

Early Hypoxia-regulated Genes in Rice
and *Arabidopsis thaliana*

Dissertation

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Abbreviations

(b)HLH	(basic) Helix-loop-helix
$A_{\langle \text{Wavelength} \rangle}$	Absorbance at given wavelength
ABA	Abscisic acid
ABRE	ABA-responsive element
ACC	1-Aminocyclopropane-1-carboxylic acid
ACO	ACC oxidase
ACS	ACC synthase
ADH	Alcohol dehydrogenase
AFB	Auxin signaling F-box protein
AGI	Arabidopsis genome identifier
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
AOX	Alternative oxidase
AP2	Apetala 2
ARD	Acireductone dioxygenase
ARF	Auxin response factor
BLAST	Basic local alignment search tool
bp	Base pairs
bZIP	Basic leucine zipper
CaMV	<i>Cauliflower mosaic virus</i>
CCA	Circadian clock associated
CDS	Coding sequence
CIPK	Calcineurin B-like interacting binding kinase
CML	Calmodulin-like
COX	Cytochrome <i>c</i> oxidase
Ct	Cycle threshold
Cyc	Cycles of PCR amplification
DUF	Domain of unknown function
E	Exon-positioned insertion
eEF	Eukaryotic translation elongation factor
EREBP	Ethylene responsive element binding protein
ERF	Ethylene responsive factor
<i>E</i> -value	Expect value
FCh	Fold change
GA	Gibberellic acid
gDNA	Genomic DNA
GOLLUM	Growth in different oxygen levels influences morphogenesis
HIF	Hypoxia-inducible factor
HRE	Hypoxia responsive ERF
HREF	Hypoxia responsive EF protein

IAA	Indole-3-acetic acid
ID	Identifier
JA	Jasmonic acid
JAZ	Jasmonate-ZIM domain
LDH	Lactate dehydrogenase
LHY	Late elongated hypocotyl
LOES	Low oxygen escape syndrome
LOQS	Low oxygen quiescence syndrome
1-MCP	1-Methylcyclopropene
MKK	MPK kinase
MPK	Mitogen-activated kinase
mRNA	Messenger RNA
MS	Murashige and Skoog
mtETC	Mitochondrial electron transport chain
NAC	Protein domain defined by the <i>NO APICAL MERISTEM</i> , <i>ATA</i> and <i>CUP-SHAPED COTYLEDON</i> genes
NCBI	National Center for Biotechnology Information
OPR	12-Oxophytodienoate reductase
P	Promoter or 5'-UTR-positioned insertion
P4H	Prolyl-4-hydroxylase
PCR	Polymerase chain reaction
PDC	Pyruvate decarboxylase
PFM	Position frequency matrix
PG56	Pin Gaew 56
PLD	Phospholipase D
PRR	Pseudo response regulator
qPCR	Quantitative real time PCR
RAP	Rice annotation project
RAP2	Related to AP2
RHDP	Rhodanese-domain containing protein
ROP	Rho-like GTPase of plants
ROS	Reactive oxygen species
RTE	Reversion to ethylene sensitivity
RT-PCR	Reverse-transcription PCR
SAPK	Osmotic stress/ABA-activated protein kinase
SD	Standard deviation
SK	Snorkel
SLR	Slender rice
SLRL	Slender rice like
SnRK	Sucrose non-fermenting 1-related kinase
SUB	Submergence
T _{Ann}	Annealing temperature

T-DNA	Transfer DNA
t_{elong}	Elongation time
TIFY	See ZIM
T_m	Melting temperature
tZ	<i>trans</i> -Zeatin
U	3'-UTR positioned insertion
UTR	Untranslated region
v/v	Volume per volume
w/v	Weight per volume
WIF	Wound-responsive / Wound-induced family protein (gene)
WT	Wild type
ZIM	ZnF protein expressed in inflorescence meristem (also named TIFY after a typical amino acid sequence)
ZnF	Zink finger (domain)

In addition to the abbreviations listed here, symbols and nomenclature as defined by the International Union of Pure and Applied Chemistry and the International System of Units were used without further introduction.

A. Zusammenfassung

Pflanzen unterliegen während ihres Lebenszyklus Hypoxie in unterschiedlich schwerer Ausprägung. Semiaquatische Spezies, wie beispielsweise Tiefwasserreis, haben eine verbesserte Anpassung an Überflutung und den damit einhergehenden Hypoxiestress entwickelt. Viele der Antworten auf und Anpassungen an Hypoxie wurden bereits beschrieben. In dieser Arbeit wurden die unterschiedlich an Hypoxie und Überflutung angepassten Arten *Arabidopsis thaliana* (L.) Heynh. und *Oryza sativa* L. untersucht. Microarray-Analysen wurden mit Tiefwasserreis durchgeführt, um Gene zu identifizieren, die eine regulatorische Rolle bei der Antwort auf Hypoxie spielen könnten. Es waren 1228 Gene nach 1 h Behandlung mit 5% Sauerstoff differenziell reguliert, unabhängig von funktioneller Wahrnehmung des Phytohormons Ethylen. Sie wurden klassifiziert, um Gene zu finden, die möglicherweise eine Rolle in Signaltransduktion und Regulation bei Hypoxie spielen. Mutanten von fünf Genen, die induziert waren, wurden untersucht. Zusätzlich wurden homologe Arabidopsis-Gene gesucht und ebenfalls auf eine Regulation bei Hypoxie analysiert. Von Arabidopsis-Homologen, die ebenfalls durch Hypoxie reguliert waren oder bei denen vorhergegangene Arbeiten auf eine Rolle in der hypoxischen Anpassung hindeuteten, wurden T-DNA-Insertionsmutanten charakterisiert und auf ihre Fähigkeit, Sauerstoffmangel zu widerstehen, untersucht. Mutanten eines *WOUND-RESPONSIVE FAMILY PROTEIN* (At4g10270), eines bHLH-Transkriptionsfaktors (At2g42280) und von Arabidopsis *CALMODULIN-LIKE 39* (At1g76640) wiesen Veränderungen in der Hypoxie-induzierten ADH-Aktivität (At4g10270 und At1g76640) und im Überleben nach 2 d annähernder Anoxie (<0,1% O₂) auf. Überexpressionsmutanten der Prolylhydroxylase *AtP4H7* (At3g28480) sowie von *RHODANESE-DOMAIN CONTAINING PROTEIN* (At2g17850) waren ebenfalls in der Überlebensfähigkeit nach diesem Stress verändert. Um die Funktionen dieser Gene bei Hypoxie verstehen zu können, müssten sie zukünftig genauer in Bezug auf ihre Rolle in der Hypoxieantwort analysiert werden. Mehrere ERF-Transkriptionsfaktoren der Gruppe VII sind an der Regulation der Hypoxieantwort in Reis und Arabidopsis beteiligt. Es konnte gezeigt werden, dass fünf weitere ERFs der Gruppe VII, die in Reis aus 15 Mitgliedern besteht, nach 1 h bei 5% O₂ heraufreguliert waren. Zusätzlich wurden ERF71 und ERF73 aus Arabidopsis in Wurzeln analysiert und es konnte gezeigt werden, dass *ERF73* und *ERF71* durch Hypoxie induziert werden. Zudem wurde gezeigt, dass *ERF73*, aber nicht *ERF71*, durch Ethylen induziert werden kann, sowie dass Ethylen die ADH-Aktivität in Arabidopsiswurzeln zum Teil über *ERF73* moduliert.

B. Summary

Plants are exposed to hypoxia during their life cycle to differing extents. Some plants, like deepwater rice cultivars, have evolved to be adapted to more severe hypoxia occurring during flooding events. Several responses and adaptations to hypoxia have been described earlier. Here, two species very different in the extent of their adaptation to hypoxia and flooding, *Arabidopsis thaliana* (L.) Heynh. and *Oryza sativa* L. were analyzed. Whole genome microarray analyses were performed using hypoxia-treated deepwater rice with the goal to identify genes potentially having a regulatory role in hypoxia responses. After 1 h at 5% oxygen, 1228 genes were differentially regulated irrespective of functional perception of the phytohormone ethylene. The genes were classified to find genes with a potential role in signaling and regulation during hypoxia. Mutants of five induced genes were analyzed. In addition, homologous *Arabidopsis* genes were identified and analyzed for regulation by hypoxia. For homologs regulated by hypoxia or proposed to potentially be involved in hypoxia adaptation according to previous studies, T-DNA insertion mutants were characterized. They were examined for their ability to withstand low oxygen stress. Mutants of *WOUND-RESPONSIVE FAMILY PROTEIN* (At4g10270), *bHLH FAMILY PROTEIN* (At2g42280), and of *Arabidopsis CALMODULIN-LIKE 39* (At1g76640) were altered in hypoxia-induced alcohol dehydrogenase (ADH) activity (At4g10270 and At1g76640) and in respect to their survival capacity after 2 d of near anoxia (<0.1% O₂). Overexpression of prolyl hydroxylase *AtP4H7* (At3g28480) as well as *RHODANESE-DOMAIN CONTAINING PROTEIN* (At2g17850) led to altered survival after the same stress treatment. These genes should be analyzed in the future to determine their role in the response to hypoxia. Group VII ERF transcription factors are known to be involved in the regulation of the response to hypoxia in rice and *Arabidopsis*. It was shown that 5 additional ERF genes of the total of 15 genes in group VII were induced after 1 h of hypoxia in rice. Additionally, we analyzed *Arabidopsis* ERF71 and ERF73 in roots and showed that *ERF73* and *ERF71* are induced by hypoxia. It was further shown that *ERF73* but not *ERF71* expression is regulated by ethylene and that ADH activity is modulated by ethylene partially through *ERF73* in *Arabidopsis* roots.

1. Introduction

1.1. Hypoxia in plants

In almost any multicellular organism, a decrease in oxygen concentration to a point where normal cellular metabolism is disturbed or compromised occurs in its life cycle to differing degrees (Kennedy et al., 1992). This state, hypoxia, can be defined as an oxygen partial pressure at which cells, tissues or the entire organism start to adapt to lower oxygen concentrations. Oxygen is vital as final electron acceptor in the oxidative phosphorylation in mitochondria of eukaryotes as well as in several other metabolic pathways involving oxygen-dependent enzymes (Geigenberger, 2003).

Intensity and frequency of hypoxic conditions vary depending on species and environmental conditions. Plants lack active oxygen distribution and rely on diffusion and ventilation through tissues and intercellular air spaces. Due to this, even at normoxia in the surrounding air space, oxygen can be limiting at the cellular level. Some cells are strongly isolated from the outside like the stele of roots with its Casparian strip (Geigenberger et al., 2000) or in seeds, isolated by the seed coat (van Dongen et al., 2004). Additionally, in tissues with high cell density and high metabolic activity, for example inside of bulky storage organs like potato tubers, diffusion can limit oxygen supply. There, oxygen concentrations can drop down to 2% (Geigenberger et al., 2000) or lower (Armstrong and Beckett, 2011).

In addition to this risk of hypoxic conditions in some tissues, plants are prone to hypoxia due to their immobility and environment: floods, excessive rain or stagnant water resulting of frozen soils can result in waterlogged or flooded plants. As diffusion of oxygen in water is about 10^4 -fold lower than in air (Gibbs and Greenway, 2003), submerged plant organs soon suffer from reduced oxygen concentrations. Additionally, turbid waters often add to the stress by limiting photosynthesis (Mommer and Visser, 2005). The effects of hypoxia can be damaging to plants, but to which degree depends on whether or not they are adapted to this stress.

1.2. Adaptations to hypoxia and flooding

Altered oxygen supply can be divided into three phases: hypoxia with a steady decrease in oxygen levels due to oxygen consumption and diffusion limitations, anoxia when all oxygen is consumed, and reoxygenation, that is when flooding declines and the plant reemerges and is again exposed to atmospheric oxygen (Blokhina et al., 2003). This is an approximation, as it is not likely that all cells of a plant indeed suffer

from the same extent of oxygen deficiency at any given time. A continuum of hypoxia with 'anaerobic cores' and 'aerobic sleeves' (Berry and Norris, 1949) throughout the plant is a better image, also supported by simulations (Armstrong and Beckett, 2011). Each of the low oxygen stress phases can be characterized by unique conditions and adaptations.

Irrespective of the low K_m of cytochrome *c* oxidase (COX; 0.013% O₂; Millar et al., 1994), respiratory oxidative phosphorylation, the major ATP producing process in respiring plant tissues, is inhibited at moderate ambient oxygen concentrations due to diffusion limitation through tissues and liquid phase boundaries (Armstrong et al., 2009). At hypoxia, ATP is produced by glycolysis instead of respiration, but NAD⁺ must be regenerated to sustain this. As glycolysis is much less efficient than respiration, ATP levels decrease (Raymond et al., 1985). Over time, storage carbohydrates are depleted and total depletion of sugars results in cessation of glycolysis and ultimately cell death (Bailey-Serres and Voesenek, 2008). Plants have evolved a conserved set of metabolic adaptations to withstand the perils of oxygen deprivation on a cellular metabolic level. The need to regenerate NAD⁺ is accomplished through fermentation of pyruvate either to lactate via lactate dehydrogenase (LDH) or to ethanol by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH; Roberts et al., 1984). This switch to fermentation in place of respiration is termed the Pasteur Effect. Lactate fermentation is proposed to lead to an acidification of the cytosol to a point where ethanolic fermentation is favored (Roberts et al., 1984). In maize root tips, it has been shown that a decline to pH lower than 7 favors PDC activity and promotes ethanol production (Morrell et al., 1990). Lack of ADH activity results in cell death and reduced viability during hypoxia (Roberts et al., 1989). Ethanol is not harmful as it can diffuse out of cells which, however, is a loss of fixed carbon to the plant. In addition to the switch from respiration to fermentation, ATP consumption is reduced by the preferred use of pyrophosphate as energy donor (Carystinos et al., 1995; Huang et al., 2008) and changes in the sucrose hydrolysis pathway (Geigenberger, 2003). Energy consuming processes such as translation and ribosome biogenesis are generally reduced (Branco-Price et al., 2008).

A strong induction of alanine aminotransferases, aspartate transferase, mitochondrial glutamate dehydrogenase, and mitochondrial Ca²⁺/Calmodulin-regulated glutamate decarboxylase has been observed (Bailey-Serres and Voesenek, 2008) with an accompanying accumulation of alanine (Good and Muench, 1993; Rocha et al., 2010), γ -aminobutyric acid, and in some cases succinate (Rocha et al., 2010). Alanine accu-

mulation could be important to counteract cytosolic acidosis or to increase the osmotic potential of cells during hypoxia (Reggiani et al., 1988). Induction of nitrate reductase as was found in *Arabidopsis* (Klok et al., 2002) during hypoxia promotes nitrite (NO_2^-) accumulation and could provide NH_4^+ , needed for alanine transamination (Igamberdiev and Hill, 2004). It has also been proposed that at hypoxia, NO_2^- could be used as an electron acceptor to oxidize NADH and NADPH reducing NO_2^- to nitric oxide (NO) catalyzed by COX and complex III in mitochondria (Stoimenova et al., 2007). The NO produced could be scavenged by the hypoxia-induced cytosolic hemoglobin that is able to catalyze the conversion of NO back to nitrate (Igamberdiev et al., 2010).

Waterlogged plants and especially flooded or submerged plants are challenged by water deficiency due to a decrease in hydraulic conductivity of roots. *Arabidopsis* plants downregulate root water uptake via aquaporin gating resulting from cytosolic acidification (Tournaire-Roux et al., 2003). Apart from changes in the affected plant itself, the root surroundings are strongly affected by flooding events and hypoxia. The soil becomes reduced. The redox, ion, and pH status of the soil changes (Pezeshki, 2001). This leads to a reduced nutrient uptake and to the formation of phytotoxic compounds that threaten the plant like Fe^{2+} , Mn^{2+} , reduced sulfur species and organic acids normally detoxified by oxidation due to radial oxygen loss from the root (Armstrong and Armstrong, 2005). In addition, reduced diffusion out of the plant may lead to accumulation of toxic metabolic products like lactate, acetaldehyde, CO_2 and others (Dat et al., 2004). In flooded or submerged plants, stress due to soil reduction can outlast decline of water levels.

In addition to the rapid metabolic adaptations to the impaired ability for oxidative phosphorylation and photosynthesis, plants have evolved strategies to cope with flooding-induced hypoxia at the anatomical and morphological levels. These responses have been broadly classified into the low oxygen quiescence syndrome (LOQS) and the low oxygen escape syndrome (LOES) by Bailey-Serres and Voesenek (2008) although some responses are conserved in all flooding-adapted plants, irrespective of strategy.

Plants following the LOQS are adapted to environments with short-term flooding conditions. After the stress is perceived, these plants commit to saving energy by reducing energy consuming translation and growth (Bailey-Serres and Voesenek, 2008). Plants well adapted to long-term flooding like the semi-aquatic deepwater rice (cultivars of *Oryza sativa* ssp. *indica* Kato) or *Rumex palustris* Sm. employ the LOES strategy. They increase the rate of elongation of aerial organs like stems, petioles or leaves and

thereby keep at least parts of the plant in contact with the atmosphere (Voesenek and Blom, 1989; Kende et al., 1998). In addition, irrespective of strategy, aeration of submerged tissues is improved by the formation or extension of aerenchyma in stems and roots that facilitate gas diffusion throughout the plant (Evans, 2003). Oxygen loss is further reduced by a barrier formed to reduce radial oxygen loss to the environment, especially in roots (Colmer, 2003). This barrier can also protect the plants from uptake of toxic compounds from the soil (Armstrong and Armstrong, 2005). Additionally, gas films are formed around leaves of submerged rice plants, increasing the interface area with the flood water and thereby improving gas exchange between leaves and water and improving photosynthesis rates in flooded plants (Pedersen et al., 2009). It has been found that in submergence-adapted plants such as *Rumex palustris*, *Oenanthe aquatica* and others, leaf morphology adapts to the flooding conditions to improve gas exchange in between the surrounding water and the plant. The acclimated newly formed leaves are elongated, thinner and therefore have an increased leaf mass area (Mommer and Visser, 2005; Mommer et al., 2007). Cell wall and cuticle thickness of the adaxial side of leaves is reduced (Mommer et al., 2007). These adaptations improve CO₂ uptake for underwater photosynthesis (Mommer and Visser, 2005). In addition to that, many plants such as rice (Lorbiecke and Sauter, 1999), maize (Drew et al., 1979), sunflowers (Wample and Reid, 1979) and also woody perennials like *Platanus occidentalis* L. (Tsukahara and Kozlowski, 1985) and poplar (Guo et al., 2011) enhance or start growth of adventitious roots upon waterlogging or flooding. Growth and emergence of these roots are induced upon flooding and provide a shorter route between aerated leaves and roots than between leaves and the soil borne roots (Steffens et al., 2006). It has to be noted that plants not well adapted to flooding like flooding intolerant lowland rice cultivars, in most cases also pursue the LOES strategy which can result in plant death if energy reserves are depleted before emergence (Bailey-Serres and Voesenek, 2008). Some traits of a plant following LOES are also present to a lesser degree in plants following the LOQS. As an example, aerenchyma are present in deepwater as well as in tolerant quiescent rice varieties (Parlanti et al., 2011).

Reactive oxygen species (ROS) form when plants reemerge into the atmosphere and are again exposed to high levels of oxygen (Gibbs and Greenway, 2003). Scavenging of these molecules by superoxide dismutase (Monk et al., 1987), catalases, peroxidases and the detoxifying ascorbate glutathione system as well as tocopherols (Blokina et al., 2003) improve recovery upon reoxygenation (Ushimaru et al., 1992;

Gibbs and Greenway, 2003). In addition, upon reoxygenation, ethanol left in the cells as a fermentation product is converted into acetaldehyde which is potentially damaging to the cells (Bailey-Serres and Voeselek, 2008). Irrespective of the strategy used for hypoxia survival, some plants adapted to flooding also evolved mechanisms to prepare for the reoxygenation stress by preemptively increasing ROS scavenging systems during hypoxia like superoxide dismutase and components of the ascorbate/glutathione system (Monk et al., 1987; Blokhina et al., 2000). In addition, alternative oxidase (AOX) appears to play a role for plants to be better prepared for reaeration (Amor et al., 2000). Translation of many transcripts was interrupted during hypoxia. Upon return to normoxia, transcripts are stabilized and swiftly recruited to polysomes to resume translation (Branco-Price et al., 2008).

1.3. Perception and signal transduction of flooding and hypoxia

Arguably, the perception and signal transduction of hypoxic stress in higher eukaryotes is best understood in metazoa, and especially so in *Homo sapiens*. Although some discussion is still ongoing, it is now consensus that oxygen-consuming prolyl hydroxylases (P4Hs) regulate the proteasomal degradation of HYPOXIA-INDUCIBLE FACTOR-1 α , a subunit of a heterodimeric transcription factor (HIF) that regulates hypoxia responses (Jaakkola et al., 2001). However, this process seems to be regulated to some degree by ROS released by mitochondria under hypoxic conditions and be influenced – if not by COX itself – by the electron transport chain (Guzy and Schumacker, 2006).

In plants, however, the mechanism of perception of oxygen concentration or hypoxia is unknown. Intuitively, as in mammals, COX in the electron transfer chain in mitochondria (mtETC) as the most important oxygen consuming enzyme in a cell is a good candidate for an oxygen sensor. Again, a problem with this concept arises when noting that the K_m of COX for O₂ is below an equivalent of 0.1% O₂ (Millar et al., 1994). Adaptation to hypoxia starts at much higher concentrations, for example at 8% O₂ in *Arabidopsis* (van Dongen et al., 2009). It has been shown that *Pisum sativum* L. reacts by respiratory adaptation linearly already at concentrations marginally below atmospheric O₂ levels (Zabalza et al., 2009). Bailey-Serres and Chang (2005) argue that a direct sensor must not necessarily exist in plants and that adaptation to low oxygen may only be mediated by indirect changes in cell homeostasis. As mentioned before, modeling by Armstrong and Beckett (2011) indicates that even at relatively high external O₂ concen-

tration, anoxia can occur in cells located at the center of a tissue. Induction of metabolic changes could be regulated by the cytosolic acidification (Greenway and Gibbs, 2003).

Release of ROS from mitochondria and a subsequent spike in cytosolic Ca^{2+} probably originating from the mitochondria can be observed in plants (Subbaiah et al., 1998). It has been proposed that due to the lower affinity of AOX for oxygen, inhibition of this enzyme could cause ROS accumulation (Rhoads et al., 2006) and that ROS production and the increase in Ca^{2+} are important for regulating the hypoxic response. A role of Ca^{2+} has been described for *ADH1* induction in rice (Tsuji et al., 2000) and *Arabidopsis* (Chung and Ferl, 1999). It has to be noted that not all responses to hypoxia are regulated in a Ca^{2+} -dependent manner (Tsuji et al., 2000). Calmodulins and Calmodulin-like proteins are also thought to play a role in regulating aerenchyma formation (He et al., 1996) and AOX expression (Tsuji et al., 2000). A partial signaling pathway explaining *ADH1* induction in *Arabidopsis* involving monomeric RHO-LIKE GTPASE OF PLANTS2 (*ROP2*) which is activated by hypoxia has been proposed (Bailey-Serres and Fukao, 2004). The proposed model gives no explanation for the perception of hypoxia. Flooding itself can be detected by ethylene which accumulates due to reduced diffusion (Jackson, 1985). Changes in nutrient availability as well as osmotic changes due to altered cellular water homeostasis could be detected in the roots of flooded plants (Dat et al., 2004).

1.4. Ethylene and ethylene responsive factors (ERFs)

The gaseous phytohormone ethylene plays a role in developmental processes like germination, growth of roots, hypocotyl, leaves and flowers, in senescence, fruit ripening and abscission as well as in responses to numerous stresses (Schaller and Kieber, 2002). A permanent cross-talk with other phytohormones in these processes occurs (Kendrick and Chang, 2008; Yoo et al., 2009). During flooding, ethylene rapidly accumulates in plant tissues due to reduced diffusion in water (Jackson, 1985; Banga et al., 1996; Kende et al., 1998). Ethylene synthesis increases, especially but not exclusively in flooding adapted plants like deepwater rice (Rzewuski and Sauter, 2008) or is stabilized as was observed in *Rumex palustris* even though the last step in ethylene biosynthesis, the oxidation of 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC oxidases (ACO) is an O_2 -dependent process (Banga et al., 1996). It was proposed that increased ACO and ACC synthase (ACS) expression found in *Rumex palustris* could balance reduced ACO activity thereby maintaining ethylene production (Voeselek et al.,

2003). In contrast to other plants, semi-aquatic plants following the LOES strategy elongate upon increased ethylene concentration (Jackson, 1985). Ethylene signaling has been studied to some degree and the main components of ethylene perception and signal transduction are known (Kendrick and Chang, 2008). Ethylene modulates the levels of the hormones abscisic acid (ABA) and gibberellic acid (GA) in deepwater rice and *Rumex palustris* by inducing GA biosynthesis and responsiveness and repressing ABA biosynthesis (Hoffmann-Benning and Kende, 1992; Voesenek et al., 2003; Nagai et al., 2010). It has to be noted that hormone interactions can differ depending on species and adaptation strategy used (Dat et al., 2004).

Despite their name, only a subgroup of the APETALA 2 (AP2)/Ethylene Response Factor (ERF) superfamily of transcription factors, also called AP2/Ethylene responsive element binding protein (AP2/EREBP) superfamily, are related to signaling and regulation by ethylene (Nakano et al., 2006; Sharoni et al., 2011). Several genes of group VII of the ERF family of AP2/ERF play an important role in the ethylene-mediated response of rice and Arabidopsis to hypoxic stress (Nakano et al., 2006). This was shown for a rice gene of the *SUBMERGENCE1* locus (*SUB1A*) and for the *SNORKEL1* and *SNORKEL2* (*SK1*, *SK2*) genes of rice (Xu et al., 2006; Hattori et al., 2009). Among other processes, SUB1A, SK1 and SK2 regulate ethylene regulated differential adaptation of rice plants to flooding and submergence (Xu et al., 2006; Hattori et al., 2009). The *SNORKEL* genes induce ethylene-dependent internode elongation in deepwater rice by regulating GA responses (Hattori et al., 2009). The presence of SUB1A-1 in non-elongating rice following the LOQS inhibits shoot elongation induced by ethylene accumulation, ethylene synthesis induction, as well as expansins, and it reduces carbohydrate consumption making it a key regulator in the distinction between LOES and LOQS strategies (Fukao et al., 2006). Regulation is mediated by restricting GA responses through the GRAS domain proteins SLENDER RICE-1 (SLR1) and SLR1 LIKE-1 (SLRL1) by SUB1A-1 (Fukao and Bailey-Serres, 2008).

In Arabidopsis, the closest homologs to SUB1A-1, SK1 and SK2 are RELATED TO APETALA2.2 (*RAP2.2/ERF75*, At3g14230), *RAP2.12 (ERF74*, At1g53910), *ERF71*, also named *HYPOXIA RESPONSIVE ERF 2 (HRE2*, At2g47520) and *ERF73 (HRE1*, At1g72360; Hinz et al., 2010). It was shown that *RAP2.2* is induced by hypoxia and by ethylene and improves anoxia survival (Hinz et al., 2010). *RAP2.12* regulates *ADH1* expression and ADH activity (Papdi et al., 2008). Licausi et al. (2010) showed that *ERF73* and *ERF71* are important for hypoxia survival in Arabidopsis by analyzing dou-

ble knock out *erf71 erf73* mutants. It was further observed that *ERF73* expression is induced by ethylene and hypoxia and that knock down or knock out of *ERF73* expression leads to altered responses to hypoxia, in particular altered induction of ADH (Hess et al., 2011, Section 5; Yang et al., 2011).

1.5. Regulation of energy consumption

Regulation of energy consumption is of mayor importance during hypoxia or flooding, especially if long-term flooding is expected (Colmer and Voesenek, 2009). Generally, sensing of energy status requires SUCROSE NON-FERMENTING 1-RELATED KINASE 1 class proteins (SnRK1s) which regulate changes in growth, metabolism and development under stress conditions as well as during darkness (Baena-González and Sheen, 2008). In rice seeds, SnRK1A is phosphorylated and activated by CALCINEURIN B-LIKE INTERACTING BINDING KINASE 15 (CIPK15) which links calcium signaling to metabolic responses (Lee et al., 2009). In the context of hypoxia adaptation, the regulation of carbohydrate consumption is central to the ability of a plant to survive hypoxic stress by either rapidly mobilizing energy to grow in the LOES strategy or saving energy to withstand hypoxic phases in LOQS-employing plants.

In this study, we performed microarray analysis of deepwater rice plants after treatment for 1 h with 5% O₂ to study genes that were regulated early in rice at these conditions. This project aimed to find signal transduction components related to hypoxia not yet identified in this plant. By analyzing putative *Arabidopsis thaliana* orthologs, the potentially conserved functions of these proteins with respect to hypoxia adaptation were studied.

2. Materials and Methods

Plant material and growth conditions

Oryza sativa ssp. *indica* Kato cultivar Pin Gaew 56 (PG56) plants were cultivated as described in Sauter (1997). For hypoxic treatments, sections of 20 cm length were excised 2 cm below the second node of 12-week-old plants and contained the youngest internode (Supplemental Figure 1). Rice mutants carrying the *Tos17* retrotransposon insertion were obtained from the National Institute of Agrobiological Sciences (Japan). These mutants were in the ssp. *japonica* cultivar Nipponbare background.

Seeds from *Arabidopsis thaliana* ecotype Columbia (Col-0) were originally obtained from the Arabidopsis Biological Resource Center (The Ohio State University, USA). Seeds of Arabidopsis mutant lines were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, United Kingdom). After 3 days of stratification at 4°C, plants were grown at long-day conditions (16 h light), at 21-22°C and 60-95 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ of photons. Plants to be grown on soil were placed on the surface of water-soaked soil ('Torfkultursubstrat 2':fine sand; 3:2) in Arabaskets in Araflats (Betatech, Ghent, Belgium) and kept under a mini greenhouse cover for the first 3 days of growth. Plants to be grown on plates were planted on sterile 1/2-strength Murashige-Skoog (MS) medium containing 1.5% (weight per volume, w/v) sucrose (Carl Roth, Karlsruhe, Germany) and 0.38% (w/v) Gelrite (Duchefa Biochemie, Haarlem, The Netherlands) at pH 5.8. Of the sterilized medium, 50 mL per plate was poured into square plates with a surface area of 144 cm², a height of 17 mm and venting ribs on the lids (Greiner Bio-One, Frickenhausen, Germany). Seeds to be germinated on plates were surface-sterilized by shaking at 1400 rounds per minute for 20 min in 1% NaOCl (volume per volume, v/v) in water, washed five times with sterile distilled water and 15-17 seeds were placed on the medium evenly distributed 2.5 cm from the top of the plate. Plates were sealed using micropore tape (3M, St. Paul, USA). After stratification, plates were placed in a climate chamber with the conditions described above at an angle of 68°.

Mutants were tested for homozygosity by polymerase chain reaction (PCR) using genomic DNA (gDNA) isolated from leaves according to Weigel and Glazebrook (2002) with the primers and conditions listed in Supplemental Table 2. To test for expression changes of the respective genes in mutants, RNA was isolated as described below from roots and/or leaves. Expression was tested by reversely transcribing mRNA in a reverse-transcription polymerase chain reaction (RT-PCR) using *Moloney murine*

leukemia virus reverse transcriptase (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. Per one sample, 500-2000 ng of RNA was used in a 20 μ L reaction mixture. For the subsequent PCR, 0.5, 1 and 2 μ L of the resulting cDNA were used in 20 μ L reactions. Primers and conditions used are listed in Supplemental Table 2. The amount of reversely transcribed RNA corresponding to the cDNA used per PCR are given in the respective figures. For expression analysis, primers were designed to encompass at least one intron to be able to detect gDNA contamination in cDNA samples if possible. All PCRs were analyzed using 1-3% agarose gels in TAE buffer (40 mM Tris, 5.71% (v/v) acetic acid, 50 mM ethylenediaminetetraacetic acid in water) containing 5 ng·mL⁻¹ ethidium bromide (all chemicals Carl Roth, Karlsruhe, Germany).

Cloning of transgenic constructs and plant transformation

To create overexpression lines for wound-responsive family gene here named *WOUND INDUCED FAMILY 2* (*WIF2*, At4g10270), *AtP4H7* (At3g28480), and the rhodanese domain containing protein gene, here named *RHDP* (At2g17850), the respective coding sequences were amplified by PCR using the primers and conditions listed in Supplemental Table 2 for this purpose and a proof-reading *Pfu* polymerase. Reversely transcribed messenger RNA (mRNA) of Arabidopsis was synthesized as described above and used as template. The amplified fragments were purified from an agarose gel after electrophoresis using NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and ligated into pENTR1A (Life Technologies, Carlsbad, USA). To achieve this, the amplified insert and the corresponding vector were digested with appropriate restriction enzymes, the vector was dephosphorylated using FastAP (Fermentas, St. Leon-Roth, Germany) and insert and pENTR1A were purified from an agarose gel after electrophoresis. Ligation was performed using T4 DNA ligase (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. The ligation product was transformed into *Escherichia coli* strain DH5 α chemically competent cells. The resulting kanamycin-resistant colonies were screened for the presence of the correct insert by colony PCR and isolation of plasmid DNA using GeneJet plasmid isolation kit (Fermentas, St. Leon-Roth, Germany) followed by restriction analysis. The insert sequence of plasmid DNA of candidate clones was sequenced (GATC Biotech AG, Konstanz, Germany) to confirm accuracy of the cloned coding sequence (CDS). Then, the respective CDS were transferred into the destination vector pB7WG2 (Karimi et al., 2002) via a recombination reaction using LR clonase enzyme (Life Technologies, Carlsbad, USA).

This vector contained the *Cauliflower mosaic virus* (CaMV) 35S transcript promoter to drive the expression of the recombined insert, the CaMV 35S transcript terminator (Odell et al., 1985), and a resistance gene against *DL*-phosphinothricin in its transfer DNA (T-DNA). The plasmids were then transformed into DH5 α cells. Presence of the correct plasmid was confirmed by plasmid isolation performed as described before and subsequent restriction analysis. The isolated plasmid was transformed into chemically competent *Agrobacterium tumefaciens* strain EHA105 cells. Arabidopsis wild type plants (WT) were transformed with these Agrobacteria using an improved floral dip method (Logemann et al., 2006) and seeds were collected after ripening. These seeds were planted on soil as described above and the seedlings were selected for successful transformation by spraying with 0.2 mM *DL*-phosphinothricin in water starting at the age of 6 days. Overexpression of the transgene driven by the 35S promoter in resistant plants was confirmed by RT-PCR using mRNA isolated from leaves as described below and using primers listed for expression analysis of the respective gene in Supplemental Table 2.

Hypoxia, anoxia and flooding treatments of rice and Arabidopsis

Up to 10 stem sections of 12-week-old PG56 rice plants were placed in a 150 mL Griffin form beaker filled with 30 mL of distilled H₂O and placed in gas-tight acrylic glass cylinders with a gas inlet at the bottom and an outlet at the top with a total gas volume of 2 L. To create hypoxia, the sections were treated for 5 min with a mixture of 5% O₂ and 0.04% CO₂ in N₂. The gas flow rate was adjusted to 1 L·min⁻¹ and the gas was conducted through gas washing bottles filled with water before and after the cylinder to stabilize humidity in the cylinders and prevent atmospheric back pressure. Stem sections at normoxia were not treated but were kept in closed acrylic glass cylinders to mimic the conditions of treated samples. The cylinders were open to the surrounding air via an inlet hole. Other sections were pre-treated with 5 ppm 1-methylcyclopropene (1-MCP; AgroFresh, The Netherlands) for 5 min before gassing with a mixture of 5% O₂ and 0.04% CO₂ in N₂ at 1 L·min⁻¹. Sections were incubated for 1 h starting from the end of the pre-treatment and gassing. Then, a 1.5 cm section of the youngest internode above the second node containing the intercalary meristem and the basal part of the elongation zone was rapidly harvested and immediately frozen in liquid nitrogen and used for RNA isolation and microarray analysis. Three independent experiments were performed per treatment condition. Microarray treatments and RNA isolation of the rice section as is described below were performed by Guillaume Rzewuski and Simone Peikert.

For hypoxia treatments of *Arabidopsis* seedlings for quantitative PCR (qPCR) analysis, seedlings were grown on sterile ½-strength MS medium for 10 days as described above. Plates were placed in a gas-tight acrylic glass box of a total volume of 7.3 L and aerated for 15 min at 2 L·min⁻¹ with a mixture of 5% O₂ and 0.04% CO₂ in N₂. Control plants were kept in similar containers in air. After treatment in the light for the times indicated, roots were harvested and frozen in liquid nitrogen and later used for RNA isolation and qPCR. Only roots that grew at the surface of the medium were used to avoid unwanted hypoxia.

To assay survival at near anoxic conditions, *Arabidopsis* seedlings were grown on ½-strength MS medium as described above. Seven-day-old seedlings were placed in a gas-tight acrylic glass box with a volume of 7.3 L in the dark and were treated with pure N₂ at 2 L·min⁻¹ for 60 min. O₂ levels were kept low by gassing again approximately every 12 h for 8 min. The seedlings were kept at anoxic conditions for 48 h. Plates were then returned to air and long-day conditions. After five days of recovery, damage to the plants was classified in four categories. Category I consisted of green seedlings with no recognizable growth defects. Category II consisted of seedlings with one yellow leaf/cotyledon or two partially yellow leaves/cotyledons or green seedlings with growth defects representing minor damage. Category III seedlings had growth defects in combination with yellow leaves/cotyledons but still some green tissue, summarized as severe damage. Category IV seedlings were dead with no green tissue or growth visible.

Hypoxic treatments of *Arabidopsis* seedlings for ADH activity assays were performed as follows: *Arabidopsis* seedlings were grown on sterile ½-strength MS medium as described above. After 10 days of growth at long-day conditions, plates were opened and placed in a gas-tight acrylic glass box with a volume of 7.3 L. Plants were treated with 5% O₂ in N₂ at 2 L min⁻¹ for 20 min. Controls were placed in similar boxes but not treated. Then, they were incubated for 4 h in the light. After that, root tissue was rapidly harvested, flash frozen in liquid N₂, and stored at -80 °C until use.

Arabidopsis plants were analyzed for their reaction to waterlogging. For this, seeds were planted on soil as described above. Seeds of different genotypes were distributed randomly on one flat to prevent experimental errors by tilting of the flat and resulting different levels of submergence depending on plant placement on it. After 16 days at the long-day conditions described above, waterlogging treatment was started by flooding the plants without submerging the shoots and keeping this level of water for 14 days. Otherwise, growth conditions were unchanged during the treatment. On the

day of harvest, the flat was emptied of water and the shoots were harvested, flash frozen in liquid N₂, weighed, and stored at -80°C until used for chlorophyll and anthocyanin determination described below. For one experiment, $n=2-34$ plants were used. Due to a limited number of experiments in the overexpressing lines, experiments of several lines of one overexpressed gene were pooled for statistical analysis. For details on data and statistical analysis see Supplemental Table 3G.

For flooding experiments of rice, 7- to 24-week-old rice plants were entirely submerged in a 600 L plastic tank filled with tap water for three days at long-day conditions as described by Sauter (1997). Controls were placed next to the tank and watered normally. Then, the intercalary meristem and elongation zone region of the 1st internode described above was excised, flash frozen in liquid N₂, and stored at -80°C until used for ADH activity assay or RNA isolation.

Microarray analyses

For microarrays, the sections isolated from the hypoxia-responsive intercalary meristem and elongation regions of rice sections treated for 1 h in 5% O₂, sections pretreated with 1-MCP and 1 h at 5% O₂, and control rice sections were ground in liquid nitrogen and total RNA was extracted using TRI reagent (Sigma-Aldrich, Munich, Germany) according to the manufacturer's instructions with a subsequent digestion with DNase I (Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. Next, phenol/chloroform purification and ethanolic precipitation were performed. Of the isolated RNA, between 7304 and 21032 ng per sample were sent to the MicroArray Facility at the Flanders Institute for Biotechnology (Leuven, Belgium). RNA quality assessment, hybridization using the GeneChip Rice Genome Array (Affymetrix, Santa Clara, USA) and statistical analysis were performed at that facility. RNA quality was tested by RNA degradation plots and quality control of the chips. The present calls were comparable ($42\pm 1.0\%$) and the background did not differ excessively between samples (40 ± 1 units). To find differentially regulated genes, log scale robust multi-array analysis (RMA) expression values (Irizarry et al., 2003) were compared using the Limma package of the Bioconductor project (Smyth, 2004). For each contrast of interest, it was tested whether it deviates significantly from 0 with a moderated t -statistic. Resulting P values were corrected for multiple testing using the Benjamini-Hochberg procedure to control for the expected proportion of the false discovery rate. Probe sets with $P < 0.05$ and a fold change larger than 2 ($|\log_2\text{-ratio}| > 1$) were used for further analysis.

Bioinformatical analyses

For bioinformatical analyses of the microarrays, all data was used with the exception of genes that were predicted not to be significantly regulated at 5% O₂ with 1-MCP treatment in comparison to the 21% O₂ control. For genes represented more than once in the set, only the strongest regulation was further used. To compare with other microarray data, these were retrieved and formatted to facilitate comparison. All identifiers (IDs) found were converted to TIGR/MSU rice annotation project (Ouyang et al., 2007) IDs when possible using either Affymetrix Affy-IDs and a respective database retrieved from Affymetrix (Santa Clara, USA, <http://www.affymetrix.com/analysis/downloads/na31/ivt/Rice.na31.annot.csv.zip>, retrieved 04/02/2011), rice annotation project (RAP) IDs and a conversion table retrieved from the RAP database site (<http://rapdb.dna.affrc.go.jp/download/latest/RAP-MSU.txt.gz>; retrieved 12/24/2009), TIGR locus IDs provided in the data or, in some cases, by matching provided cDNA sequences to the rice genome by basic local alignment search tool (BLAST) analysis and obtaining the TIGR IDs this way. The data was then rescreened for redundant entries. These were removed and the resulting list of TIGR IDs was compared to the data used here and vice versa. For convenience, for all locus IDs given in the text, figures and tables, the 'LOC_' prefix was omitted.

For promoter analysis, the sequences 1 kilobases upstream of annotated rice gene models ($n=67372$) were retrieved from the TIGR/MSU website (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_6.1/all.dir/all.1kUpstream; retrieved 04/06/2011). Start codon ATGs included in those sequences were removed. To find conserved motifs of the coregulated genes, the command line version of Weeder 1.3 (Pavesi et al., 2004) was used with a rice background model provided by Giulio Pavesi (University of Milano) at "medium" setting (i.e., searching for motifs of 6, 8 and 10 nucleotides with 1, 2, and 3 mutations, respectively), allowing multiple occurrences of motifs in one sequence, not assuming that all sequences include a motif and with a maximum of 100 motifs found per run and searching on both strands. Logos of the resulting motifs were drawn using Weblogo (Crooks et al., 2004) after converting the output position frequency matrix (PFM) files of Weeder into a multiline raw sequence file employing a custom Perl script. For comparisons with known motifs, the STAMP tool (Mahony and Benos, 2007) was used after converting the PFM result files from Weeder with PSSM convert (<http://www.phisite.org/pssm-convert/pssm-convert.htm>). The number of occurrences of

motifs in the datasets was determined using MotifScanner (Thijs et al., 2002) for each of the PFMs with the default options, but including both strands.

For comparison of the entire set of regulated genes with known transcription factors and/or genes annotated or predicted to be involved in signaling, a table of rice genes was first assembled using the GRASSIUS rice transcription factor database (Yilmaz et al., 2009) and information from publications describing individual gene families in rice or genes described in other microarray analyses (Xiong et al., 2005; Li et al., 2006; Nakano et al., 2006; Murakami et al., 2007; Lasanthi-Kudahettige et al., 2007; Ross et al., 2007; Hirano et al., 2008; Ramamoorthy et al., 2008; Rzewuski and Sauter, 2008; Nuruzzaman et al., 2010; Sharoni et al., 2011). In cases of contradicting annotations, publications overruled databases and newer publications were considered more accurate than older ones. Annotations by Nakano et al. (2006) were preferred over the ones by Sharoni et al. (2011) for simplification. Functional grouping was preferred over structural, for example several rice basic leucine zipper (OsZIP) factors were put in the ABA-related group and not with the other bZIP factors. The assembled list contained 3039 items and was compared to the set of selected genes and a heat map with the corresponding annotations was created (see below).

For a detailed manual analysis, probe sets with a fold change larger than 3 were further analyzed. For genes represented more than once in the resulting dataset, again only the strongest fold change was used for further analysis. To facilitate analysis, genes were manually assigned to categories (Supplemental Table 1) using the automated gene ontology assignments provided by the TIGR/MSU Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>; Ouyang et al., 2007). Where none of the categories applied or the annotation was unclear or no information was available, genes were declared as 'expressed protein'. Candidates for further analysis were selected according to high fold change and predicted function. Genes not regulated by both 5% O₂ and 5% O₂ including 1-MCP treatment were not analyzed. Genes with a fold change larger than 5 were only further analyzed if the expression at 5% O₂ with 1-MCP treatment did not differ to expression at 5% O₂ by more than 25%.

In order to identify putative Arabidopsis orthologs of rice candidate genes, a list of putative ortholog groups that was created using OrthoMCL (Li et al., 2003) was retrieved from the TIGR/MSU project homepage (Ouyang et al., 2007). For all rice loci in the dataset, putative orthologs from Arabidopsis were automatically retrieved in form of Arabidopsis genome identifiers (AGIs). Additionally, BLASTP analysis was performed

using the protein sequence of each rice gene on the *Arabidopsis thaliana* RefSeq protein database at the National Center for Biotechnology Information (NCBI) website. BLASTP results were compared to the results of the OrthoMCL list to verify these. For genes not appearing in that list or where no putative orthologs had been found using the list, BLASTP results were scored according to the following criteria: (1) Sequence coverage: if homology was confined to a part of the rice sequence, e.g. a DNA binding domain and the remaining sequence exhibited no similarity, the *Arabidopsis* gene encoding for that protein was discarded. (2) BLAST score: proteins with Expect (*E*)-values above $1 \cdot 10^{-3}$ were not included. In cases where the scores of the first one to three results were orders of magnitude better than those of the next ones, the genes corresponding to these proteins were also selected. For example, for Os09g36220 the two *Arabidopsis* genes encoding proteins with the highest similarity were At5g24470 and At2g46790. They had *E*-values of $3 \cdot 10^{-58}$ and $2 \cdot 10^{-54}$, respectively. However, the third-best hit had an *E*-value of $4 \cdot 10^{-45}$, nine orders of magnitude below that of the first two proteins and was not included in the selection.

Quantitative real time PCR (qPCR)

Arabidopsis genes were selected for qPCR analysis if there were no more than three homologs found using BLASTP analysis. In cases with more homologs found, only a maximum of two genes encoding for the proteins with the highest sequence similarity to the rice homolog were selected and analyzed. Oligonucleotide primers for qPCR analysis were designed using OligoPerfect Designer (Life Technologies, Carlsbad, USA; <http://tools.invitrogen.com/content.cfm?pageid=9716>) and the Primer-BLAST tool at the NCBI homepage. Oligonucleotides were designed to be specific, if possible to span an intron to detect genomic DNA contamination, to have a melting temperature T_m of 60°C and to yield products of a size between 150 and 200 base pairs (bp). All oligonucleotides used are listed in Supplemental Table 2. Seedlings were treated with hypoxia and RNA was isolated as described above. Two to four independent experiments were carried out. qPCR was performed with the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using 50 ng of RNA per reaction. Transcripts of At1g76940 and At3g18380 were selected as controls as they were determined by the RefGenes tool (Hruz et al., 2011) to be non-regulated under various stress conditions including hypoxia and anoxia. qPCR was carried out in a 7300 Real-Time PCR-System (Life Technologies, Darmstadt, Germany). In contrast to other PCR reaction conditions listed in Supplemental Table 2, the PCR program was as

follows: initially 30 min at 50°C for reverse transcription followed by 15 min of heat-activation of the polymerase and inactivation of the reverse transcriptase at 95°C. Then, 40 cycles of 20 s denaturation at 94°C, 60 s annealing at 60°C and 30 s of elongation at 72°C. The run ended with a dissociation run to test for one single PCR product. All cycle threshold (Ct) data was normalized to transcript levels of At1g76940 and At3g18380 and analyzed in comparison to untreated controls using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Statistical analysis was performed as described below and was performed on ΔCt values separately normalized against the two reference genes used. In cases where comparison to only one of these analyses resulted in significant regulation, this is nevertheless presented in the results. For detailed statistics see Supplemental Table 3A.

Rice RNA was isolated as described above from the intercalary meristem and basal elongation zone of the youngest internode of plants that were flooded and control plants that were not flooded as described above. The isolated RNA was used in a qPCR reaction that was performed as for the Arabidopsis RNA described above. Ct values were normalized against expression of the rice *EUKARYOTIC TRANSLATION ELONGATION FACTOR 1 α* (*OseEF1 α* , Os03g08020) gene and analyzed by comparing expression in flooded and non-flooded plants.

Statistical analysis

All data recorded as percentages were converted by arcsine transformation before statistical analysis. Equal variances of two sample populations was tested by F-test and Levene's test and was assumed if both resulted in $P > 0.05$. Data was additionally tested for normal distribution by Anderson-Darling normality test and was assumed if it resulted in $P > 0.05$. Statistical analysis was performed by the two-sample Student's *t*-test with equal variances assumed if applicable. If normal distribution did not apply, the confidence level demanded was increased from 95% to 99%. For *P*-values lower than or equal to 0.05, significance was assumed and is marked by an asterisk (*) in the figures. Genes which showed a relative expression fold change in qPCR analyses of stronger than 2 in the same direction in all repetitions are marked by a cross (+) in Figure 12. All statistical analysis was performed using MINITAB 14 (Minitab Inc., State College, USA). Detailed statistical data for every type of experiment is listed in Supplemental Tables 3A-3G.

Alcohol dehydrogenase (ADH) activity assay

To determine ADH enzyme activity, soluble protein was extracted from roots of 10-day-old Arabidopsis seedlings treated as described above or from the section of the first internode of rice plants described above in by grinding in liquid N₂. The ground tissue was resuspended in ice-cold extraction buffer (100 mM Tris-HCl, pH 8.0, 25% (v/v) glycerol, 0.8% (v/v) β-mercapto ethanol, 2% (w/v) polyvinyl pyrrolidone, 5 mM dithiothreitol) by vortexing. It was centrifuged at 16 000×g for 15 min at 4°C to remove cell debris and tissue clumps. The supernatant was transferred to a new reaction vessel and kept on ice until use. Protein concentrations were determined by Bradford assay (Roti-Quant, Carl Roth, Karlsruhe, Germany). Protein was diluted to 1 μg·μL⁻¹ in 50 μL extraction buffer and water was added to a final volume of 150 μL. The enzyme reaction was started by adding 800 μL activation buffer (62.5 mM Tris-HCl, pH 8.5; 1 mM NAD⁺ in water) and 50 μL ethanol. The absorbance at 340 nm ($A_{340\text{nm}}$) was recorded every 15 s for 3 min in a quartz glass cuvette with a path length d of 10 mm in a spectrophotometer (DU720, Beckman Coulter, Krefeld, Germany). Enzyme activity was calculated from the increase in absorbance at 340 nm over time using Equation 1:

$$\text{Activity} = \frac{\Delta A_{340\text{nm}} \cdot \text{Volume}_{\text{Cuvette, total}}}{\epsilon_{\text{NADH}} \cdot d \cdot \text{Volume}_{\text{Protein solution added}} \cdot t \cdot C_{\text{Protein solution}}} \quad (1)$$

Statistical analysis was performed as described above with 3-15 independent experiments for Arabidopsis, except for *prp9-1*, which was tested twice. For each line, 15-34 seedlings were used per experiment. For details, see Supplemental Table 3E for Arabidopsis statistical analysis data and Supplemental Table 3F for additional rice data.

Anthocyanin and chlorophyll determination

Shoots of 30-day-old Arabidopsis plants were harvested after the plants had been growing on soil and were waterlogged for 14 days. For anthocyanin determination, a protocol modified from Neff and Chory (1998) was used. The shoots were ground in liquid N₂. Of the ground tissue, 75-200 mg was thawed by adding 0.6 mL 1% (v/v) HCl in methanol and 0.4 mL H₂O and mixing at 1400 rounds per minute and 25°C for 3 min. This suspension was incubated rotating overnight at 4°C. Then, 1 mL chloroform was added, mixed vigorously for 30 s and centrifuged for 5 min in a table-top centrifuge at 12 000×g. The aqueous supernatant was diluted 1:10 in 0.6% (v/v) HCl in 60% (v/v) methanol in H₂O and the absorbances at 530 nm ($A_{530\text{nm}}$) and $A_{657\text{nm}}$ were measured in a DU530 spectrophotometer (Beckman Coulter, Krefeld, Germany) in polystyrene cuvettes with a path length of 10 mm. The $A_{657\text{nm}}$ was subtracted from the $A_{530\text{nm}}$ and the

result divided by the tissue mass tested to calculate the relative amount of anthocyanin per mg fresh weight. For chlorophyll determination (Arnon, 1949), 100-200 mg of ground tissue was thawed in 80% (v/v) acetone in 50 mM Tris pH 7.5 by shaking for 3 min at 25 °C and rotated overnight at 4 °C. Then, the suspension was passed through a fluted cellulose filter with a particle retention of 13 µm to remove tissue clumps and cell debris, diluted 1:10 in 80% acetone and absorbances were measured at 645 nm, 663 nm and 750 nm (A_{645nm} , A_{663nm} , A_{750nm}) with a DU530 spectrophotometer (Beckman Coulter, Krefeld, Germany) in a quartz glass cuvette with a path length of 10 mm. The relative chlorophyll content per mg was calculated as follows (m : tissue mass applied):

$$\text{Chlorophyll content} = 8.02 \cdot (A_{663nm} - A_{750nm}) + 20.2 \cdot (A_{645nm} - A_{750nm}) \cdot 10 \cdot m^{-1} \quad (2)$$

Generation of heat maps

To create the heat maps in Figures 3, 5, 6 and 11, a custom Perl script was written that (1) sorted the input data into groups, (2) ordered the groups by the columns of data selected (e.g. fold change) and (3) assigned colors to numerical data either on a logarithmic or non-logarithmic scale in a given range. The resulting groups of data were plotted in a scalable vector graphics image and this file was further edited for presentation using Inkscape version 0.49 (www.inkscape.org).

3. Results

Rice genes specifically regulated by short-term hypoxia treatment

To determine early differences in gene expression at hypoxic conditions in rice, stem sections including the 2nd node and the youngest internode of 12-week-old plants of rice cultivar PG56 (Supplemental Figure 1) were prepared. These were kept for 1 h at normoxic or hypoxic (5% O₂) conditions or hypoxic conditions in the presence of the ethylene perception inhibitor 1-MCP. RNA was extracted from the area of these sections containing the intercalary meristem and the basal part of the elongation zone of the youngest internode, typically 0-1.5 cm above the 2nd node. Previous experiments had shown that this region is highly responsive to hypoxia (Sauter and Kende, 1992). Whole genome array chip analysis was performed with these samples. Three independent experiments were carried out. Microarray data was statistically analyzed as described in Materials and Methods. Genes that were not regulated significantly at 5% O₂ with 1-MCP treatment in comparison to the control were omitted to only accept genes that were hypoxia-regulated irrespective of ethylene signaling (1367 genes; 555 up, 812 down). To simplify analysis, if for one gene different probe sets appeared in the results only the probe set with the strongest regulation was included and the others were ignored (removed 139). The remaining 1228 genes were analyzed bioinformatically (Figure 1A, shaded area). Only 337 genes were regulated by hypoxia only and 20

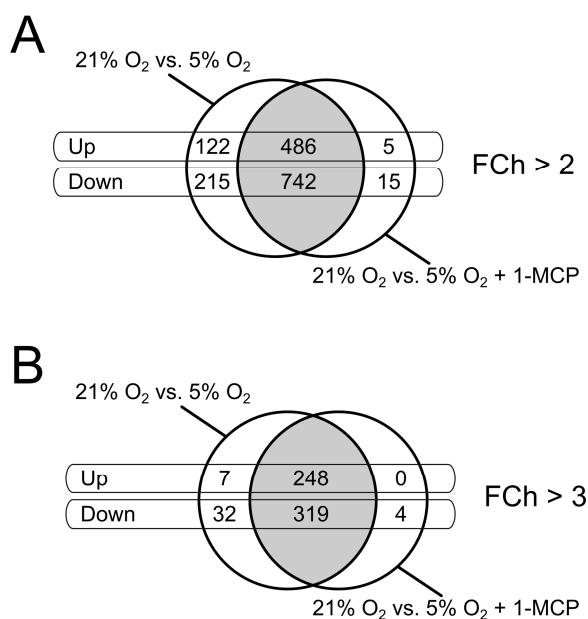


Figure 1: Genes regulated after one hour of hypoxia or hypoxia and 1-MCP treatment, grouped by up- and downregulation and fold change: (A) Fold changes larger than ± 2 , these genes were used for bioinformatical analyses; (B) Fold changes larger than ± 3 , these genes were used for manual detailed analysis, homolog screen and mutant selection.

genes were regulated by hypoxia in the presence of 1-MCP but not by hypoxia alone (Figure 1A, non-shaded areas)

For in-depth manual analysis, only genes with changes of three-fold and higher up- or downregulation were considered (Figure 1B, shaded area; 567: 248 up, 319 down). The remaining genes were manually assigned to categories. To classify the genes in a general way, the detailed categories were in turn assigned to larger meta-categories (Supplemental Table 1) shown in Figure 2.

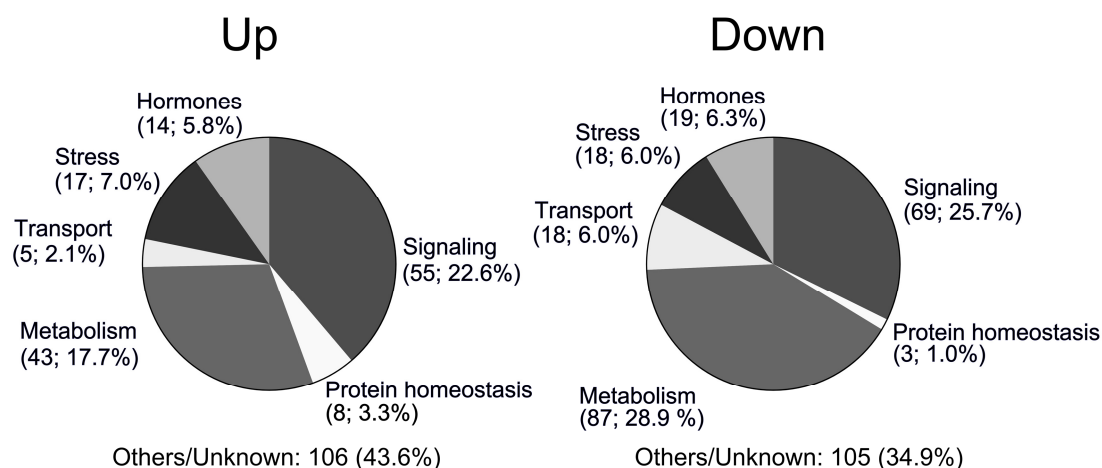


Figure 2: At hypoxia, 567 genes were regulated more than 3-fold irrespective of ethylene perception, separated in up- or downregulated genes and categorized in broad categories. Genes without clear annotation or not belonging to one of the categories listed are given separately at the bottom (211 genes, 37%).

To facilitate analysis, genes belonging to categories expected to be important for the hypoxia response (signaling- and metabolism-related categories) were extracted from the data, grouped in the corresponding categories and plotted in a heat map. Each category was sorted by regulation intensity (Figure 3).

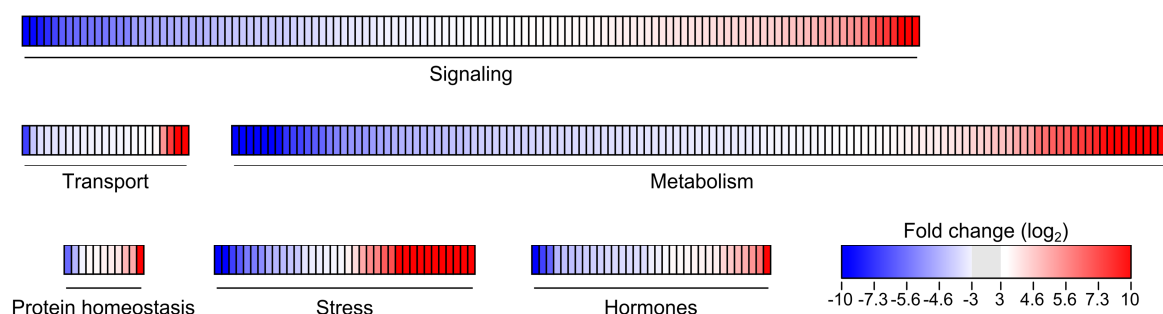


Figure 3: Heat map of unique rice genes regulated more than 3-fold after one hour of hypoxia and irrespective of 1-MCP treatment, grouped into categories (see Supplemental Table 1). Genes belonging to other categories are omitted in this figure. Each of the tiles represents one gene with the fold change visualized by color. Note that the color scheme is logarithmic (\log_2).

The most extreme regulation was found in the 'stress' category. Here, strong upregulation was visible. Metabolism-related genes were for the most part downregulated in expression. For signaling- and hormone-related genes, most genes were not regulated as strongly as in the aforementioned categories (mostly between ± 3 and ± 5).

In silico analysis of the non-redundant set of hypoxia-regulated genes

To verify that the expression pattern observed in the microarray experiment were in accordance with a hypoxia response, the results (see above, fold change stronger than ± 2) were screened for previously described responses typical for hypoxia. The most conserved responses to hypoxia were associated with metabolic adaptations to this stress (Bailey-Serres and Voeselek, 2008; Mustroph et al., 2010). A selection of genes that were found to be regulated in this study associated with hypoxia-induced changes to metabolic regulation is shown in Figure 4. Glycolysis-associated genes coding for phosphofructokinase, aldolase, triose-phosphate isomerase and pyruvate phosphate dikinase enzymes were found to be regulated as well as fermentation-associated genes encoding for lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC) and

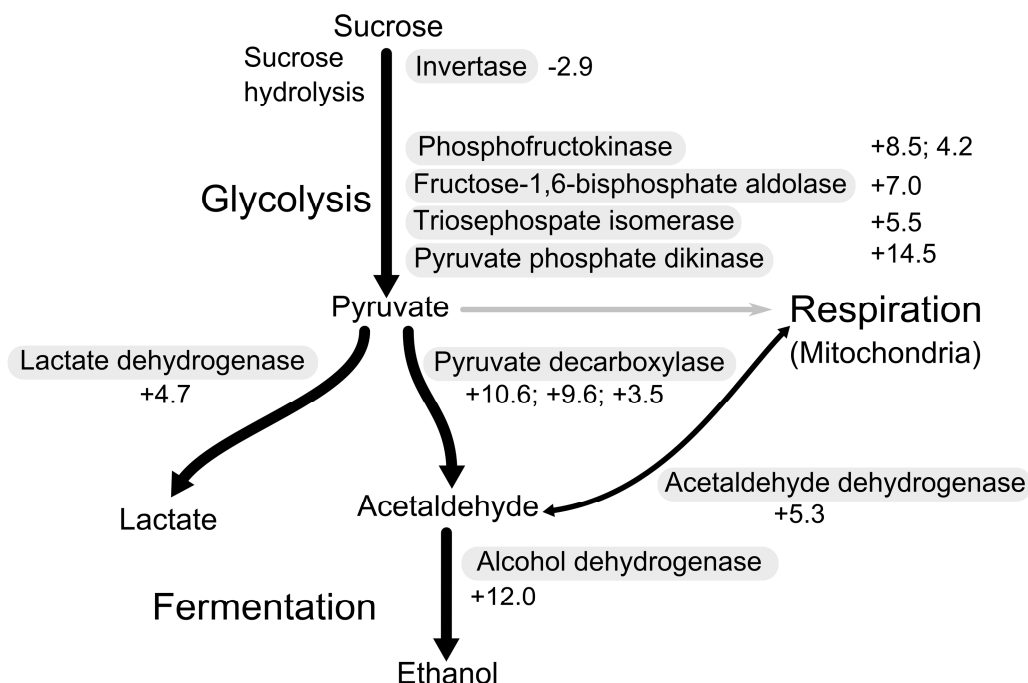


Figure 4: Genes coding for central metabolic pathway enzymes are regulated after 1 h hypoxia in rice. The increase in glycolytic activity and fermentation called Pasteur Effect can be observed on the level of gene expression. For enzymes with more than one gene coding for them, all regulations found are presented in the form of fold changes of the genes annotated to encode the respective enzymes.

alcohol dehydrogenase (ADH).

In addition to these, nitrate reductase (Os08g36480, fold change (FCh) +8.4; for all locus IDs given in this study, the 'LOC_' prefix was omitted for clarity), alanine and aspartate aminotransferases (Os10g25130, +8.9; Os01g55540, +2.4, respectively), glutamate decarboxylase (Os03g13300, +25.4) and α -amylase (Os08g36910, +3.0) were induced. Downregulation was found for genes related to energy-consuming processes which are known to be generally downregulated under hypoxia to save ATP (Bailey-Serres and Voesenek, 2008). Several ATP-dependent transport-associated genes (see also Figure 2), cell division and cell wall maintenance genes, carbohydrate storage genes, for example encoding a starch synthase (Os07g22930, -3.5), and numerous genes involved in the synthesis of secondary metabolism compounds were downregulated. Pyrophosphate is used as an energy source during hypoxia, especially by the alternate pathway of sucrose hydrolysis shown to be favored during hypoxia (Mustroph et al., 2005). Gene expression of pyrophosphatases was reduced (Os05g36260, -5.1; Os05g02310, -3.2; Os06g43660, -2.4), which is therefore also in accord with known reactions to hypoxia.

To get an idea of the type of transcription factors and putative regulatory proteins involved in early hypoxic regulation, the non-redundant set (see above, $-2 < \text{FCh} < +2$) was further bioinformatically analyzed. First, the set was compared to 3039 known transcription factors and hormone-related genes. These genes ($n=147$) were grouped according to hormone pathway and/or gene family (Figure 5).

Hormone-related, other signaling processes

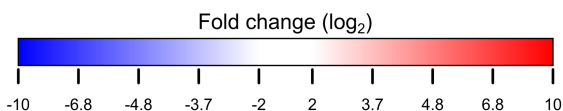
Fold change	Name (if available)	Group or family	Category or family	Superfamily
9.9	<i>OsZIP12</i>	bZIP	ABA related	
2.9	<i>OsZIP62</i>			
-4.6	<i>OsZIP42</i>			
3.2	<i>SAPK9</i>			
-3.2	<i>OsPLDa7</i>			
-4.1	<i>OsPLDa6</i>			
3.2	<i>OsARF15</i>	ARF	Auxin related	
3.3	<i>OsYUCCA7</i>			
3.4	<i>OsIAA25</i>	AUX/IAA		
2.5	<i>OsIAA27</i>			
2.5	<i>OsIAA24</i>			
2.5	<i>OsIAA18</i>			
-2.5	<i>OsIAA18</i>			
2.3		IIIc	ERF	AP2/ERF
-3.2	<i>OsERF27</i>			
-2.2	<i>OsERF36</i>			
-5.3	<i>OsERF37</i>			
-2.4	<i>OsERF40, OsDREB2A</i>			
-4.6	<i>OsERF92</i>			
-7.1	<i>OsERF91</i>			
-6.8	<i>OsERF96</i>			
-4.4	<i>OsERF83</i>			
-10.3	<i>OsERF136</i>			
-2.0	<i>OsERF77</i>			
12.5	<i>OsERF67</i>			
8.8	<i>OsERF60, OsEBP89</i>			
6.9	<i>OsERF68</i>			
4.3	<i>OsERF62</i>			
3.4	<i>OsERF61</i>			
6.7	<i>OsACO7</i>	Synthesis	Ethylene related	
3.0	<i>OsARD1</i>			
-3.5	<i>OsACS2</i>	Signaling		
-2.0	<i>OsRTE2</i>			
-2.9	<i>OsAOS2</i>	P450		
-2.7	<i>OsJAZ2, OsZIM16⁹</i>			
-2.9	<i>OsJAZ4, OsZIM6⁹</i>	ZIM	JA related	
-3.4	<i>OsJAZ5, OsZIM4⁹</i>			
-4.0	<i>OsJAZ9, OsZIM7⁹</i>			
-4.7	<i>OsJAZ7, OsZIM13⁹</i>			
-10.2	<i>OsJAZ6, OsZIM2⁹</i>			
-2.2	<i>OsOPR7</i>			
-2.7	<i>OsAOC</i>			
-3.0	<i>OsDAD1;3</i>			
4.6	<i>OsPCL1</i>	G2-like		
-5.0	<i>OsCCA1, OsMYBR6⁹</i>			
6.5	<i>OsPRR95</i>	MYB-related	Circadian clock associated	
3.4	<i>OsFKF1</i>			
2.0	<i>OsGI</i>			

Additional transcription factors

-2.5	<i>OsABI42</i>	ABI3VP1	
5.6	<i>OsHLH081</i>	bHLH	
4.3	<i>OsHLH080</i>		
4.1	<i>OsHLH111</i>		
3.3	<i>OsHLH035</i>		
2.8	<i>OsHLH40⁹</i>		
2.8	<i>OsHLH077</i>		
2.6	<i>OsHLH153</i>		
2.4	<i>OsHLH044</i>		
-2.3	<i>OsHLH058</i>		
-2.3	<i>OsHLH2⁹</i>		
-2.5	<i>OsHLH088</i>		
-2.7	<i>OsHLH010</i>		
-2.8	<i>OsHLH120</i>		
-2.9	<i>OsHLH127</i>		
-3.3	<i>OsHLH024</i>		
-4.0	<i>OsHLH006</i>		
-6.8	<i>OsHLH020</i>		
-6.9	<i>OsHLH148</i>		
4.8	<i>OsZIP2</i>	E	
2.4	<i>OsZIP71</i>	K	bZIP
2.3	<i>OsZIP22</i>		
-2.9	<i>OsZIP25</i>	V	
2.4	<i>OsCOL9</i>	C2C2-CO-like	
2.4	<i>OsGATA15</i>	C2C2-GATA	
2.2	<i>OsGATA23</i>		
2.4		C2H2	
-2.3			
-2.5			
-8.8			

Additional transcription factors, continued

Fold change	Name (if available)	Group or family
6.7	<i>OsC3H38</i>	C3H
2.3	<i>OsC3H2</i>	
2.1	<i>OsC3H22</i>	
-2.1	<i>OsC3H54</i>	
-2.4		DBP
-2.1	<i>OsE2F7</i>	E2F-DP
2.9		FAR1
3.1		G2-like
2.3		
-2.5		GNAT
3.1	<i>OsGRAS3</i>	GRAS
6.9	<i>OsGRF3</i>	GRF
4.7	<i>OsGRF6</i>	
4.1	<i>OsHSF7</i>	HSF
2.2	<i>OsHSF23</i>	
-2.8	<i>OsHSF24</i>	
3.0	<i>OsHB56</i>	Homeobox
2.4	<i>OsHB78</i>	
2.2	<i>OsHB57</i>	
-2.2	<i>OsHB94</i>	
5.9	<i>OsJMJ12</i>	JUMONJI
2.2	<i>OsJMJ8</i>	
-9.9		LOB
-2.2		MBF1
2.1	<i>OsMYB35</i>	MYB
-2.4	<i>OsMYB71</i>	
-3.7	<i>OsMYB55</i>	
-3.9	<i>OsMYBR17</i>	
-6.4	<i>OsMYB56</i>	
3.1	<i>OsMYBR9</i>	MYB-related
-2.4	<i>OsMYBR4</i>	
-2.8	NAC_Putative	OMNAC
-2.3		
-2.1	NAC67	SNAC
-2.3		
-3.2	<i>NAC48, OsNAC6</i>	
-3.7	<i>NAC68</i>	
-6.4	NAC_Putative	
2.6		OFF
-2.4		
4.5	<i>OsOrphan40</i>	Orphans
3.2	<i>OsOrphan21</i>	
3.0	<i>OsOrphan11</i>	
2.9	<i>OsCOL15</i>	
2.8	<i>OsOrphan11</i>	
2.1		Orphans
2.1		
-2.1		
-2.1	<i>OsOrphan19</i>	
-2.2		Orphans
-2.3	<i>OsOrphan102</i>	
-2.8	<i>OsSBP9</i>	SBP
-4.5	<i>OsSBP2</i>	
5.5		SET
4.4		SRS
2.7		Sigma70-like
2.0	<i>OsTCP10</i>	TCP
-4.3		TRAF
-2.3		TUB
-3.0		Trihelix
-5.9		ULT
-4.3	<i>OsZIM14</i>	ZIM
2.6	<i>OsWRKY012</i>	WRKY
-2.1	<i>OsWRKY001</i>	
-2.6	<i>OsWRKY055</i>	
-2.7	<i>OsWRKY007</i>	
-2.7	<i>OsWRKY053</i>	
-3.3	<i>OsWRKY010</i>	
-4.5	<i>OsWRKY021</i>	
-5.7	<i>OsWRKY067</i>	



(Figure legend on following page)

Figure 5 (*previous page*): Genes regulated more than 2-fold in rice after 1 h at 5% O₂ were grouped in transcription factor families and hormone-related groups. One tile corresponds to one regulated gene (Fold change included). The color scheme has a logarithmic (log₂) scale. Annotations according to GRASSIUS database (Yilmaz et al., 2009) are marked by ^(g).

The transcription factors listed in Figure 5 include 16 AP2/ERF genes. The five differentially regulated ERFs of the group VIIa were strongly upregulated throughout, whereas all other ERFs regulated here were reduced in expression. This is of interest due to the importance of group VII ERFs SNORKEL1, SNORKEL2, and SUB1A for hypoxia responses in rice. No probe sets for the corresponding genes are on the microarray chip used here. Mostly, auxin-, ABA-, ethylene- and jasmonic acid (JA) associated genes were regulated with no tendency for up- or downregulation. Only a fraction of the genes known and annotated for having a role in hormone signaling or homeostasis were found to be regulated here. Of the 53 known and annotated genes related to cytokinins or the 29 annotated genes related to brassinosteroids, none were found to be regulated. Remarkably, all JA related signaling genes that were identified were downregulated. Six of the ten JA-related genes coded for transcription factors of the ZnF protein expressed in inflorescence meristem (ZIM) family, also called TIFY or jasmonate ZIM-domain (JAZ) family. Four genes of the ethylene synthesis pathway were regulated, the ACC oxidase *OsACO7* (FCh +6.7), acireductone dioxygenase *OsARD1* (+3.0) and the ACC synthase *OsACS2* (-3.5). Except for the ERF genes, which are not necessarily involved in ethylene signaling, *REVERSION TO ETHYLENE SENSITIVITY 2* (*OsRTE2*; -2.0) was the only known ethylene signaling gene regulated. Five genes involved in circadian clock regulation were differentially regulated, including core rice clock components *CIRCADIAN CLOCK ASSOCIATED1* (*OsCCA1*; -5.0) and *PSEUDO RESPONSE REGULATOR95* (*OsPRR95*; +8.5). One hundred additional transcription factor genes were regulated with mostly intermediate regulation between fold changes of ± 2 to ± 3 .

Comparison of microarray data of this study with previously published microarray data

For a more detailed analysis, the differentially expressed genes were compared to genes identified in previous microarray studies. When comparing the genes regulated here with results from coleoptiles of anoxia-treated rice seedlings (Lasanthi-Kudahettige et al., 2007), 645 (50.5%) genes regulated more than 2-fold in the internode sections

studied here were also found to be regulated in coleoptiles. Almost all of these 565 genes (46.0%) were regulated in the same direction (up- or downregulation). Otsuka et al. (2010) analyzed hypoxia responses of 8-day-old rice seedlings of the flooding intolerant lowland ssp. *japonica* Nipponbare cultivar after a 30 min treatment in N₂-saturated Kimura's B solution. Of the 226 genes regulated more than 2-fold (downregulated genes were not reported), 226 (18.4%) genes were also found to be regulated in this study. Of these, 195 genes, that are 15.9% of the genes regulated in this study, were regulated in the same direction. When only looking at genes regulated more than 3-fold in our study, 132 (53.2%) were also regulated in seedlings (Otsuka et al., 2010) and all were upregulated. The total number of upregulated genes was higher in seedlings nevertheless. Approximately one third of the differentially expressed genes found in seedlings were also regulated in the internode sections used in this study ($n=221$, 31.3%; 26.9% were upregulated here). When focusing on EF-hand proteins as was done by Otsuka et al. (2010), 4 of the 8 EF-hand family protein genes described were also found to be regulated here (Figure 6). One of the most extensive studies on hypoxia-responsive genes compared 21 organisms and used published microarray data to find conserved hypoxia-responsive genes (Mustroph et al., 2010). When comparing our dataset with the list of

Locus ID	FCh near anoxia	FCh 21% vs. 59% O ₂	FCh 21% vs. 59% O ₂ + 1-MCP	Annotation
Os09g31040	152.3	64.5	63.5	EF hand family protein, putative (<i>OsHREF1</i>)
Os09g31000	148.0	12.7	14.1	EF hand family protein (<i>OsHREF2</i>)
Os09g30506	49.6	3.7	2.9	EF hand family protein (<i>OsHREF3</i>)
Os08g39290	30.7	2.1	1.7	EF hand family protein (<i>OsHREF4</i>)
Os05g31620	12.0	-1.3	-1.1	<i>OsCML15</i> - Calmodulin-related calcium sensor protein
Os09g30490	9.5	1.8	2.0	EF hand family protein (<i>OsHREF5</i>)
Os08g39290 ¹	6.2	1.6	1.5	EF hand family protein (<i>OsHREF4</i>) ¹
Os04g42430	4.0	1.9	1.7	EF hand family protein, putative
Os08g44390	2.6	1.1	1.2	EF hand family protein, putative

Figure 6: Regulation of EF-hand family protein genes as reported previously, (1st column, Otsuka et al., 2010) and found in our experiments under hypoxia or hypoxia with 1-MCP treatment (2nd and 3rd columns). As the microarray probe number was used for comparison, rice *HYPOXIA-RESPONSIVE EF PROTEIN 4* (*OsHREF4*) is represented twice (marked by ¹).

conserved genes regulated in poplar, rice and Arabidopsis (Supplemental Table 9 in Mustroph et al., 2010), 16.3% of genes upregulated and 13.6% of the downregulated genes in this study belonged to the group of conserved regulated genes. In the reverse comparison, the genes differentially regulated after hypoxia here were present to a lower proportion in that study (up: 12.3%; down: 9.5%). This discrepancy may be due to the different durations of hypoxia and anoxia used in the studies compared by Mustroph et al. (2010). It was shown that longer treatments led to a larger number of differentially

expressed genes, which could explain the differences to the results obtained in our study. To summarize, several genes found to be differentially expressed in this study are in general accordance with previously published data on hypoxia responses. Nevertheless, the majority were previously not described in this context.

To see if regulation patterns discovered here might have been described in other studies, the non-redundant genes regulated more than 2-fold were compared to several other whole genome expression experiments not related to oxygen deficiency. When comparing our data with Arabidopsis genes regulated by KIN10 and under sugar starvation stress (Supplemental Table S4 in Baena-González et al., 2007) 65 of 604 genes (10.8%) regulated were homologous to rice genes regulated by hypoxia in this study. The search strategy for homologous Arabidopsis genes is described below. Additionally, 18 of 24 (75%) close rice homologs of Arabidopsis SnRK1 target genes involved in general stress signaling (Table 1 in Baena-González and Sheen, 2008) were differentially regulated in this study. This overlap points to a modification in central energy regulation through SnRK1-responsive genes by hypoxia. Drought stress in this context is of interest because it is an abiotic stress with some similarities to submergence and hypoxia stress (Fukao et al., 2011). When analyzing different rice tissues at drought stress conditions (Wang et al., 2011), one gene commonly upregulated there, *OsZIP12* (Os01g64730) was also found to be regulated in our study (FCh +7.7). When comparing a list of 27 AP2/ERF family genes regulated by drought with genes regulated under hypoxia in this study, 7 genes were found that were also regulated. Not all were regulated in the same direction (see Table 1). Of these genes, five belonged to the group VII of ERF transcription factors, members of which were shown to be important for submergence responses in rice (see Introduction). As noted above, the known hypoxia-related *SNORKEL* and *SUB1A* genes could not be detected as they were not part of the microarray chip used and *SUB1A* additionally is not found in deep water rice varieties.

Table 1: Comparison of AP2/ERF genes regulated by hypoxia found in this study that were also regulated under drought stress conditions in aerial plant organs (Wang et al., 2011).

Name (ERF subgroup ¹)	TIGR locus IDs	Regulation: hypoxia	Regulation: drought
<i>OsERF60, OsEBP89</i> (VIIa)	Os03g08460	9.84	4.06 ^{a,b}
<i>OsERF67</i> (VIIa)	Os07g47790	8.46	5.06 ^{a,b}
<i>OsERF68</i> (VIIa)	Os01g21120	5.52	5.06 ^{a,b}
<i>OsERF62</i> (VIIa)	Os03g08470	4.86	3.68 ^c
<i>OsERF61</i> (VIIa)	Os05g29810	2.65	3.84 ^{a,b,d}
<i>OsERF77</i> (VIIIa)	Os04g52090	-2.18	2.97 ^a
<i>OsERF40, OsDREB2A</i> (IVa)	Os01g07120	-2.38	4.68 ^c

1: according to Nakano et al. (2006). a: FCh presented is induction in leaves at booting stage; b: also induced in tillering stage roots (FCh not shown); c: FCh in panicle leaves at the elongation stage; d: Also induced in booting stage panicles.

Of 298 auxin-regulated rice genes (Jain and Khurana, 2009), 12 (4%) were similarly regulated by hypoxia. This is 1.0% of all hypoxia-regulated genes. There were 32 (2.6%) genes regulated by hypoxia that were regulated in the opposite direction by auxin; that is 10.7% of the auxin-regulated genes. Comparison with a microarray in which excised rice roots and leaves were treated with the cytokinin *trans*-zeatin (tZ) for 30 min (Hirose et al., 2007) revealed that 388 of the genes regulated here (31.6%) were regulated with a fold change larger than 2 in roots after 30 min tZ treatment. Of these, 30 were not regulated in the same direction here as when treated with tZ. Of the genes found to be regulated (FCh>2) in leaves treated with tZ, 250 genes were also regulated here (20.4%) with 21 of these genes varying in the direction of regulation. This represents a total of 20.3% (roots) and 11.3% (leaves) of all genes regulated after 30 min tZ treatment that were also regulated in this study in the intercalary meristem tissue used.

To summarize, it was shown that several genes that are regulated after 1 h of treatment with 5% O₂ were also regulated by treatments with hormones not associated with hypoxia like cytokinin or auxin. Subsets of genes were regulated similarly at drought stress. This indicates a possible overlapping regulation of genes by different external and internal cues, which was also recently confirmed for *SUB1A* (Fukao et al., 2011). In the light of the importance of energy regulation at hypoxia, the high overlap between homologs of genes regulated in this study and targets of the Arabidopsis central energy regulator SnRK1 (Baena-González and Sheen, 2008) has to be noted.

Promoter analysis of regulated genes

Of the 1228 genes found to be regulated more than 2-fold, the sequences 1000 nucleotides upstream of the start codon ATG were retrieved and used to find motifs

overrepresented in the promoters of the differentially regulated genes. An enumerative pattern discovery method algorithm tool (Pavesi et al., 2004) was used to screen the sequences for common motifs in the coregulated genes not expected to be randomly occurring in this set in comparison to the rice whole genome background. This search was performed separately for all genes regulated ($FCh \geq \pm 2$), for genes with FCh stronger than ± 3 , and for both lists separately in up- and downregulated genes. Statistically overrepresented motifs are shown in Figure 7. A motif with the consensus sequence CACGCA was found when scanning all genes regulated, as well as only in genes regulated with stronger than 3-fold up or downregulation and $FCh < -3$ (Figure 7A, G, J). When looking at the distribution in the sets (charts next to sequence logos), this

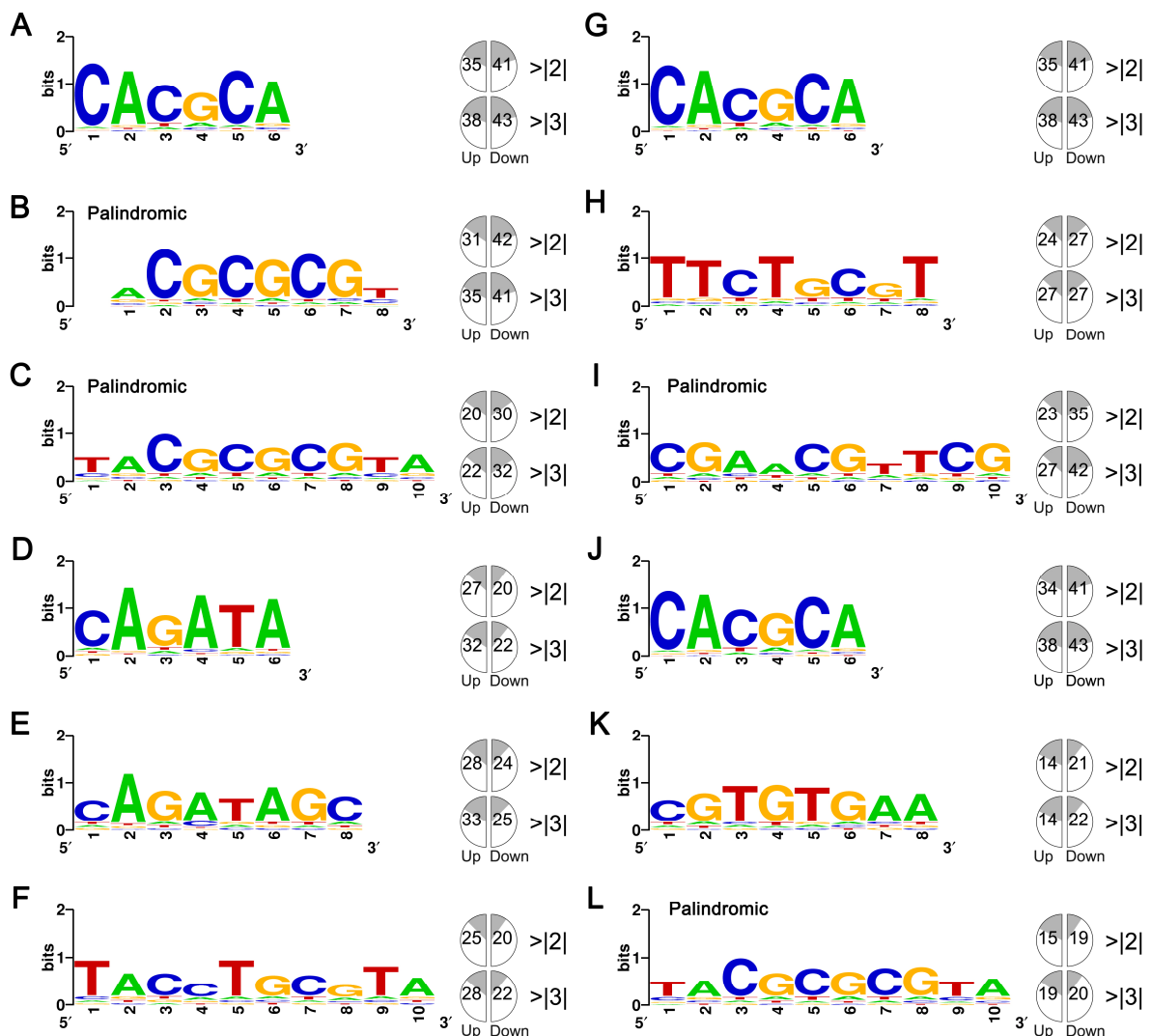


Figure 7: Motifs found to be overrepresented in hypoxia regulated genes. Displayed are motifs found in promoters in the gene with (A-C) FCh stronger than ± 2 , (G-I) FCh strong than ± 3 , (D-F) $FCh > 3$ and (J-L) $FCh < -3$. Motifs with a palindromic consensus sequence are labeled accordingly. Occurrence of the motifs in genes regulated with FCh stronger than ± 2 and in genes regulated stronger than 3-fold are presented in percent as text and shaded area in pie charts, separated for up- and downregulated genes next to the motif logos.

motif was found in more than a third of the genes in all subsets and representation increased to 40% when only considering the genes regulated stronger than 3-fold (e.g. Figure 7A). In the entire genome, only 26% of promoters contained this motif. More promoters of downregulated genes contained this motif than of upregulated genes. This is underlined by the fact that, when searching the >3-fold upregulated genes, this motif was not statistically overrepresented. The palindromic motif ACGCGCGT (Figure 7A), which is in turn a part of the also found motif TACGCGCGTA (Figure 7B) was also found when searching the entire list. The latter was also identified when only searching the promoters of genes downregulated more than 3-fold (Figure 7L). Another palindromic motif with the consensus sequence CGAACGTTTCG was identified in the >|3|-fold regulated genes (up and down; Figure 7I).

Transcription factor binding sites similar to the ones found were determined using STAMP (Mahony and Benos, 2007). The CACGCA motif bears resemblance to two plant transcription factor binding sites: a motif found in *Glycine max* small auxin regulated GRETCHEN HAGEN3 promoter D4 region needed for transcription induction (CACGCAAT, E -value $4.0 \cdot 10^{-9}$) and an Arabidopsis NO APICAL MERISTEM, ATA and CUP-SHAPED COTYLEDON (NAC) type binding site (TAACACGCAT, E -value $2.7 \cdot 10^{-9}$) of NAC factors found to bind to a drought stress and abscisic acid responsive *cis*-element (Tran et al., 2004).

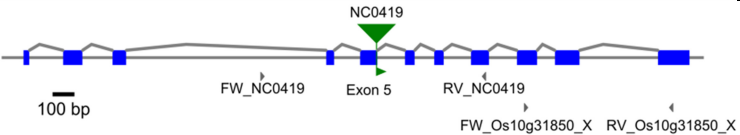
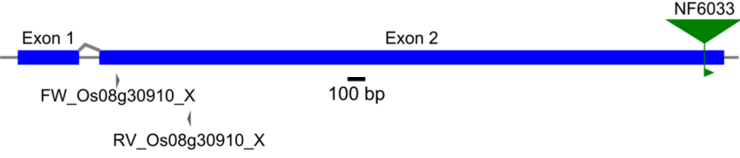
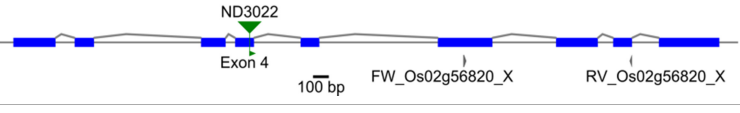
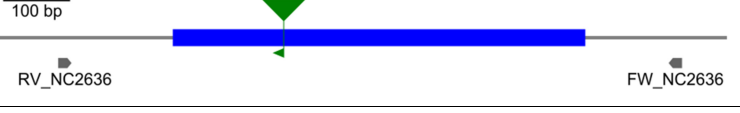
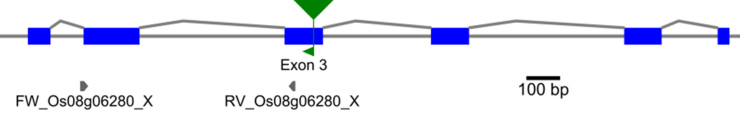
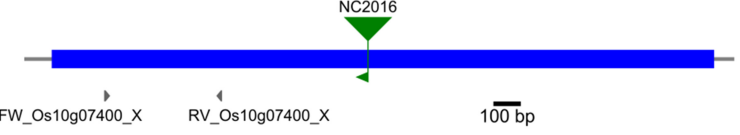
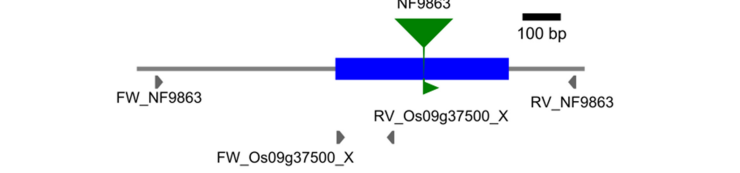
When searching for common motifs in the genes upregulated more than 3-fold, a core CAGATA motif was found, an extended version of which (CAGATAGC; Figure 7E) is related (E -value $6.2 \cdot 10^{-5}$) to an Arabidopsis gene promoter binding site recognized by Arabidopsis *LATE ELONGATED HYPOCOTYL* (*AtLHY*) and *AtCCA1*, two negative regulators of the circadian clock in Arabidopsis containing a SHAQKYF-type Myb DNA-binding domain (Hazen et al., 2005). A MYB1 binding site was found with similar probability (E -value $6.2 \cdot 10^{-5}$). For the TACGCGCGTA motif mentioned above, mammalian zinc finger binding site for Zinc finger E-box-binding homeobox 1 (E -value $1.7 \cdot 10^{-6}$) and tobacco As-1-like sequence binding factor site were described as most similar (E -value $9.5 \cdot 10^{-6}$). When comparing to the AthaMap database at STAMP, however, an E2F transcription factor binding motif (E -value $1.7 \cdot 10^{-3}$) and the same NAC-binding site mentioned above with a lower score (E -value $3.1 \cdot 10^{-3}$) were identified. Although the Weeder tool used here outperformed others, bioinformatical predication of transcription factor binding sites is still a challenge and reliability of results is not very high (Tompa et al., 2005). Therefore results will have to be confirmed by other methods. The fact that cir-

circadian clock gene recognition site like motifs were detected is of interest in the context that the circadian clock has a high impact on energy regulation (Graf et al., 2010) and that energy consumption is a core process modified by hypoxic stress adaptation.

Hypoxia-regulated genes were further analyzed

Of the unique hypoxia-regulated genes that with a fold change larger than ± 3 , 121 genes were selected for more in-depth analysis. Selection criteria were as follows: (1) Fold change: Genes with a fold change of greater than 20 were selected as candidates if they did not belong to a class of proteins already characterized such as generally stress-associated (e.g. nodulins). Also, genes previously identified and described as associated with hypoxia responsive gene expression were omitted (e.g. *OsERF60/OsEBP89*; ADH genes).

Table 2: List of rice *Tos17* transposon insertion mutants. For further information on the gene, consult Supplemental Table 4. For detailed information on primers used for analysis, see Supplemental Table 2.

Shortened annotation Locus ID	Line name (# of insertions)	Insertion position and gene structure (primers shown were used for homozygosity and/or expression evaluation)
<i>CHY ZNF FAMILY</i> Os10g31850	NC0419 (3)	
<i>YDG/SRE DOMAIN CONTAINING</i> Os08g30910	NF6033 (8)	
<i>F-BOX CONTAINING</i> Os02g56820	ND3022 (6)	
<i>OsSAUR53</i> Os09g37480	NC2636 (5)	
<i>LSD1 ZNF LIKE</i> Os08g06280	NC0550 (6)	
<i>DISEASE RESISTANCE RPP13-LIKE</i> Os10g07400	NC2016 (1)	
<i>OsSAUR55</i> Os09g37500	NF9863 (8)	

Genes with fold changes larger than 5-fold that were differentially regulated during hypoxia in the presence of 1-MCP with a difference larger than 25% in comparison to hypoxia only were omitted (11 genes in the pre-filtered list). The resulting 64 up- and 57 downregulated genes are listed in Supplemental Table 4. For these genes, seven rice transposon insertion mutants were identified in the rice *Tos17* insertion mutant database (Hirochika et al., 2004) and ordered (Table 2). More than the ones ordered were not available at the time of search. Four additional mutants were ordered later but could not be analyzed in time.

Rice insertion mutants were screened for homozygosity (Supplemental Figure 3). For the *OsSAUR53* mutant, no homozygous plant could be identified. Expression in

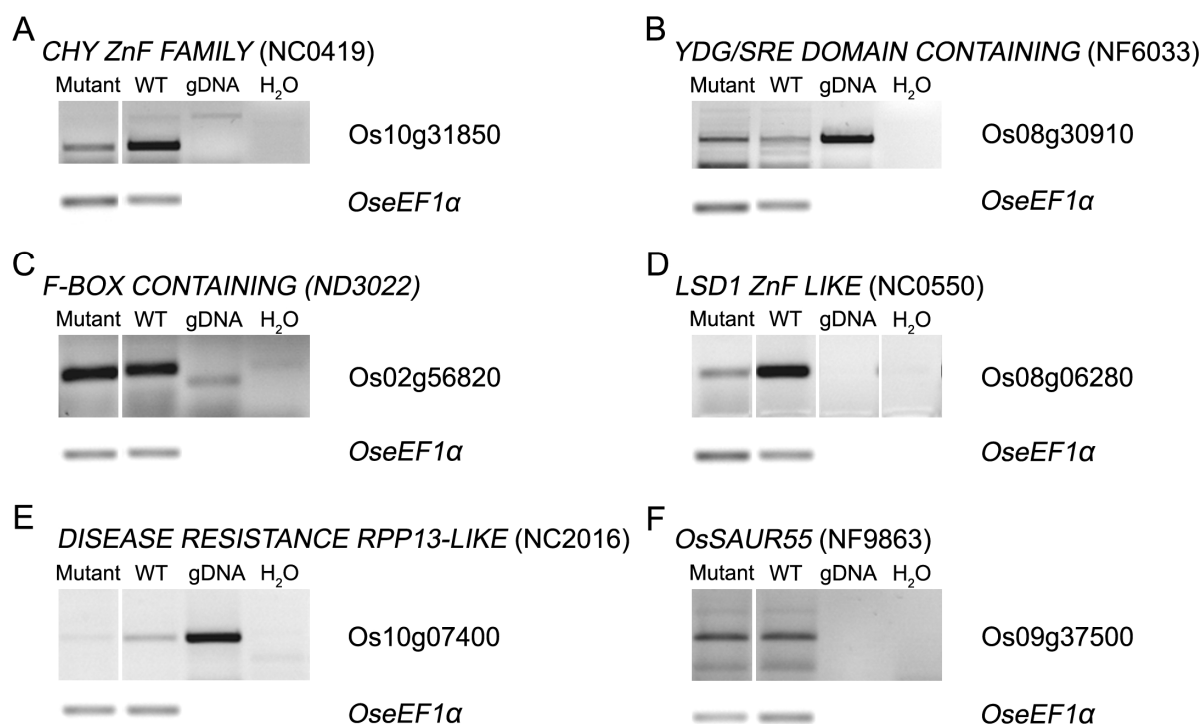


Figure 8: Expression analysis of rice *Tos17* insertion mutants by RT-PCR. After isolation of RNA of mutant and WT plants, 2 μ g were reversely transcribed in a 20 μ L reaction. Of the resulting cDNA 1 μ L corresponding to 100 ng of transcribed RNA was used as template in all PCRs with gene-specific primers (upper panels) and *OseEF1 α* primers as reference (lower panels). All primers and conditions used are listed in Supplemental Table 2. Genomic WT DNA was included to show differing PCR product size for gDNA for some genes to exclude gDNA contamination. This was not possible for the *OseEF1 α* control as primers were not intron-spanning. Therefore, no gDNA lanes were included for *OseEF1 α* . Some lanes were omitted for clarity and these breaks in gels are marked by white lines. Note that for presentation, all photographs were inverted. Reduced expression was observed in (A) the *CHY ZnF FAMILY* mutant, (D) the *LSD1 ZnF LIKE* mutant; and (E) the *DISEASE RESISTANCE RPP13-LIKE* mutant. Expression in the other mutants was unaltered. It must be noted that in the PCR for (A) the *CHY ZnF FAMILY* mutant, residual gDNA was detected. Because the primers were intron-spanning and contamination was low, expression levels were reliably detected nonetheless.

homozygous mutants was tested by RT-PCR (Figure 8). Reduced expression was observed in the CHY ZnF FAMILY mutant, the *DISEASE RESISTANCE RPP13-LIKE* mutant, and the *LSD1-LIKE ZnF DOMAIN* mutant. It is possible that the phenotypes observed are due to effects of genome insertions other than the one in the gene of interest. Additional insertions exist in all mutants except for the *DISEASE RESISTANCE RPP13-LIKE* gene mutant (NC2016). For the *LSD1-LIKE ZnF DOMAIN* (NC0550) and the *OsSAUR55* (NF9863) mutant lines, homozygous plants did not yield any seeds. The latter mutant was seemingly unable to develop any ears whereas the former had developed ears but only blank kernels were produced. To measure the effects of the insertions in the mutants, homozygous 7- to 24-week-old plants were flooded for 3 days and controls were not flooded. Internodal sections as the ones used for microarray analysis were isolated. Protein was extracted from the growth region of the youngest internode. ADH activity was measured (Figure 9). A relative decrease in ADH activity after flooding

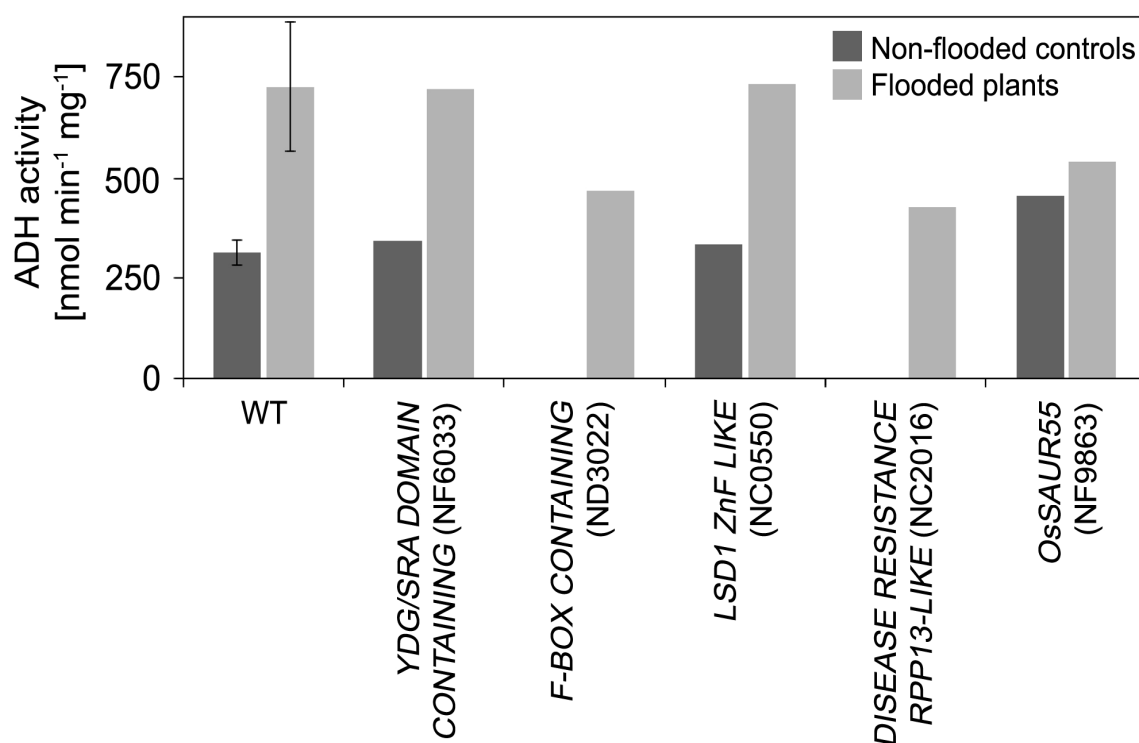


Figure 9: ADH activity in rice *Tos17* retrotransposon insertion mutants. Plants were flooded for 3 days and control plants were not flooded but otherwise kept at the same conditions. Due to limited material, the number of experiments per plant was limited; see Supplemental Table 3B for details.

in the *F-BOX CONTAINING*, *RPP13-LIKE*, and *OsSAUR55* insertion mutant lines in comparison to the WT was observed. The other mutants had similar ADH activities as the WT. Due to a very low tissue yield in the Nipponbare cultivar, which was the background of the mutants, sufficient repeats for statistical analysis could not be made,

some samples could not be obtained, and the changes observed remain to be confirmed before drawing conclusions. In addition to the ADH assays, RNA was extracted from the hypoxia-responsive intercalary meristem and elongation zone tissue of the flooded plants and qPCR was used to determine the expression of *OsADH2* and *OsCML31* as markers of transcriptional adaptation to hypoxia (Figure 10). Both genes were previously described to be typically hypoxia-inducible (Mustroph et al., 2010) and were induced in the microarray presented here. Again, material limitations allowed only 1-2 experiments for each genotype and therefore, the data remains inconclusive. Given that between-experiment variation was high, no convincing differences were found. The strong upregulation of *OsADH2* expression in the *DISEASE RESISTANT RPP13-LIKE* mutant could be an exception but has to be further verified, especially as it contrasts with a reduced ADH activity measured (Figure 9). The downregulation of *OsADH2* visible in the samples of the flooded WT was the result of one outlier sample value of a fold change of -11.1, which explains the large error. When omitting this sample, no significant change in comparison to the non-flooded WT was observed, with a fold change of $+1.2 \pm 2.0$. Such a low or absent induction is in line with recently published regulation of *OsADH2* in flooded Nipponbare cultivar rice (Kudahettige et al., 2011).

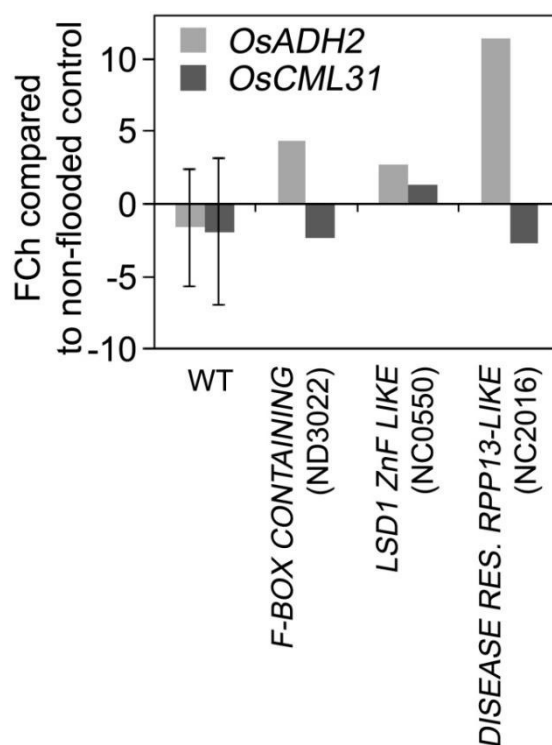


Figure 10: qPCR analysis of *OsADH2* and *OsCML31* expression in WT and selected rice mutants after three days of flooding. Data presented are expression FCh in flooded plants in comparison to non-flooded plants of the respective genotype. The number of experiments was limited; the details are listed in Supplemental Table 3F. Therefore, only error bars for WT are given and represent \pm SD. For primers used, see Supplemental Table 2. The fold changes are listed in Supplemental Table 3B.

3.1. Identification of 284 homologs in the Arabidopsis genome

To be able to analyze the function of the selected genes genetically more easily and, more importantly, to possibly find genes conserved in the hypoxia response in plants, a search for close Arabidopsis homologs of the 121 rice candidates selected was performed using a list of predicted orthologous rice genes created by OrthoMCL (Li et al., 2003) and manual BLAST analyses.

Using the predictions on homology by Li et al. (2003), 163 Arabidopsis genes were classified to be homologous with a maximum of 9 Arabidopsis homologs per rice gene. Manual BLAST search using criteria specified in Materials and Methods like high sequence coverage and high scores confirmed most of these, but there were 121 potentially relevant additional homologs found. The combined searches were assembled as a list of homologous genes. Of the homologs identified, 39 genes were selected for qPCR expression analysis (Table 3).

Table 3: Arabidopsis candidate genes selected for qPCR analysis. The Arabidopsis genome identifier (AGI) is shown with the homologous rice gene TIGR ID, its annotation in the TIGR database and the fold change measured in the microarray.

AGI	TIGR ID	Rice annotation	FCh after 1 h 5% O ₂
At4g17670	Os06g03520.1	DUF581 domain containing protein	223.5
At2g44670			
At5g25940	Os06g04940.1	Early nodulin 93 ENOD93 protein, putative	100.06
At4g10265	Os04g54310.1	Conserved hypothetical protein	61.05
At4g10270			
At3g28480	Os07g09630.1	Oxidoreductase, putative	60.82
At5g18900			
At5g48540	Os08g04210.1	Cysteine-rich repeat secretory protein 55 precursor, putative	21.53
At3g22060			
At5g27760	Os11g02080.1	Expressed protein	9.44
At2g17850	Os04g17660.1	Rhodanese-like domain containing protein, putative	9.09
At2g21045			
At5g24470	Os09g36220.1	Response regulator receiver domain containing protein	8.47
At2g46790			
At5g50915	Os09g33580.1	HLH-DNA-binding domain containing protein	5.62
At1g32230	Os03g63770.1	<i>RCD1</i> , putative	5.35
At2g35510			5.35
At3g58120	Os01g11350.1	bZIP transcription factor domain containing protein	4.84
At3g46640	Os01g74020.1	MYB family transcription factor, putative	4.57
At5g59570			
At4g10150	Os01g11460.1	ZnF, C3HC4 type domain containing protein	4.21
At1g05805	Os04g41229.1	HLH-DNA-binding domain containing protein	4.05
At2g42280			
At5g05280	Os01g60730.1	RING-H2 finger protein, putative	3.93
At3g10910			3.93
At3g61060	Os10g37830.2	OsFBX391 - F-box domain containing protein	3.78
At1g32540	Os08g06280	LSD1 zinc finger domain containing protein	3.43

AGI	TIGR ID	Rice annotation	FCh after 1 h 5% O ₂
At1g29490	Os09g37500.1	<i>OsSAUR55</i> - Auxin-responsive SAUR gene family member	3.29
At1g29460			
At1g75540	Os02g43170.1	B-box zinc finger family protein, putative	3.19
At4g39070			
At1g01060	Os08g06110.1	Myb-like DNA-binding domain, SHAQKYF class family protein / <i>OsCCA1</i>	-4.99
At5g43650	Os03g53020.1	Helix-loop-helix DNA-binding domain containing protein	-6.89
At5g42380	Os01g72530.1	<i>OsCML31</i> - Calmodulin-related calcium sensor protein	-8.14
At1g76640			
At1g76650			
At1g73680	Os12g26290.1	α -DOX2, putative	-19.86
At5g10090	Os02g51730.1	DNAJ homolog subfamily C member 7, putative	-24.89
At5g65160			

Selection was in most cases based on Arabidopsis microarray data previously published on Genevestigator (Hruz et al., 2008). Genes described there to be regulated under hypoxic and/or anoxic conditions were selected. In addition, strong regulation intensity in the rice microarray was also used as a selection criterion (e.g. for At4g17670, At2g44670 and At5g25940). Expression of the selected genes was analyzed by qPCR using RNA isolated from roots as they are very responsive to hypoxic stress in Arabidopsis (Ellis et al., 1999). Of 10-day-old seedlings treated for 1, 2, or 4 h with 5% O₂, most genes were not regulated stronger than ± 2 -fold with some notable exceptions (Figures 11, 12). For the rice gene Os04g54310, annotated as 'conserved hypothetical protein' (up 61.1-fold), two Arabidopsis genes with high sequence similarity were found by BLASTP analysis: At4g10265 and At4g10270, named *WIF1* and *WIF2* in this study for convenience. Coverage was 37% and highest similarities were found to be with the first exon of the rice homolog, where a sequence belonging to the domain of unknown function 3774 (DUF3774) superfamily was detected, also present in the two Arabidopsis homologs. The two Arabidopsis genes were highly similar with 80% identity at the protein level. They are placed adjacent to each other on the q arm of chromosome 4. In contrast to the rice gene that has a predicted size of 215 amino acids, the homologous Arabidopsis genes encode predicted proteins of a length of 90 amino acids without any introns. When comparing only to exon 1 of the rice protein sequence, coverage was 97% and it stretched over 81 (for *WIF2*) and 74 (for *WIF1*) amino acids of the respective predicted Arabidopsis protein sequences. Interestingly, when performing BLAST search on the rice genome using the predicted sequence encoded by At4g10270, the best hit, Os04g54240, was also regulated in the microarray (FCh +3.3). Both Arabidopsis genes were upregulated under hypoxia (Figure 12A, B) after 1, 2 or 4 h of treatment at 5% O₂

in comparison to the control. Upregulation of the two Arabidopsis homologs was detected in all replicates. Statistically significant regulation however was found only for *WIF1* at all times studied and for *WIF2* at 4 h. Other Arabidopsis homologs to upregulated rice genes were downregulated as was the case for the Os01g11350/*AtbZIP61* (At3g58120) pair (Figure 12E). Both genes contain a basic leucine zipper domain (bZIP) but in contrast to upregulation of the rice gene (+4.84-fold) *AtbZIP61* was significantly downregu-

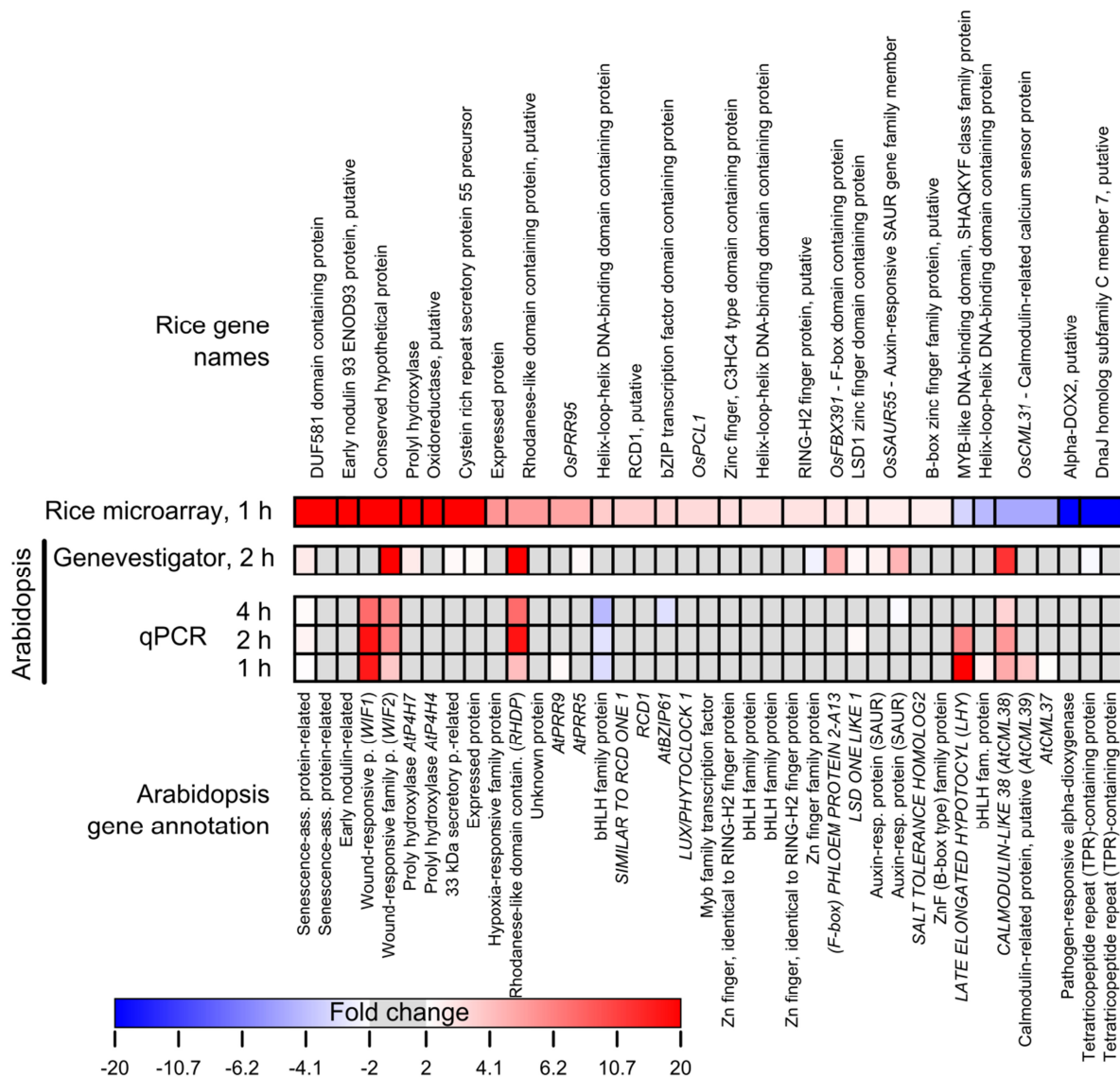


Figure 11: qPCR of candidate Arabidopsis homologs. 10-day-old seedlings of Arabidopsis grown on sterile $\frac{1}{2}$ -strength MS medium at long-day conditions were treated for 1, 2 or 4 h of hypoxia (5% O_2) and expression levels of the genes were determined by qPCR. Regulation stronger than ± 2 -fold is visualized by color in the bottom three lines. For comparison, public microarray data from similar experiments at the center line and the regulation of the rice homologs in the upper line is shown. Note that the color intensity scale is logarithmic (\log_2). Individual qPCR results for Arabidopsis homologs are shown in Figure 12. For statistics, see Supplemental Table 3A.

lated after 4 h of hypoxia. After 1 h of hypoxia, no regulation stronger than 2-fold was detected. The reverse was found for two homologs of the downregulated rice *CALMODULIN-LIKE 31* (*OsCML31*; Os01g72530, -8.1-fold). Three of the homologs were tested for their expression at hypoxia by qPCR, *AtCML37* (At5g42380), *AtCML39* (At1g76640) and *AtCML38* (At1g76650). Of these, *AtCML39* and *AtCML38* were upregulated (Figure 12G, H) after 1 h (although not significantly) and 2 h (*AtCML39*) or after 1, 2, and 4 h of hypoxia (*AtCML38*).

For homologous Arabidopsis genes regulated, T-DNA insertion mutants were ordered when available. In addition to these mutants, mutants for the prolyl-hydroxylase *AtP4H7* (At3g28480) were ordered. This gene is of interest due to a possible role in O₂ sensing similar to the HIF prolyl-hydroxylase in humans. Although regulation was not

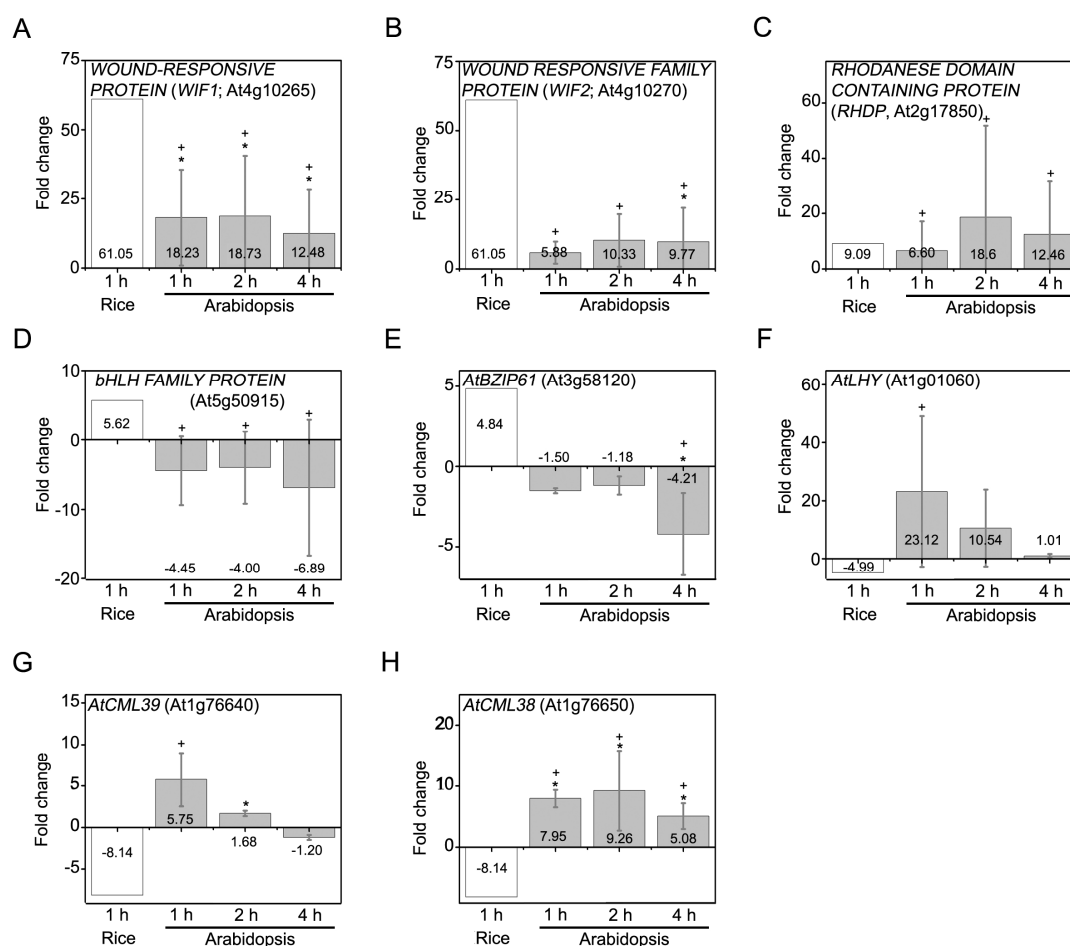


Figure 12: Expression of Arabidopsis homologs regulated by treatment with 5% O₂. Expression was analyzed using 50 ng of RNA isolated from roots of 10-day-old seedlings grown on sterile 1/2-strength MS medium after 1, 2 or 4 h of treatment with 5% hypoxia. The rice microarray results for the respective homologs are presented for comparison (white bars). Results are means of three independent experiments ±SD. Significant differences (Student's two-sample *t*-test, *P* ≤ 0.05) are marked by an asterisk (*). Expression values that were larger than ±2-fold in all three repeats are additionally marked by a cross (+). Details of statistical analysis are given in Supplemental Table 3A.

strong, the Arabidopsis *PSEUDO-RESPONSE REGULATOR 9* (*AtPRR9*; At2g46790) and the *LATE ELONGATED HYPOCOTYL* (*AtLHY*) genes, the latter of which was regulated under hypoxia (Figure 12F) are described to play a central part in circadian clock regulation (Farré et al., 2005; Nakamichi et al., 2010) and were chosen for analysis to test if they play a role in the hypoxia response of Arabidopsis as well, especially when considering the finding of a motif similar to circadian clock gene binding sites and the role of the circadian clock in energy consumption regulation (Graf et al., 2010). A total of 19 mutants for 11 Arabidopsis genes were ordered (Table 4). Mutants were selected for homozygosity.

Table 4: Arabidopsis mutants with T-DNA insertions in genes related to or regulated by hypoxia. In addition to the Arabidopsis homolog, the respective rice gene and its regulation in the rice 1 h hypoxia microarray are given.

Arabidopsis gene Rice homolog locus (Fch in rice microarray)	Mutant line	Insertion position
<i>PROTEIN OF UNKNOWN FUNCTION</i> At4g17670 Os06g03520 (+223.5)	SALK_125677C	Exon 1
<i>WOUND INDUCED FAMILY protein</i> (here termed <i>WIF2</i>) At4g10270 Os04g54310 (+61.05)	<i>wif2-2</i> (SALK_077800C)	Promoter (-200 bp)
	<i>wif2-1</i> (GK127A06)	Exon
	<i>wif2-3</i> (SALK_130465C)	3'-UTR
<i>AtP4H7</i> At3g28480 Os07g09630 (+60.82)	SALK_004428	Exon 1
	SALK_004422	Exon 1
	SALK_004421	Exon 1
<i>RHODANESE-DOMAIN CONTAINING PROTEIN</i> (here: <i>RHDP</i>) At2g17850 Os04g17660 (+9.09)	<i>rhdp-1</i> (SALK_078020C)	5'UTR, promoter
	<i>rhdp-2</i> (SALK_115776C)	Exon 1
<i>AtPRR9</i> At2g46790 Os09g36220 (+8.47)	<i>prp9-1</i> (SALK_007551C)	knock out previously confirmed (Eriksson et al., 2003)
<i>PHLOEM PROTEIN 2-A13</i> At3g61060 Os10g37830 (+3.78)	SALK_101611C	Exon 1
	SALK_127470C	Exon 1
At4g16440 Os03g53750 (-3.01)	SALK_151803(C)	5'UTR, promoter
<i>AtLHY</i> At1g01060 Os08g06110 (-4.99)	SALK_149287	Exon 6
<i>bHLH FAMILY PROTEIN</i> At2g42280 Os04g41229 (-4.05)	SAIL_567_D07	promoter
<i>AtCML39</i> At1g76640 Os01g72530 (-8.14)	<i>cml39-1</i> (SALK_078400C)	5'UTR, promoter
	<i>cml39-2</i> (GK-272A04)	Exon
<i>AtCML38</i> At1g76650 Os01g72530 (-8.14)	SALK_066538C	Exon
	SALK_001571C	5'UTR, promoter

Homozygosity was confirmed in 10 of the mutants (Supplemental Figure 2). The *prp9-1* mutant was previously described (Eriksson et al., 2003) and not retested. Homozygosity confirmation was performed by PCR using primers hybridizing 5' and 3' of the insertion site and a third primer that binds to the inserted T-DNA. Results of these tests are given in Supplemental Figure 2. Homozygosity was confirmed in the 2nd generation (not shown). Homozygosity could not be confirmed in the *At4g17670*, *At4g16440*, *AtLHY*, and *AtCML38* mutants either due to lethality of homozygous plants or errors in mutant annotation. In addition, it was tested whether expression of the genes was reduced in the homozygous mutants. Expression was not detectable in the *WIF2* mutant (Figure 13B) with the insertion in the coding region (GK127A06), from here on called *wif2-1*. The insertion in the promoter of this gene did not alter the expression notably (Figure 13A). In the mutant with the insertion in the 3'-UTR of this gene, from here on called *wif2-3*, expression was slightly reduced (Figure 13C). No change in expression was detected for the only *AtP4H7* mutant available. Expression in both *RHDP* mutants was severely reduced (Figure 13E, F). The *PHLOEM PROTEIN 2-A13* mutant had reduced gene expression (Figure 13G). No expression change was observed for the *BASIC HELIX LOOP HELIX (bHLH) FAMILY PROTEIN* mutant SAIL_567_D07 (Figure 13H). Interestingly, insertion in the promoter of *AtCML39*, in the mutant called from here on *cm139-1*, led to a slight reduction in expression of this gene (Figure 13I), but insertion in the coding region in mutant *cm139-2* did not. Rather, a slight increase in expression was observed (Figure 13J). Although in some of the mutants tested, expression of the gene carrying the insertion was unaltered or only slightly reduced, all were used for further testing. Apart from downregulation, especially for T-DNA insertions in coding sequences, protein expression was abolished in 70 cases, 88% of cases tested (Wang, 2008). This can be true even if transcripts were still detectable or expression was unaltered (Wang, 2008).

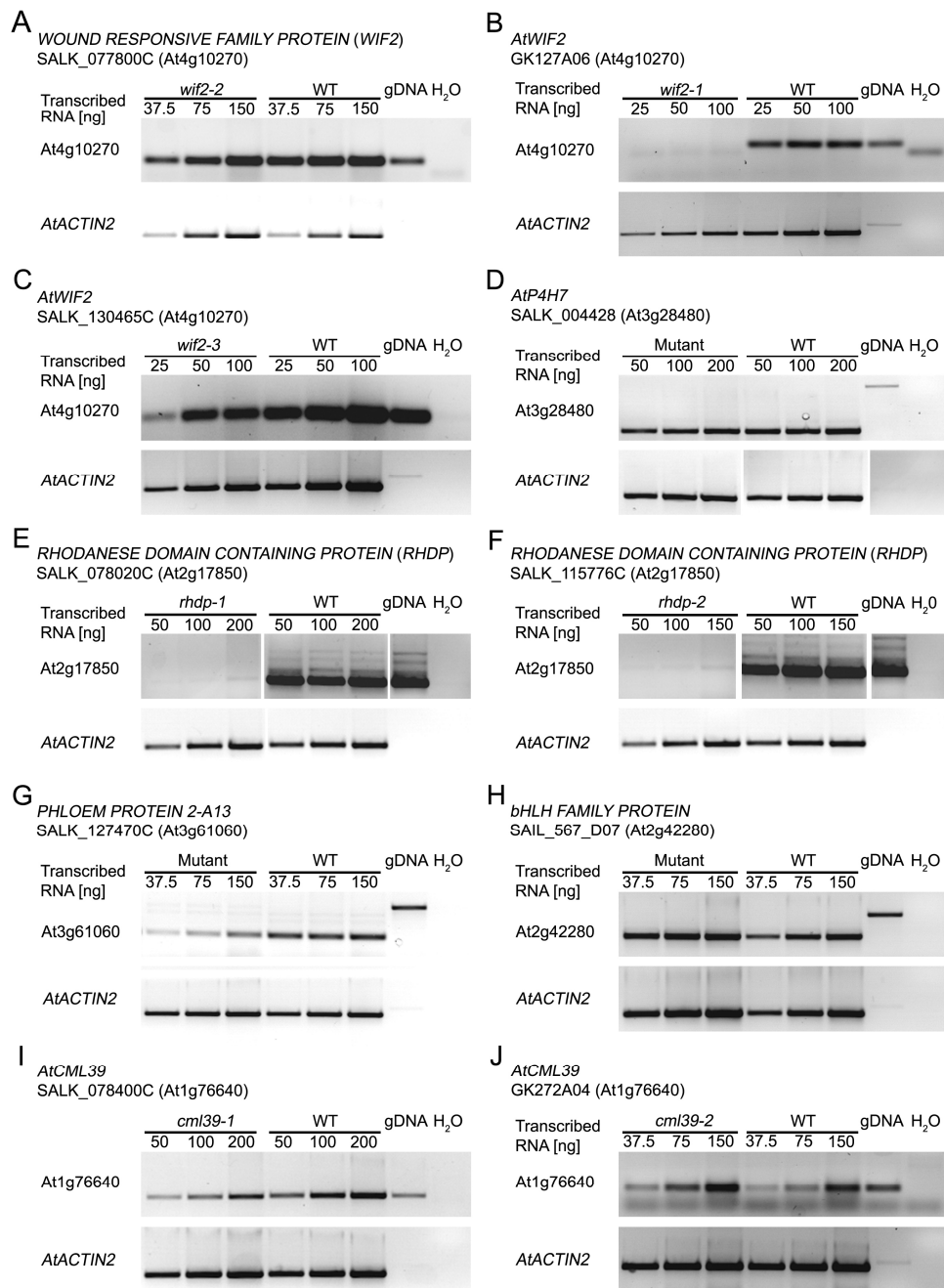


Figure 13: Expression analysis of Arabidopsis T-DNA insertion mutants. RNA of mutant and WT plants was reversely transcribed. The resulting cDNA was used as template in three dilutions in PCRs with gene-specific primers (upper panels) and *AtACTIN2* primers as a reference (lower panels). All primers used are listed in Supplemental Table 2. The amount of transcribed RNA corresponding to the cDNA amount used as template per 20 μ L PCR reaction is given on top of the gel images. Genomic WT DNA (gDNA) was included to show differing PCR product size for gDNA for some genes to exclude gDNA contamination. Some lanes were omitted for clarity and these breaks in gels are marked by white lines. gDNA and H₂O control lanes for *AtACTIN2* in (D) were taken from a separate gel showing PCRs where the same master mix was used. Note that for presentation, all photographs were inverted. Reduced expression was observed in (B) the exon-located *AtWIF2* mutant *wif2-1*, and (E, F) the *RHDP* mutants; slight reduction in (G) the *PHLOEM PROTEIN 2-A13* and (I) the *cml39-1* mutants and no change was observed in (A) the promoter-located *wif2-2*, (C) the *wif2-3*, (D) *AtP4H7*, and (H) the *bHLH FAMILY PROTEIN* mutants. (J) The exon-located *AtCML39* insertion mutant *cml39-2* appeared to have an elevated *AtCML39* expression.

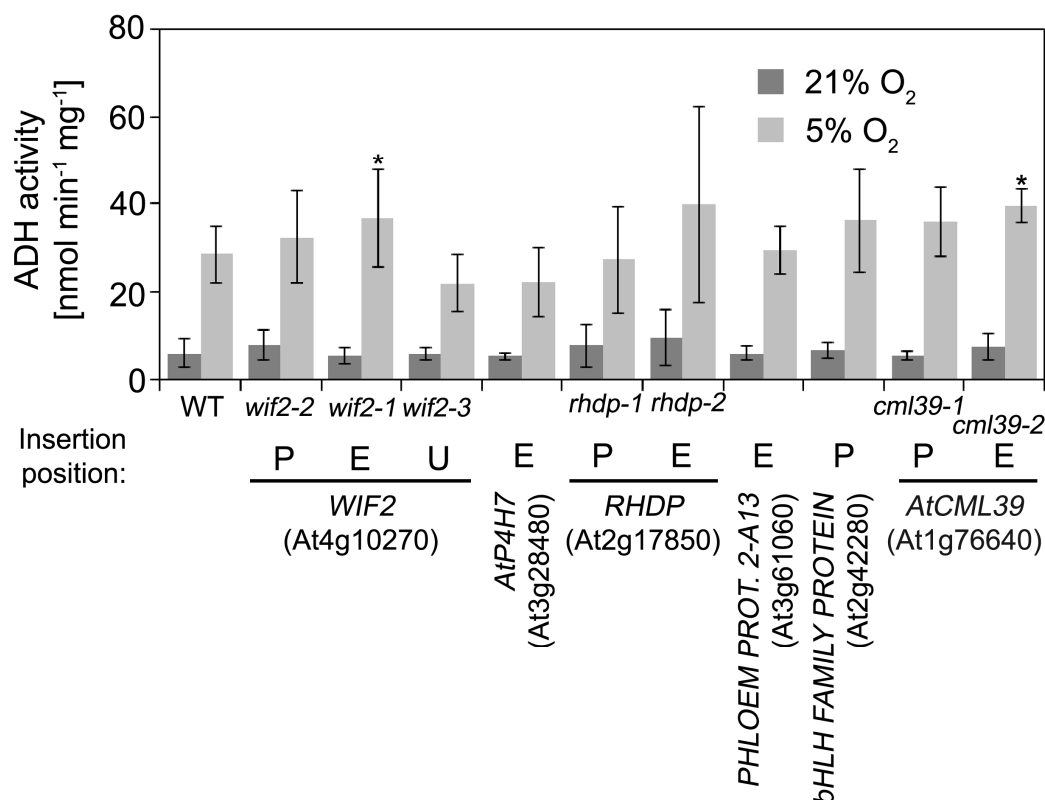


Figure 14: ADH activity in the roots of Arabidopsis T-DNA insertion mutants. 10-day-old seedlings grown at long-day conditions on sterile $\frac{1}{2}$ -strength MS medium were treated for 4 h with 5% O₂. Controls were treated with air and ADH enzyme activity was determined on root protein extracts. Bars are the means \pm SD of $n=3-7$ experiments for mutants and $n=15$ for WT. For each experiment, 15-37 seedlings were used. Statistically significant differences are marked by an asterisk (*) except for the differences between hypoxia and control plants which were significant in all mutants except for the exon insertion *RHDP* mutant *rhdp-2*. See text for details. Mutant insertion positions are marked below the chart with P: insertion in promoter/5'-UTR; E: insertion in exon; U: Insertion in 3'-UTR. For details on statistics and number of plants used, consult Supplemental Table 3E.

To test the response of mutants to hypoxia, several different approaches were used. The Arabidopsis root reacts more intensely to hypoxia than the shoot in some aspects, also with respect to ADH induction (Ellis et al., 1999) which is conceivable, because the root is the first organ to be affected by flooding. Therefore, ADH activity induction after 4 h of 5% O₂ in roots of 10-day-old seedlings of the T-DNA insertions mutants grown in the light on $\frac{1}{2}$ -strength MS was determined for all mutants except *prp9-1* (Figure 14). No significant differences in ADH activity were detected in the mutants in comparison to the WT at normoxia. In all mutants, a significant induction after hypoxia treatment was observed except for the *RHDP* exon insertion mutant *rhdp-2* (SALK_115776C). If one outlier measurement of that sample population was removed, however, hypoxic induction was also significant. ADH activity in hypoxia-treated *wif2-1*

and exon-located insertion mutant of *AtCML39*, *cml39-2*, was significantly higher than in the WT. For the other mutants, no significant difference in ADH activity at hypoxia in comparison to the activity in WT at hypoxia was observed.

To test the Arabidopsis T-DNA insertion mutants for survival of near anoxia, seedlings grown on sterile 1/2-strength MS medium on square plates at long-day conditions were treated with N₂ at near anoxia (below 0.1% O₂) for 48 h in the dark. After a recovery phase of another five days at long-day conditions, survival rates were measured by classifying the seedlings into four categories (Figure 15A): (I) Undamaged seedlings which resumed growing after the end of treatment without any chlorosis. (II) Slight damage: Chlorosis of one cotyledon/leaf or delayed restart of growth or other growth defects or two cotyledons/leaves partially chlorotic. (III) Severe damage: Two or more chlorotic cotyledons/leaves, or one chlorotic cotyledon/leaf and growth defects at the same time or severe growth defects. (IV) Dead seedlings with no green spots visible and no growth observable. Controls were kept in air at otherwise same conditions. In all controls, 100% of seedlings were undamaged (Category I; not shown). Three or more independent experiments were performed for each mutant line available (for detailed statistics, see Supplemental Table 3C). As previously reported (Hinz et al., 2010), the *rap2.2* mutant had a significantly lower survival rate under hypoxia than WT, that is a significantly lower rate of Category I (Figure 15B). A similarly reduced survival rate was observed for the *wif2* mutants (At4g10270). A reduction in Category I for *wif2-2* (SALK_077800C) and an increase in Category IV for *wif2-1* (GK127A06) were observed. A tendency for a reduced number of unharmed seedlings in the exon-positioned *wif2-1* mutant was also observed and the *P* value was close to the significance threshold (Supplemental Table 3C). A similar decline in survival fitness was observed for the mutant of *bHLH FAMILY PROTEIN* (At2g42280) and the mutant with the exon-positioned T-DNA of *AtCML39*, *cml39-2* (GK272A04). For the insertion mutant of *PHLOEM PROTEIN 2-A13* (At3g61060), significantly more seedlings than in the WT survived the treatment without apparent damage. For details on categories II and III, see Supplemental Table 3C. Generally, these other categories confirm the results presented in Figure 15B, with an increased number of Category III seedlings in mutants with a high number of dead seedlings and a higher number of only slightly damaged seedlings (Category II) in mutants with a larger number of undamaged (Category I) seedlings.

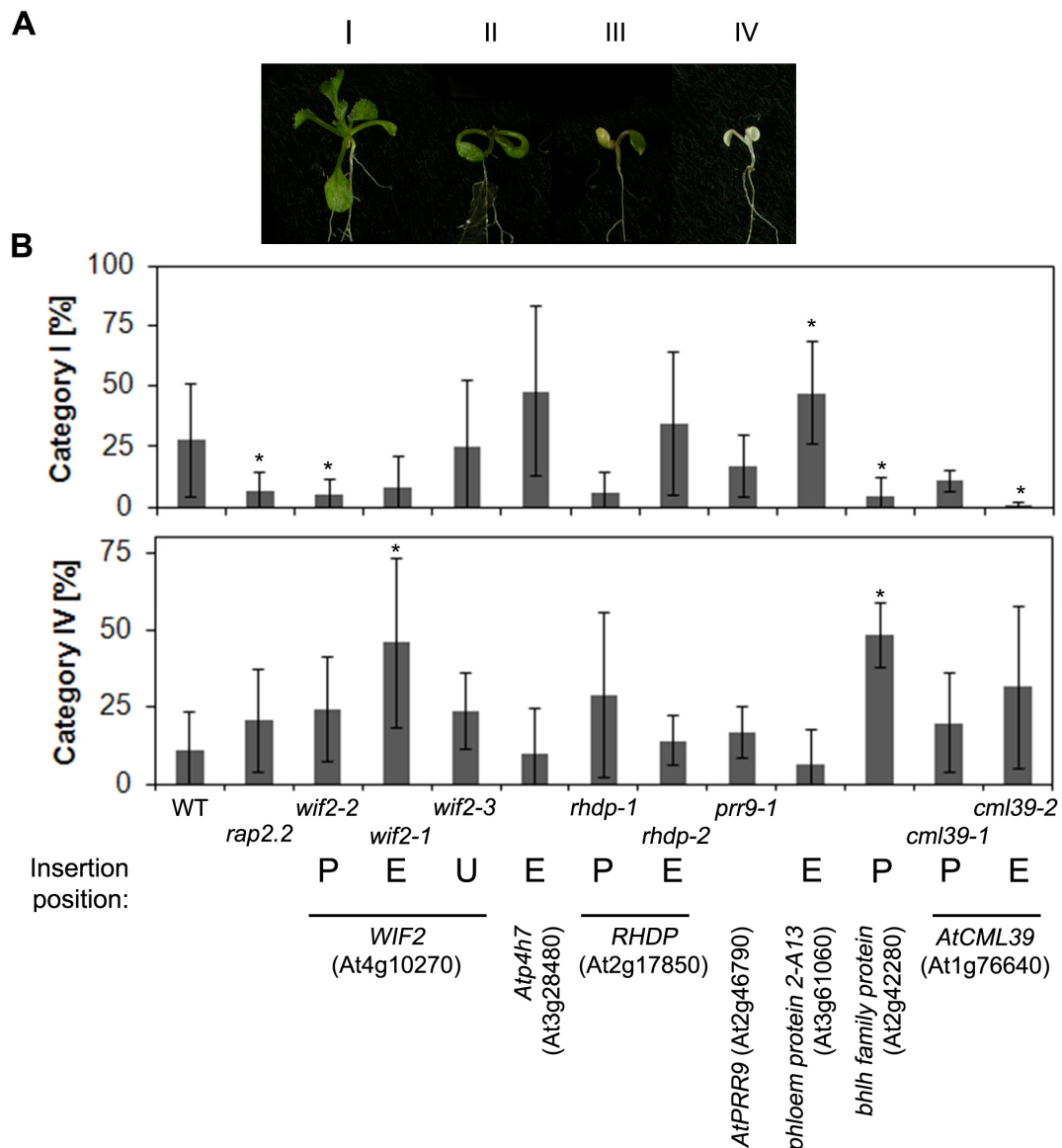


Figure 15: Hypoxia survival assays. Plants grown at long-day conditions on sterile $\frac{1}{2}$ -strength MS medium were incubated for 48 h in the dark at O_2 below 0.1%. Seedlings were analyzed for survival and damage 5 days after reoxygenation and return to long-day conditions. (A) Categories I-IV used for analysis; see text for explanation. (B) Percentage of seedlings that were assigned to Categories I (no damage; upper panel) and IV (dead seedlings; lower panel) after 5 days of recovery. Bars are means \pm SD of $n=4-7$ independent experiments for mutants and $n=25$ for WT. For each experiment, 5-34 seedlings were used. In the WT, about 25% of seedlings were unharmed after hypoxia. This is reduced in the *rap2.2* mutant previously described to have a reduced survival rate (Hinz et al., 2010). The *wif2-2* mutant with the promoter insertion had fewer unharmed seedlings and, in addition, a significantly larger number of dead seedlings were observed in the *wif2-1* mutant with the exon-located T-DNA insertion. The *PHLOEM PROTEIN 2-A13* mutant performed better after the treatment than the WT. The *bHLH FAMILY PROTEIN* mutant tested had a reduced survival rate, similar to *cml39-2*. Other categories are not shown for clarity and due to sometimes ambiguous categorization in intermediate degrees of damage. The T-DNA insertion position is highlighted in the lower part; P: promoter or 5'-UTR; E: Exon; U: 3'-UTR. Significant changes in comparison to the WT (2-sided *t*-test with $P \leq 0.05$) are marked by an asterisk (*). For details on statistical analysis, see Supplemental Table 3C. For a chart with all categories, see Supplemental Figure 4.

To test for the mutants' ability to cope with flooding stress, 16-day-old plants grown on soil were waterlogged for 14 days, aerial organs were harvested and fresh weight as well as chlorophyll and anthocyanin content as an indicator for stress or stress responses (Chalker-Scott, 1999) were measured (Figure 16). Variation was high in these assays and no significant differences in fresh weight, anthocyanin content or chlorophyll content were found. In chlorophyll content measurements, between-experiments variation was low but no differences were observed nonetheless.

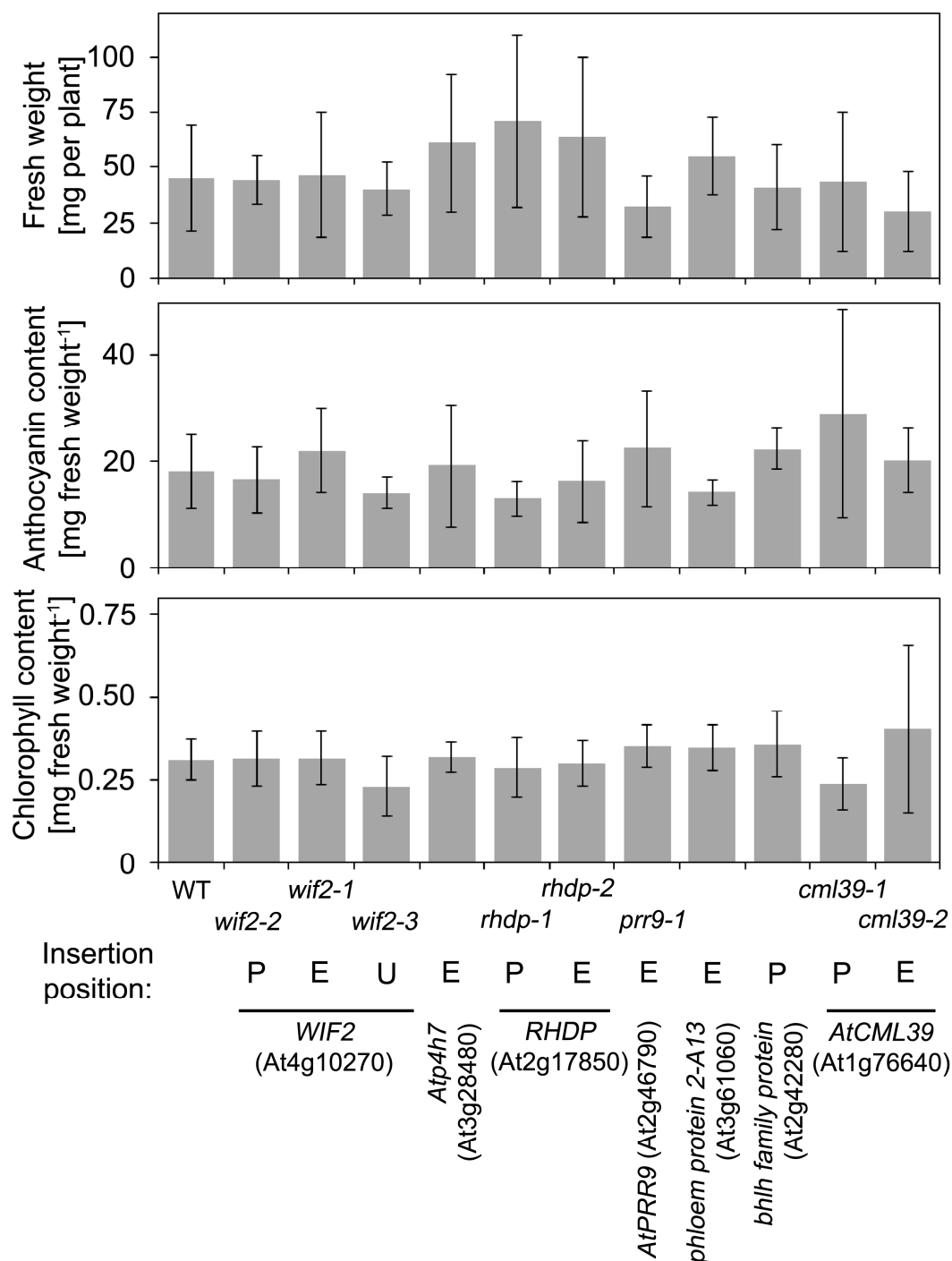


Figure 16: Analysis of waterlogged *Arabidopsis* plants. At the age of 16 days, plants were waterlogged for 14 days and fresh weight, anthocyanin and chlorophyll content per mg fresh weight of plant material was determined afterwards on aerial organs. Position of the T-DNA insertion in the mutants is listed below the charts; P: insertion in promoter/5'-UTR; E: insertion in exon; U: insertion in 3'-UTR. At the bottom, the genes with IDs carrying the insertions or mutant names are given. None of the mutants was significantly altered in any of the assays in comparison to WT. For each mutant line, $n=3-6$ independent experiments were performed, $n=13$ for WT. For each experiment, 2 to 34 plants were used. For details on statistical analysis, see Supplemental Table 3G.

3.2. Transgenic *Arabidopsis* mutants overexpressing hypoxia-related genes

For some T-DNA insertions mutants, altered hypoxia and anoxia resistance was observed and some genes studied above were regulated upon hypoxia (Figure 11). To further analyze the function of these genes and the encoded proteins, overexpression

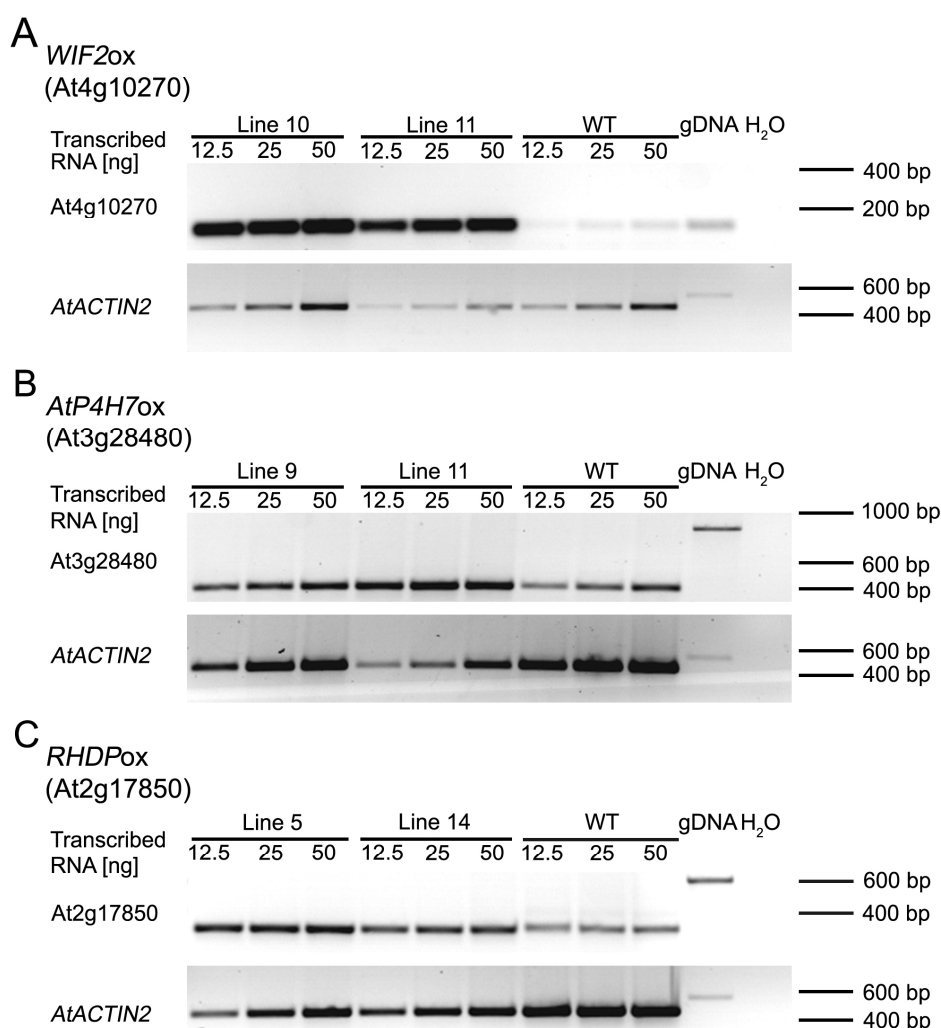


Figure 17: Expression analysis of transgenic *Arabidopsis* plants overexpressing hypoxia-related genes. RNA of mutant and WT plants was reverse transcribed. The resulting cDNA was used as template in three dilutions in PCRs with gene-specific primers named at the left side (upper panels) and *AtACTIN2* primers as reference (lower panels). All primers and conditions used are given in Supplemental Table 2. The amount of transcribed mRNA used as cDNA template per PCR reaction is given on top of the gel images. Genomic WT DNA (gDNA) was included to show differing PCR product size for gDNA for some genes to exclude gDNA contamination. For presentation, all photographs were inverted. For each gene, the two lines used in this study are shown. The expected product sizes are (all in bp): *AtACTIN2*: 427 (cDNA), 513 (gDNA); (A) *WIF2*: 122 (cDNA/gDNA); (B) *AtP4H7*: 389 (cDNA), 1088 (gDNA); (C) *RHDP*: 314 (cDNA), 626 (gDNA). In the (C) *RHDPox* panel, the WT samples were saturated when the *AtACTIN2* primers were used. As the *RHDP*-specific primers produced weaker signals than the WT, overexpression can be assumed nevertheless.

mutants of *Arabidopsis* for *WIF2* (At4g10270), *AtP4H7* (At3g28480) and *RHDP* (At2g17850) were generated and named *WIF2ox*, *AtP4H7ox* and *RHDPox*. In these, expression of the coding sequence (CDS) of the genes was driven by the near-constitutive CaMV 35S promoter. Overexpression in transformed plants was verified by RT-PCR (Figure 17). Survival assays (Figure 18) as well as waterlogging experiments (Figure 19) were carried out as described before for T-DNA insertion mutants. It has to be noted, however, that homozygous plants were not yet available and therefore, a mix of homozygous, heterozygous and WT plants (1:2:1) was analyzed in the following experiments. An exception were the waterlogging experiments, where transgenic plants were selected before flooding by phosphinothricin spraying, removing plants of WT genotype.

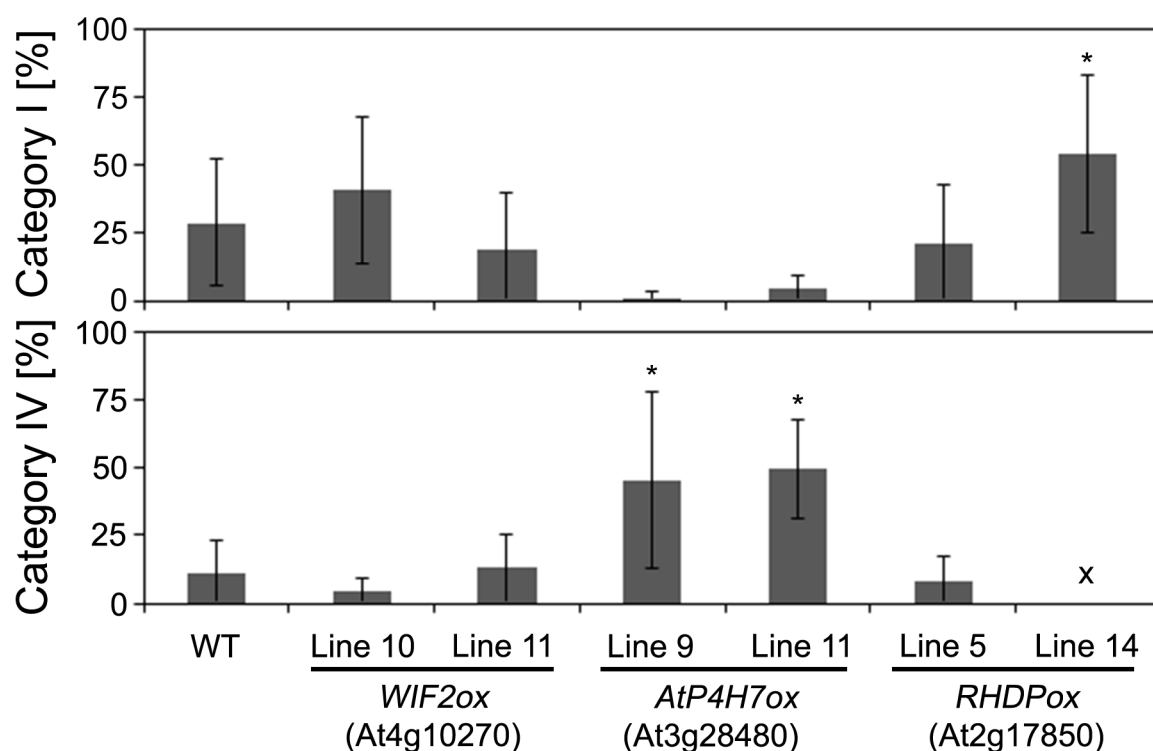


Figure 18: Survival assays with *Arabidopsis* plants overexpressing *WIF2*, *AtP4H7*, and *RHDP*. Seedlings were grown as detailed in Figure 15, treated with near anoxia for 48 h in the dark and were analyzed after a recovery period of 5 days as described in Figure 15. The bars represent means \pm SD of $n=4-7$ experiments for mutants and $n=25$ experiments for WT with 5-34 seedlings used per experiment. Significantly altered percentages in comparison to the WT are marked by an asterisk (*). For *RHDPox*-14, marked by a cross (x), no seedlings were sorted into the Category IV (dead seedlings) and therefore, no statistical analysis could be performed. Seedlings from both *AtP4Hox* lines analyzed had a reduced survival rate; significantly more dead seedlings were counted than in the WT. The *RHDPox* line 14 had a significantly larger number of seedlings that were unharmed than the WT (Category I). For details on statistical data see Supplemental Table 3D. For all categories, see Supplemental Figure 5.

Plants overexpressing *WIF2* were not significantly altered in their ability to survive a low oxygen treatment of 48 h in the dark (Figure 18). Overexpression lines of *AtP4H7*, in contrast, were altered in their survival capacity in the sense that a larger number of dead seedlings were observed after the treatment. Also, a tendency to fewer unharmed seedlings was observed but was not statistically significant. See Supplemental Figure 5 for all categories analyzed. The *RHDPox* line 14 had significantly higher number of seedlings than WT that were unharmed. In addition, no dead seedlings were found in any of the treatments of this mutant. This prevented statistical analysis of Category IV for this mutant. In addition, the seeds of this line had a very low germination rate and possibly, this could have led to non-representative data.

When analyzing waterlogged plants, as for the T-DNA insertion mutants, no significant changes were found in the relative anthocyanin and chlorophyll content of the stressed overexpressing plants (Figure 19). *WIF2ox* and *RHDPox* mutants had a reduced fresh weight in comparison to the waterlogged WT plants (Figure 19A). For

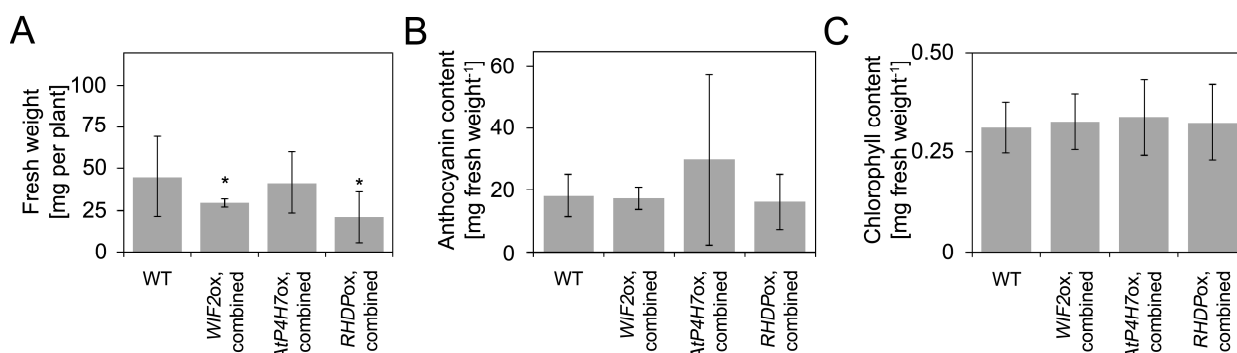


Figure 19: Analysis of waterlogged *Arabidopsis* plants overexpressing *WIF2*, *AtP4H7*, and *RHDP*. At the age of 16 days, plants were waterlogged for 14 days and fresh weight, anthocyanin and chlorophyll content per mg fresh weight of plant material was determined afterwards on aerial organs. Bars represent means of \pm SD 3-8 independent experiments for the mutants and 11-18 experiments for WT. For each experiment, 3-25 plants were used. At the bottom, the names of the overexpression lines are given. Data for individual lines were combined to allow for statistical analysis, details of which are listed in Supplemental Table 3G. *WIF2ox* and *RHDPox* plants had a significantly reduced fresh weight per plant after the treatment. No other significant changes were observed.

RHDPox line 14 seeds, a reduced germination rate was observed that was not found in the *RHDPox*-5 seeds although overexpression was similar if not higher in line 5 (Figure 17C). Possibly, this was due to the seed batch, perhaps through reduced reserve accumulation during seed formation and not necessarily an effect of the overexpression. To summarize, overexpressing *WIF2* led to a reduced fresh weight in waterlogged adult plants but no changes were observed in survival fitness after anoxic treatment of seed-

lings. Overexpression of the prolyl hydroxylase *AtP4H7* resulted in a larger number of dead seedlings after anoxia stress and a tendency fewer unharmed seedlings but did not alter growth during waterlogging conditions. Overexpression of the rhodanese-domain containing *RHDP* gene resulted in better survival after anoxia treatment in one of the lines but reduced fresh weight per plant after waterlogging for 14 days when combining the two lines tested.

4. Discussion

The intercalary meristem and elongation zone of the youngest internodes of deepwater rice plants are suitable tissues to study adaptations of a semi-aquatic plant to flooding and hypoxia as they rapidly respond to the stress by growth through increased cell division and elongation (Sauter and Kende, 1992; Kende et al., 1998). Seedlings are also being studied but adaptations are different from the ones in adult plants as seedlings can survive and grow at anoxic conditions (e.g. Carystinos et al., 1995; Lasanthi-Kudahettige et al., 2007; Otsuka et al., 2010).

To my knowledge, whole genome expression analysis of the hypoxia-responsive growth region of rice internodes has not been studied to date at early time points during hypoxic stress. We therefore performed microarray analyses using tissue of the hypoxia-responsive intercalary meristem and the basal part of the elongation zone of the youngest internodes of deepwater rice plants with the aim to identify early hypoxia-regulated genes. By this approach, we aimed to find genes involved in regulating the hypoxic response and involved in signaling in the pathways important for the response. Differential expression of 1228 genes with a fold change larger than 2 was observed irrespective of ethylene perception inhibition by pre-treatment with 1-methylcyclopropene (1-MCP). For 26517 probe sets, no present calls were detected. A probe set is a mix of probes spotted on the microarray chip to test for a specific gene or sequence. Although most of probe sets not detected will likely correspond to genes not expressed in the tissue at the time point studied, it is conceivable that some information was lost due to low expression or other reasons for failed detection. Additionally, the GeneChip used contained mostly probes for *ssp. japonica* genes and the rice variety used was of the *indica* subspecies. When comparing the set of regulated genes found in hypoxia-related studies published previously, a significant overlap in regulation was observed. Genes known to be induced in their expression by low O₂ were found in all of these studies. The previously described switch of metabolism to a stronger emphasis on

pyrophosphate consumption (Carystinos et al., 1995; Mustroph et al., 2005; Huang et al., 2008) as well as the induction of genes coding for the fermentation enzymes lactate dehydrogenase (LDH), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are examples of known hypoxia-induced responses (Peng et al., 2001; Klok et al., 2002) that were observed here. We were thereby able to show that after 1 h at 5% O₂, hypoxia responses described previously were induced. When focusing on regulation and signaling, the first step was to identify genes possibly involved therein. Transcription factors and genes encoding for DNA binding domains as well as genes coding for kinases and receptor-like domains were selected and categorized. Due to the host of available genome data on rice, several genes were classified according to their previously described or automatically annotated role in hormone signaling or homeostasis.

The importance of ethylene for the response of deepwater rice to flooding has been described (Métraux and Kende, 1983; Van Der Straeten et al., 2001) and the observed induction of enhanced ethylene synthesis was attributed to feedback regulation by the hormone itself due to accumulation during flooding (Vriezen et al., 2003). Acireductone dioxygenase *OsARD1* is involved in the recycling of the ethylene precursor *S*-adenosyl methionine and rapid induction of its expression by submergence has been shown previously (Sauter et al., 2005). Here, expression levels of *OsARD1* and a gene encoding the final ethylene biosynthesis enzyme aminocyclopropane-1-carboxylic acid (ACC) oxidase, *OsACO7*, were induced after 1 h at 5% O₂ +3.0-fold and +6.7-fold, respectively. Expression of a gene coding for ACC synthase, which produces the final ethylene precursor ACC, *OsACS2*, was reduced -3.5-fold irrespective of 1-MCP treatment and therefore, in the absence of ethylene perception. This extends the induction mechanism described above to a direct induction of ethylene biosynthesis by hypoxia alone. Ethylene accumulation during the experiment is unlikely as the volume used for treatment was large enough to allow for diffusion away from the plant. This, and more significantly, the fact that the regulations were observed irrespective of treatment with the ethylene perception inhibitor 1-MCP suggests that the regulation of ethylene biosynthesis genes, seen here by the upregulation of *OsACO7* and *OsARD1* is hypoxia- and not ethylene-regulated. The downregulation of *OsACS2* contradicts this hypothesis. However, five other genes coding for ACS are present in the rice genome (Rzewuski and Sauter, 2008), of which only one was also detected on the microarray chip and was not regulated (*OsACS6*). The others were either not detected (*OsACS1*, *OsACS3*, *OsACS5*) or were not included in the GeneChip used (*OsACS4*). Apart from changes in

the expression of genes coding for ethylene synthesis components, a putative ethylene insensitivity enhancer, *REVERSION-TO-ETHYLENE-SENSITIVITY-2* (*OsRTE2*; Rzewuski and Sauter, 2008) was downregulated. The close homolog *OsRTE1* was shown to complement Arabidopsis *rte1-2*. *AtRTE1* interacts with the Arabidopsis ethylene receptor *ETR1* and influences it positively (Rzewuski and Sauter, 2008). The downregulation points to enhanced ethylene signaling. All of the ERF genes that were upregulated can be sorted into group VIIa according to Nakano et al. (2006) or B group (Sharoni et al., 2011). This group contains multiple members that were previously identified as being important for hypoxia responses like *SNORKEL1* (*SK1*), *SK2* (Hattori et al., 2009) and the *SUB1* genes (Fukao et al., 2006) in rice and *AtERF73* and *AtRAP2.2* in Arabidopsis (Hinz et al., 2010; Licausi et al., 2010; Hess et al., 2011). The role of the rice ERFs regulated here is not known. It cannot be excluded that the probes for these ERFs on the microarray chip cross-detect *SUB1* genes. There are no dedicated probe sets for *SK1* or *SK2* on the Affymetrix GeneChip as it contains mostly *ssp. japonica* genes. All other ERF genes identified to be regulated belonged to groups III, IV, VIII and IX according to Nakano et al. (2006) and were all downregulated. All of these were previously described as being regulated under different other abiotic stresses (Sharoni et al., 2011). It can be hypothesized that the regulatory balance between different ERFs is likely to be a means to achieve the correct stress response depending on sensory and other inputs. It is not known if the differentially expressed ERFs are positive or negative transcriptional regulators. Direction of differential regulation of expression does not imply the direction of a regulatory effect on target genes of these ERFs. It can be concluded that, considering the aforementioned importance of the group VII ERFs, it would be valuable to study the ERFs regulated here in more detail in the future.

Several genes involved in auxin and jasmonic acid (JA)-mediated regulation were downregulated in their expression under low O₂. Several of the genes coding for jasmonate ZIM domain (JAZ) proteins were previously shown to be induced by wounding and JA and repressed by abscisic acid treatment (Ye et al., 2009). As JAZ proteins are thought to be transcriptional repressors of the JA response (Fonseca et al., 2009), this could point to a downregulation of JA-responsive genes at hypoxia in the tissue studied. In contrast to that, several genes that were originally annotated as wound-induced were upregulated here including the genes of the Arabidopsis homologs termed *WIF1* and *WIF2* which were studied here and are discussed later. Additionally, JA biosynthesis is likely reduced as can be determined from the reduced expression of

OsOPR7 coding for the 12-oxophytodienoate reductase important in JA biosynthesis (Tani et al., 2008), of the allene oxide cyclase gene *OsAOC*, and the allene oxide synthase *OsAOS2*. This could point to hypoxic stress activating a response involving specific JA-responsive genes without an involvement of the hormone itself. In the light of the importance of ethylene signaling at hypoxic conditions, the recent finding that JAZ proteins can suppress ethylene-induced ETHYLENE INSENSITIVE 3/ ETHYLENE INSENSITIVE 3-LIKE 1 signaling in Arabidopsis (Zhu et al., 2011) is of interest. Reduction of JAZ proteins as found here would thereby enhance ethylene signaling further.

Although 3.6% of the genes regulated here were also regulated in a whole-genome microarray after auxin treatment (Jain and Khurana, 2009), this overlap is small. In addition, 5 genes were found that encode for proteins with a potential role in auxin metabolism and signaling. The 3.3-fold upregulated *OsYUCCA7* is involved in the tryptophan-dependent auxin biosynthesis pathway (Yamamoto et al., 2007) whereas the role of three upregulated and one downregulated rice auxin and indole-3-acetic acid responsive *AUX/IAA* (*OsIAAs*) genes and of rice *AUXIN RESPONSE FACTOR 15* (*OsARF15*) was not yet studied in detail. IAA genes are for the most part repressors interacting with ARFs which are transcriptional activators in most cases (Vanneste and Friml, 2009). It was shown that expression of *OsIAA27* is reduced but expressions of *OsIAA24*, *OsIAA25*, and *OsIAA18* are induced by the natural auxin indole-3-acetic acid (Song et al., 2009). This does not match the regulation observed here, where *OsIAA18* was downregulated, and the other three *OsIAAs* were upregulated. A hormonal inducer of expression which has been shown to elicit a regulation of *OsIAA24*, *OsIAA25* and *OsIAA27* similar to the one observed in this study is gibberellic acid (GA; Song et al., 2009) which is in accord with the regulation of hypoxic and flooding responses in deep-water rice by this hormone (Sauter and Kende, 1992). Although a role for auxin has been observed previously in the induction of adventitious roots in different species including rice (Wample and Reid, 1979; Visser et al., 1995; Xu et al., 2005) and it was discussed to mediate reorientation of cellulose microfibrils (Kende et al., 1998), it does not induce the specific growth response to hypoxia in internodes (Lorbiecke and Sauter, 1999). The induction of typically auxin-responsive *AUX/IAA* and *ARF* genes could be explained by a common regulation of genes by different pathways, in this case, by GA-mediated regulation.

Three group A bZIP transcription factors which were differentially regulated by low O₂ have previously been proposed to be ABA-responsive element (ABRE) binding

proteins. They were shown to be induced after ABA and ACC treatment except for *OsZIP42* (Lu et al., 2009), which was downregulated here. ABA induction is unlikely as ABA concentration was shown to be reduced at hypoxic conditions (Hoffmann-Benning and Kende, 1992). This would mean that these genes are more likely to be induced by a hypoxia-dependent regulation pathway which would also be inducible by ACC and ethylene. This is further underlined by a contrasting finding that *OsZIP42* and *OsZIP62*, also named *OsABF7* and *OsABF8*, respectively, are indeed *not* ABA regulated (Shobbar et al., 2008). The upregulated *OSMOTIC STRESS/ABA-ACTIVATED PROTEIN KINASE9* (*SAPK9*) is a sucrose nonfermenting1-related protein kinase 2 (SnRK2) family gene previously shown to be induced by hyperosmotic stress and ABA (Kobayashi et al., 2004). It was discussed as not necessarily regulated by ABA itself but rather being involved in retrograde signaling from mitochondria (Fujii et al., 2007). It can be hypothesized that *SAPK9* is regulated by multiple signals and that ABA regulation in the context of hypoxia responses is unlikely but regulation of genes with ABRE-containing promoters by *SAPK9* (Kobayashi et al., 2005) might be important for the hypoxic response in rice. Downregulation of two genes coding for phospholipase D (*OsPLD α 6* and *OsPLD α 7*) correspond well with reduced ABA levels in similar internode sections of submerged or ethylene-treated rice as were used here (Hoffmann-Benning and Kende, 1992), because they are thought to have an enhancing effect on ABA signaling (Li et al., 2007). In Arabidopsis, PLD α 1-derived phosphatidic acid is proposed to inhibit negative regulation of ABA responses by inhibiting ABA repressor ABI1 (Zhang et al., 2004). If a similar mechanism is in place in rice this reduction in PLD α expression could even increase the ABA quenching effect by reduced ABA-responsiveness under hypoxia in addition to reduced levels of the phytohormone (Hoffmann-Benning and Kende, 1992).

When comparing with microarrays of rice roots and leaves treated with cytokinin (Hirose et al., 2007), 32% of genes regulated here were also regulated after cytokinin treatment. This is of interest when considering the role of cytokinin in cell division (Mok and Mok, 2001). However, previous findings showed that cytokinin does not induce typical responses of submergence like internode elongation in rice (Azuma et al., 1990; Lorbiecke and Sauter, 1999). In preliminary tests, transcripts of key hypoxia-induced genes like ADH were not modified by cytokinin treatment (data not shown). It is unlikely that this was an artifact due to submergence during the treatment with cytokinin by Hirose et al. (2007). Similar regulation of a group of genes at hypoxia and after cytokinin

treatment could result from the induced cell division at hypoxia (Sauter and Kende, 1992). As cytokinin has a role in cell division (Mok and Mok, 2001), similar genes could be regulated during hypoxic stress as during cytokinin treatment, both inducing cell division.

Several of the genes downregulated under hypoxia have been found to be induced by drought or ABA (Hruz et al., 2008). This suggests similarities between drought and flooding responses as a similar set of genes is modified. This was also shown for some genes regulated by the ERF transcription factor SUB1A that have a role in dehydration during drought as well as during desubmergence (Fukao et al., 2011).

To summarize, regulation of some known hormone signaling components has been observed, but these represent only some of the 124 transcription factors, kinases and other signaling components that were found to be regulated more than 3-fold and an even smaller fraction of the ones regulated between 2- and 3-fold. In addition to the nearly 22% of 3-fold or stronger regulated genes likely to be involved in signaling (transcription factors, kinases etc.) 37% of the more than 3-fold regulated genes are of unknown function. This limited understanding of many rice genes is an obstacle for the understanding of the hypoxic response.

The promoter regions of the genes regulated were analyzed to find overrepresented sequence motifs potentially involved in regulation of the genes at hypoxia. Eight core motifs were found (GACGCA, ACGCGCGT, TTCTGCGT, CGAACGTTTCG, CAGATA, CGTGTGAA, TACCTGCGTA, TACGCGCGTA) and were compared with known plant motifs. The CAGATAGC motifs found in 33% of the stronger than 3-fold upregulated genes in comparison with an occurrence of only 23% when looking at the entire genome is similar to a circadian clock components binding site with an E -value of $6.20 \cdot 10^{-5}$. This is of interest when discussing a possible influence of the circadian clock, see below. The TACGCGCGTA motif found in 26% of stronger than 2-fold regulated genes' promoters, which was only found in 19% of all rice promoters was shown to be similar to Arabidopsis NAC transcription factor binding sites with an E -value of $3.09 \cdot 10^{-3}$. Intriguingly, a NAC domain transcription factor, ANAC102, has been shown to play a role in Arabidopsis seed viability after hypoxic treatment (Christianson et al., 2009). A total of 4 downregulated NAC domain factors were found. It remains to be tested whether one or several of these factors indeed do regulate the expression of the 26% genes that contain the TACGCGCGTA motif. Several possibilities could explain why the motifs found here did not resemble any previously published motifs related to hypoxia

(Olive et al., 1991; Licausi et al., 2011). These motifs could not be conserved sufficiently to be detected by the software used, or the genes selected were not specific enough for the primary response to find the respective motifs. They might have been induced or repressed by secondary regulators thereby allowing for differing binding sites. In most previous studies, longer submergence or hypoxia treatments were performed, which could explain the lack of similarities in the motifs found. It cannot be excluded that the rice genes regulated here do contain motifs described previously or variants thereof but they might be too unspecific to be separated from the background by the scanning method used here. For example, the latter is likely to be true for the GC-motif previously found in genes modified under hypoxia conditions (Olive et al., 1991) which was identified in multiple variants (e.g. GCGCCC/GCCCC/GGCCCGG/GCGCC in Hossain et al., 1996) which likely cannot be distinguished from the background by the algorithm used here. The Weeder algorithm was chosen because it performed better when compared against similar algorithms (Tompá et al., 2005).

A dedicated oxygen sensor has to date not been found in plants, although Cavazza et al. (2008) propose that the Arabidopsis NARFL-like [FeFe]-hydrogenase-like GROWTH IN DIFFERENT OXYGEN LEVELS INFLUENCES MORPHOGENESIS (*AtGOLLUM*, At4g16440) is able to perceive and signal hypoxia. A homolog was down-regulated here (Os03g53750; -3.0-fold). Recently, Armstrong and Beckett (2011) presented a simulation showing that in roots, downregulation in respiration at ambient O₂ concentrations well above the K_m of COX can be explained by diffusion limitations inside the organ by cell walls, fluidic spaces, and organ specific structures like the Casparian strip rather than a sensor-controlled downregulation. This explanation would not require a dedicated sensor but rather rely on detection of energy status through the ATP/ADP ratio to explain the responses to hypoxia observed. Other candidates for possible oxygen sensors are prolyl-4-hydroxylases (P4Hs). In metazoans, HIF prolyl hydroxylases are important for oxygen sensing and subsequent signaling (Berra et al., 2003) and overexpression of one of the 13 Arabidopsis P4Hs (Vlad et al., 2007), *AtP4H1* led to a phenotype resembling plants grown under hypoxia and upregulation of hypoxia-associated genes (Asif et al., 2009). A T-DNA insertion mutant of Arabidopsis *P4H7*, one of the close homologs to another rice P4H strongly upregulated in the hypoxia treatment (Os07g09630) was studied here. *AtP4H7* had previously been identified to be upregulated under hypoxia (Vlad et al., 2007). The upregulation was not reproduced in qPCR analyses performed in this study. The insertion mutant of *AtP4H7* studied was

not significantly affected in its ability to cope with anoxia. Fresh weight, chlorophyll and anthocyanin content after waterlogging treatment were not altered. At normoxia, the mutant did not have a phenotype similar to the one observed in *AtP4H1* overexpression plants (Asif et al., 2009). For other mutants of this gene with exon-located T-DNAs, no homozygous plants were identified. The absence of other viable homozygous insertion mutants suggests that in the insertion mutant studied, the functional P4H7 protein could have been present as in the WT without being affected by the T-DNA insertion. This is underlined by the fact that *AtP4H7* expression was found to be unaltered in this mutant. Also, *AtP4H7* function could be redundant and performed by other P4Hs. Plants overexpressing this gene have a significantly lowered survival rate after 48 h of near anoxic treatment. The reason for this remains unknown. If *AtP4H7* indeed is involved in sensing hypoxia, a continuous induction of hypoxic responses in overexpressing plants even at normoxia might result in fitness losses when the real stress ensues. The phenotype might as well be explained without any hypoxia signaling-related effect of *AtP4H7* through other unknown fitness losses induced by the overexpression that become relevant during stress.

4.1. Regulation of metabolic adaptation

It was shown here that the expression of circadian clock genes is modulated by hypoxia. The circadian clock tightly controls starch consumption and, therefore, energy distribution during night/day cycles (Graf et al., 2010). Arabidopsis *LHY/CCA* expression peaks at dawn (Mizoguchi et al., 2002). It was shown here that the rice orthologue to these genes, *OsCCA1* (Murakami et al., 2007) was downregulated -5.0-fold. At the same time, upregulation of evening genes *OsPHYTOCLOCK1* and *OsGIGANTEA* as well as 8.5-fold upregulation of *OsPRR95* was shown. It was determined by qPCR that Arabidopsis *LHY* was strongly upregulated under the same conditions. In Arabidopsis, the *cca1/lhy* double mutation results in excessive starch degradation during the night (Graf et al., 2010). We observed that the CAGATAGC motif enriched in genes upregulated more than 3-fold exhibits similarities with a binding site of *AtCCA1* and *AtLHY*. This fits with the downregulation of *OsCCA1* observed here, as *CCA1/LHY* in Arabidopsis are known to downregulate evening genes (Pruneda-Paz et al., 2009). Given that the upregulated genes indeed contain a motif that could transduce repression by *OsCCA1*, upregulation of these genes following downregulation *OsCCA1* might be possible. As the Arabidopsis *PSEUDO-RESPONSE REGULATOR 9* (*AtPRR9*) gene was determined to be upregulated under hypoxia, the Arabidopsis *prp9-1* knock out mutant

was studied here to determine if disruption of this gene leads to altered survival under hypoxic conditions, which was not the case. *AtPRR9* is being expressed early during the day (Murakami et al., 2007) but as its knock out did not modify the fitness of *Arabidopsis* at low oxygen stress conditions it likely does not play an important role in *Arabidopsis* hypoxia responses. Alternatively, its role could also be mediated by the genes expressed later during the clock cycle, *AtPRR7* and *AtPRR5*. A modification of circadian clock genes by stress responses is in line with previous findings that have shown induction of *CCA1/LHY* by several abiotic stresses like salt stress and osmotic pressure (Kreps et al., 2002; Kant et al., 2008). The importance of the regulation of clock genes for hypoxia adaptation remains to be determined. A time lapse of circadian clock gene expression in the rice tissue studied here during hypoxia and flooding experiments would be of interest to test for the role of the circadian clock during this stress.

It is conceivable that regulation of metabolism-associated genes shown here, although measured 1 h after onset of hypoxia, is downstream of a central regulation of energy consumption during stress. It has already been shown that there are central energy and stress sensors of the Sucrose nonfermenting 1-related protein kinase 1 (SnRK1) family, KIN10 and KIN11 in *Arabidopsis* (Baena-González and Sheen, 2008), and OsSnRK1A (Os05g45420) and, less importantly, OsSnRK1B (Os08g37800; Lu et al., 2007) in rice. These rice proteins are regulated under hypoxia by Calcineurin B-like-interacting protein kinase 15 (CIPK15, Lee et al., 2009). The regulators were shown to play a central role in regulating sugar starvation responses and reduction of ATP consumption during stresses. Several target genes of SnRK1 were also regulated under hypoxia in this study. Neither the rice SnRK1-family genes themselves nor *OsCIPK15* were regulated, but as they are known to be regulated post transcriptionally (Lu et al., 2007), this is not necessarily expected.

The regulation of target genes like metabolic genes of the glycolysis/fermentation pathway was detected within 1 h after onset of hypoxia. This was helpful to confirm that hypoxia responses were indeed taking place. Nevertheless, it could be useful to study even earlier time points to exclude response genes and put a stronger focus on the regulatory genes differentially expressed.

Of signaling-related genes regulated more than +3-fold after 1 h of 5% O₂, for seven genes, *Tos17* retrotransposon insertion mutants were available. Homologous plants were identified for five of those. These consisted of one gene belonging to the class of small early auxin upregulated genes, *OsSAUR55*. In addition, there was an F-

box protein coding gene, a nucleotide binding site and leucine rich repeat containing *DISEASE-RESISTANCE RPP13-LIKE* gene, a zinc finger containing gene named *LSD1 ZnF LIKE*, and a gene containing a YDG/SRA domain which in plants is associated with histone methyl transferases according to Pfam (Finn et al., 2010). The analyses of these rice *Tos17* retrotransposon mutants should be considered very carefully due to the small number of experiments that were possible. In the WT deepwater rice ssp. *indica* PG56 cultivar used for the microarray analysis, *ADH2* expression was induced. In contrast, expression of this gene appeared to be reduced in the ssp. *japonica* Nipponbare cultivar after three days of flooding. However, as the variation was high and this gene was indeed induced in all of the *Tos17* insertion mutants tested, this is not a reliable result. It has to be considered that one outlier measurement (FCh -11.08) of the four WT samples tested extensively distorted the result. When omitting this sample, a very low induction of +1.2-fold was observed. Such a low induction is in line with recently published regulation of *OsADH2* in flooded Nipponbare cultivar rice (Kudahettige et al., 2011). It may be noteworthy that in the *DISEASE RESISTANCE RPP13-LIKE* mutant, expression induction of *OsADH2* was higher and *OsCML31* was lowered more strongly in comparison to the other mutants studied. This is of interest since this mutant is annotated to have only one *Tos17* insertion in the gene of interest in contrast to the other mutants which contained multiple insertions, so this difference could be relevant. Analyses of these mutants and the WT will have to be repeated in the future to verify the altered expression preliminarily observed here.

4.2. Differences and similarities to rice in Arabidopsis hypoxia adaptation

We determined for several genes which were regulated after 1 h at 5% O₂ in rice, that the expression of Arabidopsis homologs was not modified at low O₂. A reason could lie in different adaptation strategies to flooding. Arabidopsis is not adapted to flooding to the extent that rice is as it does not encounter frequent flooding events (Vashisht et al., 2011). It is also possible that selection by protein sequence homology did not yield bona fide orthologs. If this was the case, no regulation at hypoxia would be expected. Especially transcription factors and other regulatory proteins like F-box proteins could fulfill hypoxia-unrelated functions even if sequence similarities are relatively high. Some of the homologous genes studied were regulated in a similar fashion in Arabidopsis as in rice, or for a few genes, a reversed regulation was observed and was already discussed or is mentioned below. Two wound-induced family protein genes,

termed *WIF1* and *WIF2* for convenience, and *RHODANESE-DOMAIN CONTAINING PROTEIN (RHDP)* were upregulated more than 5-fold. The rice homologs of these genes were induced 61-fold and 9-fold, respectively. There is significant cross-talk between hormone responses during stress (Ludwig et al., 2005; Yoo et al., 2009), therefore it is not surprising that the *WIF* proteins were also associated with wounding. They contain a domain which is found in proteins that are often annotated as wound-induced according to the InterPro database (Hunter et al., 2009). When consulting public microarray data, however, the genes were most strongly expressed at low oxygen stress treatments but not when treated with other abiotic stresses (Hruz et al., 2008). It is shown here again that there is a broad range of genes regulated by different types of stress treatments and the balance of differential expression determines the correct response to the present type of stress (Kreps et al., 2002; Singh et al., 2002; Rabbani et al., 2003) and this is likely to be true for the two *WIF* genes studied here as well. They encode sequences that are homologous to exon 1 of the rice gene used for the homology search. It must be noted that several other predicted rice proteins can be found when performing a BLAST search on the rice genome using the predicted protein sequence of the rice gene regulated in the microarray, making it part of a group or family of genes in rice. To further study its role in the response of Arabidopsis to hypoxia, T-DNA insertion mutants for *WIF2* were characterized. No mutants for *WIF1* were available. In addition to significant induction of +28% of ADH activity levels in the *wif2-1* knock out mutant in comparison to the WT at hypoxia, it was found that after 2 d of near anoxic stress, significantly more seedlings of the mutants were dead after recovery with an increase of +32% over the WT seedlings. This is in contrast to the upregulation of ADH activity in *wif2-1*, which points to a repressive effect of *WIF2* on ADH. A repressive role of *WIF2* contradicts the strong induction of these genes at hypoxia as ADH activity is induced under these conditions. Perhaps, more complex regulation patterns could explain the effects observed. Although no function can be assigned to the *WIF* genes, it can be stated that a role in signal transduction by directly modulating transcription or taking part in a phosphorylation cascade would be surprising as these genes encode peptides of 90 amino acids and no DNA-binding or protein modification related domain is encoded. It can, however, not be excluded that *WIFs* are involved in a yet unknown novel form of regulatory pathway or that they encode a signaling peptide. These small proteins are homologous only to the exon 1 of the rice conserved hypothetical protein-encoding gene found to be upregulated 61-fold and encoding for the domain of un-

known function DUF3774. It could be hypothesized that they act on a process for which only this domain is important and that this might also be the case for exon 1 of the homologous rice gene. As both *WIF* genes were upregulated after 1, 2, and 4 h of hypoxia in *Arabidopsis* roots, *WIF1* could have filled in for the *WIF2* function in the *wif2-1* mutant to some degree. If mutants of *WIF1* were available, studying these mutants and, more importantly, double knock out mutants would be of interest. This would be possible by using RNAi to silence both genes.

The role of the *RHDP* gene is similarly unclear. No statistically significant differences in survival after anoxia or in fresh weight, chlorophyll or anthocyanin content after partial flooding were found in *rhdp* mutants although its expression was induced at hypoxia. However, genes with redundant functions could prevent an observable phenotype. One of the overexpressing lines of this gene performed significantly better after anoxia stress although in both of the lines studied, expression was increased. Possibly, the T-DNA inserted into a gene involved in a related process, which would explain the differing phenotype of the mutant lines and the low germination observed in line 14. In contrast to the improved survival, after waterlogging experiments the overexpressing mutants of *RHDP* were reduced in fresh weight in comparison to the WT. When considering the different strategies employed for hypoxia adaptation, reduced growth, and the resulting reduced fresh weight could improve long term survival. It would be interesting to study ADH activity in the overexpression lines to see if it is modulated there.

For two other genes, mutants were studied for a role in hypoxia adaptation. An F-box domain containing *PHLOEM PROTEIN 2-A13* (At3g61060) gene, homolog to the upregulated *OsFBX391* was not found to be significantly induced in the qPCR analyses performed here, but variation was high and it was shown as induced in Genevestigator public microarray data (Hruz et al., 2008). The mutant studied featured a significantly higher number of +26% of undamaged and normally growing seedlings in comparison to the WT after 2 d of near anoxic stress. F-box proteins are mostly part of SCF complexes involved in marking proteins for degradation by ubiquitination (Jain et al., 2007). Therefore the possibility exists that this protein regulates protein degradation of a component of the hypoxic or a general stress-related signaling pathway. Other proteins might redundantly step in for this protein's function in the insertion mutant. The second mutant contained a T-DNA insertion in the promoter of a bHLH-domain containing gene (At2g42280). Although the insertion was located in the promoter region and no apparent alteration in transcription was detected in the mutant, a definite reduction in survival in

comparison to the WT was observed after anoxia treatment. A possible explanation would be a modified promoter that inhibits induction of this gene at a specific phase in hypoxia when its expression is important. It has to be noted that this mutant was selected albeit it is not significantly regulated under hypoxia. This was due to an induction in qPCR assays found in initial experiments that were later relativized. The change in anoxia fitness is nevertheless valid. As this is likely a transcription factor involved in some part of the hypoxic response, it would be interesting to determine the genes regulated by this protein either by yeast-one-hybrid analysis (Deplancke et al., 2004) or by chromatin immunoprecipitation (Bowler et al., 2004).

The role of calcium in hypoxia signaling has been repeatedly mentioned here. In the microarray experiments conducted, a calmodulin-like protein gene, *OsCML31*, was found to be downregulated whereas the closest Arabidopsis homologs, *AtCML38* and *AtCML39* were upregulated. *AtCML38* has been found to be regulated by hypoxia previously (Lee et al., 2011) whereas *AtCML39* was to my knowledge not yet described in the literature to have a role in hypoxia. Expression of *AtCML39* was +6.0-fold induced after 1 h at 5% O₂ in Arabidopsis roots. In addition, an exon-positioned T-DNA insertion in this gene led to severely and significantly reduced survival rates after 2 d of near anoxia treatment. In comparison to the WT, a reduction of -14% in the number of unharmed seedlings was observed. ADH activity at hypoxia was induced by 38% in this mutant in comparison to WT. If *AtCML39* would repress ADH activity during hypoxia, the mutation could induce too early or too strong of an induction of ADH, which might result in reduced fitness. It is also conceivable that *AtCML39* modulates other processes that, for example, could reduce energy requirements. In the mutant, this would result in a need for enhanced fermentation and therefore an induction of ADH activity. In a previous study examining *AtCML37-39* in detail, an induction of *AtCML38* and *AtCML39* during salt, *Pseudomonas syringae*, drought, and wounding stress treatments had been observed and Ca²⁺ binding was shown for these proteins (Vanderbeld and Snedden, 2007). In the same study, it was shown by microarray data mining that *AtCML39* was more strongly induced by abiotic stresses in roots than in shoots (Vanderbeld and Snedden, 2007). The expression results presented here were also gained from experiments with the Arabidopsis root, confirming the induction of *AtCML39* in this organ. How could the *AtCMLs* regulated here fit into a hypoxia-responsive signaling pathway? Vanderbeld and Snedden (2007) propose that these *CMLs* could be involved in stress signaling pathways induced by ROS due to the finding that *AtCML38* is downstream of

mitogen-activated kinase (MAPK) kinase 2 (MKK2) signal transduction (Teige et al., 2004). Interestingly, a protein microarray approach predicted an interaction between AtCML38 and AtMPK6 (Popescu et al., 2009), the latter of which was described to modulate ethylene synthesis (Liu and Zhang, 2004). Unfortunately, AtCML39 was not tested for protein interactions there. In rice, expression of the homologous *OsCML31* was downregulated. The closest rice AtMAP6 homolog, OsSIPK/OsMPK1/OsMAPK6, was shown to be involved in O₃ stress signaling and ethylene synthesis induction and was itself activated by Ca²⁺ (Cho et al., 2009). To my knowledge, no studies on hypoxia signaling of OsSIPK and no studies on OsCML31 action have been performed to date. It is clear from other whole genome microarrays that *OsCML31* expression is modified by other stresses like salt stress and biotic stresses (Hruz et al., 2008). To conclude, altered survival after 2 d of near anoxia and induced ADH activity in the *AtCML39* mutant was observed. In addition, the expression data collected here and published data on this gene make a role of this gene and the encoded protein in a ROS-responsive calcium signaling pathway as proposed by Vanderbeld and Snedden (2007) conceivable. In this case, *OsCML31* in rice as well as *AtCML38* in Arabidopsis might be involved in a similar pathway.

To summarize, it was shown in this study that at 1 h of hypoxia, a large part of the rice response on the level of regulation of gene expression is mediated by low oxygen stress, irrespective of functional ethylene perception. Several members of the group VII of ERF transcription factors were hypoxia-induced. This is of interest due to previously characterized hypoxia regulators also found in that ERF group (Xu et al., 2006; Hattori et al., 2009; Hinz et al., 2010; Licausi et al., 2010; Hess et al., 2011; Yang et al., 2011). Circadian clock genes were regulated by hypoxia in rice and Arabidopsis and one predicted promoter element found bore similarities to a circadian clock regulator binding site. Several Arabidopsis homologs of rice genes regulated at low oxygen conditions were also regulated by hypoxia. Using mutant analyses, the role of *WIF2* and *AtCML39* genes during hypoxia was studied. It was shown that low oxygen responses like survival after 2 d of near hypoxia and ADH activity after 4 h of 5% O₂ were altered in these mutants. Further studies are needed to determine the extent of the involvement of these genes and the encoded proteins and their possible role in signal transduction during early hypoxic stress.

5. Arabidopsis *ERF71* and *ERF73* are differentially regulated by ethylene

5.1. Summary and own contribution

Several members of group VII of the ERF family of transcription factors are being regulated by and are important for the response to flooding and hypoxia (Section 1.3), with *SUB1A*, *SNORKEL1* (*SK1*) and *SK2* being the most prominent in rice and *RAP2.2*, *RAP2.12*, *ERF71* and *ERF73* in Arabidopsis. This is underlined by the fact that in the rice microarray experiment performed in this study, rice group VII ERFs *OsERF60*, *OsERF61*, *OsERF62*, *OsERF67*, and *OsERF68* were all induced more than 3-fold in response to hypoxia. *SUB1A*, *SK1* and *SK2* were not analyzed. In Arabidopsis, it was shown before that *ERF71* and *ERF73* are upregulated in roots under hypoxia and that *ERF73* plays an important part in the response to low oxygen stress. In the following study, we showed that *ERF73* expression in the roots of Arabidopsis seedlings was not only regulated by hypoxia but also by ethylene. This was shown by expression analyses in the constitutive ethylene signaling mutant *ctr1* and mutants compromised in ethylene signaling *ein2* and *ein2-1* as well as by ethylene and hypoxic stress treatments. In addition, analyses of overexpression and knock out lines for *ERF73* and *ERF71* showed that hypoxia-induced ADH activity is regulated by *ERF73* but not *ERF71* in roots of Arabidopsis seedlings. A regulation of ADH by ethylene was still detectable in *erf73* lines pointing to redundant factors regulating ADH activity. This was also shown in the rice microarrays above by the large number of rice genes that were differentially regulated by hypoxia irrespective of 1-MCP ethylene perception inhibitor treatment (Section 3).

Work on the following study was performed by Natalia Hess during her diploma work including cloning, expression studies and ADH assays. I provided technical assistance. I performed the qPCR assays on the ethylene signaling mutants presented in Figure 1C and D, prepared the figures for presentation and conducted the statistical analysis of qPCR and ADH activity assay data. Some assays were performed by Marco Anders.

5.2. Article: ‘The hypoxia responsive transcription factor genes *ERF71/HRE2* and *ERF73/HRE1* of Arabidopsis are differentially regulated by ethylene’

(Starting on following page)

The hypoxia responsive transcription factor genes *ERF71/HRE2* and *ERF73/HRE1* of *Arabidopsis* are differentially regulated by ethylene

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The AR2/ERF transcription factor genes *ERF71/HRE2* and *ERF73/HRE1* were induced at hypoxic conditions in *Arabidopsis thaliana* roots. *ERF73/HRE1* but not its related gene *ERF71/HRE2* was furthermore regulated by ethylene. Treatment with 1 ppm ethylene promoted *ERF73/HRE1* expression fivefold. This induction did not occur in the presence of the ethylene receptor inhibitor 1-methylcyclopropene. *ERF73/HRE1* expression positively regulated alcohol dehydrogenase (ADH) activity, which was analyzed as a marker enzyme for metabolic adaptation to hypoxic stress. The knock out lines *erf73/hre1-1* and *erf73/hre1-2* showed lowered ADH activity; the overexpressing lines *ERF73/HRE1-ox1* and *ERF73/HRE1-ox5* displayed elevated ADH activity. Treatment of wild-type *Arabidopsis* with 5% O₂ and 1 ppm ethylene resulted in higher induction of ADH activity than that observed with 5% O₂ or 1 ppm ethylene alone. *ERF73/HRE1-ox1* and *ERF73/HRE1-ox5* plants that were exposed to 5% O₂ did not show enhanced ADH activity after treatment with ethylene, indicating that the ethylene response with respect to ADH activity was saturated in the *ERF73/HRE1ox* lines. In contrast, *erf73/hre1-1* and *erf73/hre1-2* lines displayed ethylene-dependent ADH activity pointing to redundant factor(s) that can mediate ethylene regulation of ADH activity in the *Arabidopsis* root. Our data show that ethylene regulates metabolic adaptation to low oxygen stress in the *Arabidopsis* root through *ERF73/HRE1*.

Introduction

Hypoxia in plants results in impaired mitochondrial respiration and thus poses a severe problem (Fukao and Bailey-Serres 2004). Soil water logging or flooding are common causes of hypoxia in plants that primarily affect roots. As roots do not generally have photosynthetic oxygen evolution, they are readily affected by hypoxic stress. To deal with hypoxic or anoxic stress, plants resort to a number of adaptive strategies that improve oxygen supply and reduce loss of oxygen such as the formation

of gas spaces, enhanced shoot elongation and barriers to radial oxygen loss (Bailey-Serres and Voesenek 2008, Colmer 2003, Jackson and Armstrong 1999). Alternatively, plants display a quiescence response with inhibition of shoot growth as described for lowland rice (Xu et al. 2006). In addition, plant metabolism has evolved to produce ATP in the absence of mitochondrial respiration through various metabolic pathways including fermentation to ensure survival of cells (Bailey-Serres and Voesenek 2008, Geigenberger 2003). Activation of alcoholic fermentation can be

Abbreviations – 1-MCP, 1-methylcyclopropene; ABRC, Arabidopsis Biological Resource Center; ADH, alcohol dehydrogenase; PCR, polymerase chain reaction; qPCR, quantitative reverse transcription polymerase chain reaction; RT-PCR, real-time polymerase chain reaction.

considered a hallmark of low oxygen stress adaptation in plants (Mustroph et al. 2010).

Arabidopsis possesses four pyruvate decarboxylase genes (*PDC1–4*) and one gene that encodes alcohol dehydrogenase, *ADH1*. The *ADH1* promoter is under control of numerous stress-related transcription factors mediating low oxygen, drought or cold stress (de Bruxelles et al. 1996, Dolferus et al. 1994). An anaerobic response element was shown to mediate hypoxic induction of *ADH1* through binding of the MYB2 transcription factor (Dolferus et al. 1994, Kranz et al. 1998). MYB2 itself is induced at hypoxic conditions (Hoeren et al. 1998). It was further shown that *Arabidopsis ADH1* is regulated by ethylene (Peng et al. 2001). Recently, involvement of the apetala 2/ethylene response factor (AP2/ERF) type transcription factor RAP2.2 in *ADH1* regulation in the *Arabidopsis* shoot was shown (Hinz et al. 2010). *RAP2.2* expression was positively regulated by ethylene and evidence was provided that overexpression of *RAP2.2* improved hypoxia survival of *Arabidopsis* seedlings.

Arabidopsis RAP2.2 belongs to subgroup VII of the AP2/ERF transcription factor family (Nakano et al. 2006) along with the previously characterized rice *SUB1A* gene cluster and the rice *SNORKEL* genes (*SK1*, *SK2*). *SUB1A-1*, *SK1* and *SK2* ensure survival during flooding stress in rice (Hattori et al. 2009, Xu et al. 2006). In *Arabidopsis*, *RAP2.2*, *ERF71/HRE2* (At2g47520) and *ERF73/HRE1* (At1g72360) are closely related to these rice proteins. Licausi et al. (2010) showed that *ERF71/HRE2* and *ERF73/HRE1* genes were induced under hypoxic conditions in *Arabidopsis* roots. Similar to *RAP2.2* overexpression, overexpression of *ERF73/HRE1* improved hypoxic stress survival in *Arabidopsis* seedlings. This study pursued the question if *ERF73/HRE1* is an ethylene-regulated transcription factor that acts as a link between ethylene signaling and the metabolic response of *Arabidopsis* roots to low oxygen stress.

Materials and methods

Plant material and treatments

All analyses were carried out on *Arabidopsis thaliana* ecotype Columbia-0. Plants were raised at long day (16 h light) conditions in soil or on plates containing half strength Murashige–Skoog medium with 1.5% sucrose. *ctr1*, *ein2* and *ein2-1* were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University Columbus, OH). Treatments with 5% O₂ were carried out in a gas-tight acrylic glass box by gassing with an appropriate O₂ concentration in a mixture of 0.04% CO₂ and variable N₂ or with ambient air as a control.

Treatments with ethylene and 1-methylcyclopropene (1-MCP) were likewise carried out in a gas-tight acrylic glass box and plants were incubated in the light for the times indicated.

Expression analysis using northern and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted by grinding frozen root tissue in liquid nitrogen with a mortar and pestle using Tri-Reagent (Sigma-Aldrich, Munich, Germany) according to the manufacturer's instructions. For northern analysis, 20 µg of RNA per lane were separated on a denaturing agarose gel with 1% (w/v) agarose in 40 mM 3-(*N*-morpholino)-propanesulphonic acid buffer (MOPS) and 6% (v/v) formaldehyde. Ribosomal RNA was visualized with ethidium bromide and used as a control for RNA loading. RNA was transferred to a nylon membrane (Hybond N⁺; GE Healthcare, Munich, Germany) and hybridized to ³²P-labeled probes obtained using Ready-To-Go dCTP labeling kit (GE Healthcare). Hybridization was performed overnight at 68°C in hybridization solution [5% (w/v) dextran sulfate, 1 M NaCl, 1% (w/v) SDS, 0.1 mg/ml heat-denatured salmon sperm DNA]. Blots were washed two times in 2 × SSC/1% (w/v) SDS for 1 and 10 min, once in 1 × SSC/1% (w/v) SDS for 10 min and once in 0.5 × SSC/1% (w/v) SDS for 10 min. The gene-specific primer pairs used to generate the PCR probes from genomic DNA were 5'-TGGGCGGCTGAGATACGTGA-3' and 5'-CCGTA AAA GGCATCTGCGAGAA-3' for *ERF73/HRE1*; 5'-CAGGAA GAAGCGTAAACCC-3' and 5'-TCGGCAACCGGAATCT GATAGA-3' for *ERF71/HRE2* and 5'-TCGGCAACCGGA ATCTGATAGA-3' and 5'-CTTGGTCTGAATCTTTAGAG T-3' for *ADH1* (At1g77120).

Quantitative reverse transcription polymerase chain reaction (qPCR) was performed with QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The reverse transcription products were amplified using gene-specific primers 5'-GATTGATACACAATGGCTCGAA-3' and 5'-CGAGAAATATTCGGTCTGGTTC-3' for *ERF73/HRE1*; 5'-GGACTCGGTTCAAAAACGAA-3' and 5'-TTCCTCA CTGAGACGGGTT-3' for *ERF71/HRE2*; 5'-AATGTCTAC CACCGGACAGATTA-3' and 5'-CCCAGAAGTAAACAT CCGTGTG-3' for *ADH1*; 5'-CCGATCAAATCCGTAAGC TC-3' and 5'-ACCCTCTCATCGAGAAAGCA-3' for *ERF1* (At3g23240) and 5'-GAGCGTGCAATCAGGAGAAG-3' and 5'-TCTGGGGGATCAAAACCAAT-3' for At1g76940, which was used as a control. At1g76940 was identified as a gene that is not regulated under various stress conditions using the REFGENES tool (Genevestigator, Hruz et al.

2011). Reactions were performed with a 7300 real-time PCR-system (Life Technologies, Darmstadt, Germany). All other data were normalized to At1g76940 and analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Three independent biological experiments were performed except for the analysis of *ctr1* shown in Fig. 1C, D, where values are derived from two biological experiments with technical repeats included. Technical replicates were averaged. Statistical analysis was performed using MINITAB. Comparison of means was analyzed for statistical significance either by analysis of variance (ANOVA) and two-way ANOVA pairwise comparison including Tukey's test or by two-sample Student's *t*-test. Constant variance and normal distribution of data were verified before statistical analysis and the *P* value was set to $P < 0.001$ if either of these conditions did not apply. The *P* value for the Pearson product moment correlation and the statistical method used is indicated in the figure legends.

Identification of T-DNA insertion lines and cloning of overexpression constructs for *ERF71/HRE2* and *ERF73/HRE1*

T-DNA insertion lines SALK_052858C (*erf71/hre2-1*), SALK_039484C (*erf73/hre1-1*) and SALK_018181 (*erf73/hre1-2*) were obtained from the ABRC (Alonso et al. 2003). Genomic DNA was extracted from frozen leaf tissue of *Arabidopsis* T-DNA insertion lines using the method described by Weigel and Glazebrook (2002) and insertion of the T-DNA was verified by PCR. Primer combinations were as follow: 5'-ACCGCTTGCAACTCTC-3', 5'-CTCGGCTCTGTAGCAGCAGGA-3' and 5'-TCGGCAACCGGAATCTGATAGA-3 for SALK_052858C (*erf71/hre2-1*); 5'-ACCGCTTGCAACTCTC-3', 5'-CATAGCGCCGGAGAAGATTGCG-3' and 5'-TCCGTAAGGCATCTGCGAGA-3' for SALK_039484C (*erf73/hre1-1*) and SALK_018181 (*erf73/hre1-2*).

Transcript levels in the homozygous insertion lines *erf71/hre2-1*, *erf73/hre1-1* and *erf73/hre1-2* were analyzed by real-time polymerase chain reaction (RT-PCR) using M-MuLV-reverse transcriptase (Fermentas, St. Leon-Rot, Germany). The reverse transcription products were amplified using gene-specific primers 5'-GGACTCGGTTCAAAAACGAA-3' and 5'-TTCCTACTGAGACGGGTT-3' for *ERF71/HRE2*; 5'-CTCAATTTCCCTAACGAATCCTCT-3' and 5'-CCGTAAAGGCATCTGCGAGA-3' for *ERF73/HRE1* and 5'-CAAAGACCAGCTCTCCATCG-3' and 5'-CTGTGAACGATTCTGGACCT-3' for *Actin2*, which was used as a control for RNA input.

To constitutively overexpress *ERF73/HRE1*, a cDNA fragment encompassing the coding region was amplified

from *Arabidopsis* Columbia-0 using the gene-specific primers 5'-CACCATGTGCGGAGGAGCTGTAAT-3' and 5'-TCAGGACCATAGACCCATGTC-3'. The fragment was cloned into the vector pENTR/D-Topo (Life Technologies) to generate the entry vector pENTR/D-Topo-ERF73 from where the sequence of interest was recombined into the destination vector pB7WG2 (Karimi et al. 2002) using the LR reaction mix II (Life Technologies) to obtain the expression *35S::ERF73*. This plasmid was transformed into *Arabidopsis* Columbia-0 using the floral dip method (Clough and Bent 1998). T₀ seeds were screened for glufosinate resistance and RNA was isolated from the leaves of resistant plants. Transcript levels were analyzed by RT-PCR using M-MuLV-reverse transcriptase (Fermentas). The reverse transcription product was amplified with the gene-specific primers 5'-ACATAGCGCCGGAGAAGATTGCG-3' and 5'-CGAGAAATATTCGGTCTGGTTC-3'. Amplification of *Actin2* transcripts was used as a control with the primers described above.

ADH activity assay

For ADH enzyme activity measurements, soluble protein was extracted from the roots of 10-day-old *Arabidopsis* seedlings in cold extraction buffer [100 mM Tris-HCl, pH 8.0, 25% (v/v) glycerol, 0.8% (v/v) β -mercaptoethanol, 2% (w/v) polyvinylpyrrolidone (PVP), 5 mM dithiothreitol (DTT)] and centrifuged at 15 000 g for 15 min at 4°C. Protein concentrations were determined by Bradford assay (Roti-Quant; Roth, Karlsruhe, Germany). Fifty micrograms of protein were diluted in 50 μ l extraction buffer and water was added to a final volume of 150 μ l. The enzyme reaction was started by adding 800 μ l activation buffer (62.5 mM Tris-HCl, pH 8.5; 1 mM NAD⁺) and 50 μ l ethanol. Activities were calculated from the increase in absorbance at 340 nm over time. Assays were performed in triplicate with at least two technical replicates each. Statistical analysis was performed as described in the section Expression analysis using northern and quantitative reverse transcription polymerase chain reaction.

Results

The ERF transcription factor genes *ERF71/HRE2* and *ERF73/HRE1* were previously shown to be induced at hypoxic conditions in *Arabidopsis* roots, a regulation that was confirmed in this study (Fig. 1A–D). To test if these ERF genes were ethylene regulated, we used *Arabidopsis* mutants with altered ethylene signaling and studied gene expression in two ways, northern analysis (Fig. 1A, B) and qPCR (Fig. 1C, D). In *ctr1*, with

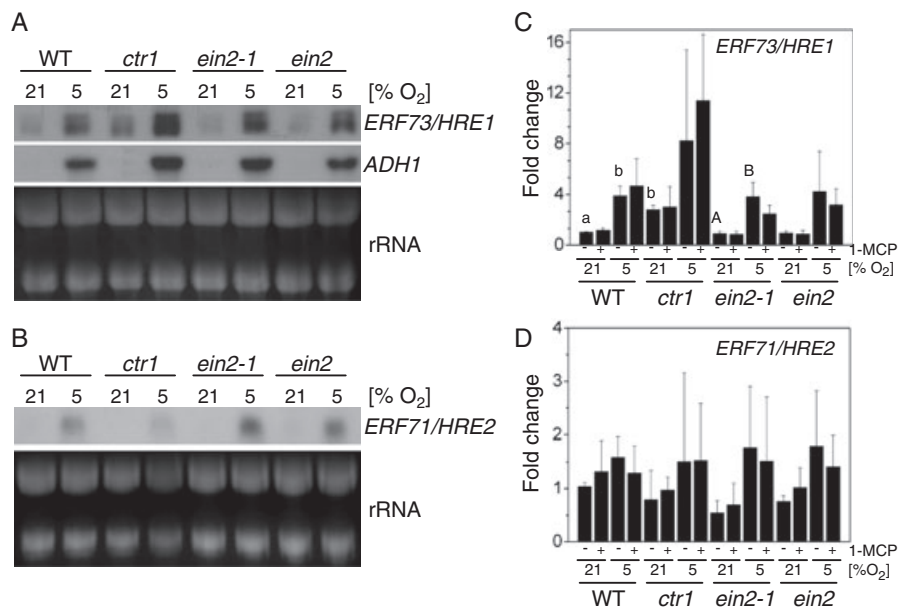


Fig. 1. *ERF73/HRE1* expression is enhanced by constitutive ethylene signaling at hypoxic conditions. Analysis of *ERF73/HRE1* and *ERF71/HRE2* expression by (A, B) northern blot and (C, D) qPCR was performed on roots of 10-day-old wild type (WT), *ctr1*, *ein2-1* and *ein2* *Arabidopsis* seedlings that were treated for 4 h in a gas atmosphere containing 5% O₂, 0.04% CO₂ and N₂, or were kept in air as a control. *ADH1* expression in (A) was included as a control for hypoxia treatment. Staining of ribosomal RNA (rRNA) is shown as a control for RNA loading. (C, D) Seedlings were treated at normoxic or hypoxic conditions in the presence or absence of 5 ppm 1-MCP, an ethylene receptor inhibitor, and transcript abundance of (C) *ERF73/HRE1* and (D) *ERF71/HRE2* was quantified using qPCR. Different letters indicate statistically different values as compared with normoxic wild type (WT; a, b) or normoxic *ein2-1* (A, B) seedlings at $P < 0.05$ (*t*-test).

constitutively activated ethylene signaling, expression levels of *ERF73/HRE1* were elevated at both normoxic and hypoxic conditions in comparison to the wild type (WT). qPCR data were not statistically different likely because of the reduced number of samples analyzed in this genotype. However, northern analysis and qPCR showed highly similar regulatory patterns. In the ethylene-insensitive mutants *ein2-1* and *ein2*, transcript levels were unchanged compared with wild type at either normoxic or hypoxic conditions, indicating that basal expression and hypoxic induction occurred independent of ethylene signaling (Fig. 1A, C). Expression of *ERF71/HRE2* was slightly increased by hypoxia but was not significantly affected by mutations in ethylene signaling (Fig. 1B, D). Treatment with the competitive ethylene receptor inhibitor 1-MCP affected neither *ERF71/HRE2* nor *ERF73/HRE1* expression (Figure 1C, D), indicating that basal expression and hypoxic induction of *ERF71/HRE2* and *ERF73/HRE1* are not under positive or negative control by ethylene. As ethylene signaling in *ctr1* is constitutively activated, an effect of 1-MCP was not expected. We next analyzed the dose dependence of ethylene on *ERF73/HRE1* expression by treating plants with defined concentrations of ethylene at normoxic conditions. Quantitative RT-PCR indicated significant

induction of *ERF73/HRE1* transcript abundance at 0.1 ppm and higher concentrations of ethylene (Fig. 2). In the presence of the ethylene perception inhibitor 1-MCP, the induction of *ERF73/HRE1* expression by 1 ppm ethylene was fully inhibited, indicative of a true ethylene response. In contrast, *ERF71/HRE2* was not induced at any ethylene concentration applied. Neither was the basal expression level of *ERF71/HRE2* affected by treatment with 1-MCP, confirming that *ERF71/HRE2* is not subject to regulation by ethylene in the *Arabidopsis* root as was concluded from the analysis of ethylene signaling mutants.

We next analyzed the expression of *ADH1* in response to ethylene signaling in the root. *ADH1* expression at normoxic conditions was unaffected in the ethylene signaling mutants, whereas transcript levels were elevated in *ctr1* at hypoxic conditions compared with wild type and compared with *ein2-1* and *ein2* (Fig. 1A). qPCR analysis supported a combined effect of low oxygen and ethylene signaling on *ERF73/HRE1* (Fig. 3A) and *ADH1* expression (Fig. 3B). *ERF1* was previously identified as an ethylene-regulated gene and was used as a control in the qPCR expression study (Fig. 3C; Fujimoto et al. 2000). As expected, *ERF1* expression was enhanced after treatment with 1 ppm ethylene, but unlike

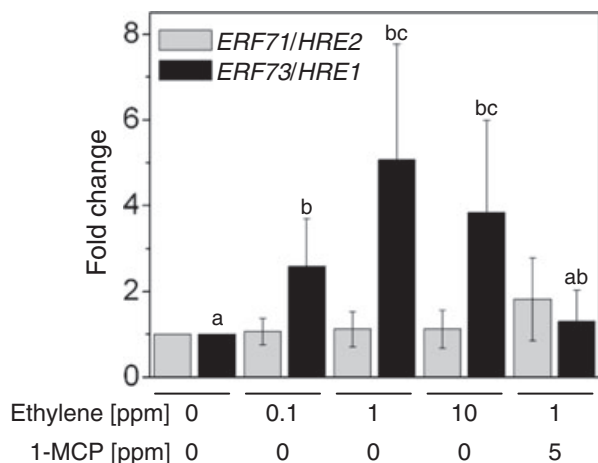


Fig. 2. *ERF73/HRE1* but not *ERF71/HRE2* expression is regulated by ethylene. qPCR analysis was performed on RNA isolated from roots of 10-day-old wild-type *Arabidopsis* plants treated with ethylene and 1-MCP for 4 h at the concentrations indicated. The relative transcript level without treatment was set to 1 and all other values were calculated as fold changes. Fold changes for *ERF71/HRE2* did not differ significantly. For *ERF73/HRE1*, letters indicate statistically significant expression fold changes at $P < 0.001$ (ANOVA with Tukey's test).

ERF73/HRE1, *ERF1* was not induced by low oxygen conditions. Rather, a slight repression of ethylene-induced gene expression was observed at 5% O_2 .

To functionally link *ERF73/HRE1* activity to ADH activity, expression of *ERF73/HRE1* was genetically altered. Knockout mutants of *ERF73/HRE1* were identified (Fig. 4A) and verified (Fig. 4B, C). As a control, a knockout line of *ERF71/HRE2* was identified (Fig. 4A, D). Furthermore, overexpression lines were generated with *ERF73/HRE1* expression driven by the constitutive 35S promoter (Fig. 4E). These lines were subsequently analyzed for ADH activity (Fig. 5). Down-regulation of *ERF73/HRE1* expression in the *erf73/hre1-1* and *erf73/hre1-2* lines resulted in significantly lowered ADH activity at hypoxic conditions (Fig. 5A), whereas overexpression of *ERF73/HRE1* enhanced ADH activity at hypoxic conditions. However, hypoxic induction of ADH activity was not abolished in *erf73/hre1-1* and *erf73/hre1-2* lines again supporting the idea that regulation of *ADH1* by hypoxia is in part independent of *ERF73/HRE1*. At normoxia, no differences in ADH activity were detected between wild type and mutants (Fig. 5A). The *ERF71/HRE2* T-DNA insertion line *erf71/hre2-1* (Fig. 4A, D) did not show altered ADH activity at normoxic conditions or after hypoxia treatment for 4 h as compared with wild type (Fig. 5B).

We observed that *ADH1* gene expression was increased after treatment with 1 ppm ethylene at hypoxic conditions in *Arabidopsis* roots (Fig. 3B). A slight

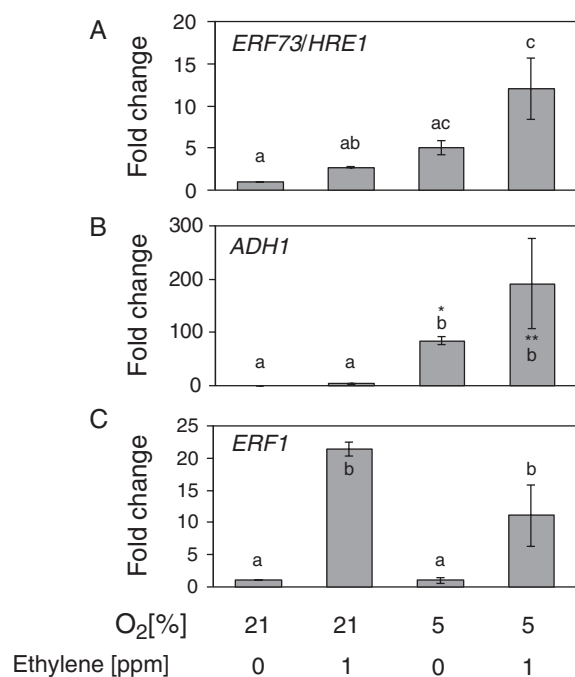


Fig. 3. Regulation of *ERF73/HRE1* and *ADH1* gene expression by hypoxia and ethylene. qPCR analysis was performed on RNA isolated from the roots of 10-day-old wild-type *Arabidopsis* plants treated with 1 ppm ethylene at normoxic or hypoxic conditions for 4 h. Expression levels at normoxic conditions without ethylene were set to 1 for each gene analyzed. Transcript levels of the known ethylene responsive gene *ERF1* were analyzed as a control. Statistically significant different values at $P < 0.001$ (ANOVA with Tukey's test) are indicated by different letters. The asterisks in (B) indicate a significant increase in the expression at $P < 0.05$ (t-test) when plants were treated with ethylene and hypoxia in comparison to treatment with hypoxia only.

increase in ADH activity observed at these conditions was not statistically significant possibly because elevation of activity lags behind gene expression (Fig. 6A). At normoxic conditions, neither *ADH1* expression nor ADH activity was notably elevated (Figs 2A and 6A). To further study the role of *ERF73/HRE1* in hypoxia adaptation, *ERF73/HRE1ox* seedlings and *erf73/hre1* knockout lines were analyzed for ADH activity at low oxygen conditions and treatment with ethylene or 1-MCP (Fig. 6B). Treatment with 1 ppm ethylene appeared to promote ADH activity in wild type and in the *erf73/hre1* knockdown lines, even though the differences were not statistically significant. In the *ERF73/HRE1-ox1* and *ERF73/HRE1-ox5* lines with constitutively elevated ADH activity, ethylene treatment did not further enhance ADH activity, indicating that ethylene-dependent activation of ADH was saturated. Treatment with 1-MCP at hypoxic conditions did not alter ADH activity compared with that observed at hypoxic conditions without effector treatment in any of the genotypes. This observation supports

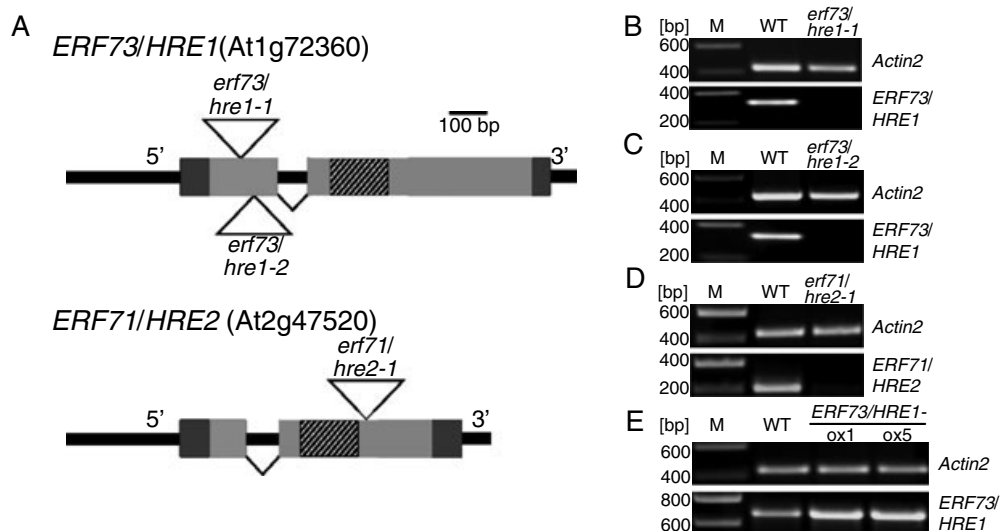


Fig. 4. *ERF71/HRE2* and *ERF73/HRE1* mutants. (A) T-DNA insertion mutants for *ERF71/HRE2* and *ERF73/HRE1* were identified and insertion of the T-DNA was verified by PCR. Indicated is the location of the T-DNA in lines *erf73/hre1-1*, *erf73/hre1-2* and *erf71/hre2-1*. The dark gray 5' and 3' areas indicate untranslated regions. The shaded area indicates the position of the single AP2 domain. (B) RT-PCR analysis of *ERF73/HRE1* expression in wild type and in the *erf73/hre1-1* T-DNA insertion line. (C) RT-PCR analysis of *ERF73/HRE1* expression in wild type and in the *erf73/hre1-2* T-DNA insertion line. (D) RT-PCR analysis of *ERF71/HRE2* expression in wild type and in the *erf71/hre2-1* T-DNA insertion line. (E) RT-PCR analysis of *ERF73/HRE1* expression in wild type and in the overexpressing lines *ERF73/HRE1-ox1* and *ERF73/HRE1-ox5*.

the view that hypoxic induction of ADH activity occurred independent of ethylene signaling.

In summary, our results support the view that ethylene contributes to ADH regulation at hypoxic conditions in the *Arabidopsis* root through *ERF73/HRE1*. Licausi et al. (2010) showed that overexpression of *ERF73/HRE1* improves anoxia survival in *Arabidopsis*. Our results suggest that low oxygen tolerance in *Arabidopsis* roots may in part be because of ethylene signaling through *ERF73/HRE1*.

Discussion

Several AP2/ERF-type transcription factors were previously shown to be involved in the hypoxic response of plants. In certain lowland rice varieties that survive flash floods through a quiescence strategy, the ERF transcription factor *SUB1A-1* is responsible for growth inhibition in response to submergence (Xu et al. 2006). *SUB1A-1* expression is activated by ethylene that accumulates in submerged tissue. Growth inhibition by *SUB1A-1* occurs through inhibition of gibberellin signaling (Fukao et al. 2006). Introduction of the *SUB1* locus into the submergence-intolerant lowland rice cultivar M202 resulted in inhibition of shoot elongation, elevated PDC and ADH activities and enhanced submergence tolerance (Fukao et al. 2006). In deepwater rice, the related ERF genes *SNORKEL1* (*SK1*) and *SNORKEL2*

(*SK2*) promote the opposite response, that is stem elongation, as an escape response to submergence (Hattori et al. 2009). As was observed for *SUB1A*, expression of *SK1* and *SK2* is positively regulated by ethylene.

AP2/ERF transcription factors are a large family with 121 members in *Arabidopsis* (Nakano et al. 2006). Despite their original designation as ethylene response factors, not all ERFs are regulated by ethylene. Among the subgroup VII ERFs which play a role in the low oxygen stress response, rice *SUB1A*, *SK1* and *SK2* and *Arabidopsis* *RAP2.2* were shown to be under the control of ethylene (Fukao et al. 2006, Hattori et al. 2009, Hinz et al. 2010). Regulation of the hypoxia responsive subgroup VII ERFs *ERF71/HRE2* and *ERF73/HRE1* of *Arabidopsis* by ethylene is described in this study. While *ERF71/HRE2* transcript levels were not subject to regulation by ethylene in the root, *ERF73/HRE1* transcripts clearly increased in response to ethylene signaling in the root. In accord with this result, Yang et al. (2011) recently described regulation of *ERF73/HRE1* by the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) in 14-day-old *Arabidopsis* seedlings.

Adaptation to low oxygen conditions occurs at various levels. Morphological changes such as the growth of adventitious roots (Vergara et al. 1976) and anatomical adaptations such as the formation of aerenchyma (Shiono et al. 2008) were shown to be controlled by ethylene signaling, indicating that ethylene plays a central role

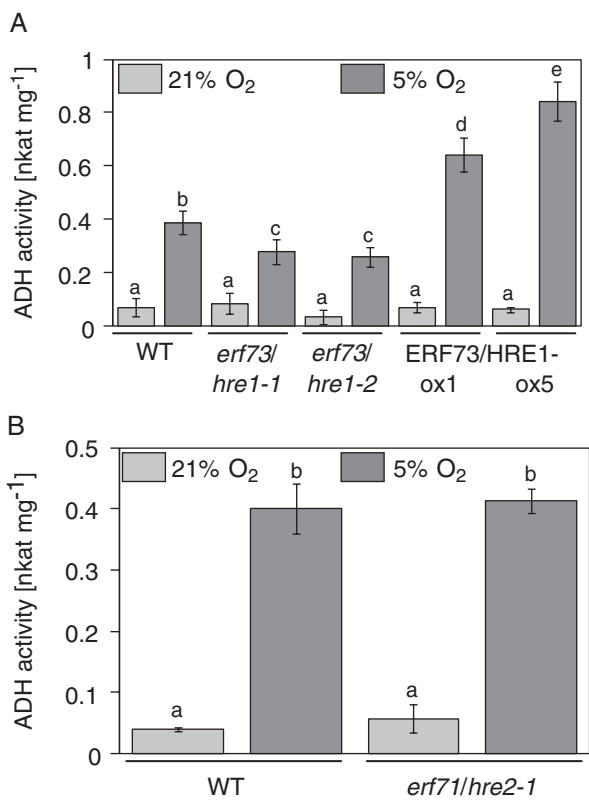


Fig. 5. ADH activity is altered in *ERF73/HRE1* expression mutants at hypoxic conditions. (A) The 10-day-old plants were kept for 4 h at normoxic or hypoxic conditions. ADH activity is significantly reduced at hypoxic conditions in the roots of *erf73/hre1-1* and *erf73/hre1-2* knockout lines and is significantly enhanced in *ERF73/HRE1-ox1* and *ERF73/HRE1-ox5* overexpression lines. (B) Knockout of *ERF71/HRE2* expression in *erf71/hre2-1* does not alter ADH activity. Significantly different values at $P < 0.001$ (ANOVA with Tukey's test) are indicated by different letters.

in low oxygen stress adaptation. Low oxygen conditions often arise upon water logging or flooding, conditions at which the gaseous ethylene is trapped within the plant tissue. As no degradation mechanisms exist for ethylene in plants, ethylene will accumulate in submerged plant parts even if rates of synthesis are low. In rice and other plants, ethylene rapidly accumulates to physiologically active levels upon submergence (Bailey-Serres and Voesenek 2008, Kende et al. 1998). Plants appear to have adapted to the consequence of limited gas diffusion in an aqueous medium by using ethylene as an indicator for submergence conditions and as a signal that promotes morphological, anatomical and metabolic changes to ameliorate the stress. Metabolic adaptation to low oxygen conditions comprises many pathways. The general response of plants to limited oxygen supply is to downregulate energy-consuming secondary metabolism and transport processes and to switch to primary

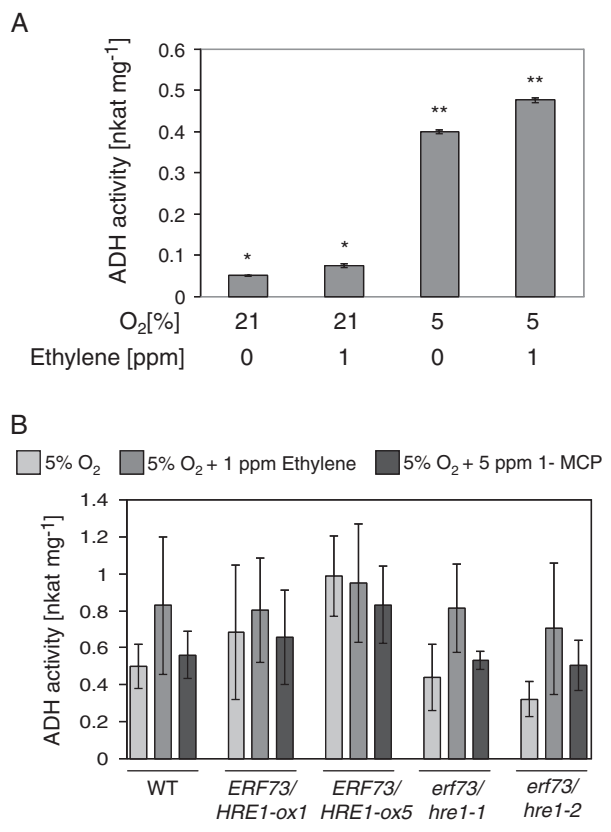


Fig. 6. Regulation of ADH activity by ethylene in *Arabidopsis* roots. (A) Wild-type plants were treated at normoxic or hypoxic conditions with 1 ppm ethylene for 4 h and ADH activity was measured in roots. Significant differences between plants treated with normoxia and hypoxia at $P < 0.05$ (t-test) are indicated by asterisks. (B) Wild-type, *erf73/hre1-1*, *erf73/hre1-2*, *ERF73/HRE1-ox1* and *ERF73/HRE1-ox5* plants were kept at hypoxic conditions for 4 h and treated in addition with 1 ppm ethylene or 5 ppm 1-MCP.

metabolic pathways that allow regeneration of NAD⁺ from NADH in the absence of mitochondrial respiration (Branco-Price et al. 2008, Liu et al. 2005, van Dongen et al. 2009). The switch from respiration to fermentation is a core response in the hypoxic stress response, and ADH activity can be considered as a marker enzyme of metabolic adaptation (Mustroph et al. 2010).

ADH gene expression and enzyme activity are induced in response to low oxygen. In *Arabidopsis*, the Myb transcription factor MYB2 was shown to bind to the *ADH1* promoter and to mediate gene expression (Hoeren et al. 1998). MYB2 itself is induced by hypoxia. Peng et al. (2001) showed that *ADH1* is regulated not only by hypoxia but is also subject to regulation by ethylene. The ERF transcription factor RAP2.2 was recently shown to mediate ethylene signaling of *ADH1* induction in the *Arabidopsis* shoot (Hinz et al. 2010). The data indicated that RAP2.2 requires a hypoxic signal to

promote *ADH1* expression. Expression of *Arabidopsis ERF73/HRE1* is induced at hypoxic conditions in the root. Genetic manipulation of *ERF73/HRE1* expression showed that *ERF73/HRE1* regulates expression of an array of metabolic genes including *ADH1* (Licausi et al. 2010). *ERF73/HRE1* function was further shown to improve survival of anoxic conditions, indicating that metabolic regulation is physiologically relevant. This study and a related study (Yang et al. 2011) revealed that *ERF73/HRE1* is regulated not only by hypoxia but is also induced by ethylene with the two signals likely acting in a synergistic manner. Furthermore, we and others (Licausi et al. 2010) showed that *ADH* activity is regulated by *ERF73/HRE1* at hypoxic conditions. Treatment with ethylene under hypoxic conditions resulted in higher *ADH* activity than was observed at hypoxic conditions in the absence of ethylene. Furthermore, it was observed that knockout of *ERF73/HRE1* reduced hypoxic induction of *ADH* activity and overexpression of *ERF73/HRE1* enhanced *ADH* activity at hypoxic conditions, functionally linking *ERF* activity to metabolic changes. It must be noted that regulation of *ADH* activity is only in part *ERF73/HRE1* dependent as revealed by the still significant induction of *ADH* activity in *erf73/hre1* mutants. Taken together, it can be concluded that the effect of ethylene in hypoxia adaptation at the metabolic level in roots occurs through *ERF73/HRE1*. Roots suffer from oxygen shortage during water logging and partial submergence. These conditions favour accumulation of ethylene. Thus, plants use two gaseous signals, oxygen and ethylene, to promote a metabolic stress response. This is in contrast to other adaptations, such as adventitious root growth and aerenchyma formation, which are induced by ethylene even at normoxic conditions. The molecular mechanisms and the physiological significance of this differential regulation remain to be studied.

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7. Supplemental Data

Supplemental Table 1: Categories used for analyzing transcript data and corresponding meta categories used for simplified presentation in Figure 2.

Category	Meta category	Category	Meta category
ABA homeostasis	Hormones	Kinase	Signaling
ABA signaling	Hormones	Nucleic acid binding	Signaling
Auxin homeostasis	Hormones	Pathogen resistance	Signaling
Auxin signaling	Hormones	Phosphatase	Signaling
CK homeostasis	Hormones	Receptor	Signaling
CK signaling	Hormones	Receptor kinase	Signaling
Ethylene homeostasis	Hormones	Signaling	Signaling
Ethylene signaling	Hormones	mRNA turnover	Signaling
GA homeostasis	Hormones	Transcription factor	Signaling
GA signaling	Hormones	Redox regulation	Stress
Hormone metabolism	Hormones	ROS metabolism	Stress
JA homeostasis	Hormones	Senescence	Stress
JA signaling	Hormones	Stress	Stress
Electron carrier activity	Metabolism	Transport	Transport
Electron transport	Metabolism	cDNA	Unknown/Others
Inositol metabolism	Metabolism	Cell cycle	Unknown/Others
Metabolism	Metabolism	Cell wall maintenance	Unknown/Others
Photosynthesis	Metabolism	Cell-cell adhesion	Unknown/Others
Primary metabolism	Metabolism	Expressed protein	Unknown/Others
Secondary metabolism	Metabolism	Hemoglobin	Unknown/Others
Storage	Metabolism	Histone	Unknown/Others
Peptidase	Protein homeostasis	Hypothetical protein	Unknown/Others
Protein homeostasis	Protein homeostasis	No blast match	Unknown/Others
Protein modifying	Protein homeostasis	Transposon	Unknown/Others
Chaperone	Protein homeostasis	Unknown	Unknown/Others
Histone	Signaling		

Supplemental Table 2: Oligonucleotides used for qPCR analysis, RT-PCR expression analysis and homozygosity determination. PCR cycle conditions were as follows: Initial denaturation for 60 s at 95 °C. A number of cycles (*Cyc*) of: 20 s at 94 °C, 40 s at annealing temperature (T_{Ann}), a varied time of elongation (t_{elong}) at 72 °C. This was followed by a final extension for 6 min at 72 °C. The number of cycles used depended on the amount of transcribed mRNA (cDNA) used in a 20 μ L PCR reaction mixture for expression analyses. The typical amount used with a defined number of cycles is given in parentheses after the number of cycles. For homozygosity detection, 1 μ L of isolated gDNA typically of one leaf was put into the PCR reaction mix. Reaction conditions for qPCR are listed in Materials and Methods in more detail. The primers were used for qPCR, determination of homozygosity in T-DNA insertion and *Tos17* retrotransposon insertion mutants (Homozygosity analysis), determination of expression levels of genes in insertion and overexpression mutants by RT-PCR (Expression analysis) or for cloning of overexpressing lines (cloning ox lines). All primers for expression analysis for Arabidopsis were located 3' of the insertion site except for the SALK_130465C mutant where the insertion is located at the very 3' of the transcript of At4g10270. For the rice mutants, the expression analysis primers were located 5' of the insertion site for NF6033, NC0550 and NC2016 and 3' of the insertion site for the other mutants tested. The conditions given for the homozygosity analysis were used for a PCR where the gene specific primers were put into the same reaction as the respective T-DNA left border primers to produce two bands (WT and mutant) with gDNA of heterozygous and one band (WT or mutant) with gDNA of homozygous or WT plants.

Gene & Mutant	Purpose	Name(s)	Sequence(s) (5' > 3')	Conditions
<i>Tos17</i> left border	Homozygosity analysis	TOS17 LB	ATTGTTAGGTTGCAAGTTAGTTAAGA	See gene-specific primers for conditions.
<i>CHY ZnF FAMILY</i> Os10g31850 (NC0419)	Homozygosity analysis	FW_NC0419 RV_NC0419	AGGGAAATGACAAAGGCATG GGACAGTTGTGATGCATTGC	$T_{Ann}=58^{\circ}\text{C}$ $t_{elong}=65\text{ s (+1/cycle)}$ $Cyc=35$
<i>YDG/SRE DOMAIN CONTAINING</i> Os08g30910 (NF6033)	Homozygosity analysis	FW_NF6033 RV_NF6033	CAACTCCAATGGTGCAATTG TCAACTGCAGTCCTGTCCAG	$T_{Ann}=58^{\circ}\text{C}$ $t_{elong}=65\text{ s (+1/cycle)}$ $Cyc=35$
<i>F-BOX CONTAINING</i> Os02g56820 (ND3022)	Homozygosity analysis	FW_ND3022 RV_ND3022	CATGTTTCATGCCTAAATGCC CAGTCTCCCTGTCCAACCAC	$T_{Ann}=56^{\circ}\text{C}$ $t_{elong}=75\text{ s (+1/cycle)}$ $Cyc=37$
<i>OsSAUR53</i> Os09g37480 (NC2636)	Homozygosity analysis	FW_NC2636 RV_NC2636	AACCACATAGCTTGGCCTTG ACCTGAACGAATCATTTGCC	$T_{Ann}=58^{\circ}\text{C}$ $t_{elong}=65\text{ s (+1/cycle)}$ $Cyc=35$
<i>LSD1 ZnF LIKE</i> Os08g06280 (NC0550)	Homozygosity analysis	FW_NC0550 RV_NC0550	CCTGCACAGAAATTCCAGGT TTGCATGGAAGATGCTTTTG	$T_{Ann}=58^{\circ}\text{C}$ $t_{elong}=65\text{ s (+1/cycle)}$ $Cyc=35$
<i>DISEASE RESISTANCE RPP13-LIKE</i> Os10g07400 (NC2016)	Homozygosity analysis	FW_T08402T RV_T08402T	GCTTTGATTAATGCCTGGG TGGTGAGAGTTGCAAGCATC	$T_{Ann}=58^{\circ}\text{C}$ $t_{elong}=65\text{ s (+1/cycle)}$ $Cyc=35$
<i>OsSAUR55</i> Os09g37500 (NF9863)	Homozygosity analysis	FW_NF9863 RV_NF9863	ACAAGATGCCTAATTGCATGG CAGCTTTGCCAGAATTTTCAG	$T_{Ann}=58^{\circ}\text{C}$ $t_{elong}=65\text{ s (+1/cycle)}$ $Cyc=35$

Gene & Mutant	Purpose	Name(s)	Sequence(s) (5' > 3')	Conditions
pBIN-pROK2 left border (SALK lines)	Homozygosity analysis	SALK_LBb1a	ACCGCTTGCTGCAACTCTC	See gene-specific primers for conditions.
Left border GK T-DNA	Homozygosity analysis	GK_LB1	ATATTGACCATCATACTCATTGC	See gene-specific primers for conditions.
<i>WIF2</i> At4g10270 SALK_077800C	Homozygosity analysis	FW_SALK_077800C RV_SALK_077800C	TTTGGTTTTCGATTTTGTGTTTG TGGAATTGCAATGTTGATTAGG	T _{Ann} =60°C t _{elong} =95 s Cyc=32
<i>WIF2</i> At4g10270 GK127A06	Homozygosity analysis	FW_GK12706_GSP RV_SALK_077800C	CGTTCTTAAAAGGCTAACAACGTC TGGAATTGCAATGTTGATTAGG	T _{Ann} =56°C t _{elong} =70 s (+1/cycle) Cyc=34
<i>WIF2</i> At4g10270 SALK_130465C	Homozygosity analysis	FW_SALK_130465C RV_SALK_130465C	AAACCAGGTGCACATGATAGC TTGCATTTATGGACTGAGTTGTG	T _{Ann} =60°C t _{elong} =95 s Cyc=32
<i>AtP4H7</i> At3g28480 SALK_004428	Homozygosity analysis	FW_SALK_004428 RV_SALK_004428	TGGGTCCAAAATTCCTCTACC TTCCTCATCAGAAAGAAACCC	T _{Ann} =58°C t _{elong} =70 s (+1/cycle) Cyc=34
<i>RHDP</i> At2g17850 SALK_078020C	Homozygosity analysis	FW_SALK_078020C FW_SALK_115776C	CGGAAGAAACAAGAAATTTGG CAAGAGCTGAAAAGCCTCAAAC	T _{Ann} =59°C t _{elong} =75 s Cyc=34
<i>RHDP</i> At2g17850 SALK_115776C	Homozygosity analysis	FW_SALK_115776C RV_SALK_115776C	CAAGAGCTGAAAAGCCTCAAAC TTACCAGGATGAGATGATCGG	T _{Ann} =57.5°C t _{elong} =75 s Cyc=33
<i>PHLOEM PROTEIN 2-A13</i> At3g61060 SALK_127470C	Homozygosity analysis	FW_SALK_127470 RV_SALK_127470	CATAAGCAGCTGACTGGAACC GCTCAGGAGAATTGACCTTTTG	T _{Ann} =59°C t _{elong} =75 s Cyc=35
<i>bHLH FAMILY PROTEIN</i> At2g42280 SAIL_567_D07	Homozygosity analysis	FW_SAIL_567_D07B RV_SAIL_567_D07B	TAAGTTGTACCATGCGAATGG GTCTCGTGACGTTTCAGGAAG	T _{Ann} =59°C t _{elong} =75 s Cyc=34
<i>AtCML39</i> At1g76640 SALK_078400C	Homozygosity analysis	FW_SALK_078400C RV_SALK_078400C	TTTGACCTCATCTTTGCGTTG AGAGAGTTTAAACCGCAGCCTC	T _{Ann} =56°C t _{elong} =70 s (+1/cycle) Cyc=34
<i>AtCML39</i> At1g76640 GK272A04	Homozygosity analysis	FW_GK272A04 RV_GK272A04	AAGAAGAGCCCAGAGCAGAAC CGTGTTTTAAACATGGGGATG	T _{Ann} =52°C t _{elong} =80 s Cyc=34
<i>eEF1α</i> Os03g08020	Expression analysis & qPCR	qFW_Os_eEF1a qRV_Os_eEF1a	ACTCTTGGTGTGAAGCAGATGA TGGACCTCTCAATCATGTTGTC	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=22 (100 ng)
Os10g31850 (NC0419)	Expression analysis	FW_Os10g31850_X RV_Os10g31850_X	CTTGCACTGCGGGCACACGA GGCACTTCTGCGCCAGGACG	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=26 (100 ng)
Os08g30910 (NF6033)	Expression analysis	FW_Os08g30910_X RV_Os08g30910_X	GCCCGGGCGCGGTTCTCCT ACGGGCAGGCGCCCATCCG	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=31 (100 ng)
Os02g56820 (ND3022)	Expression analysis	FW_Os02g56820_X RV_Os02g56820_X	TCGGGGGACGCCAATCCACT GCGAACCTCGAGTCGGCGAG	T _{Ann} =60°C t _{elong} =30 s (+1/cycle) Cyc=28 (100 ng)
Os08g06280 (NC0550)	Expression analysis	FW_Os08g06280_X RV_Os08g06280_X	TGCAAACCTGAAGCGGTTTCTTTTCATGT GGCACCCAGAGCACACGAGC	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=26 (100 ng)
Os10g07400 (NC2016)	Expression analysis	FW_Os10g07400_X RV_Os10g07400_X	TGCACTCAAGGAATGGCTGCGA GCCTCCAAGCCCAAGCAGAGG	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=22 (100 ng)
Os09g37500 (NF9863)	Expression analysis	FW_Os09g37500_X RV_Os09g37500_X	GCCATGATCAGCGCCAAGAGGA AGTGACCCTTGCCGGCCACC	T _{Ann} =60°C t _{elong} =35 s (+1/cycle) Cyc=28 (100 ng)

Gene & Mutant	Purpose	Name(s)	Sequence(s) (5' > 3')	Conditions
<i>WIF2</i> At4g10270	Expression analysis (including ox lines)	FW_At4g10270_X4 RV_At4g10270_X4	CAAGCAAAGCATGGACAGTG AACGTTGTTCCGGAGATGTT	T _{Ann} =60°C t _{elong} =30 s (+1/cycle) Cyc=29 (50 ng)
<i>wif2-1</i> (GK127A06) At4g10270	Expression analysis	FW_At4g10270_X6 RV_At4g10270_X5	AGAGCGAGAAGGCTAAGAAGGCT AGGACCCCAACAGCTCAAGTACA	T _{Ann} =60°C t _{elong} =25 s (+1/cycle) Cyc=31 (100 ng)
<i>AtP4H7</i> At3g28480	Expression analysis	FW_At3g28480_X RV_At3g28480_X	TTATCAAATTGGCGAAAGGGAAG TTCGGTCCAGCTATCGTCTT	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=28 (50 ng)
<i>RHDP</i> At2g17850	Expression analysis	FW_At2g17850_X RV_At2g17850_X	ACCATCGATGTAAACCAAGCTCAGA ACCCAAGCAATGTAAACCACCGTCCA	T _{Ann} =57°C t _{elong} =60 s (+1/cycle) Cyc=30 (25 ng)
<i>PHLOEM PROTEIN 2-A13</i> At3g61060	Expression analysis	FW_At3g61060_X FW_SALK_127470C	TTGCACACAAGGTGTTTCGATG CATAAGCAGCTGACTGGAACC	T _{Ann} =60°C t _{elong} =30 s (+1/cycle) Cyc=30 (150 ng)
<i>bHLH FAMILY PROTEIN</i> At2g42280	Expression analysis	FW_At2g42280_X RV_At2g42280_X	CTCTCTTCAAGGCCACCTTCT TTTGTTGCGCCCTATATTGAC	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=28 (150 ng)
<i>AtCML39</i> At1g76640 for SALK_078400	Expression analysis	FW_At1g76640_X RV_At1g76640_X	ACATGGATGCAAACAGAGACGGTAG AGCGCATCATGAGGGCGAACT	T _{Ann} =60°C t _{elong} =30 s (+1/cycle) Cyc=27 (100 ng)
<i>AtCML39</i> At1g76640 for GK272A04	Expression analysis	FW_At1g76640_X2 RV_At1g76640_X	AGCTTGGTGAGTCTAGAACCACTGA AGCGCATCATGAGGGCGAACT	T _{Ann} =60°C t _{elong} =25 s (+1/cycle) Cyc=28 (150 ng)
<i>AtActin2</i> At3g18780	Expression analysis	FW_AtAct2 RV_AtAct2	CAAAGACCAGCTCTTCCATCG CTGTGAACGATTCTTGACCT	T _{Ann} =60°C t _{elong} =40 s (+1/cycle) Cyc=23 (25 ng)
<i>AtP4H7</i> At3g28480	Cloning ox lines	FW_P4H7_CDS RV_P4H7_CDS	ATGGATCCGAAAATGGATTCTCG- GATTTT ATCTCGAGCTTAAGATGAGCAAGCTT- TGCAAC	T _{Ann} =54.5°C t _{elong} =120 s (+1/cycle) Cyc=35 (100 ng)
<i>RHDP</i> At2g17850	Cloning ox lines	FW_At2g17850_CDS_2 RV_At2g17850_CDS_S TP	ATGGATCCTCAAAAATGGATTCTTTA- CACGTTCTTAG ATCTCGAGTCAAAGCTCATCATACTT- GAGTTCC	T _{Ann} =49°C t _{elong} =65 s (+1/cycle) Cyc=38 (100 ng)
<i>WIF2</i> At4g10270	Cloning ox lines	FW_At4g10270CDS RV_At4g10270STP	AGGATCCGAAAAGATGAGTT- CTACAAGCAAAGC ATCTCGAGTTAATTAGGAC- CCCAACAGCTCAAG	T _{Ann} =55°C t _{elong} =60 s Cyc=30
<i>OsADH2</i> Os11g10510	qPCR	qFW_OsADH2 qRV_OsADH2	ATATCCTGCTTCAATGTGTCC TCTTCATGTACAGCTCGACGAC	T _{Ann} =60°C t _{elong} =30 s Cyc=40
<i>OsCML31</i> Os01g72530	qPCR	qFW_CML31 qRV_CML31	TGCTGTGCGAGGCGGAGTTC AGATCATGGCGCGGAGTTCG	same as above
At1g76940	qPCR control gene	qFW_At1g76940_2 qRV_At1g76940_2	GAGCGTGCAATCAGGAGAAG TCTGGGGATCAAAACCAAT	same as above
At3g18380	qPCR control gene	qFW_At3g18380 qRV_At3g18380	ATGATGTCAGAGTTGTCGTTG GAGTTAGCCAAATCGTTTACGG	same as above
At4g17670	qPCR	qFW_At4g17670 qRV_At4g17670	TTTCTTGGACTCTTGCTTCCTC GCTTCGCTTTTCATTTCTTCTC	same as above
At2g44670	qPCR	qFW_At2g44670 qRV_At2g44670	TGATCTTTCTATGGATCGGAGA CAACCCAGATTACAAAACCAT	same as above
At5g25940	qPCR	qFW_At5g25940 qRV_At5g25940	AGTAGCTGCCGTTTCGTATGTTT CTTTAACTGAGCCTCGGTGTTT	same as above
<i>WIF1</i> At4g10265	qPCR	qFW_At4g10265 qRV_At4g10265	TGCGAATCAGTATCTACGCAAC CATTAAC TAGACCCCAACAGC	same as above
<i>WIF2</i> At4g10270	qPCR	qFW_At4g10270 qRV_At4g10270	GGGAAAAGTTCTCTTCGTCTT TGGAATTGCAATGTTGATTAGG	same as above

Gene & Mutant	Purpose	Name(s)	Sequence(s) (5' > 3')	Conditions
<i>AtP4H7</i> At3g28480	qPCR	qFW_At3g28480 qRV_At3g28480	GTTGAGGGAGAGAAATGGTCAG ATGGTCTTTGTCTGAACCAACC	same as above
At5g18900	qPCR	qFW_At5g18900 qRV_At5g18900b	AGGAAAGGGGATGCTTTGTT GCCCATCTCTCACAGCTCTC	same as above
At5g48540	qPCR	qFW_At5g48540 qRV_At5g48540	AGTTGCTATGTCCGGTACGAGT AGCAAAGAGCCTTATTTTGCAC	same as above
At3g22060	qPCR	qFW_At3g22060 qRV_At3g22060	AAATGGAATTATTGGGGAGCTT AATTAAGACCGTGTGGCATT	same as above
At5g27760	qPCR	qFW_At5g27760 qRV_At5g27760	ACATAAGCTTCGTACCGTTGGT CAGTGGCTCCAGATTTGTGAT	same as above
<i>RHDP</i> At2g17850	qPCR	qFW_At2g17850 qRV_At2g17850	TGGCTACACGTTTCTTGATGTC TGAGATGATCGGTTTGGTTACA	same as above
At2g21045	qPCR	qFW_At2g21045 qRV_At2g21045	GAACATTTGATCGTGGCTTGTA GTGCCGATTTAGTTTTCTTTG	same as above
At5g24470	qPCR	qFW_At5g24470 qRV_At5g24470	TTAACCAAATTCGAATGAAGC TGTTGGATCACTATGGAGCTTG	same as above
At2g46790	qPCR	qFW_At2g46790 qRV_At2g46790	TGCCCTTCTTCGTAATGCT GCTCCATTACCAAAGCAAGC	same as above
At5g50915	qPCR	qFW_At5g50915 qRV_At5g50915	ATGTCGACACAAACACAAAAGC TATGTTGAAACTCTCGGGACAA	same as above
At1g32230	qPCR	qFW_At1g32230 qRV_At1g32230	CCTTTGTGGTTCTCCTTTTGAC AGAAAACAAAGACCCAACGACAT	same as above
At2g35510	qPCR	qFW_At2g35510 qRV_At2g35510	CGAAAAGAATAGCTCCAGCAGT TTCCTTTCTCGACACCTTCTTC	same as above
At3g58120	qPCR	qFW_At3g58120 qRV_At3g58120	CATGGCGAAAAGATTCTTGACT CCCTCCACTCCATAAGAAAA	same as above
At3g46640	qPCR	qFW_At3g46640 qRV_At3g46640	CTTGTTATTGCTCGTGGAACTG TGCAACATCACCAAACAAGTG	same as above
At5g59570	qPCR	qFW_At5g59570 qRV_At5g59570	AAAATCACTGGGAAGCTTGTA ATAACCGAACAAACATGTGACG	same as above
At4g10150	qPCR	qFW_At4g10150 qRV_At4g10150	AACAGCACCAATGTTGA TGCTTGGTAGTCCCAAGAC	same as above
At1g05805	qPCR	qFW_At1g05805 qRV_At1g05805	AGATCTTGTCCCAACATGG TGACCCTACCTTTGGGTTTG	same as above
At2g42280	qPCR	qFW_At2g42280 qRV_At2g42280	ATAAGCGAGCGAATGAGGAA TTTGTGCGCCCTATATTGA	same as above
At5g05280	qPCR	qFW_At5g05280 qRV_At5g05280	CGGTTGATTCATACAGTCCTGA GAATGTGACAGCAACCAAGTGT	same as above
At3g10910	qPCR	qFW_At3g10910 qRV_At3g10910	CCTCTTGCCCCACTTGTAGA CAACAAAAACCAAGCCCACT	same as above
At3g61060	qPCR	qFW_At3g61060 qRV_At3g61060	GACTCAAATCGATTGCACTCAC GCAGCCCAGATCAAGAAAAA	same as above
At1g32540	qPCR	qFW_At1g32540 qRV_At1g32540	GGAGCAAGATCAGTGAAATGTG TGAAAAAGATTTGATGCTTCCTC	same as above
At1g29490	qPCR	qFW_At1g29490 qRV_At1g29490	TATTCCACAGAAGGACCAATC TGAAGTTGCATAGAGCATTGTG	same as above
At1g29460	qPCR	qFW_At1g29460 qRV_At1g29460	TCGTGGTTTACACGGTTGATAA CCATCCATTCTTCGTTGATA	same as above
At1g75540	qPCR	qFW_At1g75540 qRV_At1g75540	CCCACAGATCACTCCTCCTC AAACCGCAATCACACTCAA	same as above
At4g39070	qPCR	qFW_At4g39070 qRV_At4g39070	AGCTCTTCTTCGACGACCAC GCAAATCCTCCACTCTCCAA	same as above
At1g01060	qPCR	qFW_At1g01060 qRV_At1g01060	ATTTGATTATTTCCGGGAACG CATCTTTGATCTCCCAACA	same as above
At5g43650	qPCR	qFW_At5g43650 qRV_At5g43650	AGGATGATGTGCGCCGAAGATA TGGTAGTAGAGAAATGGAGAGCTAAGT	same as above

Gene & Mutant	Purpose	Name(s)	Sequence(s) (5' > 3')	Conditions
At5g42380	qPCR	qFW_At5g42380 qRV_At5g42380	TCTCCGGTGAAGAGCTACAAA CGCTTCTTTCAACTCCTTCCT	same as above
<i>AtCML39</i> At1g76640	qPCR	qFW_At1g76640 qRV_At1g76640	GGATGCAAACAGAGACGGTAG TTCCCTTTGATAAGCAGAGCA	same as above
<i>AtCML38</i> At1g76650	qPCR	qFW_At1g76650 qRV_At1g76650	AGCCGGAGAGATACAACAACA CAATCTAACAGCAGCTACGGC	same as above
At1g73680	qPCR	qFW_At1g73680 qRV_At1g73680	ACCGGACTGCTAGACTTGTGA CAAATCTTGCTCCAACCATGT	same as above
At5g10090	qPCR	qFW_At5g10090 qRV_At5g10090	CACGACTCAAGAAACTCAGTC AATCCCAAGCTTAGCGTTAC	same as above
At5g65160	qPCR	qFW_At5g65160 qRV_At5g65160	GGTCGGTTATGCTGGTTTCT GAACCGTCCAGATTTAAACAGC	same as above

Supplemental Tables 3: Detailed statistical data of experiments performed in this study. Abbreviations: *n*: number of independent experiments; SD: Standard deviation; ND: Normally distributed data; EQV: Data samples have equal variance in comparison to WT.

Supplemental Table 3A: Arabidopsis qPCR statistics (Figures 11, 12). Conditions were 0 h, 1 h, 2 h, and 4 h of 5% O₂ and the results are listed in the order presented here except for 0 h, as values were compared to that. They were sorted by the intensity of regulation of the rice homolog in the microarray. Statistical analysis was performed on Δ Ct values normalized against the two reference genes used separately. In cases where only one of these analyses resulted in significant regulation, this is nevertheless presented in the result and is marked in the table. Statistical analysis was only performed for the data presented in Figure 12. At least 8 seedlings were used per experiment. Abbreviations used are explained above.

Gene	<i>n</i> per condition 1 h / 2 h / 4 h	Mean regulation 1 h / 2 h / 4 h	Standard deviation 1 h / 2 h / 4 h	Statistical parameters (where determined)	Two-sided student's <i>t</i> -test compared to untreated
At4g10265	4 / 4 / 4	18.2 / 18.7 / 12.5	17.29 / 21.84 / 16.2	ND, EQV	$P < 0.05$ / < 0.05 / < 0.05
At4g10270	4 / 4 / 4	5.9 / 10.3 / 9.8	3.97 / 9.54 / 12.30	ND, EQV (only At3g18380 as reference)	$P > 0.05$ / > 0.05 / < 0.05 (only At3g18380 as reference)
At3g28480	4 / 4 / 4	1.3 / 1.4 / 1.9	0.41 / 0.59 / 1.64		
At5g48540	4 / 4 / 4	1.8 / 1.5 / 1.3	1.17 / 0.58 / 0.15		
At3g22060	3 / 3 / 3	1.1 / 0.7 / 0.7	0.39 / 0.31 / 0.26		
At5g24470	3 / 3 / 3	1.5 / 1.0 / 0.7	0.94 / 0.60 / 0.42		
At1g32230	3 / 3 / 3	1.2 / 0.7 / 0.8	0.5 / 0.30 / 0.29		
At2g35510	3 / 3 / 3	1.3 / 0.9 / 1.2	0.42 / 0.19 / 0.16		
At3g58120	3 / 3 / 3	0.7 / 0.8 / 0.2	0.7 / 0.40 / 0.15	ND, EQV	$P > 0.05$ / > 0.05 / < 0.05
At3g46640	3 / 3 / 3	0.7 / 1.2 / 0.7	0.11 / 0.22 / 0.13		
At5g59570	3 / 3 / 3	0.6 / 0.9 / 0.7	0.45 / 0.69 / 0.41		
At5g05280	3 / 3 / 3	1.2 / 1.1 / 0.6	0.57 / 0.13 / 0.27		
At3g61060	3 / 3 / 3	1.6 / 1.6 / 0.7	1.21 / 0.68 / 0.23		
At1g32540	4 / 4 / 4	0.9 / 2.4 / 1.7	0.14 / 0.38 / 0.47		
At1g29490	3 / 3 / 3	0.8 / 0.9 / 0.4	0.49 / 0.20 / 0.17		
At1g29460	3 / 3 / 3	0.9 / 1.1 / 0.5	0.67 / 0.33 / 0.27		
At4g17670	4 / 4 / 4	2.1 / 2.8 / 2.3	0.87 / 2.20 / 1.55		
At2g44670	3 / 3 / 3	1.3 / 0.9 / 1.3	0.33 / 0.32 / 0.10		
At5g25940	3 / 3 / 3	0.8 / 1.0 / 0.8	0.19 / 0.31 / 0.13		
At5g18900	3 / 3 / 3	0.8 / 1.0 / 0.10	0.38 / 0.29 / 0.17		
At5g27760	3 / 3 / 3	0.10 / 0.10 / 0.7	0.13 / 0.22 / 0.9		
At2g17850	4 / 4 / 4	6.6 / 18.6 / 12.5	10.58 / 33.16 / 19.47		
At2g21045	3 / 3 / 3	0.10 / 0.10 / 0.9	0.28 / 0.28 / 0.32		
At2g46790	3 / 3 / 3	2.4 / 1.7 / 0.10	1.14 / 0.86 / 0.54		
At5g50915	2 / 2 / 2	0.2 / 0.3 / 0.1	0.25 / 0.32 / 0.21		
At4g10150	2 / 2 / 2	1.7 / 1.4 / 0.10	1.12 / 1.49 / 0.64		
At1g05805	2 / 2 / 2	1.0 / 0.9 / 0.8	0.12 / 0.4 / 0.15		
At2g42280	3 / 3 / 3	1.2 / 1.7 / 1.8	0.69 / 0.99 / 0.95		
At3g10910	2 / 2 / 2	1.5 / 1.1 / 1.1	0.45 / 0.34 / 0.1		
At1g75540	2 / 2 / 2	0.9 / 0.9 / 0.9	0.7 / 0.10 / 0.3		
At4g39070	2 / 2 / 2	0.6 / 0.6 / 0.8	0.15 / 0.29 / 0.31		
At5g10090	3 / 3 / 3	1.3 / 1.1 / 1.3	0.54 / 0.34 / 0.59		
At5g65160	3 / 3 / 3	0.9 / 0.8 / 0.7	0.33 / 0.13 / 0.25		
At1g73680	3 / 3 / 3	0.9 / 0.9 / 0.7	0.45 / 0.17 / 0.6		
At5g42380	3 / 3 / 3	2.2 / 1.7 / 1.5	0.43 / 0.46 / 0.79		
At1g76640	3 / 3 / 3	5.7 / 1.7 / 0.8	3.23 / 0.33 / 0.21	ND, EQV (only At3g18380)	$P < 0.001$ / < 0.05 / < 0.05 (only At3g18380)
At1g76650	3 / 3 / 3	7.10 / 9.3 / 5.1	1.42 / 6.56 / 2.10	ND	$P > 0.05$ / < 0.05 / > 0.05 (only At1g76940)
At5g43650	3 / 3 / 3	3.1 / 0.9 / 0.8	1.33 / 0.80 / 0.89		
At1g01060	3 / 3 / 3	23.1 / 10.5 / 1.0	25.95 / 13.27 / 0.64		

Supplemental Table 3B: Rice mutants' qPCR statistics. Rice plants grown for 7 to 24 weeks were submerged for 3 days. The intercalary meristem and basal elongation zone tissues of 2-6 plants per experiment were harvested. RNA was isolated from the tissue and qPCR was performed using the respective primers listed in Supplemental Table 2. No means of fold changes can be given for the respective controls as the expression was normalized to the non-flooded plants. SD can only be given for WT. For the mutants, the number of repeats was too low. Abbreviations used are explained above.

Genotype	Conditions	Number of experiments performed (<i>n</i>)	Mean FCh <i>ADH2</i>	SD FCh <i>ADH2</i>	Mean FCh <i>CML31</i>	SD FCh <i>CML31</i>
WT	Untreated	4	-			
	Flooded	4	-1.62	4.06	-1.88	5.02
<i>F-BOX CONTAINING</i> (NC3022)	Untreated	1				
	Flooded	1	11.47		-2.66	
<i>LSD1 ZnF LIKE</i> (NC0550)	Untreated	0				
	Flooded	1	4.41		-2.33	
DISEASE RESISTANCE RPP13-LIKE (NC2016)	Untreated	1				
	Flooded	2	2.49		1.29	

Supplemental Table 3C: Statistical information on survival assays of Arabidopsis T-DNA insertion mutants (Figure 15, Supplementary Figure 4). For each experiment, 5-34 seedlings were analyzed. For one experiment, the seedlings were kept for 2 d at near anoxia in the dark and were analyzed after a recovery phase of 5 days. Categories used were described in Figure 15A. Categories II and III are given in the table below. No statistical analysis was performed for these. Mean percentages with SD are given. Abbreviations used are explained above.

Line	Data of Category I (normal growth, undamaged)					Data of Category IV (dead seedling)				
	Mean percentage \pm SD	<i>n</i> experiments	Data variation		<i>P</i> =	Mean percentage \pm SD	<i>n</i> experiments	Data variation		<i>P</i> =
WT	24.5 \pm 19.8	25	ND	-	-	11.6 \pm 12.5	25	ND	-	-
<i>rap2.2</i> ko799	6.8 \pm 7.7	6	ND	EQV	0.050	20.7 \pm 16.5	6	ND	EQV	0.170
<i>wif2-2</i> (SALK_077800C)	5.0 \pm 6.0	4	ND	EQV	0.050	24.4 \pm 16.7	4	ND	EQV	0.104
<i>wif2-1</i> (GK127A06)	8.1 \pm 13.0	7		EQV	0.027	43.3 \pm 28.9	6	ND	EQV	0.002
<i>wif2-3</i> (SALK_130465C)	25.0 \pm 27.3	4	ND	EQV	0.995	24.0 \pm 12.4	4	ND	EQV	0.080
SALK_004428	39.4 \pm 29.7	6	ND	EQV	0.257	11.8 \pm 14.9	6	ND	EQV	0.995
<i>rhdp-1</i> (SALK_078020C)	5.7 \pm 8.8	4	ND	EQV	0.053	29.1 \pm 26.6	4	ND	EQV	0.063
<i>rhdp-2</i> (SALK_115776C)	34.2 \pm 29.7	6	ND	EQV	0.229	14.3 \pm 8.1	6	ND	EQV	0.372
<i>prp9-1</i>	29.7 \pm 18.0	4	ND	EQV	0.387	17.0 \pm 8.4	4	ND	EQV	0.276
SALK_127470	50.8 \pm 22.7	4	ND	EQV	0.032	6.8 \pm 10.9	5	ND	EQV	0.235
SAIL_567_D07	4.7 \pm 7.4	4	ND	EQV	0.040	48.5 \pm 10.5	4	ND	EQV	0.001
<i>cml39-1</i> (SALK_078400)	10.8 \pm 4.2	5	ND		0.115	20.0 \pm 16.1	5	ND	EQV	0.216
<i>cml39-2</i> (GK272A04)	0.7 \pm 1.4	4	ND	EQV	0.005	31.6 \pm 26.2	4	ND	EQV	0.051

Line	Data of Category II (minor damage)		Data of Category III (severe damage)	
	Mean percentage \pm SD	<i>n</i> experiments	Mean percentage \pm SD	<i>n</i> experiments
WT	28.5 \pm 13.3	25	31.8 \pm 22.0	25
<i>rap2.2</i> ko799	19.5 \pm 19.3	6	53.1 \pm 22.6	6
<i>wif2-2</i> (SALK_077800C)	16.8 \pm 7.9	4	53.8 \pm 9.2	4
<i>wif2-1</i> (GK127A06)	12.5 \pm 15.9	7	33.6 \pm 7.5	6
<i>wif2-3</i> (SALK_130465C)	23.5 \pm 12.9	4	27.4 \pm 21.4	4
SALK_004428	12.9 \pm 11.6	6	21.9 \pm 22.2	6
<i>rhdp-1</i> (SALK_078020C)	11.6 \pm 8.8	4	53.6 \pm 22.9	4
<i>rhdp-2</i> (SALK_115776C)	28.4 \pm 14.5	6	23.2 \pm 11.10	6
<i>prp9-1</i>	20.5 \pm 9.2	4	45.5 \pm 18.0	4
SALK_127470	12.2 \pm 9.9	4	33.7 \pm 20.7	5
SAIL_567_D07	8.5 \pm 10.8	4	38.3 \pm 19.7	4
<i>cml39-1</i> (SALK_078400)	22.5 \pm 11.4	5	46.7 \pm 16.10	5
<i>cml39-2</i> (GK272A04)	6.0 \pm 7.9	4	61.7 \pm 19.9	4

Supplemental Table 3D: Statistical information on survival assays of Arabidopsis overexpressing mutants (Figure 18, Supplementary Figure 5). For each experiment, 5-34 seedlings were analyzed. Statistical analysis of *RHDPox-14* in Category IV was not possible as no seedlings were found to belong to that category in any experiment. The reduction in dead seedling number in *AtP4H7ox-9* is not significant because for data that was not normally distributed, and for samples like that a confidence level of $P \leq 0.001$ was demanded. Mean percentages with SD are given. Abbreviations used are explained above.

Line	Data of Category I (normal growth, undamaged)					Data of Category IV (dead seedling)				
	Mean percentage \pm SD	<i>n</i> experiments	Data variation		<i>P</i> =	Mean percentage \pm SD	<i>n</i> experiments	Data variation		<i>P</i> =
WT	24.5 \pm 19.8	25	ND	-	-	11.6 \pm 12.5	25	ND	-	-
<i>WIF2ox-10</i>	40.8 \pm 26.9	6	ND	EQV	0.240	4.1 \pm 4.8	6		EQV	0.145
<i>WIF2ox-11</i>	19.3 \pm 20.8	7	ND	EQV	0.438	13.0 \pm 12.6	7	ND	EQV	0.562
<i>AtP4H7ox-9</i>	1.1 \pm 2.2	4		EQV	0.004	45.4 \pm 32.5	4	ND	EQV	0.003
<i>AtP4H7ox-11</i>	4.7 \pm 4.1	4	ND	EQV	0.051	49.6 \pm 18.2	4	ND	EQV	0.001
<i>RHDPox-5</i>	21.3 \pm 21.4	6	ND	EQV	0.744	8.4 \pm 8.9	6	ND	EQV	0.728
<i>RHDPox-14</i>	54.2 \pm 28.6	6	ND	EQV	0.013	0	6	Impossible, all 0%.		

Line	Data of Category II (minor damage)		Data of Category III (severe damage)	
	Mean percentage \pm SD	<i>n</i> experiments	Mean percentage \pm SD	<i>n</i> experiments
WT	28.5 \pm 13.3	25	31.8 \pm 22.0	25
<i>WIF2ox-10</i>	23.0 \pm 9.0	6	32.1 \pm 33.6	6
<i>WIF2ox-11</i>	19.5 \pm 13.5	7	48.2 \pm 13.4	7
<i>AtP4H7ox-9</i>	6.7 \pm 2.4	4	46.8 \pm 24.1	4
<i>AtP4H7ox-11</i>	13.1 \pm 15.4	4	32.6 \pm 23.5	4
<i>RHDPox-5</i>	26.0 \pm 11.6	6	44.2 \pm 28.2	6
<i>RHDPox-14</i>	23.9 \pm 23.10	6	21.9 \pm 18.3	6

Supplemental Table 3E: Statistical information on ADH activity of Arabidopsis T-DNA insertion mutants and WT (Figure 14). In each experiment, 1-2 plates were used per genotype with 15-37 10-day-old seedlings. Hypoxia treated seedlings were incubated for 4 h in 5% O₂ in the light. The *P*-value was generated by comparing to 21% O₂ WT and 5% O₂ WT, respectively. Abbreviations used are explained above. For each line, *n* independent experiments were performed.

Line	Data of 21% O ₂ (controls)					Data of 5%O ₂ (hypoxia treatment)				
	Mean ADH activity ±SD [nmol min ⁻¹ mg ⁻¹]	<i>n</i>	Data variation		<i>P</i> =	Mean ADH activity ±SD [nmol min ⁻¹ mg ⁻¹]	<i>n</i>	Data variation		<i>P</i> =
WT	5.9 ± 3.1	15	ND	-	-	28.6 ± 6.5	15	ND	-	-
SALK_077800C	7.9 ± 3.5	7	ND	EQV	0.192	32.5 ± 10.6	7	ND	EQV	0.298
<i>wif2-1</i> (GK127A06)	5.4 ± 1.9	7	ND	EQV	0.688	36.8 ± 11.1	7	ND	EQV	0.040
SALK_130465C	5.7 ± 1.4	6	ND	EQV	0.847	21.10 ± 6.5	5	ND	EQV	0.061
SALK_004428	5.3 ± 0.8	6	ND		0.439	22.4 ± 7.9	6	ND	EQV	0.075
<i>rhdp-1</i> (SALK_078020C)	7.6 ± 4.9	3	ND	EQV	0.442	27.4 ± 11.9	3	ND	EQV	0.786
<i>rhdp-2</i> (SALK_115776C)	9.5 ± 6.5	4	ND		0.363	39.9 ± 22.3	4			0.390
<i>prp9-1</i>	5.7 ± 0.0	2	-	-	-	42.4	2	-	-	-
SALK_127470	5.10 ± 1.6	3	ND	EQV	0.983	29.5 ± 5.6	3	ND	EQV	0.824
SAIL_567_D07	6.5 ± 1.8	6	ND	EQV	0.667	36.2 ± 11.6	6	ND		0.184
SALK_078400	5.4 ± 0.9	3	ND	EQV	0.763	35.9 ± 7.8	4	ND	EQV	0.070
GK272A04	7.3 ± 2.10	6	ND	EQV	0.376	39.5 ± 3.9	6	ND	EQV	0.001

Supplemental Table 3F: Additional information on ADH activity of rice *Tos17* retrotransposon insertion mutants and WT (Figure 9) as well as qPCR analysis of these plants (Figure 10). Plants of the age of 7-24 weeks were flooded for 3 days at long-day conditions. Then, the intercalary meristem and basal elongation zone tissue of the youngest internode were harvested and flash frozen and ADH activity was determined as specified in Materials and Methods. Here, the number of experiments n and, if >2 the SD of the mean is given for WT and the insertion mutants studied. No SD is given for the non-flooded controls in the qPCR experiments as all data was compared to these making them equal to 1 by definition. Therefore, FCh and SD are given for the flooded plants. Two to six plants per genotype and treatment were used. Abbreviations used are explained above. For each line, n independent experiments were performed.

Line	Data of non-flooded controls		Data of flooded plants	
	ADH assays	qPCRs	ADH assays	qPCRs
WT	$n = 3$	$n = 4$	$n = 3$	$n = 4$
	314 ± 31 [nmol min ⁻¹ mg ⁻¹]		726 ± 159 [nmol min ⁻¹ mg ⁻¹]	FCh _{ADH2} = -1.6 ± 4.1 FCh _{CML31} = -1.9 ± 5.0
YDG/SRA DOMAIN CONTAINING (NF6033)	$n = 1$	-	$n = 1$	-
	346 [nmol min ⁻¹ mg ⁻¹]		721 [nmol min ⁻¹ mg ⁻¹]	
F-BOX CONTAINING (ND3022)	$n = 1$	$n = 1$	$n = 1$	$n = 1$
			468 [nmol min ⁻¹ mg ⁻¹]	FCh _{ADH2} = 11.47 FCh _{CML31} = -2.66
LSD1 ZnF LIKE (NC0550)	$n = 2$	$n = 1$	$n = 2$	$n = 1$
	337 [nmol min ⁻¹ mg ⁻¹]		733 [nmol min ⁻¹ mg ⁻¹]	FCh _{ADH2} = 4.4 FCh _{CML31} = -2.3
DISEASE RESISTANCE RPP13-LIKE (NC2016)	$n = 1$	$n = 2$	$n = 1$	$n = 2$
			428 [nmol min ⁻¹ mg ⁻¹]	FCh _{ADH2} = 2.49 FCh _{CML31} = 1.29
OsSAUR55 (NF9863)	$n = 2$	-	$n = 2$	-
	457 [nmol min ⁻¹ mg ⁻¹]		540 [nmol min ⁻¹ mg ⁻¹]	

Supplemental Table 3G: Statistical information on waterlogging experiments of Arabidopsis T-DNA insertion and overexpression mutants (Figures 16, 19). Data is separated in fresh weight, anthocyanin content and chlorophyll content assays. The respective data given is the mean \pm SD. The number of experiments n , the minimum and maximum number of plants tested per experiment, and the total number of plants tested are presented in parentheses. The P -value is a result of a two-sided student's t -test in comparison to WT performed as described in Materials and Methods. Note that for the overexpression lines, experiments of different transgenic lines expressing the same gene were combined for statistical analysis. Below, they are listed separately.

Fresh weight					
Line	[mg per plant]	Statistical data			
WT	45.1 \pm 24.0	$n = 18$ (5-25; 264 total)		-	-
<i>wif2-2</i> (SALK_077800C)	44.5 \pm 11.0	$n = 5$ (2-21; 71 total)	ND	EQV	$P = 0.341$
<i>wif2-1</i> (GK127A06)	46.4 \pm 28.3	$n = 9$ (5-18; 94 total)	ND	EQV	$P = 0.313$
<i>wif2-3</i> (SALK_130465C)	40.2 \pm 12.2	$n = 5$ (3-17; 54 total)	ND		$P = 0.073$
SALK_004428	61.0 \pm 31.0	$n = 8$ (6-20; 93 total)	ND	EQV	$P = 0.980$
<i>rhdp-1</i> (SALK_078020C)	70.9 \pm 38.8	$n = 6$ (6-16; 63 total)	ND	EQV	$P = 0.429$
<i>rhdp-2</i> (SALK_115776C)	63.7 \pm 36.1	$n = 5$ (6-16; 64 total)	ND	EQV	$P = 0.725$
<i>prp9-1</i>	32.1 \pm 13.9	$n = 5$ (8-19; 92 total)	ND	EQV	$P = 0.146$
SALK_127470	55.1 \pm 17.9	$n = 5$ (7-13; 46 total)	ND	EQV	$P = 0.726$
SAIL_567_D07	41.0 \pm 18.8	$n = 4$ (11-17; 56 total)	ND	EQV	$P = 0.315$
<i>cml39-1</i> (SALK_078400)	43.6 \pm 31.5	$n = 4$ (3-34; 70 total)	ND	EQV	$P = 0.639$
<i>cml39-2</i> (GK272A04)	30.2 \pm 17.8	$n = 4$ (3-34; 67 total)	ND	EQV	$P = 0.157$
<i>WIF2ox-10</i>	26.8	$n = 1$ (9 total)	ND		$P = 0.003$
<i>WIF2ox-11</i>	31.1	$n = 2$ (11, 15; 26 total)			
<i>AtP4H7ox-9</i>	57.2	$n = 2$ (5-11; 16 total)	ND	EQV	$P = 0.190$
<i>AtP4H7ox-11</i>	36.5 \pm 17.5	$n = 6$ (6-11; 61 total)			
<i>RHDPox-5</i>	37	$n = 1$ (4 total)	ND	EQV	$P = 0.050$
<i>RHDPox-14</i>	15.8 \pm 13.5	$n = 3$ (8 total)			
Anthocyanin content					
Line	[per mg fresh weight]	Statistical data			
WT	18.2 \pm 6.9	$n = 13$ (5-25; 189 total)	ND	-	-
<i>wif2-2</i> (SALK_077800C)	16.5 \pm 6.1	$n = 5$ (2-21; 71 total)	ND	EQV	$P = 0.341$
<i>wif2-1</i> (GK127A06)	22.1 \pm 7.8	$n = 6$ (5-18; 75 total)	ND	EQV	$P = 0.313$
<i>wif2-3</i> (SALK_130465C)	14.1 \pm 2.9	$n = 3$ (8-17; 41 total)	ND		$P = 0.073$
SALK_004428	19.2 \pm 11.6	$n = 3$ (13-20; 50 total)	ND	EQV	$P = 0.980$
<i>rhdp-1</i> (SALK_078020C)	13.1 \pm 3.3	$n = 3$ (7-16; 32 total)	ND	EQV	$P = 0.429$
<i>rhdp-2</i> (SALK_115776C)	16.3 \pm 7.6	$n = 3$ (12-16; 43 total)	ND	EQV	$P = 0.725$
<i>prp9-1</i>	22.5 \pm 10.9	$n = 5$ (8-19; 92 total)	ND	EQV	$P = 0.146$
SALK_127470	14.3 \pm 2.40	$n = 3$ (7-13; 31 total)	ND		$P = 0.726$
SAIL_567_D07	22.3 \pm 3.8	$n = 4$ (11-17; 56 total)	ND	EQV	$P = 0.315$
<i>cml39-1</i> (SALK_078400)	29.1 \pm 19.5	$n = 3$ (3-34; 57 total)	ND		$P = 0.639$
<i>cml39-2</i> (GK272A04)	20.2 \pm 6.1	$n = 3$ (3-19; 33 total)	ND	EQV	$P = 0.157$
<i>WIF2ox-10</i>	21.3	$n = 1$ (9 total)	ND	EQV	$P = 0.815$
<i>WIF2ox-11</i>	15.1	$n = 2$ (11, 15; 26 total)			
<i>AtP4H7ox-9</i>	10.3	$n = 2$ (5, 11; 16 total)			$P = 0.312$
<i>AtP4H7ox-11</i>	37.6 \pm 29.3	$n = 5$ (6-11; 50 total)			
<i>RHDPox-5</i>	8.7	$n = 1$ (4 total)	ND	EQV	$P = 0.674$
<i>RHDPox-14</i>	19.9	$n = 2$ (9, 10; 19 total)			

(continued on following page)

(Supplemental Table 3G, continued)

Chlorophyll content					
Line	[per mg fresh weight]	Statistical data			
WT	0.311 ± 0.063	<i>n</i> = 11 (7-25; 167 total)	ND	-	-
<i>wif2-2</i> (SALK_077800C)	0.315 ± 0.082	<i>n</i> = 4 (12-21; 69 total)	ND	EQV	<i>P</i> = 0.916
<i>wif2-1</i> (GK127A06)	0.316 ± 0.081	<i>n</i> = 6 (5-18; 75 total)	ND	EQV	<i>P</i> = 0.882
<i>wif2-3</i> (SALK_130465C)	0.231 ± 0.091	<i>n</i> = 3 (8-17; 41 total)	ND	EQV	<i>P</i> = 0.099
SALK_004428	0.318 ± 0.045	<i>n</i> = 4 (13-20; 66 total)	ND	EQV	<i>P</i> = 0.845
<i>rhd1-1</i> (SALK_078020C)	0.287 ± 0.088	<i>n</i> = 3 (7-16; 32 total)	ND	EQV	<i>P</i> = 0.608
<i>rhd1-2</i> (SALK_115776C)	0.300 ± 0.068	<i>n</i> = 3 (12-16; 43 total)	ND	EQV	<i>P</i> = 0.806
<i>pr1-1</i>	0.353 ± 0.065	<i>n</i> = 5 (8-19; 92 total)	ND	EQV	<i>P</i> = 0.205
SALK_127470	0.346 ± 0.067	<i>n</i> = 3 (7-13; 31 total)	ND	EQV	<i>P</i> = 0.405
SAIL_567_D07	0.359 ± 0.100	<i>n</i> = 4 (11-17; 40 total)	ND	EQV	<i>P</i> = 0.283
<i>cml39-1</i> (SALK_078400)	0.238 ± 0.078	<i>n</i> = 3 (7-34; 61 total)	ND	EQV	<i>P</i> = 0.115
<i>cml39-2</i> (GK272A04)	0.403 ± 0.251	<i>n</i> = 3 (4-34; 57 total)	ND		<i>P</i> = 0.592
<i>WIF2ox-10</i>	0.285	<i>n</i> = 1 (9 total)	ND	EQV	<i>P</i> = 0.711
<i>WIF2ox-11</i>	0.346	<i>n</i> = 2 (11, 15; 26 total)			
<i>AtP4H7ox-9</i>	0.266	<i>n</i> = 2 (5, 11; 16 total)	ND	EQV	<i>P</i> = 0.490
<i>AtP4H7ox-11</i>	0.360 ± 0.099	<i>n</i> = 6 (6-11; 50 total)			
<i>RHDPox-5</i>	0.257	<i>n</i> = 1 (4 total)	ND	EQV	<i>P</i> = 0.783
<i>RHDPox-14</i>	0.357	<i>n</i> = 2 (5, 9; 14 total)			

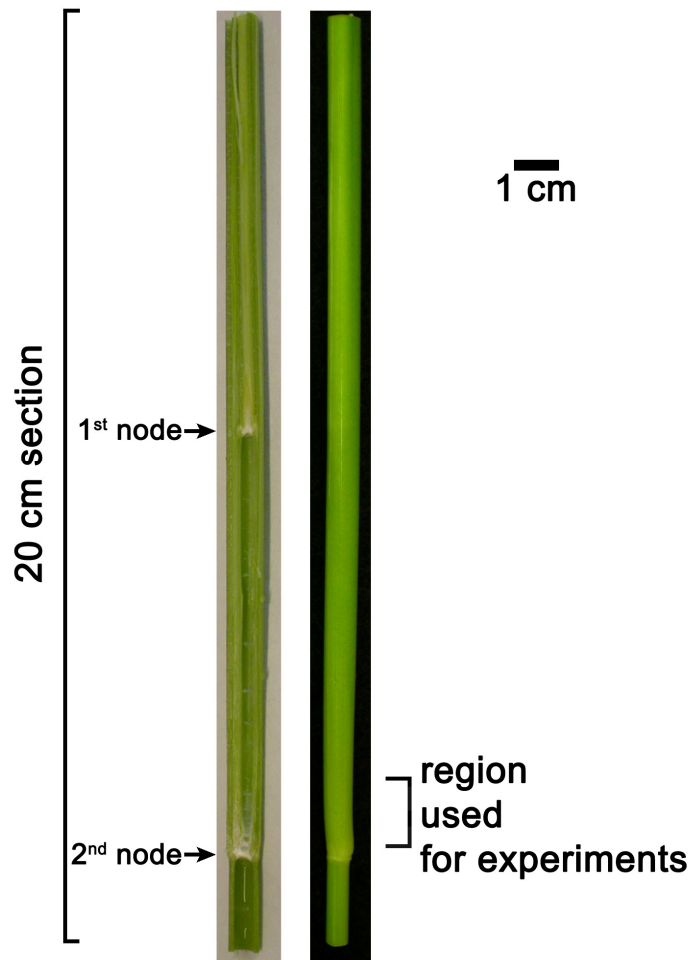
Supplemental Table 4: Rice genes that are regulated in plants that were treated with 1 h of 5% oxygen stronger than 3-fold in comparison to the expression in untreated plants were selected for detailed analysis, mutant selection and homozygosity comparisons with Arabidopsis. Genes that were selected for mutant analysis are highlighted in red.

TIGR locus ID	TIGR version 6.1 transcript assignment	FCh at 5% O ₂	Category
Os06g03520	DUF581 domain containing protein	223.5	expressed protein
Os04g54310	Conserved hypothetical protein	61.0	hypothetical protein
Os07g09630	Oxidoreductase, putative	60.8	expressed protein
Os08g04210	Cysteine-rich repeat secretory protein 55 precursor, putative	21.5	expressed protein
Os08g03420	Kelch repeat protein, putative	21.3	expressed protein
Os09g31000	EF hand family protein	12.7	signaling
Os09g37369	<i>OsSAUR41</i> - Auxin-responsive SAUR gene family member	11.0	unknown
Os03g58890	Oxidoreductase, putative	9.6	expressed protein
Os11g02080	Expressed protein	9.4	expressed protein
Os04g17660	Rhodanese-like domain containing protein, putative	9.1	expressed protein
Os09g36220	Response regulator receiver domain containing protein	8.5	signaling
Os04g42470	Regulatory subunit, putative	8.2	signaling
Os10g31850	RING finger and CHY zinc finger domain-containing protein 1, putative	6.0	transcription factor
Os09g22540	Transcription factor jumonji, putative	5.9	transcription factor
Os07g29310	<i>OsSAUR30</i> - Auxin-responsive SAUR gene family member	5.9	auxin signaling
Os08g30910	YDG/SRA domain containing protein	5.5	signaling
Os02g56820	<i>OsFBX69</i> - F-box domain containing protein	5.5	signaling
Os03g63770	RCD1, putative	5.4	signaling
Os09g37480	<i>OsSAUR53</i> - Auxin-responsive SAUR gene family member	4.9	auxin signaling
Os01g07350	Transposon protein, putative, unclassified	4.9	phosphatase
Os01g11350	bZIP transcription factor domain containing protein	4.8	transcription factor
Os08g38880	WD-40 repeat family protein, putative	4.7	expressed protein

TIGR locus ID	TIGR version 6.1 transcript assignment	FCh at 5% O ₂	Category
Os04g48510	Growth regulating factor protein, putative	4.7	transcription factor
Os02g01700	RNA recognition motif containing protein, putative	4.7	transcription factor
Os01g39890	Transposon protein, putative, unclassified	4.6	primary metabolism
Os01g74020	MYB family transcription factor, putative	4.6	transcription factor
Os04g13480	Response regulator receiver domain containing protein	4.5	CK signaling
Os03g20500	OsFBX82 - F-box domain containing protein	4.2	signaling
Os01g11460	Zinc finger, C3HC4 type domain containing protein	4.2	transcription factor
Os11g44810	Auxin-repressed protein, putative	4.2	auxin signaling
Os02g32590	HSF-type DNA-binding domain containing protein	4.1	transcription factor
Os04g41229	Helix-loop-helix DNA-binding domain containing protein	4.1	transcription factor
Os01g06560	Transcription factor HBP-1b, putative	4.0	transcription factor
Os01g60730	RING-H2 finger protein, putative	3.9	transcription factor
Os04g31340	CBS domain containing membrane protein, putative	3.9	kinase
Os10g37830	OsFBX391 - F-box domain containing protein	3.8	signaling
Os02g46690	OsFBD8 - F-box and FBD domain containing protein	3.8	signaling
Os06g46910	ZOS6-07 - C2H2 zinc finger protein	3.7	transcription factor
Os05g50190	Protein kinase domain containing protein	3.7	kinase
Os09g30506	EF hand family protein	3.7	signaling
Os03g20870	Zinc finger, C3HC4 type domain containing protein	3.5	transcription factor
Os01g37960	Hydrolase, putative	3.5	auxin signaling
Os11g34460	OsFBO10 - F-box and other domain containing protein	3.4	signaling
Os08g06280	LSD1 zinc finger domain containing protein	3.4	transcription factor
Os10g07400	Disease resistance RPP13-like protein 1, putative	3.4	signaling
Os08g01780	OsIAA25 - Auxin-responsive Aux/IAA gene family member	3.4	auxin signaling
Os05g29810	AP2 domain containing protein	3.4	transcription factor
Os01g21590	Homeodomain, putative	3.3	transcription factor
Os04g03980	Flavin monooxygenase, putative	3.3	auxin homeostasis
Os04g54240	Wound induced protein, putative	3.3	stress
Os09g37500	OsSAUR55 - Auxin-responsive SAUR gene family member	3.3	auxin signaling
Os01g06640	Basic helix-loop-helix, putative	3.3	transcription factor
Os09g28810	RNA recognition motif containing protein, putative	3.2	nucleic acid binding
Os09g25090	CAMK_KIN1/SNF1/Nim1_like.34 - CAMK includes calcium/calmodulin dependent protein kinases	3.2	kinase
Os12g39630	CAMK_CAMK_like.49 - CAMK includes calcium/calmodulin dependent protein kinases	3.2	ABA signaling
Os02g43170	B-box zinc finger family protein, putative	3.2	transcription factor
Os05g48870	Auxin response factor 15, putative	3.2	auxin signaling
Os01g65900	Chitin-inducible gibberellin-responsive protein, putative	3.1	transcription factor
Os02g08364	Protein phosphatase 2C, putative	3.1	phosphatase
Os06g24070	MYB-like DNA-binding domain containing protein	3.1	transcription factor
Os01g34060	MYB family transcription factor, putative	3.1	transcription factor
Os01g01340	Light-induced protein 1-like, putative	3.0	signaling
Os06g04850	Homeobox associated leucine zipper, putative	3.0	transcription factor
Os02g05470	CCT motif family protein	3.0	signaling
Os03g53750	Nuclear prelamin A recognition factor, putative	-3.0	signaling
Os05g49140	CGMC_MAPKCMGC_2.8 - CGMC includes CDA, MAPK, GSK3, and CLKC kinases	-3.0	kinase
Os01g48330	Protein kinase domain containing protein	-3.0	kinase
Os03g18340	Transcription factor like protein, putative	-3.0	transcription factor
Os01g37460	Zinc finger family protein, putative	-3.1	transcription factor
Os06g40180	Phospholipase D, putative	-3.2	ABA signaling
Os01g66120	No apical meristem protein, putative	-3.2	transcription factor
Os01g73770	Dehydration-responsive element-binding protein, putative	-3.2	transcription factor
Os06g10160	Tyrosine protein kinase domain containing protein, putative	-3.2	kinase
Os01g09100	OsWRKY10 - Superfamily of TFs having WRKY and zinc finger domains	-3.3	transcription factor
Os01g39330	helix-loop-helix DNA-binding domain containing protein	-3.3	transcription factor

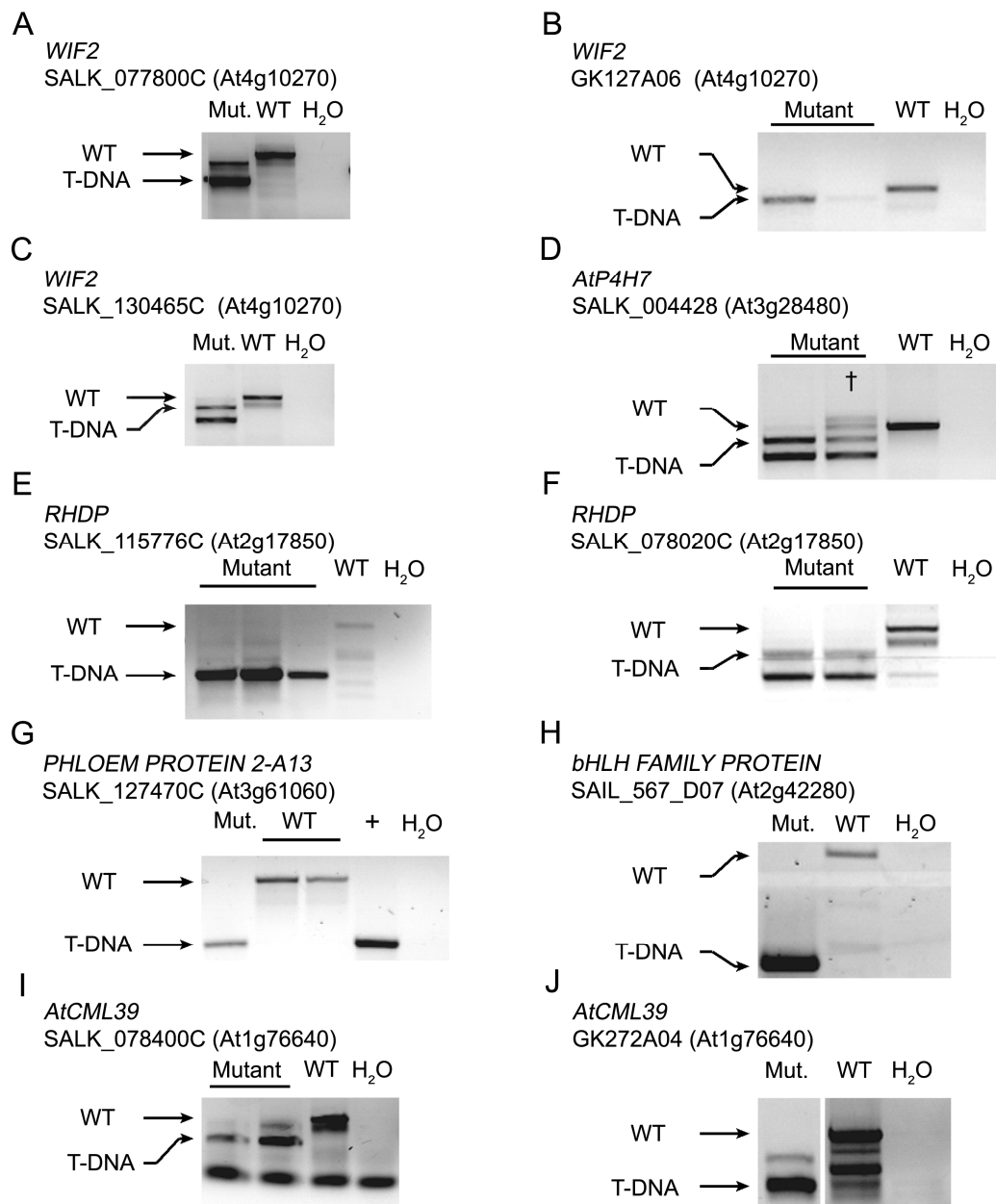
TIGR locus ID	TIGR version 6.1 transcript assignment	FCh at 5% O ₂	Category
Os12g43640	receptor-like protein kinase HAIKU2 precursor, putative	-3.4	receptor
Os06g12560	zinc finger, C3HC4 type, domain containing protein	-3.4	transcription factor
Os03g13820	protein kinase family protein, putative	-3.6	kinase
Os03g53080	zinc finger, C3HC4 type domain containing protein	-3.6	transcription factor
Os06g46950	EF hand family protein, putative	-3.6	signaling
Os04g43680	MYB family transcription factor, putative	-3.7	transcription factor
Os01g60020	NAC domain transcription factor, putative	-3.7	transcription factor
Os01g45250	DUF1645 domain containing protein, putative	-3.8	expressed protein
Os05g10740	pollen-specific protein SF21, putative	-3.8	transcription factor
Os01g64360	MYB family transcription factor, putative	-3.9	transcription factor
Os04g23550	bHLH family protein, putative	-4.0	transcription factor
Os02g45710	zinc finger, C3HC4 type domain containing protein	-4.1	transcription factor
Os06g40170	phospholipase D, putative	-4.1	ABA signaling
Os10g25230	ZIM domain containing protein, putative	-4.3	transcription factor
Os07g34070	Auxin-induced protein 5NG4, putative	-4.3	auxin signaling
Os04g40630	<i>BTBZ4</i> - Bric-a-Brac, Tramtrack, Broad Complex BTB domain with TAZ zinc finger and Calmodulin-binding domains	-4.3	transcription factor
Os01g66530	ARGOS, putative	-4.3	auxin signaling
Os03g64260	AP2 domain containing protein	-4.4	transcription factor
Os04g41160	protein kinase, putative	-4.4	kinase
Os03g19720	EF hand family protein, putative	-4.4	signaling
Os07g46920	Sex determination protein tasselseed-2, putative	-4.5	signaling
Os01g60640	<i>OsWRKY21</i> - Superfamily of TFs having WRKY and zinc finger domains	-4.5	transcription factor
Os01g69830	<i>OsSPL2</i> - SBP-box gene family member	-4.5	transcription factor
Os05g41070	bZIP transcription factor, putative	-4.6	ABA signaling
Os01g09670	Pollen-specific protein SF21, putative	-4.7	transcription factor
Os09g26780	Zinc-finger protein, putative	-4.7	JA signaling
Os06g09310	Zinc finger, C3HC4 type domain containing protein	-4.9	transcription factor
Os08g06110	MYB family transcription factor, putative	-5.0	transcription factor
Os05g46750	STE_MEKK_ste11_MAP3K.18 - STE kinases include homologs to sterile 7, sterile 11 and sterile 20 from yeast	-5.1	kinase
Os03g17700	CGMC_MAPKCGMC_2_ERK.2 - CGMC includes CDA, MAPK, GSK3, and CLKC kinases	-5.1	kinase
Os11g05660	<i>OsFBX396</i> - F-box domain containing protein	-5.1	signaling
Os04g46410	AP2 domain containing protein	-5.3	transcription factor
Os03g12390	STE_MEK_ste7_MAP2K.6 - STE kinases include homologs to sterile 7, sterile 11 and sterile 20 from yeast	-5.4	kinase
Os04g49160	ZnF, C3HC4 type domain containing protein	-5.5	transcription factor
Os05g09020	<i>OsWRKY67</i>	-5.7	transcription factor
Os04g45020	MYB family transcription factor, putative	-6.4	transcription factor
Os11g08210	No apical meristem protein, putative	-6.4	transcription factor
Os03g43770	<i>OsFBX97</i> - F-box domain containing protein	-6.5	signaling
Os10g41330	AP2 domain containing protein	-6.8	transcription factor
Os03g46860	Helix-loop-helix DNA-binding protein, putative	-6.8	transcription factor
Os03g53020	Helix-loop-helix DNA-binding domain containing protein	-6.9	transcription factor
Os01g72530	<i>OsCML31</i> - Calmodulin-related calcium sensor protein	-8.1	signaling
Os03g55540	<i>ZOS3-18</i> - C2H2 zinc finger protein	-8.8	transcription factor
Os02g45780	zinc finger, C3HC4 type domain containing protein	-9.3	transcription factor
Os08g28710	Receptor protein kinase CRINKLY4 precursor, putative	-9.8	receptor kinase
Os12g26290	Alpha-DOX2, putative	-19.9	ROS metabolism

Supplemental Figure 1:



Supplemental Figure 1: Rice sections used for microarray studies. Rice stems were cut 2 cm below the second node to create 20 cm sections of 10- to 12-week-old *Oryza sativa* ssp. *indica* Kato cultivar PG56 plants grown at long-day conditions. After a 1 h treatment with 5% O₂, a region of 0-1.5 cm directly above the second node containing the intercalary meristem and the early elongation zone was harvested and used for analysis as indicated in the figure. This region was earlier described as being responsive to submergence and ethylene (Kende et al., 1998).

Supplemental Figure 2:



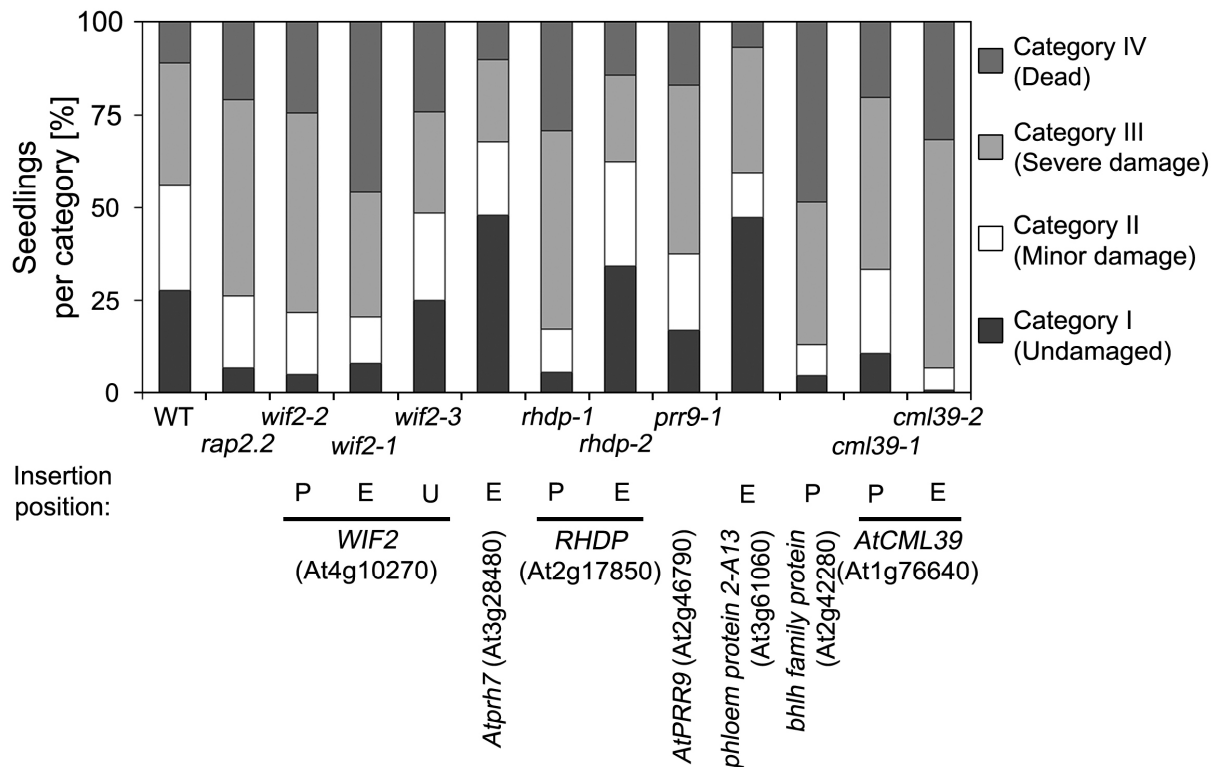
Supplemental Figure 2: Homozygosity test PCRs of Arabidopsis T-DNA insertion mutants. PCRs with genomic DNA (gDNA) template were performed using left border primers for the respective T-DNA and a gene specific primer pair in a duplex PCR setup. All primers are listed in Supplemental Table 2. Approximate expected sizes are marked with the label WT for the WT allele and with the label T-DNA for the PCR product if a T-DNA insertion is present. Mut.: Mutant gDNA as template; WT: WT gDNA as template; H₂O: Water control without gDNA as template. In (D), gDNA of heterozygous plants was tested, this is marked with a dagger (†). For (G) At3g61060, a plasmid carrying the At3g61060 coding sequence was used as a positive control, marked with a cross (+). For each mutant, all lanes shown resulted from one gel. In (J), lanes were omitted to clarify presentation and the break is marked by a white line.

Supplemental Figure 3:



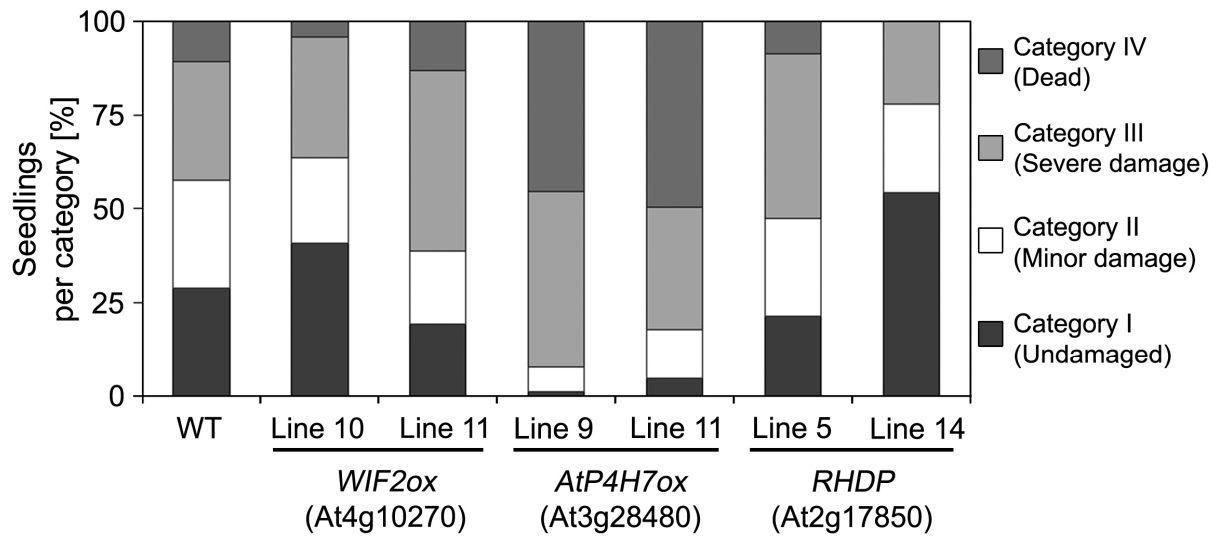
Supplemental Figure 3: Homozygosity test PCR of *Oryza sativa* ssp. *japonica* cultivar Nipponbare *Tos17* retrotransposon insertion mutants. PCRs with genomic DNA (gDNA) template were performed using left border primers for *Tos17* and a gene specific primer pair in a duplex PCR setup. For primers used see Supplemental Table 2. Approximate expected sizes are marked with an arrow and the label WT for the WT allele and an arrow with the label *Tos17* for the PCR product if a *Tos17* insertion is present. Mut.: Mutant gDNA as template; WT: WT gDNA as template; H₂O: Water control without gDNA as template. For each mutant, all lanes shown resulted from one gel. Lanes were omitted to clarify presentation and the breaks are marked by white lines. Note that 'LOC_' is omitted for clarity.

Supplemental Figure 4:



Supplemental Figure 4: Survival assay with *Arabidopsis* mutants. Seedlings were grown on sterile $\frac{1}{2}$ -strength MS for 10 days at long-day conditions. Then, they were subjected to 48 h of near anoxia (0.1% O₂) in N₂. After that period, plates were returned to long-day conditions and kept in air for additional 5 days. Seedlings were sorted into four categories, detailed in Figure 15A. Here, all categories are presented. Statistical analysis was only performed for categories I and IV due to sometimes ambiguous categorization in the other categories. For statistical data, see Figure 15 and Supplemental Table 3C. Mean percentages for each category are also given in Supplemental Table 3C. It can be seen that for mutants with lower survival rates the percentage of Category III is also much higher than that of Category II, for example in the exon-inserted mutants *wif2-1* and *cml39-2*. For the mutants, $n=4-7$ independent experiments were performed, $n=25$ for the WT. For each experiment, 5-34 seedlings were used.

Supplemental Figure 5:



Supplemental Figure 5: Survival assay with *Arabidopsis* mutants overexpressing putatively hypoxia-related genes. Seedlings were grown on sterile $\frac{1}{2}$ -strength MS for 10 days at long-day conditions. Then, they were subjected to 48 h of near anoxia ($<0.1\%$ O_2) in N_2 . After that period, plates were returned to long-day conditions and kept in air for additional 5 days. Seedlings were categorized into four categories, detailed in Figure 15A. Here, all categories are presented. Statistical analysis was only performed for Categories I and IV due to sometimes ambiguous categorization in the other categories. For statistical data, see Figure 18 and Supplemental Table 3D. Mean percentages for each category are also presented in Supplemental Table 3D. It can be seen for mutants with lower survival rates that the percentage of Category III is also much higher than that of Category II, for example in the mutant lines overexpressing *AtP4H7*. For the mutants, $n=4-7$ independent experiments were performed, $n=25$ for the WT. For each experiment, 5-34 seedlings were used.

8. Declaration of Authorship

I hereby declare that I authored this work independently and that it is, to the best of my knowledge and belief, original and the result of my own investigation, with the following exceptions: advice was given by Prof. Dr. Margret Sauter on the manuscript and during experimentation. Hypoxic treatment and RNA isolation for microarray analyses were performed by Dr. Guillaume Rzewuski and Simone Peikert. Experiments in Section 5 were partly performed by Natalia Hess and Dr. Marco Anders. The manuscript (Section 5.2) was written by Prof. Dr. Margret Sauter. PCR and transformation to clone *WIF2ox* (At4g10270) into pENTR1A as well as its LR recombination into pB7WG2 was performed by Timo Staffel. I declare that to the best of my knowledge and belief, I followed the rules for Good Scientific Practice as detailed in the recommendations of the Deutsche Forschungsgemeinschaft and the Senate of the Christian-Albrechts-Universität zu Kiel of 2002/28/05. This work has not been submitted, either in part or whole, for a degree or as part of a doctoral thesis at this or any other University.

Part of this work was previously published as follows:

Hess N, Klode M, Anders M, Sauter M (2011) The hypoxia responsive transcription factor genes *ERF71/HRE2* and *ERF73/HRE1* of Arabidopsis are differentially regulated by ethylene. *Physiol Plant* 143: 41-49

DOI: 10.1111/j.1399-3054.2011.01486.x

Kiel,

Mathias Klode

9. Curriculum Vitae

Name: Mathias Klode

Born: 1982 in Hamburg, Germany

Citizenship: German

Education: I visited the elementary schools Colegio alemán Alexander von Humboldt (Lima, Peru) from 1988 to 1989 and Fridjof-Nansen-Schule Swatten Weg (Hamburg, Germany) from 1990 to 1994. I studied at the Goethe-Gymnasium (Hamburg, Germany) from 1994 to 1996 and at the Deutsche Schule Santiago de Chile (Santiago, Chile) from 1996 to 1998. I finished school by visiting Wolfgang-Borchert-Gymnasium (Halstenbek, Germany) from then on and finished school by passing the Abitur in 2001.

I studied Biochemistry at the University of Bielefeld from 2002 to 2004 and at the University of Hamburg from 2005 to 2007, concluding with the following subjects: Molecular Biology and Biochemistry (principal subject), Plant genetic engineering (selected subject), and immunology (subsidiary subject).

I finished my diploma thesis 2007 in the group of Prof. Dr. Hans-Peter Mühlbach, at the Botanical Institute of the University of Hamburg. It carried the title: "Funktionsanalyse zweier Proteine des neuartigen European mountain ash ringspot-associated virus (EMARAV)".

Publication list

Klode M, Dahlke RI, Sauter M und Steffens B (2011) Expression and subcellular localization of *Arabidopsis thaliana* auxin-binding protein 1 (ABP1). *J Plant Growth Regul – Published online before print*

DOI: 10.1007/s00344-011-9203-2

Hess N, Klode M, Anders M, Sauter M (2011) The hypoxia responsive transcription factor genes *ERF71/HRE2* and *ERF73/HRE1* of *Arabidopsis* are differentially regulated by ethylene. *Physiol Plant* 143: 41-49

DOI: 10.1111/j.1399-3054.2011.01486.x