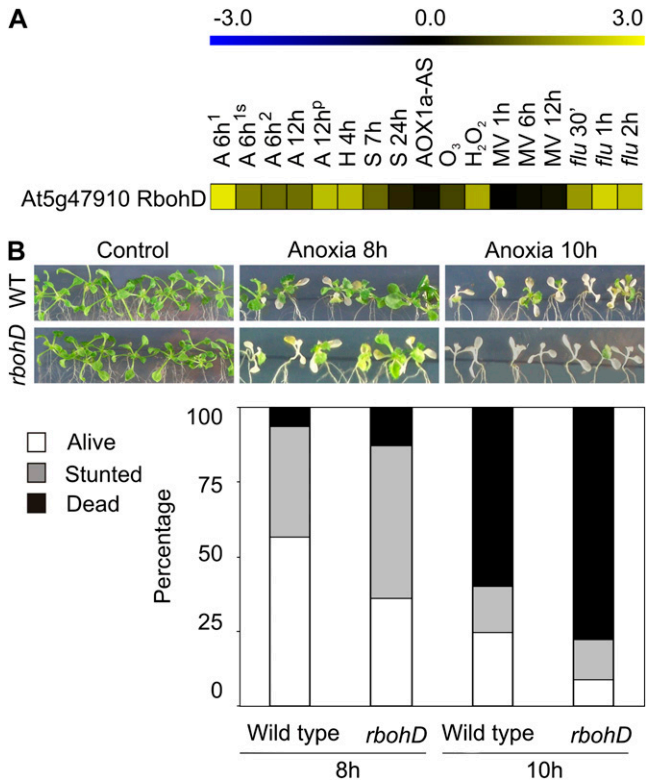


Figure 5. NADPH oxidase role under anoxia. **A**, Regulation of *rbohD* isoform in the O₂ deprivation microarray experiments. **B**, Arabidopsis wild-type plants and *rbohD* mutant survival after 8 and 10 h of anoxia; stunted plants were those showing bleaching symptoms on some of the leaves but not all. Percentage of alive plants was significantly different, paired Student's *t* test ($P < 0.05$, $n = 6$). A 6h¹, 6 h of anoxia treatment without or with 90 mM Suc (s) added before the treatment (Loreti et al., 2005); A 6h², 6 h of anoxia treatment (Banti et al., 2010); A 12h, 12 h of anoxia treatment, total and polysomal (p) RNA (Branco-Price et al., 2005); H 4h, 4 h of hypoxia treatment at 1% O₂ (Licausi et al., 2010); S 7/24h, 7 and 24 h of submergence treatment (Lee et al., 2011); *flu* 0.5 (30')/1/2h, *flu* mutant shift from dark to light (op den Camp et al., 2003); AOX1a-AS, AOX1a-AS mutant (Umbach et al., 2005); MV 1/6/12h, MV 1, 6, 12 h treatment (Dr. Kirch, NASCAArray repository); O₃, 1 h of O₃ fumigation at 200 nL L⁻¹ (Dr. Shirras, NASCAArray repository); H₂O₂, 1 h of H₂O₂ treatment at 20 mM (Dr. Mittler, NASCAArray repository).

functional RBOHD. ZAT12 has been found to be potentially involved in ROS because its expression is up-regulated under several stresses including heat, high light, and cold (Rizhsky et al., 2004; Davletova et al., 2005a; Miller et al., 2008; Doherty et al., 2009). Moreover, knockout and overexpressor ZAT12 plants were found to be altered in response to different stresses (Rizhsky et al., 2004; Davletova et al., 2005b).

Recently, mitochondria-associated ROS production has been found to be involved in Arabidopsis response to O₂ deprivation (Chang et al., 2012). O₂ deprivation and also reoxygenation promote the rapid and transient activation of mitogen-activated protein kinases 3, 4, and 6 that seem to be dependent on mitochondrial ROS. The overexpression of mitogen-activated protein kinase 6 leads to increased survival to anoxia, but the classic

transcripts related to the anaerobic metabolism were not significantly modulated, suggesting a promotion in plant survival probably related to a pathway different from that involving the activity of proteins encoded by the anaerobic core genes (Chang et al., 2012). The link between mitochondrial and membrane-derived ROS production under anoxia is currently unknown. However, a link between functional mitochondria and HSP synthesis has been demonstrated. Mitochondrial dysfunction down-regulates HSP production during mild

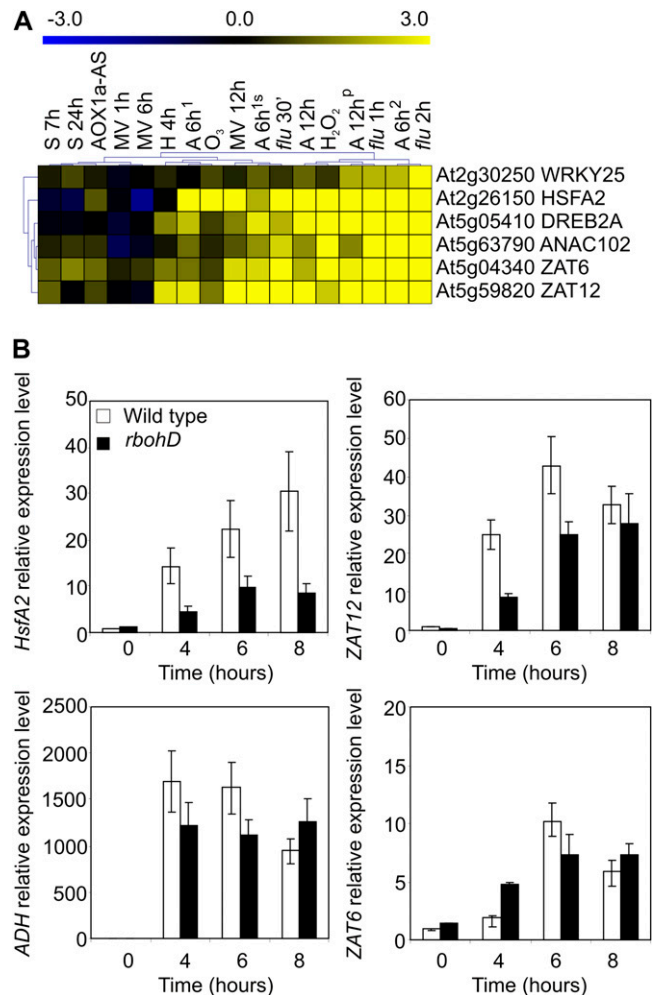


Figure 6. ROS-related TF regulation under anoxia. **A**, Regulation of ROS-related TF in the O₂ deprivation microarray experiments. **B**, Regulation of *HsfA2*, *ZAT12*, *ZAT6*, and *ADH1* gene in wild-type and *rbohD*⁻ plants under anoxia in a time course up to 8 h. A 6h¹, 6 h of anoxia treatment without or with 90 mM Suc (s) added before the treatment (Loreti et al., 2005); A 6h², 6 h of anoxia treatment (Banti et al., 2010); A 12h, 12 h of anoxia treatment, total and polysomal (p) RNA (Branco-Price et al., 2005); H 4h, 4 h of hypoxia treatment at 1% O₂ (Licausi et al., 2010); S 7/24h, 7 and 24 h of submergence treatment (Lee et al., 2011); *flu* 0.5 (30')/1/2h, *flu* mutant shift from dark to light (op den Camp et al., 2003); AOX1a-AS, AOX1a-AS mutant (Umbach et al., 2005); MV 1/6/12h, MV 1, 6, 12 h treatment (Dr. Kirch, NASCAArray repository); O₃, 1 h of O₃ fumigation at 200 nL L⁻¹ (Dr. Shirras, NASCAArray repository); H₂O₂, 1 h of H₂O₂ treatment at 20 mM (Dr. Mittler, NASCAArray repository).

heat shock in Arabidopsis cell (Rikhvanov et al., 2007). Moreover, in mammals' pulmonary arteries hypoxia-driven mitochondrial ROS production has been suggested to trigger NADPH oxidase activity, suggesting a mechanism by which mitochondria and cytosol both contribute to the increase in ROS production during low O₂ (Rathore et al., 2008). Future studies will elucidate whether this is the case in plants.

CONCLUSION

The balance between cellular ROS production and ROS scavenging rate enables a rapid and dynamic change in tissue and subcellular ROS levels (Mittler et al., 2011). These are necessary features if a signaling molecule is to be efficient. ROS were recently shown to be required in response to diverse abiotic stimuli for rapid cell-to-cell communication over long distances (Miller et al., 2009). Data describing the existence of ROS production under O₂ deprivation (Baxter-Burrell et al., 2002; Chang et al., 2012), together with the overlap with the molecular response to heat (Banti et al., 2010), suggest a role for ROS in plant adaptation to low O₂. In this work, we identified a group of genes regulated by anoxia but not hypoxia, most of which are heat related. These genes are not part of the group of anaerobic core genes that are regulated by ERFs under the control of the N-end pathway for O₂ sensing recently described (Gibbs et al., 2011; Licausi et al., 2011). Furthermore, a set of genes that respond to anoxia overlaps with a group of ROS-regulated genes and we observed an early, transient peak of H₂O₂ production under anoxia but not hypoxia. We propose that *RbohD* is partially involved in the signaling cascade responsible for the induction of ROS-related TF under anoxia, a mechanism that allows Arabidopsis to survive longer under O₂ absence.

MATERIALS AND METHODS

Microarray Analysis

Publicly available Arabidopsis (*Arabidopsis thaliana*) microarray databases were screened for ROS and O₂ deprivation related experiments (Tables I and II). CEL files were downloaded from the Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/gds>) or the European Arabidopsis Stock Centre's International Affymetrix Service (<http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl>). A total of six experiments related to O₂ deprivation (6 h anoxia ± Suc feeding, 12 h anoxia, 0.5, 2 and 48 h of 1%, 4%, and 8% of O₂, 4 h of 1% O₂, and 7 and 24 h of submergence) and another five related to ROS-generating systems (MV treatment, O₃ fumigation, H₂O₂ treatment, AOX1a-AS, and *flu* mutants) were selected for the analysis. Three of the microarrays had no biological replicates whereas the others included a minimum of two biological replicates. All the experiments were used for all analysis. For specific information on each experiment please refer to the original datasets. Information on the condition of the MV, O₃, and H₂O₂ treatment, performed by the groups of Dr. Kirch, Dr. Shirras, and Dr. Mittler, respectively, can be found in the NASCArrays repository at the respective reference number (Table II).

Data Processing

Raw intensity CEL files were imported in Robin interface for microarray (Lohse et al., 2010) and the quality of the original 133 arrays was checked.

Probe level model residual pseudo images showing potential artifacts were excluded from the analysis. Raw normalized expression values of ROS and O₂ deprivation arrays, generated from treated samples only, were analyzed using hierarchical clustering based on Pearson correlation. Two experimental groups with a similar global response in gene expression were identified in roots and seedlings/shoots, in both ROS and O₂ deprivation conditions (Supplemental Figs. S1 and S2). The two organ-specific clusters were treated separately in the subsequent analysis. For the MV treatment on shoot (NASCARRAY-143), cluster dendrogram revealed three different main clusters of genes (data not shown). The experiments related to 1, 6, and 12 h of treatment were then selected for the seedlings/shoots analysis as representative of the three clusters. Both seedlings/shoots and roots groups data were normalized and the signal intensities were estimated using the Affimetrix Microarray Analysis Suite 5.0. Average expression values and their adjusted *P* values were calculated using the Benjamini-Hochberg adjustment method (Reiner et al., 2003). For the experiment of Branco-Price et al. (2005), polysomal RNA fold-change in expression values was calculated on the basis of the row CEL data obtained by the GSE2218 experiment. Calls resulting significantly regulated in at least one experimental condition (*P* < 0.05) were filtered, resulting in 1,762 and 68 significantly differentially expressed genes, in seedlings/shoots and roots, respectively (data not shown).

The core of O₂ deprivation, anoxic, and hypoxic related genes was arbitrarily defined to be $\log_2 \geq 1$ (*P* < 0.05) in the overall seedlings/shoots and roots experiments (Fig. 1; Supplemental Fig. S3).

The relative abundance of functional gene classes was calculated considering for each class the percentage of coregulated genes divided by the percentage of gene in the microarray (Fig. 2B).

The averaged log-normalized values of the selected probesets were hierarchically clustered using the average linkage on the Euclidean distance. The clustering analysis and heatmaps were obtained using The Institute for Genomic Research Multiple Experiment Viewer 3.1 (Saeed et al., 2003).

Plant Material and Treatment Conditions

Arabidopsis, Col-0 ecotype, was used for the experiments. The homozygous line of the transposon-tagged insertional mutant *rbohD* (N9555) was obtained from the European Arabidopsis Stock Centre, where it was donated by Jonathan Jones (John Innes Centre). Seeds were sterilized 10 min with diluted bleach (1.7% sodium hypochlorite), rinsed, and washed several times in sterile water. For the detection of H₂O₂ and the H₂O₂ treatments, the experiments were performed with 4-d-old dark-grown seedlings. Seeds were sown in liquid Murashige and Skoog (MS) one-half-strength medium supplemented with 1% Suc. Seeds were stratified for 72 h in the dark at 4°C and then transferred to 23°C in the dark with shaking.

When fed to seedlings, H₂O₂ was dissolved in MS medium of 4-d-old seedlings, added to the final concentration of 0.05, 0.5, 5, and 50 mM. For the time-course experiments, 5 mM H₂O₂ was used (Fig. 4B). Plates were incubated at 23°C in the dark with shaking for 2 h.

Anoxic and hypoxic treatments were carried out in the dark. An enclosed anaerobic workstation (anaerobic system model 1025; Forma Scientific) was used to provide an O₂-free environment for seedlings' incubation. This chamber uses palladium catalyst wafers and desiccant wafers to maintain strict anaerobiosis to less than 10 μg mL⁻¹ O₂ (according to the manufacturer's specifications). High-purity N₂ was used to initially purge the chamber, and the working anaerobic gas mixture was N₂:H₂ with a ratio of 90:10. Hypoxic treatments were carried out using a glovebox flushed with 1% O₂. The anoxic and hypoxic treatments lasted up to 3 h, collecting samples every 15 min (Fig. 4A).

Agar plates were used to evaluate plants' tolerance to anoxia and for gene expression analysis (Figs. 5B and 6B). To obtain 7-d-old plants in vertical plates, seeds were germinated on MS one-half-strength medium containing agar (0.9%) and Suc (1%). Seeds were stratified for 72 h in the dark at 4°C and then transferred at 23°C and 12 h light photoperiod, photosynthetically active radiation approximately 100 μmol m⁻² s⁻².

Anoxic treatments were performed by transferring the plates in the anaerobic work station and kept in the dark. The plates were then transferred for the postanoxic recovery to monitor the plant survival (23°C and 12 h light photoperiod, photosynthetically active radiation approximately 100 μmol m⁻² s⁻²).

H₂O₂ Quantification

H₂O₂ production was measured using the Amplex red H₂O₂/peroxidase assay kit (Molecular Probes) following the manufacturer's instructions. Plant ma-

terial extraction was performed using 50 mg of frozen tissue ground in 200 μ L of 20 mM sodium phosphate buffer (pH 6.5), according to the protocol developed by the Schachtman Laboratory (Shin and Schachtman, 2004). After centrifugation at 10,000 rpm for 10 min (4°C), 50 μ L of supernatant were used for Amplex red assay.

Molecular Analysis

Total RNA was extracted using a RNAqueous kit (Applied Biosystems), according to the manufacturer's instructions, and subjected to DNase treatment using TURBO DNA-free kit (Ambion). Five micrograms of RNA were reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems). Transcript abundance was analyzed by real-time reverse transcription PCR, using TaqMan probes (Applied Biosystems/Ambion) and primers specific for each gene (Supplemental Table S3), using an ABI Prism 7000 sequence detection system (Applied Biosystems). PCR reactions were carried out using 50 ng of cDNA and TaqMan universal PCR master mix (Applied Biosystems/Ambion) or qPCR MasterMix Plus for SYBR green I (Eurogentec), following the manufacturer's protocol. The relative expression level of each gene was quantified with the comparative threshold cycle method, as described in the ABI PRISM 7700 Sequence Detection System (User Bulletin No. 2; Applied Biosystems), using *Ubiquitin10* (At4g05320) as an internal reference. PCR reactions for each of the three biological replicates were performed in duplicate.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Hierarchical clustering based on Pearson correlation of the raw normalized expression values of ROS-related arrays, generated from treated samples only.

Supplemental Figure S2. Hierarchical clustering based on Pearson correlation of the raw normalized expression values of O₂ deprivation arrays, generated from treated samples only.

Supplemental Figure S3. Genes regulated by anoxia, hypoxia, and O₂ deprivation in Arabidopsis microarray datasets of roots.

Supplemental Figure S4. Hierarchical average linkage clustering of transcripts up-regulated in both the ROS-generating and O₂ deprivation experiments.

Supplemental Figure S5. Hierarchical average linkage clustering of transcripts down-regulated in both the ROS-generating and O₂ deprivation experiments.

Supplemental Figure S6. Hierarchical average linkage clustering of seedlings/shoots heat-stress-related genes regulated in 6 h anoxic experiments with different Suc treatment.

Supplemental Table S1. List of genes significantly up-regulated both in roots and seedlings/shoots experiments under low O₂.

Supplemental Table S2. Regulation of heat stress orthologous genes in tolerant/sensitive rice varieties under submergence.

Supplemental Table S3. List of primers used in this work.

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