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**ETHYLENE RESPONSE FACTOR VII transcription factors  
control the reshaping of the root system architecture in  
response to hypoxia**

by

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## 1 Abbreviations

1-MCP	1-methylcyclopropene
ABA	abscisic acid
ABA2/3	ABA-deficient 2/3
ABCB	ATP-binding cassette transporters
ABH2	ABA hypersensitive 2
ADH1	ALCOHOL DEHYDROGENASE1
ANOVA	analysis of variance
AP2/ERF	APETAL2/ETHYLENE RESPONSE FACTOR
AR	adventitious root
ATE	ARGINYL-tRNA PROTEINTRANSFERASE
ATP	adenosine triphosphate
AUX/LAX	AUXIN1/LIKE-AUX1
Ca <sup>2+</sup>	calcium
cDNA	complementary deoxyribonucleic acid
CTR1	CONSTITUTIVE TRIPLE RESPONSE 1
CYP707A	ABA 8'-HYDROXYLASE
DNA	deoxyribonucleic acid
EIL1	EIN3-LIKE 1
EIN2	ETHYLENE INSENSITIVE 2
EIN3	ETHYLENE INSENSITIVE 3
EIN4	ETHYLENE INSENSITIVE 4
ERFVII	ETHYLENE RESPONSE FACTOR GROUP VII
ERS1/2	ETHYLENE RESPONSE SENSOR 1/2
ETR1/2	ETHYLENE RESISTANT 1/2
FPU	food producing unit
GAPC	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C
GDP	gross domestic product

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GFP	green fluorescence protein
GUS	β-Glucuronidase
h	hour
HRE1	HYPOXIA RESPONSIVE ERF1
HUP09	HYPOXIA-RESPONSIVE UNKNOWN PROTEIN09
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
LBD41	LOB DOMAIN-CONTAINING PROTEIN 41
LC-MS	liquid chromatography – mass spectrometry
LDH	L-LACTATE DEHYDROGENASE
LR	lateral root
MDR	multidrug-resistant
MEP	2C-methyl-D-erythritol-4-phosphate
mETC	mitochondrial electron transport chain
min	minute
MT2b	METALLOTHIONEIN2b
n	number
NAD <sup>+</sup>	nikotinamid-adenin-dinukleotid
NADPH	nicotinamide adenine dinucleotide phosphate
NASC	Nottingham Arabidopsis Stock Centre
NCED	9-cis-epoxycarotenoid dioxygenase
NPA	N-1-naphthylphthalamic acid
ORFs	open reading frames
<i>P</i>	probability
PCD	programmed cell death
PCO	PLANT CYSTEINE OXIDASE
PCR	polymerase chain reaction
PDC1/2	PYRUVATE DECARBOXYLASE 1/2
PIN	PIN-FORMED

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PP2C	A type-2C PROTEIN PHOSPHATASE
PR	primary root
PRT6	E3 ubiquitin ligase PROTEOLYSIS 6
QC	quiescence center
qRT-PCR	quantitative real-time PCR
RAP	RELATED TO APETALA
RBOH	RESPIRATORY BURST OXIDASE HOMOLOG
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RSA	root system architecture
RT-PCR	reverse transcriptase PCR
SDH	SUCCINATE DEHYDROGENASE
SE	standard error
SnRK2	SNF1-RELATED PROTEIN KINASE 2
SUS1/4	SUCROSE SYNTHASE1/4
TCA	tricarboxylic acid
US	United State
w/v	weight per volume
wt	wild type
YUC6	INDOLE-3-PYRUVATE MONOOXYGENASE 6
$\alpha$ -NAA	$\alpha$ -naphthalene acetic acid

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### 3 Zusammenfassung

Staunässe und Überflutung führen bei Pflanzen zu Sauerstoffmangel und damit zu einem Verlust der mitochondrialen ATP Bildung. Wurzeln sind die ersten Organe, die dabei Sauerstoffmangel erfahren, weil Gasdiffusion in Wasser vermindert ist und Sauerstoff im Boden durch aerobe Mikroorganismen aufgebraucht wird. Wurzeln sind aber entscheidend für das Überleben der Pflanze bei Hypoxie. *Arabidopsis thaliana*, mit seiner mittleren Toleranz gegenüber Überflutung, wurde als Modellorganismus gewählt, um Veränderungen der Wurzelarchitektur bei Hypoxie zu untersuchen, und die zugrundeliegenden molekularen Regulationsmechanismen aufzuklären.

Die Ergebnisse zeigen, dass Hypoxie Krümmungswachstum der Primärwurzel auslöst, vermutlich um zu durchlüfteten Erdschichten vorzudringen. Mithilfe der Auxinreporter DII-VENUS und DR5:GUS konnte gezeigt werden, dass die Wurzelkrümmung durch Etablierung eines Auxingradienten über die Wurzelspitze erreicht wird. Der basipetale Auxintransport, vermittelt durch den Auxin-Effluxtransporter PIN2, könnte eine entscheidende Rolle in der veränderten Auxinakkumulation spielen, denn der Verlust von PIN2 in *pin2-1* Keimlingen unterdrückt die durch Hypoxie ausgelöste Krümmung. Des Weiteren ist die PIN2 Proteinmenge bei Hypoxie reduziert, erkennbar durch eine reduzierte Signalstärke des PIN2 Reporters *PIN2pro:PIN2-GFP*. Die APETAL2/ETHYLENE RESPONSE FACTOR Transkriptionsfaktoren der Gruppe VII (ERFVIIs), RAP2.2, RAP2.3, RAP2.12, HRE1 und HRE2 werden bei geringer Sauerstoffkonzentration aktiviert und wirken als Regulatoren von Hypoxieantworten. Diese Arbeit zeigt, dass RAP2.12 der Hypoxie-induzierten Primärwurzelkrümmung entgegen wirkt, indem es verhindert, dass die PIN2 Proteinmenge abnimmt. *RAP2.12ox1 x PIN2pro:PIN2-GFP* Keimlinge zeigen keine reduzierte PIN2 Abundanz bei Hypoxie und *rap2.12-1* Keimlinge weisen eine verstärkte Krümmung auf.

Bei Hypoxie stellen Lateralwurzeln nach dem Auswachsen aus der Primärwurzel ihr Wachstum vorübergehend ein. Um weiterwachsen zu können, müssen ERFVII Transkriptionsfaktoren aktiv sein, denn Lateralwurzeln von *erfVII* Keimlingen arretieren nachhaltig. Diese Ergebnisse zeigen, dass die Pflanze das Wachstum der Lateralwurzeln auch bei Energiemangel aufrechterhält. Untersuchungen an Wurzeln von *rap2.3-2*, *rap2.12-1* und *hre2-1* Keimlingen zeigen, dass diese ERFVIIs die Expression von



*CYP707A1* und *CYP707A3* fördern, die für ABA-abbauende ABA 8'-Hydroxylasen kodieren, was zu einem reduzierten ABA-Gehalt in der Wurzel führt. Da ABA Lateralwurzeln hemmt, könnte das Absenken der ABA-Konzentration das Wachstum ermöglichen.

Neben Primär- und Lateralwurzeln verändert Hypoxie das Wachstum von Adventivwurzeln. Diese werden im Vergleich zum Wildtyp bei hypoxischen Bedingungen länger, ein Effekt der auch in *HRE2ox1*-Keimlingen beobachtet werden kann, was eine Regulation durch ERFVIIIs nahelegt. Ethylen, das einige Überflutungsantworten steuert, hemmt das Längenwachstum von Adventivwurzeln bei Hypoxie sodass eine gegensätzliche Regulation durch Hypoxie und Ethylen vorliegt. Möglicherweise, wird dadurch das näher an der Oberfläche befindliche Adventivwurzelsystem gefördert, weil sich dort weniger Ethylen anreichert.

In dieser Arbeit wurden spezifische Veränderungen der Wurzelarchitektur in Antwort auf und wahrscheinlich in Anpassung an Sauerstoffmangel beschrieben und teilweise mechanistisch aufgeklärt. ERFVIIIs fungieren als Gegenspieler zu der durch Hypoxie ausgelösten Krümmung der Wurzel. Gleichzeitig fördern sie das Wachstum von Sekundärwurzeln, was zu einer insgesamt veränderten Wurzelarchitektur führt. Darüber hinaus wurde deutlich, dass es einen bisher unbekannten, ERFVII unabhängigen, Mechanismus zur Wahrnehmung geringer Sauerstoffkonzentration gibt.

## 4 Summary

Upon flooding, roots are the first organs to encounter oxygen shortage due to decreased gas diffusion and depletion of oxygen by microorganisms in the soil. Root performance is crucial for plant survival during hypoxia. *Arabidopsis thaliana*, with its intermediate tolerance to flooding, is a well-suited model organism to characterize adaptation to hypoxia at the level of root system architecture and its underlying molecular regulation.

This study shows that hypoxia triggers primary root bending likely to reach aerated soil layers. Root bending is achieved by the establishment of an auxin gradient across the root tip as quantified with the DII-VENUS auxin reporter. Basipetal auxin transport, mediated by the auxin efflux carrier PIN2, might play a crucial role in altering auxin accumulation as loss of PIN2 in *pin2-1* seedlings abolished the bending response to hypoxia. Furthermore, the PIN2 protein level is reduced by hypoxia as indicated by the reduced signal intensity of the PIN2 reporter *PIN2pro:PIN2-GFP*. The group VII APETAL2/ETHYLENE RESPONSE FACTOR (ERFVII) RAP2.2, RAP2.3, RAP2.12, HRE1, and HRE2 are activated by low oxygen and are key regulators of the low oxygen response in *Arabidopsis*. Results shown here demonstrate that RAP2.12 counteracts the hypoxia-induced primary root bending by regulating the abundance of PIN2 since *RAP2.12ox1* × *PIN2pro:PIN2-GFP* seedlings do not display reduced PIN2 abundance under hypoxia and *rap2.12-1* knock out seedlings show an exaggerated bending response.

Hypoxia furthermore causes transient arrest of lateral root growth at the post-emergence stage. Knockout of all five ERFVII in the *erfVII* mutant enhances the growth arrest indicating that ERFVII maintain lateral root elongation despite of energy limitations. RAP2.3, RAP2.12, and HRE2 redundantly upregulate expression of *CYP707A1* and *CYP707A3* encoding for ABA-degrading ABA 8'-hydroxylases and ERFVII reduce the ABA content in the root. Since ABA inhibits lateral root growth, lowering ABA levels is the molecular mechanism by which ERFVII relieve growth inhibition.

Finally, hypoxia induces elongation growth of adventitious roots in an ERFVII-dependent manner as indicated by longer adventitious roots in *HRE2ox1* seedlings compared to wild type. However, ethylene, a key hormone of the flooding response,

overrides the promotive effect of hypoxia revealing antagonistic regulation by hypoxia and ethylene.

Taken together, this work revealed distinct changes in the root system which are hypothesized to contribute low oxygen stress adaptation. ERFVIIIs antagonize hypoxia-induced root bending and enhance lateral and adventitious root elongation highlighting their regulatory role in the developmental reprogramming of the root system. The study further revealed a, yet unknown, ERFVII-independent sensing mechanism for low oxygen.

## 5 General introduction

### 5.1 Impact of flooding

Flooding is an environmental disaster caused by e.g. hurricanes, monsoons, seasonal overtopping of water from rivers and from heavy rainfalls that negatively affects plant vigor, susceptibility to biotic stress and plant reproduction (Voesenek and Bailey-Serres, 2015). Due to climate changes, the number of flooding events are increasing worldwide (Bailey-Serres *et al.*, 2012a), particularly in developing areas such as Middle- and South-America, Southeast-Asia or Middle-Africa (Voesenek and Bailey-Serres, 2015). The frequency of flooding events in the above-mentioned areas reflects the impact on agricultural value relative to the total gross domestic product (GDP) of food producing unit (FPU) estimated by Ward *et al.* (2013). Furthermore, in emerging countries, agriculture is one of the major sectors contributing to the total GDP. In India in 2017, the agricultural GDP was 17% of the total GDP (The World Factbook, 2017) therefore the consequence of environmental disasters can have a huge impact on the economy. In Asia, agriculture suffered enormous product losses causing 48 billion US dollars (\$ worth of damage by flooding between 2003 and 2013 where Indian's agriculture was the most affected. A case study shows that in 2010 extraordinary rainfall caused 50% of the total damages in agriculture including 90% loss of crop production, damaged agricultural infrastructure and enterprises which caused \$5.3 billion losses in Pakistan (FAO, 2015).

Taken together due to the unsolved climate changes and increasing population, researchers need to improve flood-sensitive species to maintain sustainable agriculture with proper livelihoods and food security.

### 5.2 Effect of flooding on plants

Flooding as abiotic stress engenders dramatic changes in the environment of the plants. Based on the oxygen availability, the environment of plants can be defined as normoxic (21% O<sub>2</sub>), hypoxic (<21% O<sub>2</sub>) or anoxic (no O<sub>2</sub>) (Bailey-Serres *et al.*, 2012a). A flooding regime is composed of multiply factors such as flooding duration (prolonged or flash flood),

the states of the water (turbid, clear, salty) and water depth (submergence or waterlogging) (Bailey-Serres *et al.*, 2012b; Sasidharan and Voesenek, 2015). In all cases, plants are exposed to oxygen deprivation due to the reduced gas diffusion in the water (Grable *et al.*, 1966) and to ethylene accumulation (Sasidharan and Voesenek, 2015). At the cellular level, both, ethylene and low oxygen act as a signal to initiate transcriptional reprogramming resulting at the end in metabolic adjustment and morphological adaptations to flooding.

### 5.3 Regulation of ERFVII transcription factors

The first crucial step in response to stress conditions is for the plant to perceive the change in the environment. The group of ETHYLENE RESPONSE FACTOR VII (ERFVII) transcription factors that include RELATED TO APETALA2.2 (RAP2.2), RAP2.3, RAP2.12, HYPOXIA RESPONSIVE ERF1 (HRE1) and HRE2 were identified as key regulators of low oxygen stress responses in *Arabidopsis thaliana*. ERFVIIs belong to the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family based on their single AP2 DNA-binding domain (Nakano *et al.*, 2006). ERFVIIs share a characteristic N-motif (NH<sub>2</sub>-MCGGAI/L), a conserved region at the N-terminus of the protein (Nakano *et al.*, 2006) which enables a protein degradation system to destabilize the ERFVIIs under normal atmospheric conditions via the so called N-end rule pathway (Licausi *et al.*, 2011; Gibbs *et al.*, 2011). After the cleavage of the first methionine (Met) residue by Met amino peptidase (MAP), the cysteine acts as an N-degradon that is oxidized to cysteine sulphenic acid by PLANT CYSTEINEOXIDASE (PCOs) utilizing oxygen as a cosubstrate. ARGINYL-tRNA PROTEINTRANSFERASE (ATEs) conjugate an arginine to the oxidized cysteine. Thereby, the arginylated protein can be recognized by E3 ubiquitin ligase PROTEOLYSIS6 (PRT6) targeting the proteins for proteosomal degradation. Targeted proteolysis of ERFVIIs enables the plant to regulate cellular responses in an oxygen-dependent manner (Licausi *et al.*, 2011; Bailey-Serres *et al.*, 2012a; van Dongen and Licausi, 2015).

In addition to post-translation modification, expression of ERFVIIs is regulated by different factors in *Arabidopsis*. *RAP2.2*, *RAP2.3*, *RAP2.12* are expressed in all developmental stages (Bui *et al.*, 2015) and are induced by osmotic stress and, to a lesser

extent, by abscisic acid (ABA) (Papdi *et al.*, 2015). In contrast to the *RAP* genes, expression of *HRE1* and *HRE2* is highly induced by hypoxia, anoxia, and ROS in a cell type-specific manner in roots (Yang, 2014; Eysholdt-Derzsó and Sauter, 2017). Ethylene, as an important signaling molecule of flooding, exclusively promotes the expression of *RAP2.2*, *RAP2.3*, and *HRE1* in roots (Hinz *et al.*, 2010; Hess *et al.*, 2011; Licausi *et al.*, 2011). The distinct expression patterns of ERFVIs indicate different functions in a tissue and cell type-specific manner. A comprehensive summary of the expression of ERFVIs is given in Table 1. Furthermore, trans-activation studies have shown functional redundancy among RAPs and provide evidence of the minor role of HRE1 and HRE2 in activation of genes involved in low oxygen response, such as LOB DOMAIN-CONTAINING PROTEIN41 (LBD41) and PCO1 (Gasch *et al.*, 2015).

Factors	<i>RAP2.2</i>	<i>RAP2.3</i>	<i>RAP2.12</i>	<i>HRE1</i>	<i>HRE2</i>	References
Constitutive	✓	✓	✓			Licausi <i>et al.</i> , 2010; Bui <i>et al.</i> , 2015
Hypoxia				✓	✓	Licausi <i>et al.</i> , 2010
Anoxia				✓	✓	Papdi <i>et al.</i> , 2015
Osmotic stress	✓	✓	✓		✓	Park <i>et al.</i> , 2011; Papdi <i>et al.</i> , 2015
ROS				✓	✓	Yang, 2014; unpublished data by Eysholdt-Derzsó
Ethylene	✓	✓		✓		Hinz <i>et al.</i> , 2010; Hess <i>et al.</i> , 2011; Licausi <i>et al.</i> , 2011
ABA	✓	✓	✓			Papdi <i>et al.</i> , 2015
Dark	✓					Hinz <i>et al.</i> , 2010

Table 1. Transcriptional regulation of ERFVIs by abiotic stresses, ROS and phytohormones.

Besides the divers expression patterns of the ERFVIs, several studies revealed a difference in localization of the proteins. *RAP2.12* is localized at the plasma membrane at normoxic conditions, is dissociated from acyl-CoA-binding proteins under low oxygen conditions and moves to the nucleus within 3 h. Within 3 h of re-oxygenation, *RAP2.12* is degraded concomitant with the return of transcript levels of hypoxia responsive genes to

normoxic levels (Kosmacz *et al.*, 2015). In contrast, the hypoxia-induced HRE1 and HRE2 proteins directly accumulate in the nucleus (Licausi *et al.*, 2010; Licausi *et al.*, 2011).

#### 5.4 Low oxygen signaling via an ERFVII-independent pathway

Recently, several papers were published that highlight the possibility for an ERFVII-independent oxygen-sensing mechanism (Wang *et al.*, 2017; Eysholdt-Derzsó and Sauter, 2017/Chapter 1; Holdsworth, 2017; Schmidt *et al.*, 2017). In principle, the pentuple *erfVII* mutant (Abbas *et al.*, 2015) is hypoxia-insensitive due to the lack of ERFVIIs. It was however shown that *erfVII* seedlings show a hypoxia-hypersensitive phenotype and that ERFVIIs and the ERFVII-independent pathway antagonistically regulate primary root slanting (Eysholdt-Derzsó and Sauter, 2017). This theory was further supported by Schmidt *et al.* (2017) concluding that besides the ‘primary oxygen sensor’ a decentralized sensing mechanism might exist in several organelles. Several O<sub>2</sub> signaling mechanisms were proposed, including ROS, calcium, energy signaling and mitochondrial dysfunction. ROS accumulate in mitochondria when plants are exposed to low oxygen due to the uncoupled complexes in the mitochondrial electron transport chain (mETC) and in the apoplast through the activation of NADPH oxidase D at the plasma membrane (RBOHD) (Pucciariello and Perata, 2017). The generated H<sub>2</sub>O<sub>2</sub> triggers a MAPK3/MAPK6 cascade forwarding the signal to the nucleus in response to low oxygen stress (Chang *et al.*, 2012). According to Wang *et al.* (2017), ERFVIIs are ‘transducers,’ but not sensors and additional protein(s) are involved in direct oxygen sensing. Potential oxygen-sensing proteins were suggested in Arabidopsis including ion channel proteins based on the homology to a mammalian oxygen-sensing protein. Six K<sup>+</sup> and Ca<sup>2+</sup> channels, tandem-pore K<sup>+</sup> channel proteins KCO1, KCO4, KCO5, KCO6, Arabidopsis K<sup>+</sup> transporter AKT2 and two-pore channel 1 TPC1 from Arabidopsis have sequence similarities with their mammalian counterparts without however containing any of the mammalian oxygen-sensing domains (Wang *et al.*, 2017). The exact role of calcium in the low oxygen response is largely unknown, but under anoxia, an organ-specific calcium flux change was observed by using a Ca<sup>2+</sup>-sensitive luminescent protein as a reporter. In the shoot, a calcium peak was observed after 5 min of anoxia treatment indicating a highly specific

early response, while in the root many smaller peaks appeared as a result of anoxia (Sedbrook *et al.*, 1996).

## 5.5 Transcriptional and metabolic changes upon low oxygen stress

As flooding signals are perceived by the plant, further signaling cascades are induced to initiate transcriptional changes as a response to low oxygen stress. Transcriptome profiling revealed that submergence (Lee *et al.*, 2011) waterlogging (Hsu *et al.*, 2011), hypoxia and anoxia (Hwang *et al.*, 2011) lead to an induced expression of 49 hypoxia core genes that encode mostly metabolic enzymes involved in starch degradation, glycolysis and fermentation such as SUCROSE SYNTHASE (SUS1/4), ALCOHOL DEHYDROGENASE (ADH1), PYRUVATE DECARBOXYLASE (PDC1/2) and L-LACTATE DEHYDROGENASE (LDH) (Mustroph *et al.*, 2009). Furthermore, waterlogged plants triggered a systemic response in the shoot resulting in upregulation of genes involved in sucrose degradation for energy mobilization in the shoot (Hsu *et al.*, 2011). As key regulators of low oxygen signaling, ERFVILs trigger transcriptional changes. RAP2.12 plays a role in the activation of hypoxia core genes such as *SUS1*, *ADH1*, *PDC1*, *LBD41*, *HUP09* (Licausi *et al.*, 2011). HRE1 induces the expression of some hypoxia marker genes including *ADH1* and *PDC2*. Additionally, HRE1 negatively affects the expression of several peroxidases (At3g03670, At1g14540) and cytochrome P450 genes (At4g31970, At5g57220 CYP82C2, CYP81F2) under hypoxic conditions (Yang *et al.*, 2011, Licausi *et al.*, 2010). Although HRE2 is strongly induced by low oxygen conditions, the expression of hypoxia markers is not altered by HRE2 (Licausi *et al.*, 2010). A small sub-set of genes, mostly responsible for mitochondrial function, is repressed by HRE2.

As the transcriptional modification indicates, plants switch from aerobic metabolism to anaerobic fermentation to maintain at least a low level of energy production during oxygen deprivation. In the mitochondria, due to the lack of electron donor oxygen, the mETC is uncoupled, therefore the respiration rate dramatically drops down at around 4% O<sub>2</sub> availability (Zabalza *et al.*, 2009). To compensate the loss of ATP production from oxidative phosphorylation, plants upregulate glycolysis and activate fermentative pathways. To fuel glycolysis, starch breakdown is necessary to mobilize sucrose via the enzymatic process preferably through sucrose synthases (SUS) (van Dongen and Licausi,



2015). In glycolysis, 2 ATP are generated while NAD<sup>+</sup> as an oxidized redox cofactor is used. The regeneration of NAD<sup>+</sup> from NADH is processed in the lactate and ethanol fermentation pathways catalyzed by either LDH or PCD and ADH1 enzymes (Bailey-Serres *et al.*, 2012a). Changes also occur in the tricarboxylic acid (TCA) cycle which generates reduced NADH for ATP synthesis in the mETC. Via <sup>13</sup>C-pyruvate, <sup>13</sup>C-glutamate and <sup>15</sup>N-ammonium labeling, metabolic analysis of the TCA cycle revealed that during oxygen deprivation the oxidizing TCA flux changes to a non-cyclic mode with an oxidative and reductive branch as a consequence of the inhibition of succinate dehydrogenase (SDH) (António *et al.*, 2016). As a result of metabolic reprogramming, plants sustain growth and prolong survival upon oxygen deprivation which was confirmed by several studies using mutants such as *pgm* defective in starch synthesis (Loreti *et al.*, 2017), overexpressor of RAP2.12 involved in oxygen sensing mechanism (Paul *et al.*, 2016) and *adh1* lacking ethanol fermentation (Ismond *et al.*, 2003).

## 5.6 Morphological adaptations under flooding stress

Besides metabolic adjustment, plants evolved morphological adaptations to improve survival upon flooding. Depending on the natural habitat of species they are exposed to highly diverse water availability and flooding regimes and therefore sensitive and tolerant species exist in nature. Unfortunately, most of the agriculturally important crop species are sensitive to flooding such as intolerant wheat (*Triticum aestivum*), maize (*Zea mays*) especially in an early developmental phase, soybean (*Glycine max*), alfalfa (*Medicago sativa*), pea (*Pisum sativum*), tomato (*Solanum lycopersicum*), and many peach and citrus rootstocks indicate their 'dryland' origin (Zhang and Davies 1987; Schaffer *et al.*, 1992; Castonguay *et al.*, 1993; Ahsan *et al.*, 2007; Hossain and Uddin, 2011; Nanjo *et al.*, 2014; Zaidi *et al.*, 2015). *Arabidopsis thaliana* shows an intermediate tolerance to flooding (Vashisht *et al.*, 2011), whereas rice (*Oryza sativa*) and marsh dock (*Rumex palustris*) as 'wetland' species, are flood tolerant (Kende *et al.*, 1998; Pierik *et al.*, 2005).

Morphological adaptations play an important role in survival upon prolonged flooding stress because they improve gas exchange between aerated and submerged organs. To improve gas exchange, plants form aerenchyma (Steffens *et al.*, 2011; Yamauchi *et al.*, 2013), adventitious roots (Lorbiecke and Sauter, 1999), and gas films (Herzog *et al.*,

2017), or induce hyponastic growth (Millenaar *et al.*, 2005; Pierik *et al.*, 2005), and stem or petiole elongation (Millenaar *et al.*, 2005, Kende *et al.*, 1998) to raise leaves above the water surface.

Aerenchyma provides a low-resistance gas diffusion path for oxygen along the stem and roots (Yamauchi *et al.*, 2017). Rice, maize, tomato, marsh dock, wheat especially in roots, soybean in stems and roots form aerenchyma to improve survival (Mühlenbock *et al.*, 2007; Hossain and Uddin, 2011; Yamauchi *et al.*, 2013; Yamauchi *et al.*, 2017). However, in soybean, there was no correlation between survival rate and aerenchyma formation because soybean generates aerenchyma in the root too late thus it cannot contribute to a better survival (Yamauchi *et al.*, 2017).

Rice, certain maize lines, tomato, marsh dock, and tolerant wheat induce aerenchyma as well as adventitious root growth upon flooding (Visser *et al.*, 1996; Lorbiecke and Sauter, 1999; Mano *et al.*, 2005; Vidoz *et al.*, 2010; Hossain and Uddin, 2011). Adventitious roots facilitate gas exchange, uptake of minerals, water, and oxygen under flooding conditions, and they anchor the plant during the post-submergence phase (Lorbiecke and Sauter, 1999).

*Arabidopsis thaliana* as a model plant tolerates flooding in an intermediate level by promoting aerenchyma formation in the hypocotyl and hyponastic leaf growth (Millenaar *et al.*, 2005; Pierik *et al.*, 2005; Mühlenbock *et al.*, 2007). However, it has not been well explored how the primary root system changes upon low oxygen stress. Until now, it was shown that inhibition of primary root growth correlates with the reduction of oxygen availability (van Dongen *et al.*, 2009) and root fresh weight is significantly reduced upon flooding (Ellis *et al.*, 1999) like in sensitive species such as alfalfa, pea and wheat that showed reduced root dry or fresh weight under oxygen deprivation (Zhang and Davies 1987; Castonguay *et al.*, 1993.; Hossain and Uddin, 2011). Detailed analysis of morphological changes of the root system is, however, necessary to obtain a better understanding of the root architecture and its underlying molecular regulation upon low oxygen stress.

## 5.7 Root development and its hormonal regulation in *Arabidopsis*

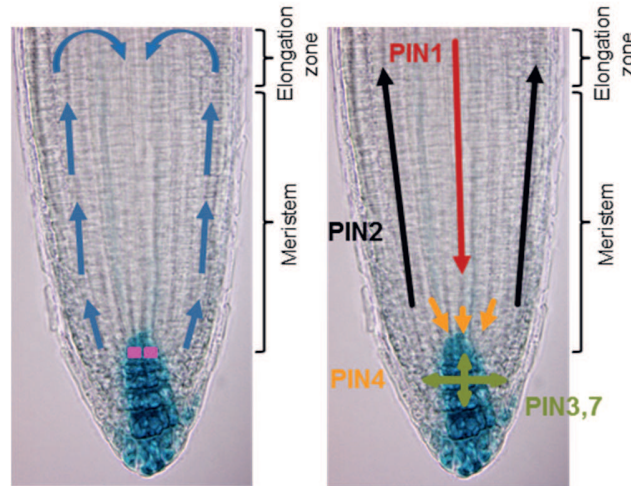
The *Arabidopsis thaliana* root system architecture (RSA) is made up of three main root types, namely primary, lateral and junction roots (Slovak *et al.*, 2016). Under certain stress conditions, additionally, adventitious roots can emerge. The root system plays a major role in anchoring the plant, supplying it with nutrients and water, and maintaining symbioses. The root system shows high plasticity under abiotic and biotic stress conditions allowing for stress-specific adaptation. Moreover, higher branching ability of LRs was correlated with an increased root surface area resulting in better water and nutrient uptake thus improved storage root growth in cassava and tuber bulking in potato (Izumi *et al.*, 1999; Wishart *et al.*, 2013; Khan *et al.*, 2016).

### 5.7.1 Primary root

The primary root is the first root type in plant development and is formed during embryogenesis. Its continuous growth allows the formation of lateral roots (LRs) which originate from the primary root highlighting its crucial role in shaping RSA (Tian *et al.*, 2014).

Auxin as a major regulatory hormone involved in many aspect of plant development such as primary root growth, gravity responses, apical hook formation in dicotyledonous seedlings, and lateral root development (Rahman *et al.*, 2007; Vandenbussche *et al.*, 2010; Band *et al.*, 2012; Jung and McCouch, 2013) where it regulates cell division, expansion, and differentiation (Mashiguchi *et al.*, 2011). The main site of auxin biosynthesis is the shoot apical meristem, from where auxin moves by polar transport towards the distal root tip via the influx carriers AUXIN1/LIKE-AUX1 (AUX/LAX) (Bennett *et al.*, 1996; Swarup and Péret, 2012), the efflux carriers PIN-FORMED (PIN1, 2, 3, 4, 7) (Blilou *et al.*, 2005) and ABCB carriers (Geisler and Murphy, 2006). In order to establish an auxin gradient for correct cell division and elongation, auxin influx and ABCB transporters take up auxin in an undirected manner and export it in a directed manner. PIN1 together with PIN4 is responsible for auxin transport to the root apex and where an auxin pool is established in the quiescence center (QC). PIN3 and 7 distribute auxin across the columella followed by a basipetal auxin transport in the lateral root cap and

epidermal cell via PIN2 towards the elongation zone (Friml *et al.*, 2002b; Blilou *et al.*, 2005; Wisniewska *et al.*, 2006) (Figure 1).



**Figure 1. Auxin maximum and its transport mechanism in the root tip**

The auxin maximum in the primary root apex is indicated by blue stain from DR5:GUS activity and auxin flux via auxin efflux carriers are visualized by colored arrows. Auxin accumulates around the quiescent center (magenta box) and in columella cells transported acropetally by PIN1 (red arrow) and PIN4 (orange arrow) and distributed in the columella cells by PIN3 and 7 (green arrows). Yellow arrows show basipetal auxin transport to the distal elongation zone via PIN2 (black arrow) (Blilou *et al.*, 2005; Swarup and Péret, 2012).

Auxin also acts in regulating plant tropic responses (Swarup *et al.*, 2005). The response to gravity is triggered during germination. Obstacle avoidance or water deficiency can cause a change in growth direction that is initiated by asymmetric cell division in the elongation zone (Rahman *et al.*, 2010). The initial step of gravitropism is sensing the change in the gravity vector which is perceived by the starch-filled amyloplasts in the root cap. The sedimentation of the starch grains in columella cells likely generates a physical signal although it is not known yet how the physical signal is converted to a physiological response. Further analysis suggests starch-independent signal transduction as well (Sato *et al.*, 2015). In order to initiate differential cell elongation, auxin levels are elevated at the upper vs. the lower side of the root within 0.5-1 hour of gravistimulation (Vandenbussche *et al.*, 2010). The auxin distribution shows a highly dynamic change

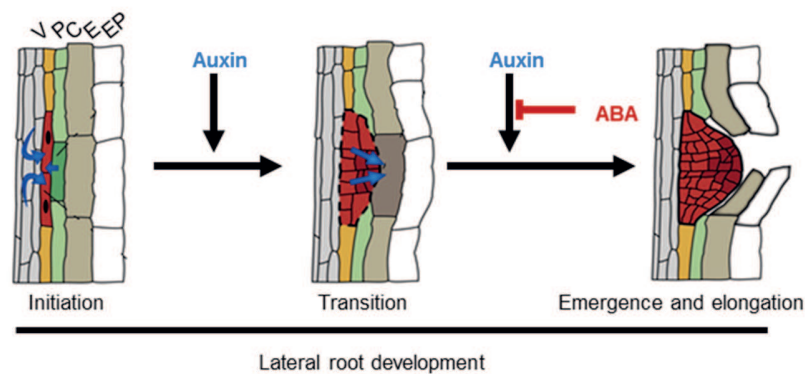
when the angle of the root reaches 40° to the horizontal such that auxin asymmetry vanishes. The asymmetric auxin accumulation is driven by auxin influx and efflux carriers. AUX1 acts synergistically with PIN2 to move auxin to the gravi-response site and plays a role in the efficient uptake of auxin into epidermal cells (Swarup and Péret, 2012). In contrast to AUX1, PIN2 facilitates directed basipetal auxin flux to the elongation zone. The loss-of-function *Atpin2::En701* mutant shows inhibited primary root growth and a strong agravitropic phenotype (Müller *et al.*, 1998). PIN1 is responsible for the auxin flux but not for establishing an auxin gradient and thus does not contribute to the gravity response (Friml *et al.*, 2002b). Analysis of *pin3* seedlings revealed a function in the gravitropic response. PIN3 protein changes locate to the lateral face of the columella cells supporting asymmetric auxin distribution (Friml *et al.*, 2002b). *pin3 pin7* double knock-out but not single knock out seedlings showed a defect in gravitropism indicating a redundancy in their function (Sato *et al.*, 2015). Friml *et al.* (2002a) provided evidence that PIN4 is involved in establishing a correct auxin gradient for root patterning. The ABC transporter MDR4 plays a main role in gravitropism via basipetal auxin transport and is essential for triggering the differential cell elongation based on the observation that *mdr4* mutant with 50 % of disrupted basipetal transport and maintained faster and enhanced curvature upon gravity stimulus. Whereas another ABC transporter MDR1 is responsible for the acropetal auxin transport but does not alter gravity response verified by using *mdr1* single knock out, in which auxin acropetal transport was 80 % less than in wild type but showed normal gravity response (Müller *et al.*, 1998; Rashotte *et al.*, 2000; Lewis *et al.*, 2007). In conclusion AUX1, PIN2 and the MDR4 establish and maintain a sufficient gravity response generating auxin gradient in the lower side of the root apex.

### 5.7.2 Lateral roots

LRs form the majority of the root biomass, enable efficient uptake of water, nutrition, maintain symbiosis and anchor the plant. LRs are formed post-embryonically from the pericycle cell layer adjacent to the xylem pole. LR development includes initiation, primordia formation, meristem activation after emergence and elongation (Jung and McCouch, 2013; Malamy and Benfey, 1997). Auxin is the main phytohormone regulating LR development. LR initiation is established by the priming of cells in the basal meristem

via the oscillation of auxin accumulation mediated by AUXIN RESPONSE FACTORS 5,6,7,8 and 19 (Lavenus *et al.*, 2013). After the establishment of the LR founder cell, asymmetric cell divisions take place driven by auxin influx and efflux carriers generating an auxin gradient in the founder cells. Multiple cell divisions form a dome-shaped primordium followed by the penetration of the primordium through the overlying cell layers (Du and Scheres, 2018). To avoid excessive damage of the primordia a coordinated auxin accumulation occurs in the cortex, endodermis, and epidermis controlled by LAX3 that triggers cell wall remodeling in the overlying cell layers prior to LR penetration (Péret *et al.*, 2009).

LR elongation has not been extensively studied, thus information about its regulation is limited. Hormonal interaction with auxin and abscisic acid it is known to control the emergence of the primordia that influences the LR elongation and branching (Figure 2) (Jung and McCouch, 2013). ABA inhibits the emergence of primordia in an auxin-independent manner through the suppression of auxin response which arrests growth prior to the activation of the meristem (De Smet *et al.*, 2003). The exact regulatory network is unknown but a recently identified *kob1* mutant of a putative glycosyl transferase, AtABI8/ELD1/KOBITO, showed properly formed but arrested LR primordia in the emergence phase (Pagant *et al.*, 2002). Additionally, the ABA biosynthesis loss-of-function mutants *aba1* and *aba3* had a larger root system (Signora *et al.*, 2001). ABA plays a major regulatory role in modulating the LR system under environmental stimuli (Harris, 2015) such as salt stress under which ABA represses LR emergence acting exclusively in the endodermis (Duan *et al.*, 2013).



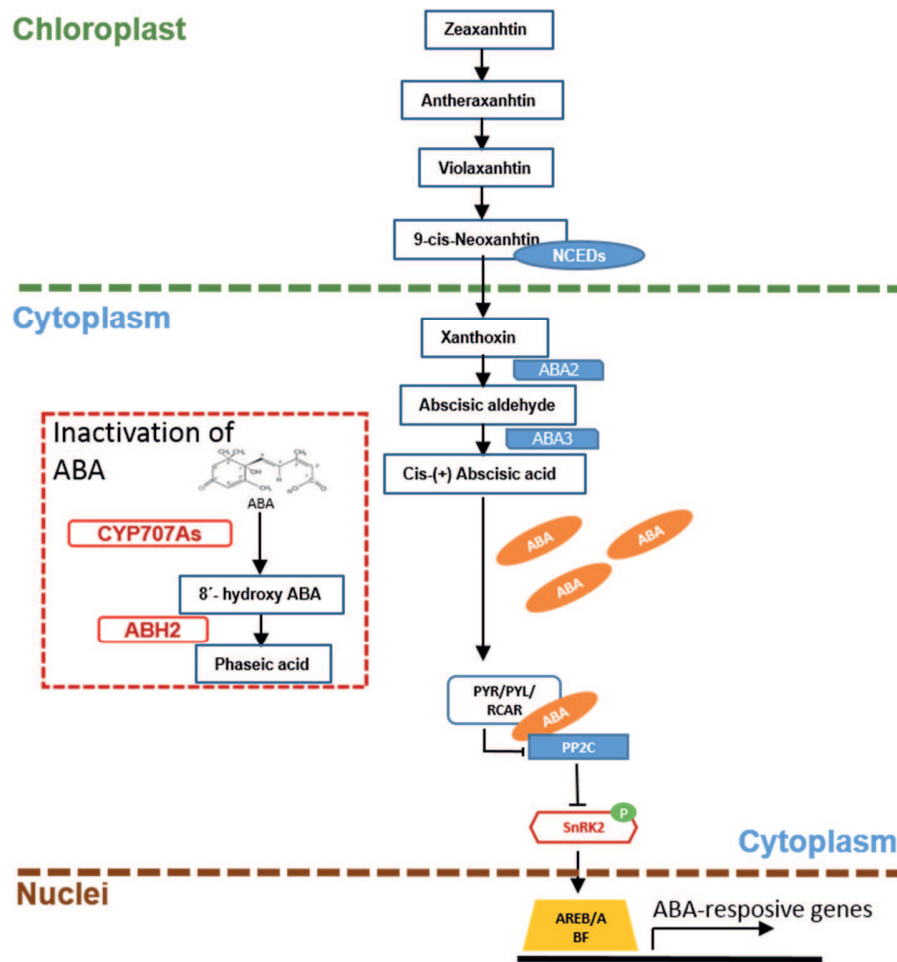
**Figure 2. Hormonal regulation of LR development by auxin and ABA.**

Blue arrows show auxin flux and accumulation controlling cell fate in all stages of LR development (initiation, transition, and emergence). V: vasculature, P: pericycle, C: cortex, E: endodermis, EP: epidermis (Based on Jung and McCouch, 2013, Vilches-Barro and Maizel, 2015 with Attribution-NonCommercial-ShareAlike 3.0 Unported (CC BY-NC-SA 3.0) license (<https://creativecommons.org/licenses/by-nc-sa/3.0/>)).

An ABA response is the result of ABA metabolism and signaling. Biosynthesis of the ABA precursor takes place in the plastid through the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway followed by carotenoid production. The key step in ABA synthesis is catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) that cleaves either 9-cis-violaxanthin or 9-trans-neoxanthin to produce xanthoxin, that is subsequently converted to ABA via ABA-aldehyde by sequential activity of ABA-deficient 2 and 3 (ABA2 and 3) in the cytosol. The cytosolic ABA either initiates ABA responses or is degraded through oxidation or conjugation pathways to eliminate bioactive ABA (Finkelstein, 2013).

For ABA inactivation, *Arabidopsis* utilizes ABA hydroxylation via ABA 8'-hydroxylases (CYP707A1-4) as a major pathway with phaseic acid as a product. Phaseic acid is converted to the non-bioactive (-)-dihydrophaseic acid by the phaseic acid reductase ABA hypersensitive 2 (ABH2) (Weng *et al.*, 2016). ABA is perceived by 15 PYR1/PYL/RCAR receptors (Ma *et al.*, 2009; Park *et al.*, 2009) that, when activated, relieve the inhibitory effect of group A type-2C protein phosphatases (PP2C) including ABI1, ABI2 or HAB2 (Hao *et al.*, 2011). ABI1 and its homolog ABI2 play a central role in ABA signaling as negative regulators of SNF1-related protein kinases 2 (SnRK2s) (Leung *et al.*, 1997). SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3 function in seed development, germination and abiotic stress responses through phosphorylation of AREB/ABF-type bZIP transcription factors that control ABA responses (Nakashima *et al.*, 2009) (Figure 3).





**Figure 3. ABA homeostasis and signaling.**

ABA biosynthesis takes place in the chloroplast through catabolic reactions via 9-cis-epoxycarotenoid dioxygenases (filled blue oval box, NCEDs), later on in the cytosol via xanthoxin dehydrogenases (filled blue round corner rectangle boxes ABA2 and 3). Biosynthesized ABA can be targeted by conjugative inactivation pathway involving ABA 8'-hydroxylases (non-filled red round corner square box, CYP707As) and phaseic acid reductase (non-filled red round corner square box ABH2). Alternatively, absciscic acid (filled orange oval box, ABA) directly initiates downstream ABA response perceived by ABA receptors (non-filled blue round corner square box, PYR/PYL/RCAR) that inactivates A type-2C protein phosphatases (filled blue square box, PP2C). As positive regulators of the ABA signaling SNF1-related protein kinases (non-filled red hexagon, SnRK) activate transcriptional regulators, such as ABF transcription factors (filled orange trapezoid box,



AREB/ABF) (Leung *et al.*, 1997; Ma *et al.*, 2009; Nakashima *et al.*, 2009; Park *et al.*, 2009; Finkelstein, 2013; Weng *et al.*, 2016).

### 5.7.3 Adventitious root

Similar to LR, adventitious roots (AR) are initiated as part of post-embryonic development but in contrast with LR, AR originates from non-root organs such as the root-shoot junction, hypocotyl, node, stem, and leaf and are formed from the closest vascular tissues (Bellini *et al.*, 2014; Steffens and Rasmussen, 2016). Several plant species including *Hedera helix* (ivy), *Fragaria × ananassa* (strawberry) or *Rhizophora spp.* (mangroves) form ARs as part of the normal developmental program to supply nutrition and physically support. On the other hand under certain environmental conditions such as etiolation, nutrient deficiency or abiotic stresses *Arabidopsis*, maize, tomato form stress-induced ARs (Steffens and Rasmussen, 2016).

Rice plants initiate AR primordia in the nodes during normal development that are induced to emerge in response to flooding (Lorbiecke and Sauter, 1999). AR emergence without tissue damages is particularly important to avoid pathogen infection under water. To ensure AR emergence without damages of the root tip, a complex regulatory network fine tunes programmed cell death (PCD) in the overlying epidermal cell layer of AR (Mergemann and Sauter, 2000). Ethylene is a primary signal in AR emergence. Ethylene as a gaseous hormone is entrapped in submergence tissue and, in addition, ethylene biosynthesis is induced (Metraux and Kende 1983; Voesenek *et al.*, 1990; Banga *et al.*, 1996; Mekhedov and Kende, 1996). Ethylene promotes growth of AR primordia mediated by reactive oxygen species (ROS). The growing primordium generates a mechanical force that acts locally on the overlying epidermal cells (Steffens *et al.*, 2012). Concomitantly, in the overlying epidermal cells, ethylene promotes ROS accumulation by inhibiting the expression of the ROS scavenger METALLOTHIONEIN2b (MT2b) (Steffens and Sauter, 2009). Force, together with an increased level of ROS, initiates cell death in the overlying epidermis cells allowing a barrier-free emergence of the AR.

In *Arabidopsis*, ARs are formed in etiolated hypocotyl that originates from the pericycle cells of the vasculature and adjacent to the xylem pole. Auxin plays a central role in AR

initiation and emergence similar to LR development and cross-talks with many other phytohormones such as cytokinin, ethylene, jasmonic acid and ABA (Bellini *et al.*, 2014).

Natural forms of auxin such as indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) accumulate due to the activity of YUCCA6 (YUC6) to establish AR initiation and later on the quiescent center of the root apical meristem. Additionally, proper efflux/influx auxin transport mediated by PIN1, PIN2, AUX1 and LAX3 is necessary for AR formation and emergence (Pacurar *et al.*, 2014; Bellini *et al.*, 2014).

In tomato and rice, ethylene induces AR emergence (Lorbiecke and Sauter, 1999; Muday *et al.*, 2012) while in *Arabidopsis* ethylene was reported to inhibit (Veloccia *et al.*, 2016) or promote it (Rasmussen *et al.*, 2017). Both studies verified the ethylene effect by exogenous ethylene treatment together with an examination of ethylene insensitive and sensitive mutant. Moreover, both ethylene biosynthesis and signaling seem to be important in regulating AR growth as confirmed by using the ethylene synthesis mutants *eto1* and *eto2* and the ethylene signaling mutants *ctr1*, *ein2*, *ein3*, and *ein3 eil1* (Veloccia *et al.*, 2016; Rasmussen *et al.*, 2017). The perception of ethylene is mediated by the receptors ETHYLENE RESISTANT 1 and 2 (ETR1/2), ETHYLENE RESPONSE SENSOR 1 and 2 (ERS1 and 2), and ETHYLENE INSENSITIVE 4 (EIN4) in the endoplasmic reticulum. They function together with CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) as a negative regulator of ethylene signaling. In the presence of ethylene, ETHYLENE INSENSITIVE 2 (EIN2) is relocated to the nucleus where it initiates the expression of ethylene response genes via the activation of ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE (EIL1) transcriptional regulators (Merchante *et al.*, 2013).

Ethylene and auxin form a complex regulatory network where they mutually control biosynthesis, transport, and responses in primary and secondary root development (Muday *et al.*, 2012). In conclusion, ethylene alters IAA biosynthesis and transport to enable fine-regulation of AR growth in a development phase-specific manner (Sukumar, 2010; Veloccia *et al.*, 2016). Further studies are however necessary to further elucidate the crosstalk between auxin and ethylene in AR development.

## 6 The aim of the study

Low oxygen conditions can occur as a consequence of flooding and are common abiotic stress that primarily affects roots and, as a consequence, plant vigor. The aim of this study is to advance our understanding of changes in root system architecture under low oxygen stress and the molecular mechanisms that regulate such adaptations. Root system architecture is the result of primary, lateral, junction and stress-induced adventitious root growth. The structure of the overall root system is characterized by the length, the angle, the rate of formation and, in the case of secondary roots, the position of the roots. In order to obtain comprehensive information about changes in the root system architecture of *Arabidopsis thaliana* under hypoxic conditions the following aspects are analyzed:

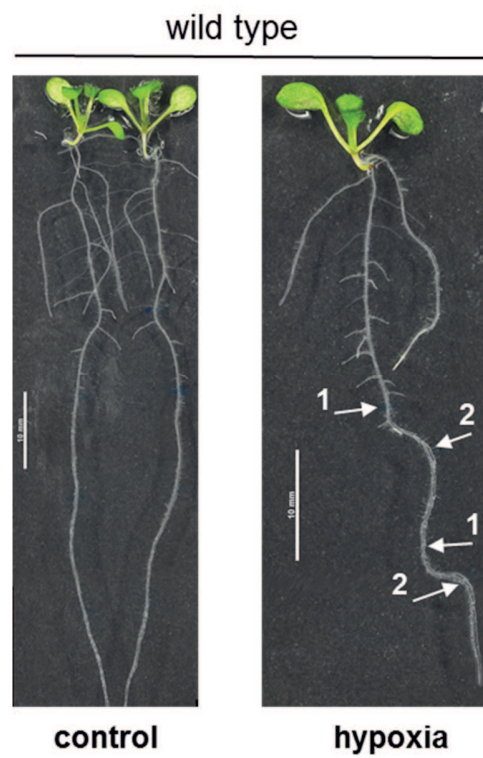
- Chapter 1: Primary root growth and angle
- Chapter 2: Lateral root formation, developmental stages, and elongation growth
- Chapter 3: Adventitious root formation and elongation

Root development is known to be under hormonal control. To understand the hormonal regulation of defined root developmental processes in response to hypoxia, the role of auxin in changing the root angle, the contribution of abscisic acid, that acts as an inhibitor of lateral root development, and the impact of ethylene on adventitious root formation and growth are analyzed using molecular-genetic and cytological approaches.

Furthermore, ETHYLENE RESPONSE FACTOR VII (ERFVII) transcription factors play a crucial role in low oxygen stress response, particularly to regulate the required metabolic changes whereas the involvement of ERFVII in root adaptations to low oxygen conditions has not been studied. For further mechanistic understanding of hypoxia signaling, the involvement of ERFVII in root system changes to low oxygen will be studied using single, and multiple knock out and overexpressing mutants. The approaches taken will add to our understanding of how root system architecture changes during hypoxia and of the regulatory mechanisms underlying these changes.

## 7 Chapter 1

### 7.1 Root bending is antagonistically affected by hypoxia and ERF-mediated transcription via auxin signaling



# Root Bending Is Antagonistically Affected by Hypoxia and ERF-Mediated Transcription via Auxin Signaling<sup>1[OPEN]</sup>

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When plants encounter soil water logging or flooding, roots are the first organs to be confronted with reduced gas diffusion resulting in limited oxygen supply. Since roots do not generate photosynthetic oxygen, they are rapidly faced with oxygen shortage rendering roots particularly prone to damage. While metabolic adaptations to low oxygen conditions, which ensure basic energy supply, have been well characterized, adaptation of root growth and development have received less attention. In this study, we show that hypoxic conditions cause the primary root to grow sidewise in a low oxygen environment, possibly to escape soil patches with reduced oxygen availability. This growth behavior is reversible in that gravitropic growth resumes when seedlings are returned to normoxic conditions. Hypoxic root bending is inhibited by the group VII ethylene response factor (ERFVII) RAP2.12, as *rap2.12-1* seedlings show exaggerated primary root bending. Furthermore, overexpression of the ERFVII member *HRE2* inhibits root bending, suggesting that primary root growth direction at hypoxic conditions is antagonistically regulated by hypoxia and hypoxia-activated ERFVII. Root bending is preceded by the establishment of an auxin gradient across the root tip as quantified with DII-VENUS and is synergistically enhanced by hypoxia and the auxin transport inhibitor naphthylphthalamic acid. The protein abundance of the auxin efflux carrier PIN2 is reduced at hypoxic conditions, a response that is suppressed by RAP2.12 overexpression, suggesting antagonistic control of auxin flux by hypoxia and ERFVII. Taken together, we show that hypoxia triggers an escape response of the primary root that is controlled by ERFVII activity and mediated by auxin signaling in the root tip.

Flooding is a common cause of hypoxia or anoxia in plants. Soil water logging and flooding are first encountered by roots, which respond to hypoxic conditions with metabolic adaptations, formation of aerenchyma, or formation of barriers to the radial loss of oxygen, that is, the reinforcement of the rhizodermis (Abiko et al., 2012; Sauter, 2013; Voisenek and Bailey-Serres, 2015). The AP2/ERF (apetala2/ethylene response factor) transcription factor family of *Arabidopsis* (*Arabidopsis thaliana*) has 147 members that can be subdivided into ten groups (Nakano et al., 2006). Members of group VII (short ERFVII) were identified as key regulators of low oxygen responses in *Arabidopsis* (Bailey-Serres et al., 2012; van Dongen and Licausi, 2015). ERFVII proteins are characterized by a conserved motif at the amino terminus that

initiates protein degradation at normoxic conditions via the N-end rule pathway (Gibbs et al., 2011; Licausi et al., 2011). At reduced oxygen levels, ERFVII are stabilized and engage in transcriptional regulation of hypoxia-responsive genes (Mustroph et al., 2009, 2010). A significant number of hypoxia-regulated genes in plants encode for enzymes of anaerobic metabolism such as alcohol dehydrogenase and lactate dehydrogenase. Fermentation and other metabolic adjustments ensure energy supply required to maintain cellular integrity in times when mitochondrial respiration is impaired. Plants, however, not only maintain basic cellular functions at low oxygen conditions, but they also invest resources to support developmental reprogramming. These are often but not always driven by ethylene that accumulates in flooded tissue (Sauter, 2013; Loreti et al., 2016).

In this study, we investigated developmental adaptation of the root system to hypoxia. Root architecture is determined by the rates of adventitious and lateral root formation, by primary and lateral root growth rates, and by the angles at which roots grow. Some plants such as *Oryza sativa* and *Solanum dulcamara* that are adapted to frequent flooding grow adventitious roots when submerged to replace soil-borne roots that become dysfunctional (Sauter, 2013; Dawood et al., 2014). However, not all plants are programmed to readily form adventitious roots upon flooding. We therefore hypothesized that such plants may adapt their root system to low oxygen conditions by alternative strategies.

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M.S. conceived the project and supervised the experiments; E.E.D. and M.S. designed the experiments; E.E.D. performed and analyzed the experiments; and M.S. wrote the manuscript with contributions from E.E.D.

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We employed *Arabidopsis* as a model plant to study developmental adaptation of roots to hypoxia and to investigate the role of ERFVIIs in root adaptation. Our studies revealed that ERFVII-dependent and ERFVII-independent hypoxia signaling control root structure by altering auxin activity.

Auxin determines root architecture by regulating growth rate, lateral root formation, and root growth direction. Auxin is a unique plant hormone in that its transport occurs in a polar manner, thereby providing a mechanism to actively generate auxin gradients. Indole-3-acetic acid (IAA), the main natural auxin in plants, is transported from cell to cell. IAA is protonated in the acidic apoplast and deprotonated in the weakly basic cytosol. Uptake of the uncharged molecule occurs by AUX1/LAX (auxin1/like AUX1) uptake carriers (Péret et al., 2012) in an undirected way. Extrusion of negatively charged IAA occurs via undirected ABCB transporter-mediated efflux (Noh et al., 2001; Geisler et al., 2005) and by PIN (pin-formed) proteins that are responsible for directed auxin movement (Friml et al., 2003; Blilou et al., 2005). The *Arabidopsis* genome codes for eight PIN proteins (Friml et al., 2003). Three of these, PIN5, PIN6, and PIN8, are localized at the ER membrane and engage in intracellular auxin transport (Adamowski and Friml, 2015). PIN1, PIN2, PIN3, PIN4, and PIN7 are localized at the plasma membrane where they support polar auxin transport. In roots PIN1, PIN3, PIN4, and PIN7 are found at the lower, root tip-facing end of cells where they drive acropetal auxin transport. PIN2 is localized at the upper side of root epidermal cells and ensures basipetal auxin movement away from the root apex toward the base. Together, the auxin transporters drive the so-called reverse fountain movement of auxin, which describes auxin transport in the central cylinder toward the tip, redistribution to the sides in the root cap, and transport toward the base in the lateral root cap and epidermis (Blilou et al., 2005). Tropic responses such as root gravitropism result from asymmetric auxin distribution across the root that can be established when movement of auxin to the elongation zone is higher on one side than the other. While AUX1/LAX transporters determine auxin levels, directional auxin transport is driven by PIN proteins (Band et al., 2014).

In this study, we describe a change in root growth direction in response to hypoxia. Our analyses showed that root slanting is subject to regulation by ERFVIIs and functionally linked to polar auxin transport, revealing a role for auxin in hypoxia adaptation.

## RESULTS

### Expression of *HRE2* in Hypoxic Roots

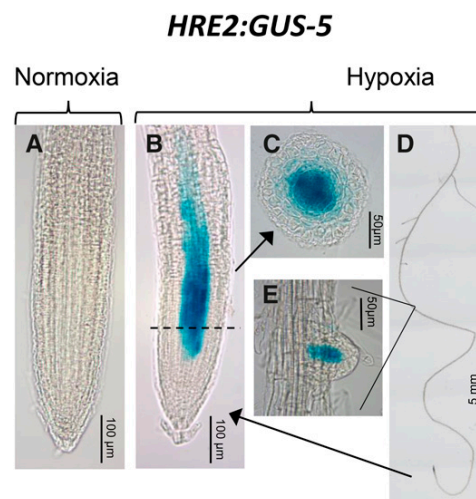
To gain insight into the role of the oxygen-sensing subgroup VII AP2/ERFs (abbreviated ERFVIIs) in regulating root development, we employed promoter:GUS lines to study expression of *HRE2* in roots at

normoxic and hypoxic conditions (Fig. 1, A–E). No activity was observed in roots of *HRE2:GUS5* seedlings at normoxic conditions (Fig. 1A). After exposure to 2% oxygen for 1 d, GUS staining was observed close to the root tip in the developing vasculature (Fig. 1, B–D). A similar pattern was visible in lateral roots (Fig. 1E). Induction of gene expression at hypoxic conditions indicated that *HRE2* may mediate root adaptation specifically to low oxygen conditions. By contrast, expression of *HRE1* was induced by low oxygen and in response to ethylene (Hess et al., 2011; Supplemental Fig. S1).

### Hypoxia Induces Root Slanting

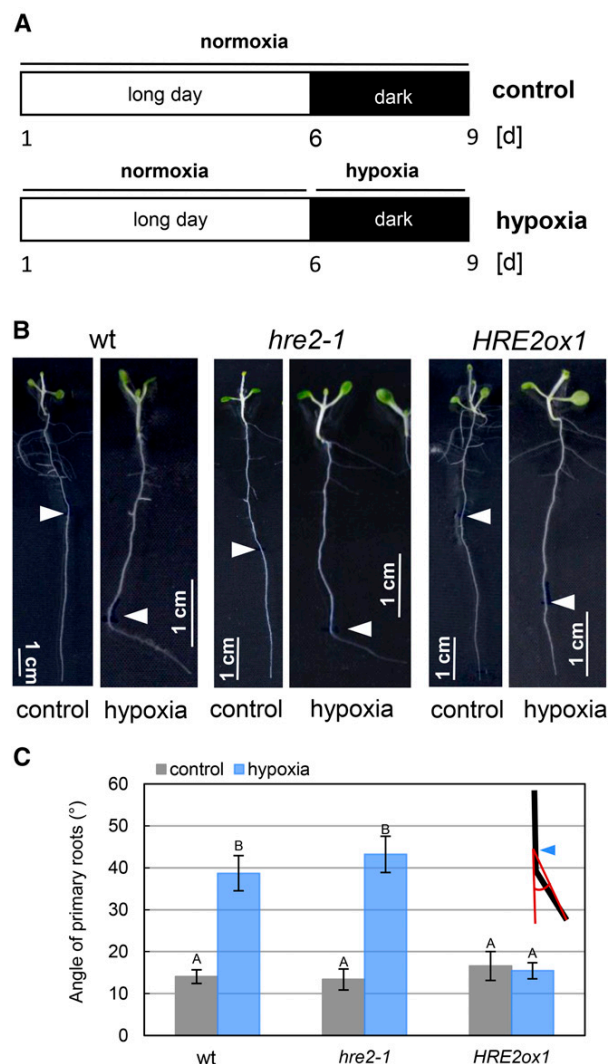
To study root development during hypoxia, we used seedlings grown for 6 d at long-day conditions on plates placed at a near-vertical angle and transferred them to the dark for 3 d to avoid photosynthetic oxygen evolution either at normoxic (21% O<sub>2</sub>) or hypoxic (2% O<sub>2</sub>) conditions (Fig. 2A). Exposure of wild-type seedlings to 2% O<sub>2</sub> caused slanting of the primary root (Fig. 2B). The primary root of seedlings kept at normoxic conditions grew downward with an average deviation of about 14° from the vertical (Fig. 2C). Exposure to hypoxia caused seedling roots to change growth direction within about 12 h. On average, the angle increased to 38.7° after 3 d of hypoxia.

We hypothesized that the change in growth direction of the primary root at low oxygen conditions was not simply due to a reduced energy supply but was a developmentally regulated process. To test that hypothesis,



**Figure 1.** *HRE2:GUS* expression is induced by hypoxia in the developing stele. Histochemical GUS analysis of 7-d-old *HRE2:GUS-5* seedlings that were exposed to (A) normoxic (21% O<sub>2</sub>) or (B–E) hypoxic (2% O<sub>2</sub>) conditions for 1 d. A, Normoxic primary root tip. B, Hypoxic primary root tip. C, Cross-section through primary root tip as indicated in B. D, Hypoxic root. E, Hypoxic lateral root after emergence.





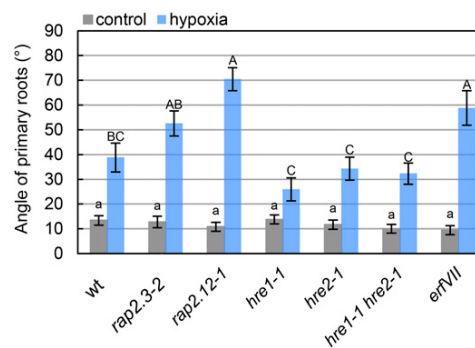
**Figure 2.** Hypoxia alters primary root growth direction. **A**, Six-day-old light-grown seedlings were transferred to the dark at control (21%  $O_2$ ) or hypoxic (2%  $O_2$ ) conditions for 3 d. **B**, Representative roots of 9-d-old wild-type, *hre2-1* and *HRE2ox1* seedlings exposed to the treatment protocols shown in **A**. Arrowheads indicate the position of the root tip at the beginning of the hypoxia treatment. **C**, Average angles by which the primary root changed its growth direction in the three genotypes exposed to normoxia or hypoxia. Results are averages ( $\pm$  se) from three independent experiments. Different letters indicate significantly different values (Kruskal-Wallis test with Dunn's test,  $n = 24-30$ ,  $P < 0.05$ ). Insert: schematic showing how the angles were determined. The blue arrowhead indicates the position of the root tip at time of transfer.

we explored the involvement of ERFVIIIs in regulating the slanting response. Since *HRE2* was induced in roots upon hypoxia, we employed *HRE2* knockout and over-expression lines to study an *HRE2* function in slanting. Six-day-old seedlings were exposed to 3 d of hypoxia or to ambient oxygen conditions in the dark (Fig. 2A). The *hre2-1* knockout line (Supplemental Fig. S2; Hess et al., 2011) showed wild-type slanting, whereas *HRE2ox1* seedlings

had lost their ability to change root growth direction in response to low oxygen (Fig. 2, B and C), suggesting that slanting was repressed by ERFVIIIs. The observation that root slanting was not increased in *hre2-1* seedlings suggested that ERFs other than *HRE2* might naturally regulate root growth direction.

To test the contribution of other ERFVIIIs in the control of root slanting, we compared the slanting response in knockout mutants of *RAP2.3*, *RAP2.12*, *HRE1*, *HRE2*, the double knockout line *hre1-1 hre2-1* and the quintuple knockout line *erfVII* (Abbas et al., 2015; Fig. 3). For *RAP2.2*, a true knockout is not available. It was therefore not analyzed. The gene models with the insertion sites of the T-DNA are shown in Supplemental Figure S2. Loss of gene expression in the knockout lines was verified by reverse transcription-PCR (RT-PCR). Compared to wild type, *rap2.12-1* seedlings displayed significantly increased root bending (Fig. 3) with a mean angle of 70.4°. A role of ERFVIIIs in the suppression of hypoxia-induced root slanting was further supported by the exaggerated slanting phenotype of *erfVII* seedlings. Loss of ERFVIIIs activity resulted in primary root growth that deviated from the gravity vector by a mean angle of 58.8°. Interestingly, while hypoxic *hre1-1*, *hre2-1*, and the *hre1-1 hre2-1* double knockout seedlings did not have a significantly different root growth angle compared to wild type, they did slant significantly less than *rap2.3-2* and *rap2.12-1* seedlings, indicating that the function of *RAP2.3* and *RAP2.12* is not fully redundant to that of *HRE1* and *HRE2*. The data further suggested that *RAP2.12* plays a key role in controlling root bending.

Imposing alternating hypoxic and normoxic conditions (Fig. 4A) revealed that primary root bending induced by hypoxia was reversible. Roots rapidly



**Figure 3.** Root slanting at hypoxic conditions is negatively controlled by *RAP2.12*. Seedlings of wild type; the single gene knockout lines *rap2.3-2*, *rap2.12-1*, *hre1-1*, and *hre2-1*; the double knockout line *hre1-1 hre2-1*, and the quintuple knockout line *erfVII* were exposed to hypoxic conditions as indicated in Figure 2A. Subsequently, the angle by which roots deviated from the original growth direction was determined. Results are averages ( $\pm$  se) from three independent experiments. Lowercase a indicates no difference between genotypes at normoxic (control) conditions; different capital letters indicate significant differences between genotypes at hypoxic conditions (Kruskal-Wallis test with Dunn's test,  $n = 25-29$ ,  $P < 0.05$ ).

returned to gravitropic growth when transferred from hypoxic to normoxic conditions and resumed slanting during a second hypoxic phase (Fig. 4B), suggesting that agravitropic root growth in response to oxygen limitation is a tightly regulated and reversible process.

### Hypoxia Triggers Asymmetric Auxin Distribution at the Root Apex

According to the Cholodny-Went theory, directed growth of plant organs such as phototropic or gravitropic growth is the result of an auxin gradient that is established across the organ (Briggs, 2014). To test if hypoxia alters auxin levels we employed the DII-VENUS reporter line to

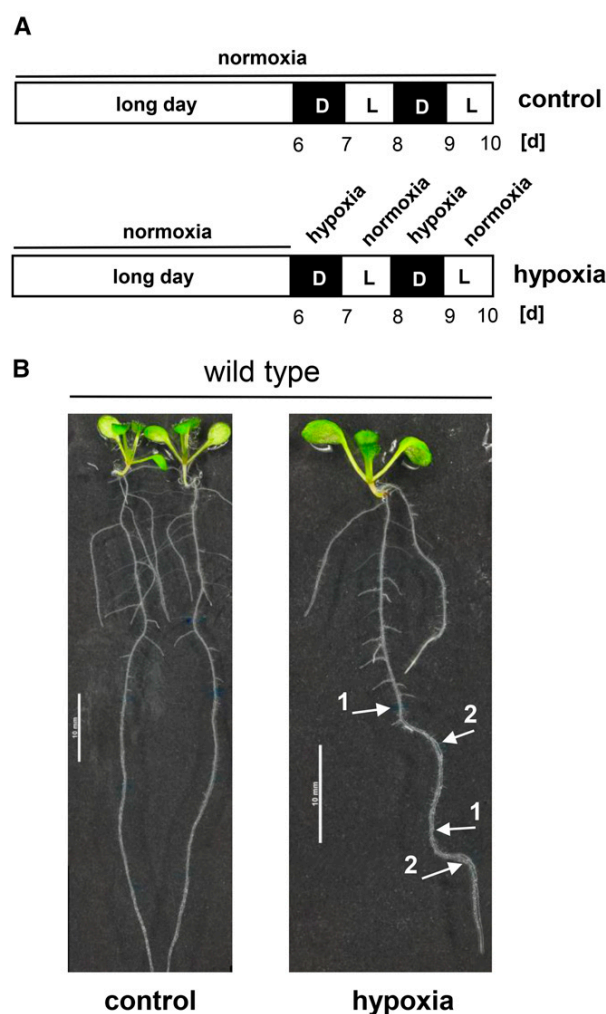
study auxin activity at the root tip (Fig. 5). The DII-VENUS protein is degraded as a consequence of SCF<sup>TIR1</sup>-mediated auxin signaling (Brunoud et al., 2012). The DII-VENUS fluorescence was visualized by confocal laser scanning microscopy (Fig. 5A). It decreased when seedlings were exposed to 2% oxygen for 7 h compared to control seedlings kept in air, indicating that auxin activity was elevated (Fig. 5, A and B). Furthermore, the distribution of the fluorescence across the root became significantly more asymmetric at hypoxic conditions revealing an auxin gradient (Fig. 5, A and C).

### Auxin Enhances Hypoxia-Induced Root Bending

To further test for a role of auxin in hypoxia-induced root slanting, we preincubated 5-d-old wild-type, *hre2-1*, and *HRE2ox1* seedlings (Supplemental Fig. S4) with the auxin analog  $\alpha$ -naphthalene acetic acid ( $\alpha$ -NAA) or the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA; Geisler et al., 2005; Nishimura et al., 2012; Zhu et al., 2016) for 1 d followed by hypoxic treatment for another 2 d or by normoxic conditions as a control in the presence of auxin or NPA (Fig. 6A). In the presence of 20 nM  $\alpha$ -NAA, wild-type roots showed significantly increased bending even at normoxic conditions, with an average growth angle of 17.2° compared to 9.1° in the control (Fig. 6, B and C). In hypoxia, the average angle increased to 33.6° in wild type.  $\alpha$ -NAA and hypoxia caused bending at an angle of 55.1°. Root bending of wild-type seedlings was promoted by 0.5  $\mu$ M NPA from 9.1° to 26.9° at normoxic and from 33.6° to 93.2° at hypoxic conditions. The response to NPA and hypoxia exceeded the responses to the single treatments, indicating that the two signals act in a synergistic manner pointing to a role of auxin transport in hypoxia-induced root slanting. *hre2-1* seedlings showed wild-type slanting behavior while slanting was reduced in *HRE2ox1* seedlings at hypoxic conditions but not fully inhibited when seedlings were exposed to auxin or NPA.

To further explore the link between hypoxia and auxin, we employed the *DR5:GUS* marker as a positive indicator for auxin activity. *DR5:GUS* seedlings were treated with 20 nM  $\alpha$ -NAA or 0.5  $\mu$ M NPA or left untreated (Fig. 6, A and D). Treatment with  $\alpha$ -NAA increased auxin activity at the root apex. NPA enhanced auxin activity, particularly at the root apical meristem as described previously (Ottenschläger et al., 2003). At hypoxic conditions GUS activity increased likewise at the root apical meristem, a pattern that was even more pronounced when hypoxic seedlings were treated with NPA, further supporting the idea that auxin distribution is a target of hypoxia signaling. An asymmetric auxin distribution across the root tip was not observed in *DR5:GUS* seedlings, likely due to the lower sensitivity of this reporter as compared to the DII-VENUS reporter (Brunoud et al., 2012).

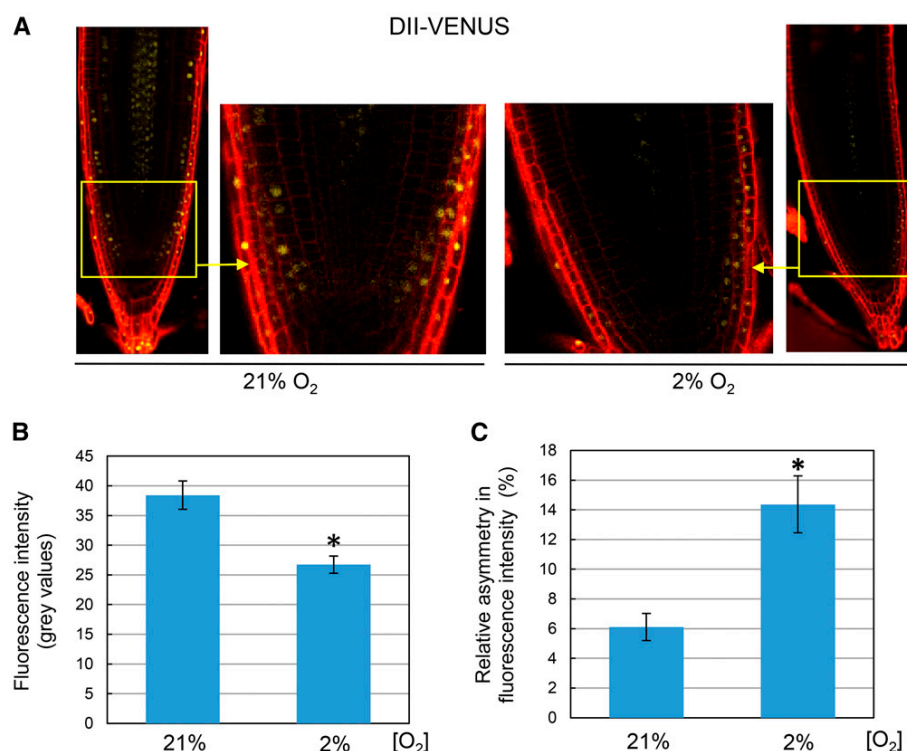
Our results revealed that root bending is synergistically enhanced by NPA and hypoxia, possibly suggesting that hypoxia signaling via ERFVII transcription factors targets auxin transport.



**Figure 4.** Hypoxia-induced slanting is a reversible growth response. **A**, Six-d-old seedlings were alternately exposed to hypoxic and normoxic conditions as indicated or kept at normoxic conditions as a control. **B**, Representative wild-type seedlings grown at normoxic conditions (control) or exposed to alternate normoxic and hypoxic conditions. Arrows indicate the position of the root tip at time of transfer to (1) hypoxia or (2) normoxia.



**Figure 5.** Hypoxia triggers asymmetric auxin distribution. A, Six-day-old DII-VENUS seedlings were exposed to normoxia or hypoxia for 7 h in the dark to examine auxin distribution at the root tip. B, Average ( $\pm$  se) fluorescence intensities at the root tip were quantified in three independent experiments. The asterisk indicates a significantly reduced value at hypoxic conditions (two-sample *t* test,  $n = 21\text{--}30$ ,  $P < 0.05$ ). C, Relative average asymmetries ( $\pm$  se) in fluorescence intensities between the two sides of the root tip. The asterisk indicates significantly different values (Mann-Whitney *U* test,  $n = 21\text{--}30$ ,  $P < 0.001$ ).



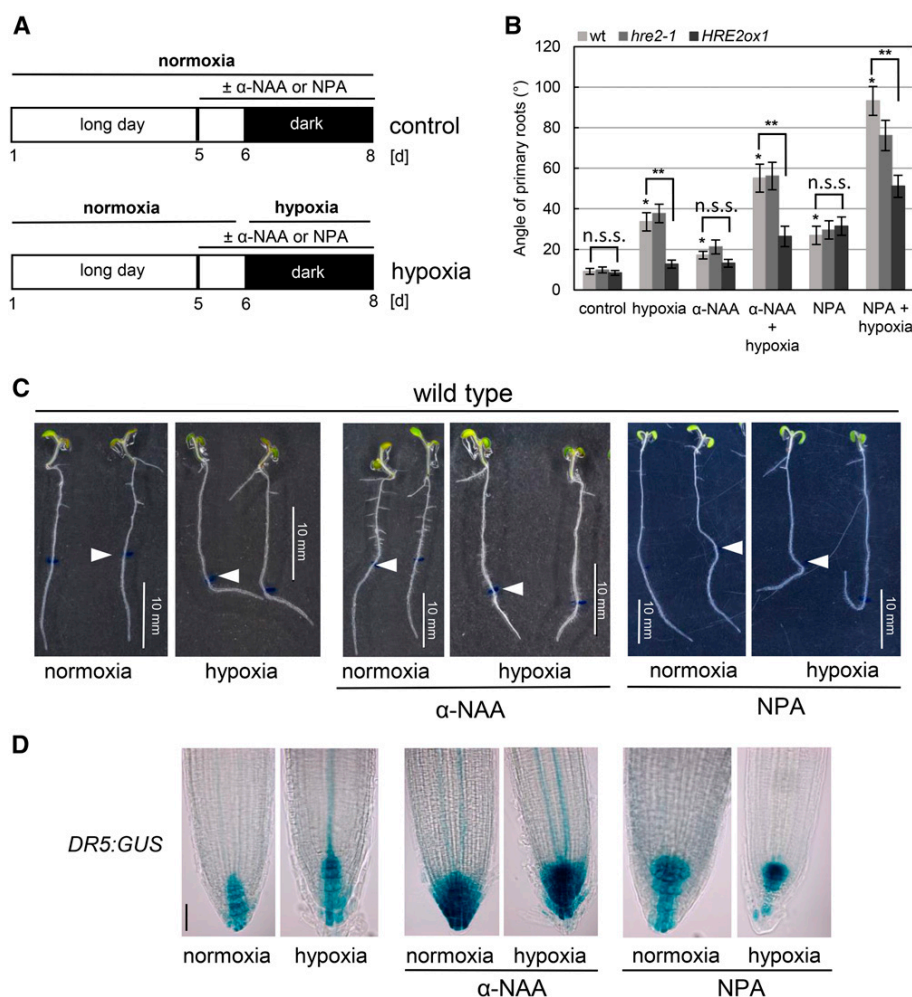
#### PIN2 Protein Abundance Is Antagonistically Regulated by Hypoxia and RAP2.12

Auxin distribution is an active process that is driven by auxin transporters. Since we observed local changes in auxin activity at hypoxic conditions and since NPA mimicked the auxin distribution at the root tip that was observed upon hypoxia, we hypothesized that hypoxia promotes root bending by acting on auxin transport proteins. Auxin transport is mediated by different auxin transport protein classes that include the PIN proteins that are responsible for polar auxin transport. To test the hypothesis that hypoxia promotes bending by altering polar auxin transport, we studied hypoxia-induced root bending in *pin1-1*, *pin2-1*, *pin3-4*, and *pin4-2* mutant seedlings.

Wild-type and *pin1-1*, *pin2-1*, *pin3-4*, and *pin4-2* knockout seedlings were pretreated with 20 nM  $\alpha$ -NAA for 1 d or left untreated and subsequently transferred to 2% O<sub>2</sub> in the dark for hypoxic treatment or kept at normoxic conditions (for the treatment regime, see Fig. 6A). The phenotypes of the wild-type and PIN mutant seedlings are shown in Figure 7A. Subsequently, the angle of primary root growth direction was determined (Fig. 7B; Supplemental Fig. S5). As observed previously, hypoxia and auxin induced bending in the wild type that was exaggerated when both treatments were combined. Among the *pin* mutants, *pin1-1*, *pin3-4*, and *pin4-2* seedlings displayed wild-type root bending at all conditions. By contrast, the *pin2-1* mutant showed root slanting at normoxic conditions, with an average root

growth angle of 26.8° as compared to 8.2° in wild type. However, neither hypoxia nor hypoxia in the presence of  $\alpha$ -NAA further increased the angle at which *pin2-1* roots grew (Supplemental Fig. S5). These findings indicate that lack of PIN2, which is responsible for transport of auxin from the root tip toward its base, renders roots unresponsive to auxin and hypoxia with regard to their growth angle.

The PIN2 gene was not regulated by hypoxia, and expression of PIN2 was not altered in the *erfVII* mutant, indicating that PIN2 is not transcriptionally regulated during low oxygen conditions (Fig. 8A). To further study a possible role of PIN2 in hypoxia signaling, we looked at PIN2 protein distribution at the root tip of *PIN2pro:PIN2-GFP* seedlings at hypoxic conditions (Fig. 8). Confocal laser scanning microscopy confirmed PIN2 localization in the epidermis and cortex. The abundance of PIN2 protein decreased within 5 h of hypoxia as compared to controls (Fig. 8C), possibly indicating that reduced basipetal auxin transport via PIN2 may be responsible for increased auxin levels in these cells (Fig. 5). On the other hand, PIN2 distribution remained symmetrical up to 6 h of hypoxia (Fig. 8D). To answer the question if PIN2 protein was regulated not only by hypoxia but also by ERFVII signaling, we crossed the *PIN2pro:PIN2-GFP* line with wild type as a control and with RAP2.12 overexpressing line *RAP2.12 OE2* (Gasch et al., 2016). Reduced PIN2 abundance by hypoxia was confirmed in the *PIN2pro:PIN2-GFP*  $\times$  wild-type line, whereas PIN2 was not reduced in



**Figure 6.** Root slanting is synergistically induced by hypoxia and the auxin transport inhibitor NPA. **A**, Treatment protocol: 6-d-old light-grown wild-type seedlings were pretreated with 20 nM  $\alpha$ -NAA or 0.5  $\mu$ M NPA and then transferred to the dark at control (21%  $O_2$ ) or hypoxic (2%  $O_2$ ) conditions for 2 d. **B**, Angles by which root growth direction deviate from the vertical vector were determined in three biological replicates. \*Significant difference between control and treatment in wild type; \*\*significant difference; n.s.s., no statistically significant difference between wild type and *HRE2ox1* (Mann-Whitney *U* test,  $n = 22-30$ ,  $P < 0.0001$ ). **C**, Representative roots of 8-d-old seedlings exposed to the treatments indicated in **A**. Arrowheads show the position of the root tip at the onset of the hypoxic treatment. **D**, Auxin activity at the root apex following the treatments indicated in **A** was visualized with the *DR5:GUS* reporter (bar = 50  $\mu$ m; applies to all pictures).

*PIN2pro:PIN2-GFP*  $\times$  *RAP2.12 OE2* seedlings (Fig. 8, E and F; Supplemental Fig. S6). Our data thus reveal an antagonistic effect of hypoxia and *RAP2.12* on *PIN2* protein abundance that may reflect a well-balanced control of auxin activity at the root apex at low oxygen conditions.

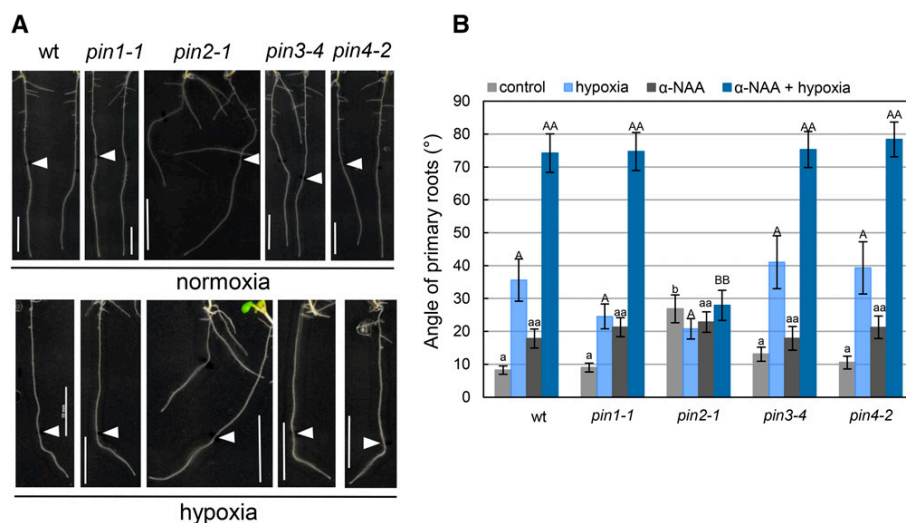
## DISCUSSION

### Hypoxia and ERFVII Antagonistically Regulate Primary Root Growth Direction

The ERFVII group of transcription factors has five members, *RAP2.2*, *RAP2.3*, *RAP2.12*, *HRE1*, and *HRE2*, that were shown previously to regulate hypoxic response genes (Licausi et al., 2010; Hess et al., 2011; Bui et al., 2015), prominently among which are genes involved in the metabolic shift from respiration to fermentation. More recently, however, *RAP2.2* and *RAP2.12* were identified as principle activators of hypoxia-responsive genes, while *RAP2.3*, *HRE1*, and *HRE2* were shown to

play minor roles (Gasch et al., 2016). The expression of the ERFVII members are differentially induced by environmental factors such as darkness, hypoxia, or ethylene (Hinz et al., 2010; Licausi et al., 2010; Hess et al., 2011), supporting the idea that ERFVII members have distinct physiological functions.

This study shows that Arabidopsis seedlings that encounter hypoxic conditions change the direction of primary root growth. This response is under negative control of ERFVII, as the quintuple *erfVII* mutant displays exaggerated root bending. A comparable phenotype was observed in *rap2-12-1* seedlings but not in *rap2.3-2*, *hre1-1*, and *hre2-1* seedlings, indicating that *RAP2.12* acts to suppress root bending in response to hypoxic conditions. *HRE2* is expressed at low oxygen conditions in the developing vasculature at the root tip, but knockout of *HRE2* does not alter the slanting response of the primary root, indicating that ERFVII activity in the developing stele is not sufficient to change root growth direction. Yet, when expressed ectopically at high levels, *HRE2* efficiently inhibited hypoxia-induced



**Figure 7.** The *pin2-1* mutant is unresponsive to hypoxia and auxin. A, Representative root phenotypes of 8-d-old wild-type, *pin1-1*, *pin2-1*, *pin3-4*, and *pin4-2* seedlings grown at normoxic or hypoxic conditions for 2 d (bar = 10 mm). B, Average angles of primary root growth direction in wild type, *pin1-1*, *pin2-1*, *pin3-4* and *pin4-2* seedlings treated as indicated in Figure 6A. Different single minor letters indicate significantly different values in the control; different capital letters indicate significantly different values in hypoxia-treated seedlings; different double minor letters indicate significantly different values in seedlings treated with 20 nM  $\alpha$ -NAA; different double capital letters indicate significantly different values in seedlings treated with 20 nM  $\alpha$ -NAA at hypoxic conditions (Kruskal-Wallis test with Dunn's test,  $n = 23$ – $25$ ,  $P < 0.01$ ). Results are averages ( $\pm$  SE) from three independent experiments. Statistically significant differences within a given genotype are shown in Supplemental Figure S5.

root bending, suggesting that it can function redundantly to RAP2.12 depending on its spatiotemporal expression.

Interestingly, hypoxia promotes slanting as well as stabilization of RAP2.12, which, in turn, inhibits slanting. In wild-type seedlings, the angle of hypoxia-induced root bending was intermediary to that observed in *HRE2ox1* and *rap2.12-1* or *erfVII* seedlings, indicating that the growth angle of hypoxic roots is regulated by ERFVII activity in a dose-dependent manner making ERFVII a potential hub for signal integration. Environmental signals other than the oxygen concentration might determine root growth direction by controlling RAP2.12 activity through transcriptional regulation, protein stability, or subcellular localization (Licausi et al., 2011; Kosmacz et al., 2015).

Slanting primary roots rapidly resumed gravitropic growth when seedlings were returned to normoxic conditions, indicating that the gravisensing mechanism was intact. Gravity sensing in roots is performed in root cap cells by amyloplasts that act as sedimenting statoliths (Baldwin et al., 2013). The molecular mechanism that underlies gravity perception is not understood. The signal is transmitted from the root cap statocytes via basipetal auxin transport to the root elongation zone mediated by the polar auxin transporter PIN2 (Müller et al., 1998). Differential auxin transport between the lower and upper sides of the root tip results in differential growth manifested as root bending. Hypoxia offsets gravitropic primary root growth while resupply of oxygen to normoxic levels restores it. Genetic data point to RAP2.12 as the major ERFVII that limits root

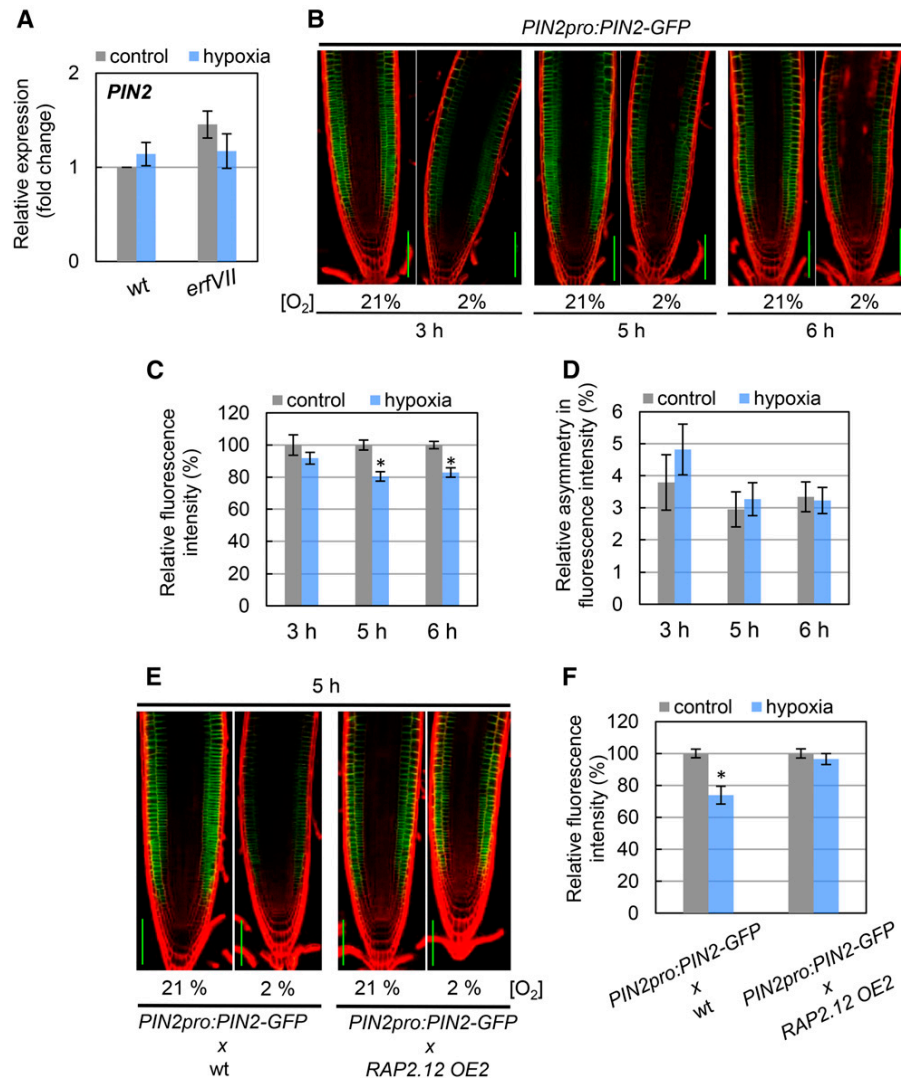
slanting during hypoxia without fully inhibiting it, suggesting that slanting serves a physiological function. Primary root slanting might be an escape response that is induced by hypoxia and at the same time restricted by hypoxia-stabilized RAP2.12.

In nature, roots encounter hypoxic conditions due to soil water logging or flooding. If oxygen availability declines with soil depth, sideways growth of the root may help to reach remaining gas spaces to maintain root oxygen supply. When the root is well supplied with oxygen positive gravitropic growth resumes to ensure anchoring and exploration of nutrients and water in deeper soil layers.

#### ERFVII and Auxin Pathways Interact to Control Root Slanting

Application of  $\alpha$ -NAA or NPA increased auxin activity at the root tip as detected by the *DR5:GUS* reporter. At the same time, the angle at which roots grew when exposed to hypoxia was exaggerated with auxin and NPA. NPA inhibits auxin flux primarily by inhibiting nonpolar ABCB19 auxin transporters and PIN-type auxin transporters, which results in a local increase in auxin concentration (Ottenschläger et al., 2003; Geisler et al., 2005; Nishimura et al., 2012; Habets and Offringa, 2014; Zhu et al., 2016). A synergistic effect of NPA and hypoxia and, to a lesser degree, of auxin and hypoxia was observed with regard to root bending, indicating that hypoxia and auxin signaling pathways





**Figure 8.** Hypoxia causes a decrease in PIN2 abundance at the root tip. **A**, Expression levels of *PIN2* in the root tips of 6-d-old wild-type and *erfVII* seedlings exposed to 21% O<sub>2</sub> (control) or 2% O<sub>2</sub> (hypoxia) for 8 h in the dark were quantified by quantitative RT-PCR. Results are means ( $\pm$  SE) of three independent biological replicates with two technical repeats each (one-way ANOVA with Tukey's test,  $P < 0.05$ ). **B**, Visualization of PIN2-GFP at the root tip of 6-d-old *PIN2pro:PIN2-GFP* seedlings that were exposed to normoxia or hypoxia for 3, 5, or 6 h in the dark. Scale bar = 100  $\mu$ m. **C**, Average ( $\pm$  SE) fluorescence intensities at the root tips of hypoxic seedlings were normalized to the control samples in three independent experiments. Asterisks indicate significantly different values (two-sample *t* test,  $n = 18$ –20,  $P < 0.05$ ). **D**, PIN2-GFP fluorescence intensities across the root tip are not significantly different. Results are averages ( $\pm$  SE,  $n = 18$ –20) from three independent experiments. **E**, PIN2-GFP fluorescence in *PIN2pro:PIN2-GFP*  $\times$  wild-type seedlings and in *PIN2pro:PIN2-GFP*  $\times$  *RAP2.12 OE2* seedlings exposed to normoxic or hypoxic conditions. Scale bar = 100  $\mu$ m. **F**, Quantification of relative PIN2-GFP fluorescence ( $\pm$  SE) in the root tips of *PIN2pro:PIN2-GFP*  $\times$  wild type and *PIN2pro:PIN2-GFP*  $\times$  *RAP2.12 OE2* seedlings reveal a regulatory role of *RAP2.12* in PIN2 abundance at hypoxic conditions. The asterisk indicates a significant difference to the control (two-sample *t* test,  $n = 14$ –17,  $P < 0.05$ ).

interact. Overexpression of *HRE2* fully repressed root bending at hypoxic conditions but only partially reduced root bending in response to auxin or NPA at hypoxic conditions, suggesting that elevated auxin activity can partly overcome ERFVII activity and may hence act downstream of ERFVII. The distribution of DR5:GUS activity at the root tip was altered in hypoxic

as compared to normoxic conditions, pointing to auxin transport as a target of hypoxia signaling.

The DR5 reporter is based on multiple tandem repeats of a highly active synthetic auxin response element (AuxRE) that are fused upstream of a minimal 35S promoter-GUS reporter gene (Ulmasov et al., 1997). *DR5:GUS* expression does not directly indicate endogenous

auxin abundance but also depends on auxin signaling capacities and rates of transcription and translation, which may vary locally. The DII-VENUS reporter is based on the constitutive expression of a fusion protein of the auxin-binding domain (DII) of an Aux/IAA protein with the fluorescent VENUS protein (Brunoud et al., 2012). Binding of auxin to its receptor TIR1 results in degradation of DII-VENUS. Due to this immediate regulation by auxin, DII-VENUS fluorescence is directly related to endogenous auxin levels in that fluorescence decreases with elevated auxin abundance, and it is more sensitive than DR5:GUS activity. Analysis of the DII-VENUS reporter line confirmed increased auxin levels in root tips at hypoxic conditions and furthermore revealed asymmetric auxin distribution in the outer root cell layers. The lateral root cap and epidermis cells are the site of shootward auxin transport and were shown to be crucial for the gravitropic response (Swarup et al., 2005). Changes in auxin abundance and distribution in hypoxic conditions clearly suggest that hypoxia signaling affects auxin transport.

### Hypoxia Increases Auxin Activity at the Root Tip

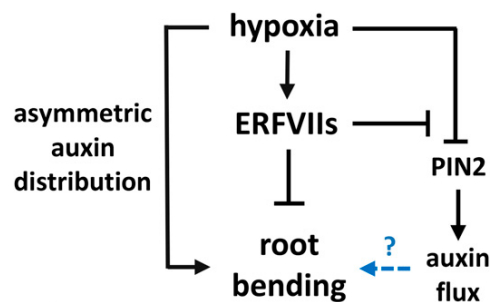
Auxin movement from cell to cell is achieved by nonpolar auxin influx mediated by AUX1/LAX carriers and by auxin efflux carriers (Band et al., 2014). Auxin efflux is mediated by the nonpolar ABCB carriers and by the polar auxin efflux carriers PIN1, PIN2, PIN3, PIN4, and PIN7 (Habets and Offringa, 2014). AUX1/LAX carriers control auxin levels, while PINs control the direction of auxin transport within tissues (Band et al., 2014). Basipetal auxin transport in roots is mediated by PIN2 that is localized in the epidermis and cortex at the root apex (Wisniewska et al., 2006). In epidermal cells, PIN2 was shown to be localized to the upper side, whereas in cortex cells PIN2 is found at the basal side (Wisniewska et al., 2006). PIN2-GFP abundance decreased at hypoxic conditions. Reduced PIN2 protein abundance may limit basipetal auxin transport and thereby contribute to the increased auxin activity that we observed at the root tip with the DR5:GUS and DII-VENUS reporters in response to hypoxia. Elevated auxin activity at the root tip has been observed in the *pin2* mutant (Boonsirichai et al., 2003). Unlike other *pin* mutants that do not have an altered root gravitropic response, the *pin2* mutant shows weakly agravitropic root growth (Müller et al., 1998) that was also observed in this study. Hypoxia did not further increase the angle of agravitropic growth of the *pin2-1* mutant. Hypoxia and  $\alpha$ -NAA promoted root bending in a nonadditive manner in wild type but, again, did not enhance root slanting of *pin2-1* seedlings. All other PIN mutants, *pin1-1*, *pin3-4*, and *pin4-2*, showed a wild-type root growth phenotype in response to hypoxia. These data indicate that PIN2 acts epistatic to hypoxia.

Down-regulation of PIN2 protein by hypoxia and suppression of this down-regulation by RAP2.12 overexpression indicate that auxin activity at the root

tip is actively controlled by modulation of PIN2 protein abundance. As elevated auxin activity at the root tip goes along with increased root bending (Fig. 6), we hypothesize that a decrease in PIN2 protein level reduces shootward auxin flux, causing auxin to accumulate at the apex, which promotes root bending (Fig. 9).

PIN2 is degraded by the ubiquitin-proteasome pathway in the vacuole (Abas et al., 2006; Leitner et al., 2012; Adamowski and Friml, 2015). Reduced PIN2 levels in roots exposed to hypoxia may indicate that hypoxia signaling targets the PIN2 protein degradation pathway. Baster et al. (2013) suggested that posttranslational regulation of PIN2 could lead to its proteasomal and vacuolar lytic degradation. The polar localization of PIN2 is regulated via phosphorylation by PINOID (PID) and its related protein kinases WAG1 and WAG2 that in turn are antagonized by protein phosphatase 2AA (Michniewicz et al., 2007; Adamowski and Friml, 2015; Santner and Watson, 2006). It is not shown here but conceivable that a similar regulatory mechanism targets PIN2 for degradation. Future work will have to unveil the mechanism by which hypoxia and RAP2.12 control PIN2 abundance.

The theory proposed by Cholodny and Went states that organ bending is caused by an auxin gradient across the organ. A difference in auxin levels across the root causes cells to elongate at different rates and the root to bend. However, while we did observe an auxin gradient, we did not observe an asymmetric distribution of PIN2 protein using the *PIN2pro::PIN2-GFP* reporter line. Since PIN2 distribution is highly dynamic, it is conceivable that polar PIN2 activity differentially changes at the two sides of the root in response to hypoxia and that we simply did not microscopically resolve this asymmetry. Alternatively, there is no PIN2 asymmetry and the auxin gradient is formed independent of PIN2 distribution.



**Figure 9.** Model: Hypoxia signaling controls primary root growth direction in an ERFVII-dependent and ERFVII-independent manner. Hypoxia induces root bending by establishing an auxin gradient across the root tip, whereas hypoxia-stabilized ERFVIIIs limit root bending. The polar auxin transporter PIN2 is antagonistically regulated by hypoxia and RAP2.12 at the protein level, suggesting control of auxin flux. Both the hypoxia-induced lateral auxin gradient and reduced basipetal auxin flux alter local auxin activities that contribute to root bending.

## Conclusions

Arabidopsis seedlings adapt to hypoxic conditions by a change in root growth direction, possibly to explore gas spaces in the soil with higher oxygen levels. The angle of root bending is restricted by the activity of the oxygen-sensing ERFVII member RAP2.12 that gets activated at hypoxic conditions (Fig. 9). PIN2 is a polar auxin transporter that drives auxin transport from the root tip to the root elongation zone. Hypoxia reduces PIN2 abundance, promotes auxin activity at the root tip, and enhances root bending, whereas the hypoxia-stabilized RAP2.12 promotes PIN2 abundance and restricts root bending, indicating tight control of the slanting response. In summary, hypoxia induces root slanting by altering auxin signaling at the root apex.

## MATERIALS AND METHODS

### Plant Material

Experiments were carried out with Arabidopsis (*Arabidopsis thaliana*) Col-0 originally obtained from NASC. All mutants are in the Col-0 background with the exception of *pin1-1*, which is in the Enkheim background (Okada et al., 1991). The T-DNA insertion lines *hre1-1* (*erf73-1*) and *hre2-1* (*erf71-1*) were described previously (Hess et al., 2011) and were used to generate the double knockout line *hre1-1 hre2-1* by crossing. Knockout of both genes was verified by RT-PCR (Supplemental Fig. S1) in seedlings exposed to hypoxic conditions. The *pin1-1*, *pin4-2* (Gälweiler et al., 1998; Friml et al., 2002a, 2002b) lines were obtained with consent from J. Friml (Institute of Science and Technology Austria, Austria). *pin4-2* was verified as a homozygous EN-1 transposon line with the gene-specific primers AT2G01420for2 and AT2G01420rev2 (Supplemental Table S1). The *erfVII* line (Abbas et al., 2015) was kindly provided by M. J. Holdsworth (University of Nottingham), the DII-VENUS marker line (Brunoud et al., 2012) was kindly provided by Tom Beeckman (Ghent University), and the *DR5:GUS* line (Ulmasov et al., 1997) was obtained from T. Ulmasov (University of Missouri). *PIN2pro:PIN2-GFP* (Swarup et al., 2008) was a kind gift from Malcolm J. Bennett (University of Nottingham). The RAP2.12 OE2 line was kindly provided by Angelika Mustroph (University Bayreuth) and was described previously by Gasch et al. (2016). We crossed *PIN2pro:PIN2-GFP* with wild type and with RAP2.12 OE2 to generate *PIN2pro:PIN2-GFP* × wild type and *PIN2pro:PIN2-GFP* × RAP2.12 OE2 lines. The expression level of RAP2.12 in wild type × *PIN2pro:PIN2-GFP* and RAP2.12 OE2 × *PIN2pro:PIN2-GFP* seedlings was verified by RT-PCR (Supplemental Fig. S6) using the primers ForAT1G53910 and RevAT1G53910 (Supplemental Table S1).

The T-DNA insertion lines *rap2.3-2* (WiscDsLox247E11) described by Marin-de la Rosa et al. (2014), *rap.2.12-1* (GK\_503A11) described by Gibbs et al. (2014), and *pin2-1* (GK\_262G07-014951) and *pin3-4* (SALK\_038609) described by Friml et al. (2003) were obtained from NASC and verified as homozygous knockout lines (Supplemental Figs. S1 and S3) with gene-specific and T-DNA insertion-specific primers (Supplemental Table S1) At3g16770For, At3g16770Rev, WiscDsLoxLB for RAP2.3, At1g53910For, At1g53910Rev, LB-GK for RAP2.12, At5g57090forRT, At5g57090revRT, LB-GK for PIN2, and AT1G70940forRT, AT1G70940revRT for PIN3.

### Growth Conditions and Analysis

Seeds were sterilized under continuous shaking (1,400 rpm) in 2% (w/v) sodium hypochlorite (Carl Roth) for 20 min at room temperature followed by three washing steps with sterilized ultra-pure water. Surface sterilized seeds were placed on square plates with 0.39% (w/v) gelrite (Duchefa), 1.5% (w/v) sucrose (Carl Roth), and half-strength Murashige and Skoog basal salt mix (Duchefa). For chemical treatments, 5-d-old seedlings were transferred to new plates supplemented with 0.5  $\mu$ M NPA (Sigma) or 20 nM  $\alpha$ -NAA (Sigma) for 1 d prior to hypoxic treatment. Hypoxic treatment was carried out in the dark in airtight acryl boxes flushed at a flow rate of 2 L/min for N<sub>2</sub> and 40 mL/min with O<sub>2</sub> to equilibrate the atmosphere at 2% oxygen. To ensure that the oxygen concentrations in the acryl boxes were as desired, they were measured by gas

chromatography at the beginning and end of the first treatment (Sasidharan et al., 2017). Controls were kept in acryl boxes in ambient air in the dark. Plates were placed upright leaning backward at an angle that deviated from the vertical by 6°. To measure the angle of primary root growth deviation, roots were imaged with an SMZ18 stereo microscope (Nikon) and a DS-U3 camera (Nikon) and analyzed with ImageJ (Schneider et al., 2012). The angles were measured between the growth direction of the primary root before transfer to normoxia or hypoxia in the dark and the line drawn from the position of the root tip at time of transfer to the position of the root tip at the end of the treatment as indicated in the cartoon in Figure 2C.

### Cloning and Plant Transformation

To generate 35S:HRE2 overexpressing lines, the open reading frame of *HRE2* was amplified by PCR with gene-specific primers (Supplemental Table S1, At2G47520\_TC\_forox, At2G47520\_TC\_revov primers) using cDNA isolated from seedlings exposed to hypoxia. The amplified fragment was cloned into the pENTR/D-Topo vector (Life Technologies) and subsequently recombined into the destination vector pB7WG2 using the Gateway cloning system (Karimi et al., 2002). This vector was transformed into *Agrobacterium tumefaciens* strain EHA105 followed by plant transformation of Col-0 with the floral dip method (Clough and Bent, 1998). T1 seeds were screened for glufosinate resistance and RNA was isolated from leaves of resistant plants. Transcript levels of *HRE2* and of *ACTIN2* as a control for RNA input were analyzed by RT-PCR with gene-specific primers (Supplemental Table S1; At2G47520forRT, At2G47520revRT, At2G47520forqPCR, and At2G47520revqPCR primers for *HRE2*; Actin2F1 and Actin2R1 primers for *Actin2*) using M-MuLV reverse transcriptase (Fermentas). Homozygous seeds were obtained after the T4 generation.

To generate *HRE2:GUS* lines, 728 bp 5' of the start codon of *HRE2* was amplified from genomic DNA with promoter-specific primers (Supplemental Table S1, erf71profor, erf71prorev primers). The amplified promoter sequence was cloned into the pBGWFS7 destination vector (Karimi et al., 2002), transformed into *Agrobacterium* followed by plant transformation as described above. T1 seeds were screened for glufosinate resistance. Homozygous seeds were obtained after the T4 generation.

### Quantitative RT-PCR Analysis

To investigate the gene expression of *PIN2* in wild type and *erfVII*, 5 mm of the root tips was collected for RNA isolation. Gene expression was analyzed by quantitative RT-PCR. The expression level in wild-type seedlings at control conditions was set to 1 and all other values are shown as multiples of that. *GAPC1* and *ACTIN2* were used for normalization. The primers AT5G57090qF and AT5G57090qR were used for the quantitative RT-PCR analysis of *PIN2*, Actin2qF and Actin2qR for *ACTIN2*, and GAPC1qF and GAPC1qR primers for *GAPC1* (Supplemental Table S1).

### Histochemical Analysis of Reporter Lines

Histochemical analysis of *HRE2:GUS* and *DR5:GUS* seedlings was performed according to Blázquez et al. (1997) with modifications. Plant material was directly collected into the  $\beta$ -glucuronidase staining solution (50 mM sodium-phosphate, 0.2% [v/v] Triton X-100, 2 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 2 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], and 2 mM 5-brom-4-chloro-3-indolyl-glucuronide [Duchefa], pH 7.5). The *DR5:GUS* reporter line was incubated for 30 min and *HRE1:GUS* and *HRE2:GUS* seedlings were incubated for 2 h at 37°C and then stored in 70% (v/v) ethanol until analysis. To better visualize the blue staining, samples were cleared in a 6:2:1 (g/mL/mL) solution of chloralhydrate:deionized water:glycerol (Carl Roth). Cleared samples were mounted on a microscope slide and visualized with bright-field illumination using an Olympus BX41 (Olympus) microscope with Color View II Kamera.

### Microscopy and Live Imaging of DII-VENUS and PIN2-GFP

Six-day-old DII-VENUS seedlings were analyzed after 7 h of hypoxia treatment or treatment at normoxic conditions, and *PIN2pro:PIN2-GFP* seedlings were analyzed after 3, 5, or 6 h of hypoxia or normoxia by taking pictures with a confocal laser scanning microscope (Leica TCS SP5). Furthermore, PIN2 protein abundance was detected in root tips of wild type × *PIN2pro:PIN2-GFP* and RAP2.12 OE2 × *PIN2pro:PIN2-GFP* seedlings after exposure to hypoxia or

normoxia for 5 h using confocal laser scanning microscopy. Fluorescence intensities in the plane of the quiescent center of each root tip were quantified with the LAS AF Lite software (Leica). The fluorescence intensities of PIN2-GFP at normoxic conditions were set to 100% and all other values are given in relation to that.

## Statistical Analysis

Statistical analyses were performed using Minitab14 software (www.minitab.com). A Student's *t* test (two-sample *t* test) was used to compare the means of two populations. One-way ANOVA with Tukey's post hoc test was applied for testing the variance of populations when they were normally distributed. The Kruskal-Wallis nonparametric one-way test was used to determine significant differences with Dunn's test for multiple comparisons among the group. A nonparametric Mann-Whitney *U* test was used for comparing two groups.

## Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers RAP2.3 – At3g16770, RAP2.12 – At1g53910, HRE1 – At1g72360, HRE2 – At2g47520, PIN1 – At1g73590, PIN2 – At5g57090, PIN3 – At1g70940, and PIN4 – At2g01420.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Analysis of HRE1-GUS expression in response to low oxygen and ethylene.

**Supplemental Figure S2.** Gene models of RAP2.3, RAP2.12, HRE1, and HRE2 indicating the T-DNA insertion sites of the knockout lines used.

**Supplemental Figure S3.** Gene model of PINs with the T-DNA or EN-1 transposon insertion sites.

**Supplemental Figure S4.** Overexpression of HRE2 prevents root slanting at hypoxic conditions.

**Supplemental Figure S5.** Average angle of primary root growth direction in wild-type, *pin1-1*, *pin2-1*, *pin3-4*, and *pin4-2* seedlings treated with 20 nM  $\alpha$ -NAA for 2 d at normoxic or hypoxic (2% O<sub>2</sub>) conditions.

**Supplemental Figure S6.** Expression of RAP2.12 in PIN2pro:PIN2-GFP lines crossed to wild type or to RAP2.12 OE2.

**Supplemental Table.** Primer names and sequences used in this study.

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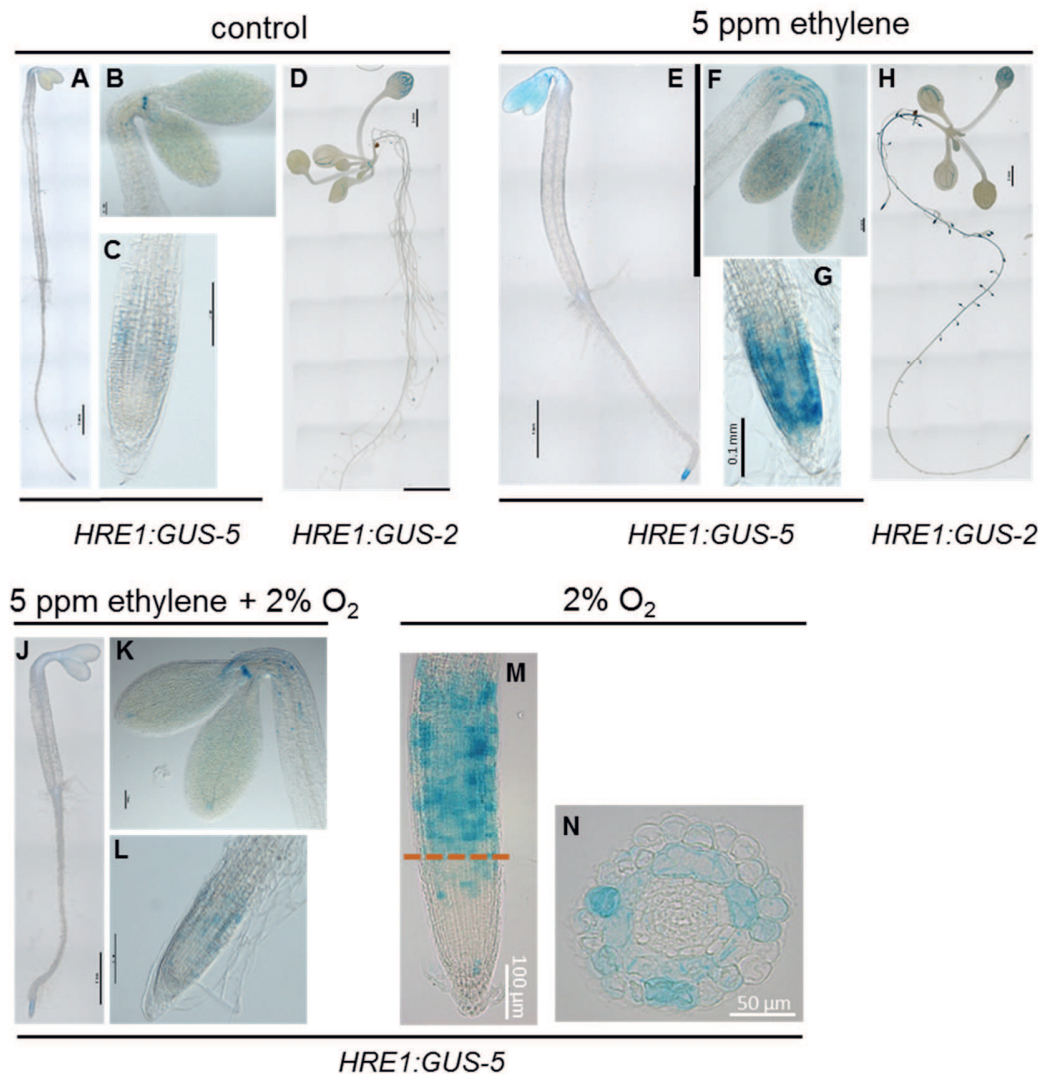
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## Supplemental Figures



**Supplemental Figure S1. Analysis of *HRE1-GUS* expression in response to low oxygen and ethylene.**

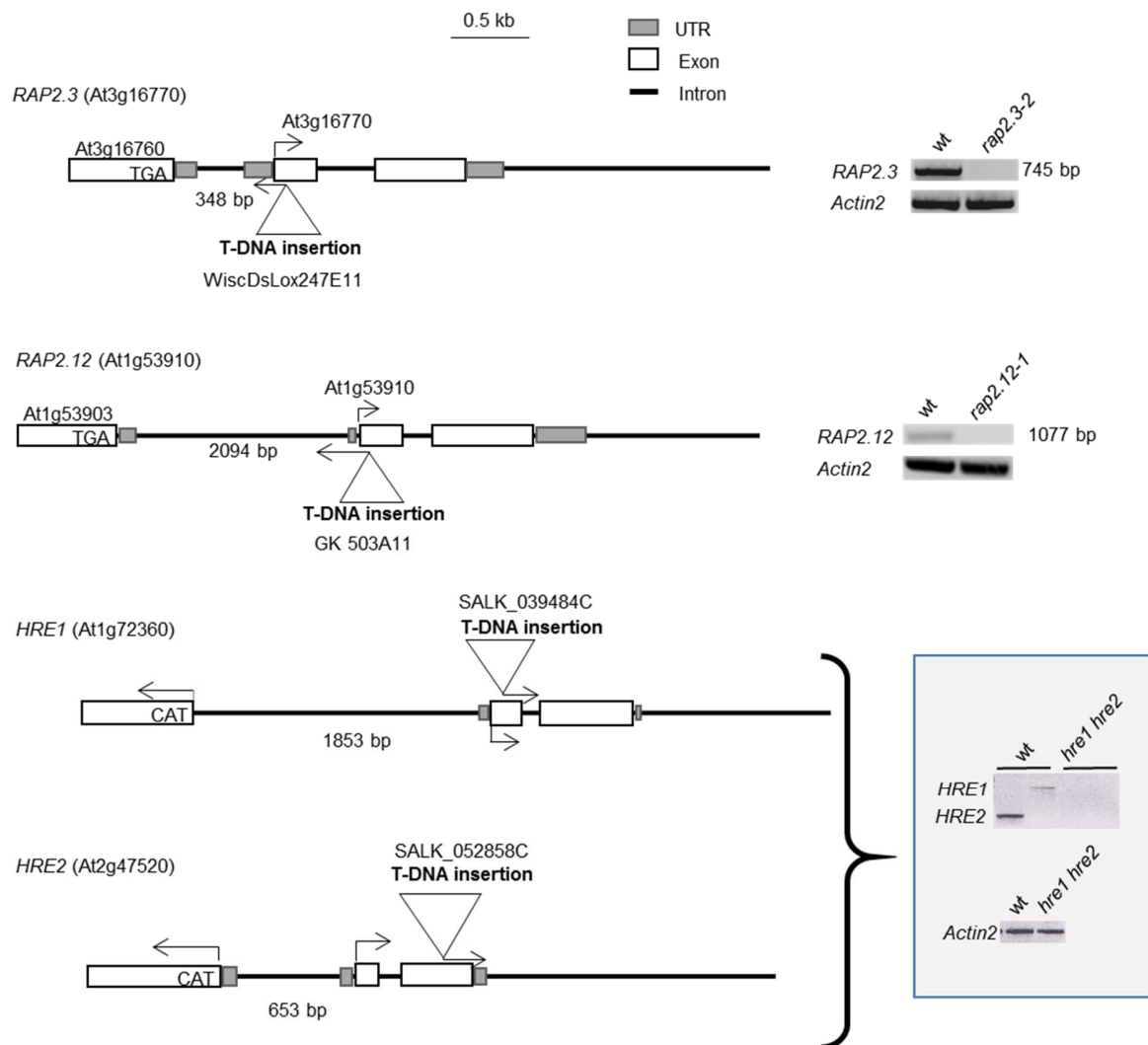
**A, E, J**, Histochemical GUS analysis of 4-d-old *HRE1:GUS-5* seedlings exposed to normoxia, 5 ppm ethylene or 5 ppm ethylene at 2% oxygen for 1 day.

**B, F, K**, Apical hook of 4-d-old seedlings.

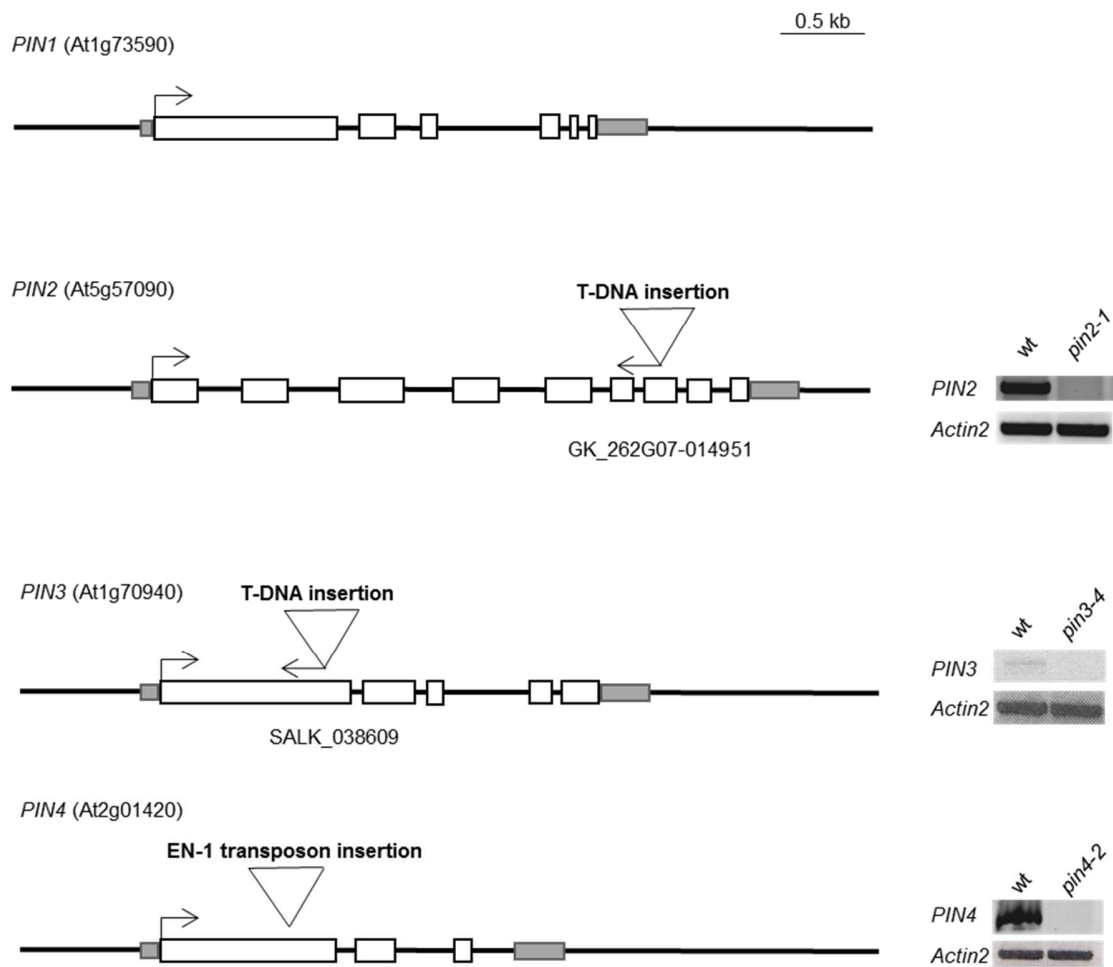
**C, G, L**, Primary root tip of 4-d-old seedlings.

**D, H**, 14-d-old *HRE1:GUS-2* seedlings.

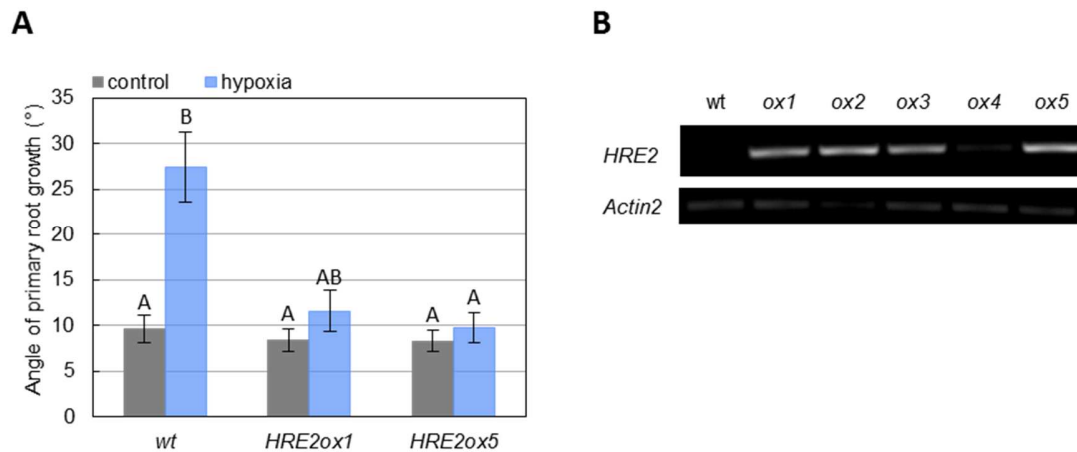
**M, N**, Primary root of 7-d-old seedlings with cross-section (N) at the position indicated by a dotted red line in (M).



**Supplemental Figure S2.** Gene models of *RAP2.3*, *RAP2.12*, *HRE1*, and *HRE2* indicating the T-DNA insertions sites of the knock out lines used. To the right, RT-PCR data show loss of gene expression in the respective T-DNA insertion lines. The expression of *HRE1* and *HRE2* were induced by applying hypoxic conditions prior to RNA isolation.



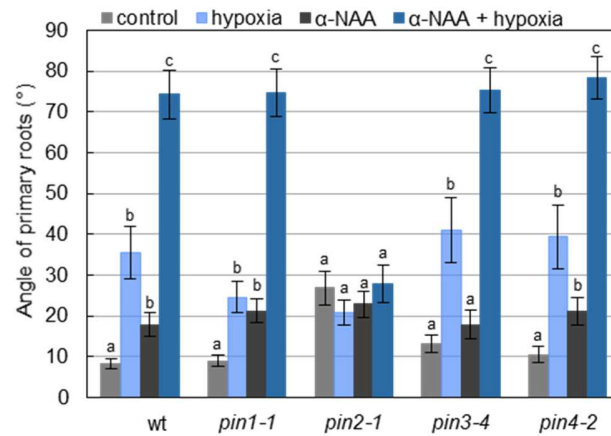
**Supplemental Figure S3.** Gene model of *PINs* with the T-DNA or EN-1 transposon insertion sites. The gels to the right show loss of *PIN2*, *PIN3* and *PIN4* transcripts in *pin2-1*, *pin3-4*, and *pin4-2*, respectively.



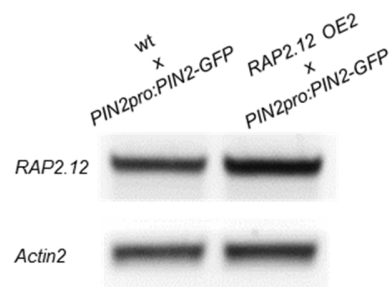
**Supplemental Figure S4.** Overexpression of *HRE2* prevents root slanting at hypoxic conditions.

**A,** Seedlings of wild type and two independent *HRE2ox* lines were exposed to normoxic or hypoxic conditions for 3 days and analyzed for root bending. Results are averages ( $\pm$ SE) of three independent experiments. Different letters indicate significantly different values (Kruskal-Wallis test with Dunn's test,  $H=30.34$ ,  $n=20-29$ ,  $P<0.01$ ).

**B,** Relative transcript levels of wild type and 5 independent *HRE2ox* lines were analyzed in seedlings kept at normoxic conditions. *ACTIN2* was used as a control for RNA input.



**Supplemental Figure S5.** Average angle of primary root growth direction in wild type, *pin1-1*, *pin2-1*, *pin3-4* and *pin4-2* seedlings treated with 20 nM  $\alpha$ -NAA for 2 days at normoxic or hypoxic (2%  $O_2$ ) conditions. Letters indicate significantly different values between treatments for each given genotype (Kruskal-Wallis test,  $n = 23-25$ ,  $P < 0.01$ ). Results are averages ( $\pm$  SE) from three independent experiments.



**Supplemental Figure S6.** Expression of *RAP2.12* in *PIN2pro:PIN2-GFP* lines crossed to wild type or to *RAP2.12 OE2*.

Relative transcript levels of *RAP2.12* in six-d-old wild type x *PIN2pro:PIN2-GFP* and *RAP2.12 OE2* x *PIN2pro:PIN2-GFP* seedlings were analyzed by RT-PCR. *ACTIN2* was used as a control for RNA input.

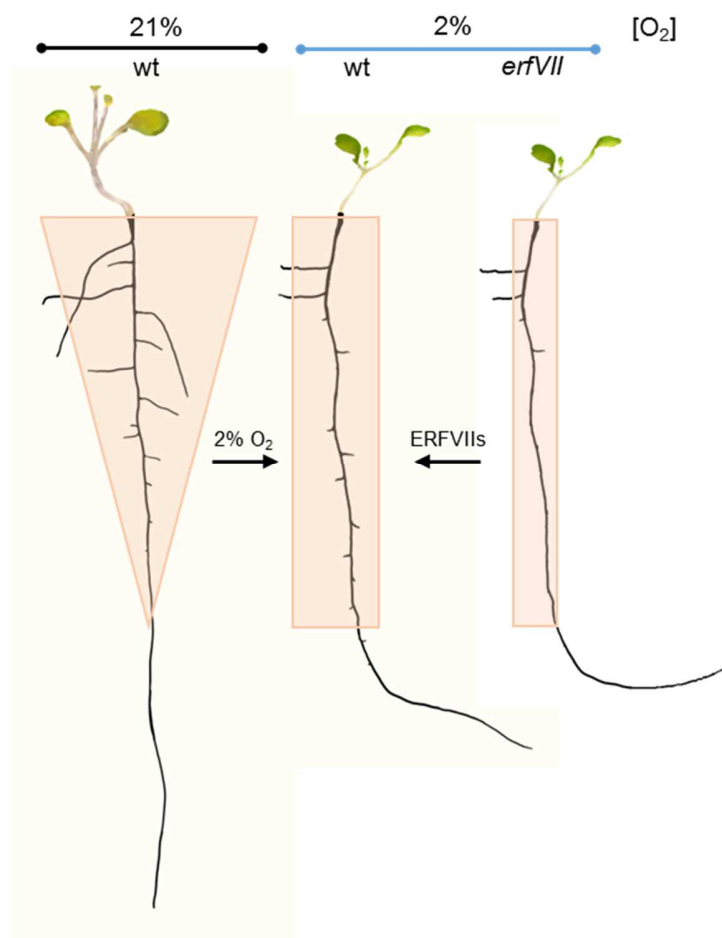
Name of primer	5'-3' sequence
At2G47520forRT	CTCGGCTCTGTTAGCAGCAGG
At2G47520revRT	TCGGCAACCGGAATCTGATAG
erf71profor	AAATTTGCGGCCGCTGTTAGTAGTTGCTCGTCTG
erf71prorev	AAATTTGATATCTTTTGTGTTTTTGTGTTTTTACGCTTCC
At2G47520 TC_forox	CACCACGTCTTGTTCTAGTTTGGAG
At2G47520 TC_revov	GATTTAATTGGAGTCTTGATAGC
At2G47520forqPCR	GGCTCTGTTAGCAGCAGGAA
At2G47520revqPCR	CGAATCTCCGCTGCCCATT
Actin2F1	CAAAGACCAGCTCTCCATCG
Actin2R1	CTGTGAACGATTCTGGACCT
At5g57090forRT	CCAACGCGAAGAATGCTATGA
At5g57090revRT	CGTAGTAGAGTACTGTTACAGGC
LB-GK	ATATTGACCATCATACTCATTGC
At1g53910For	TTACGCGTCGACATGTGTGGAGGAGCTATAATATC
At1g53910Rev	AAATTTGATATCTCAGAAGACTCCTCCAATCATG
At3g16770For	TTACGCGTCGACATGTGTGGCGGTGCTATTATTC
At3g16770Rev	AAACCGGAATTCCTTACTCATACGACGCAATGAC
WisconDsLoxLB	CGTCCGCAATGTGTTATTAAG
AT1G70940forRT	CCGATGCAGAGATAGGTGACGA
AT1G70940revRT	TTGAGGTAATGCGGCCTGAA
AT2G01420for2	GCTTCTTATTATGGAGCAGTTCCC
AT2G01420rev2	CCACTATCAAGACCGCCGATAT
Actin2qF	ACATTCCAGCAGATGTGGATCTC
Actin2qR	GATCCCATTCATAAAACCCAGC
GAPC1qF	GATTCTACAATGGCTGACAAGAAGA
GAPC1qR	ATGAAGGGGTGCTTGACAGC
AT5G57090qF	GCGTGCGGAAAATCAGTAGC
AT5G57090qR	GGAAGAGCAGCCTGAACGAT
ForAT1G53910	TTACGCGTCGACATGTGTGGAGGAGCTATAATATC
RevAT1G53910	AAATTTGATATCTCAGAAGACTCCTCCAATCATG

**Supplemental Table S1** Primer names and sequences used in Chapter 1.



## 8 Chapter 2

### 8.1 Arabidopsis group VII ETHYLENE RESPONSE FACTOR transcription factors maintain lateral root growth in hypoxia by regulating abscisic acid



Running title: Lateral root development during hypoxia

**Arabidopsis group VII ETHYLENE RESPONSE FACTOR transcription factors maintain lateral root growth in hypoxia by regulating abscisic acid**

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**Author contributions:**

MS and EED conceived the project and designed experiments, EED performed and analyzed experiments, BH performed the LC-MC analysis. MS and EED wrote the manuscript with contributions from BH.

**Keywords:** hypoxia, flooding, root system architecture, lateral root, abscisic acid (ABA), ERFVII, *Arabidopsis thaliana*

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**Abstract**

The number and extent of floods are increasing worldwide posing a severe problem in agriculture. Plant roots are the first organs to become hypoxic during flooding yet we know little about adaptations of the root system to this adverse condition. Here we show that while hypoxia generally limits root growth and development, lateral root (LR) development is actively maintained by group VII ETHYLENE RESPONSE FACTOR (ERFVII) transcription factors by relieving the growth-inhibitory activity of ABA. Hypoxia-induced ERFVIIs lower ABA levels by inducing the ABA-degrading ABA 8'-hydroxylases *CYP707A1* and *CYP707A3*. Fine tuning of ABA responses via *PYL6* and *OST1* further contributes to LR growth. *erfVII* seedlings no longer induce ABA catabolic genes and are hypersensitive to hypoxia with LRs arresting at the post-emergence stage. Hence, LR development at hypoxic conditions is not simply a result of metabolic restrictions but is a controlled process that is integrated with metabolic adjustment at the level of ERFVII.

## Introduction

Due to climate changes the number of floods is increasing worldwide resulting in reduced agricultural production (Voisenek and Bailey-Serres, 2015). Flooding can cause soil water logging, or partial or complete submergence of plants that can last for hours to months and cause oxygen shortage in the flooded plant parts (Sasidharan *et al.*, 2015). Independent of the flooding regime, roots are the first to encounter oxygen limitations (Sauter, 2013). It is therefore highly relevant for the plant to adapt the root system to conditions of low oxygen stress to improve survival.

Low oxygen conditions have a profound effect on energy metabolism since plants have to switch to alternative metabolic pathways to generate ATP thereby compensating for the decrease in respiration. Metabolic changes include higher pyruvate production through induced glycolysis and fermentation (Zabalza *et al.*, 2009, Bailey-Serres *et al.*, 2012, António *et al.*, 2016). The Arabidopsis AP2/ERF transcription factors of group VII, HRE1, HRE2, RAP2.2, RAP2.3 and RAP2.12 (ERFVIs), were identified and characterized as triggers for transcriptional changes of hypoxia core genes (Licausi *et al.*, 2011) to adjust metabolic changes (Paul *et al.*, 2016). Although metabolic adjustment is a prerequisite for plants to survive periods of limited oxygen availability, plants have evolved many strategies to cope with the stress that involve anatomical and morphological changes. For a plant to survive flooding, it has to maintain a functional root system that supplies the plant with water and nutrients. To improve their oxygen status, roots can form constitutive or flooding-induced aerenchyma that facilitates gas exchange, and suberize the rhizodermis to limit radial oxygen loss (Armstrong, 1971). However, given the plasticity of root development in response to environmental changes (Linkohr *et al.*, 2002; Xiong *et al.*, 2006; Bao *et al.*, 2014), it seems likely that adaptation to hypoxia also occurs with regard to root system architecture (RSA). In support of this hypothesis, bending of the primary root in response to hypoxia was recently reported as an escape response of Arabidopsis seedlings (Eysholdt-Derzsó and Sauter, 2017).

The RSA is characterized by the length of the primary root, density, positioning and length of lateral roots, the angle at which lateral roots (LRs) grow, and adventitious root formation (Koevoets *et al.*, 2016). In many species, including Arabidopsis, LRs provide

the main part of the root system (Per  t *et al.*, 2009). LR originate post-embryonically from the pericycle. The LR primordium grows through the overlying cell layers until it penetrates the epidermis (Lavenus *et al.*, 2013; Du and Scheres, 2018). LR initiation and emergence are tightly controlled by auxin that coordinates the initial asymmetric cell division, cell wall remodeling of the overlying layers (Lewis *et al.*, 2013) and the activation of the meristem in the emerged LR (Lavenus *et al.*, 2013). Development of LR is also under control of abscisic acid (ABA) that is required for LR initiation and suppresses activation and maintenance of the LR meristem after LR emergence (De Smet *et al.*, 2003; De Smet *et al.*, 2006).

ABA is synthesized from carotenoids with the key step catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED) in plastids. The product of the NCED activity, xanthoxin, is converted to ABA via ABA-aldehyde by sequential activity of ABA-deficient 2 and 3 (ABA2 and 3) in the cytosol. Bioactive ABA levels are further determined by either degradation via oxidation or inactivation through conjugation (Finkelstein, 2013). Degradation to phaseic acid is catalyzed by ABA 8'-hydroxylases that are encoded by four genes, *CYP707A1-4*, in Arabidopsis. Subsequent metabolism to (-)-dihydrophaseic acid is achieved by the phaseic acid reductase ABA hypersensitive 2 (ABH2) (Weng *et al.*, 2016). ABA is perceived by PYR1/PYL/RCAR receptors (Ma *et al.*, 2009, Park *et al.*, 2009) that, when activated, relieve the inhibitory effect of group A type-2C protein phosphatases (PP2C) including ABI1, ABI2 or HAB2 (Hao *et al.*, 2011). ABI1 and its homolog ABI2 play a central role in ABA signaling as negative regulators of SNF1-related protein kinases 2 (SnRK2s) (Leung *et al.*, 1997). SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3 function in seed development, germination and abiotic stress responses through phosphorylation of AREB/ABF-type bZIP transcription factors that control ABA responses (Nakashima *et al.*, 2009).

In this study, we show remodeling of the root system architecture in response to low oxygen stress and reveal a role for ABA in the control of LR development. The post-emergence arrest of lateral root development is controlled through ABA homeostasis and signaling in an auxin-independent manner. We show that hypoxia upregulates genes of the ABA degradation pathway through ERVILs resulting in lowered ABA levels. As a consequence, seedlings retain the competency for LR growth during hypoxia.

## Results

### Inhibition of root growth by low oxygen stress results from lowered mitotic activity

Root growth is determined by cell proliferation and cell elongation rates (Slovak et al., 2016). In *Arabidopsis* seedlings, we observed inhibition of primary root growth by 40 % after 4 d at 2% O<sub>2</sub> in the dark compared to seedlings kept at normoxic conditions in the dark (Figure 1A). Analysis of a *CyclinB1;1::GUS* reporter line revealed a significantly reduced number of mitotic cells in the primary root apical meristem after 8 h and 24 h at 2% O<sub>2</sub> compared to normoxic seedlings (Figure 1B, C). After 4 days, the cell division rate was still low in hypoxic seedlings but was also reduced in normoxic seedlings likely due to the dark conditions imposed during treatments to avoid photosynthetic oxygen evolution (Figure 1B, C, and Supplementary Figure S3). However, while primary root growth was impaired at hypoxic conditions, the formation of lateral roots (LRs) per unit primary root, i.e., the root density was not reduced after 2 days and mildly reduced after 4 d of hypoxia (Supplementary Figure S1A) suggesting that initiation of LRs was not impaired. In accord with this finding, mitotic activity in developing LRs at the primordia and emerged stage did not differ between normoxic and hypoxic seedlings. By contrast, elongated LRs displayed fewer *CyclinB1;1::GUS* expressing cells at hypoxic conditions (Figure 2B).

### Hypoxia inhibits lateral root elongation

Since we observed a near constant formation of LRs yet reduced cell division activity in root apical meristems of elongating LRs, we carried out a more detailed analysis of LR development in seedlings exposed to 2% O<sub>2</sub> for 2 days (Figure 2A). Thereby, we distinguished LR primordia, emerged LRs and elongated LRs (Figure 2A). The visual inspection of the LR system revealed that fewer emerged LRs elongated in hypoxia compared to normoxia, an observation that was confirmed by statistical evaluation of the data (Figure 2C, D). The number of LRs at the penetrated stage had increased significantly in wild type after 2 days at hypoxic compared to normoxic conditions, whereas the number of elongated LRs decreased significantly. The hypoxia signal is mediated by group VII ERF (ERFVII) transcription factors with five members in *Arabidopsis* (Licausi et

*al.*, 2011). To test for involvement of ERFVII in root development during hypoxia, we analyzed *erfVII* quintuple mutant seedlings (Abbas *et al.*, 2015). As a control, seedlings were kept in the air. *erfVII* seedlings showed a more severe phenotype than wild type with LR development arrested mostly at the primordia and penetrated LR stages with few LRs elongating after 2 days of treatment. To test if LR formation was limited by metabolic constraints at hypoxic conditions we induced LR formation by adding the synthetic auxin  $\alpha$ -NAA (Figure 2D, E). With auxin, the total number of LRs increased over twofold but, again, LRs arrested at the penetrated stage indicating that arrested LR development was a genetically controlled process rather than a result of limited resources.

After 4 days of hypoxia (Figure 3), more of the emerged LRs had elongated in wild type indicative of a recovery process. By contrast, LRs of *erfVII* seedlings did not elongate (Figure 3B, C). In the presence of auxin, with overall more LRs, a similar observation was made. In hypoxic wild type, emerged roots had elongated, whereas roots of hypoxic *erfVII* seedlings remained at the penetrated stage supporting the conclusion that LR development is decelerated at hypoxic conditions and recovers over time as a result of ERFVII activity.

### **ABA controls lateral root elongation during hypoxia**

Since hypoxia did not limit LR growth through metabolic limitations only but rather through an ERFVII-controlled process, we looked into a possible downstream regulatory mechanism. Saline stress repression of cell division in LRs is regulated by ABA (Duan *et al.*, 2013). To test if ABA might be a target of hypoxia signaling, we analyzed the effect of 5  $\mu$ M ABA in combination with low-oxygen on LR elongation. While ABA did not significantly reduce the number of elongated LRs at normoxic conditions, this number dropped significantly at 2% O<sub>2</sub> suggestive of an interaction between hypoxia and ABA (Figure 4A, B). We next looked at post-emergence LR growth in response to hypoxia and ABA (Figure 4C). The average length of LRs was reduced nearly three fold by hypoxia when compared to normoxia (Figure 4C). Addition of 5  $\mu$ M ABA inhibited LR growth at normoxic conditions 4.5-fold whereas combined treatment with hypoxia and 5  $\mu$ M ABA did not further inhibit post-emergence LR growth, a finding that is in accord with the idea that ABA acts downstream of hypoxia signaling (Figure 4C). As 5  $\mu$ M is a high ABA



concentration, we extended the study of root elongation to lower ABA concentrations (Figure 4D, E, F). The growth of the primary root was not impaired by ABA at concentrations of 0.01 to 5  $\mu\text{M}$  at either normoxic or hypoxic conditions (Figure 4E).

While the number of elongated LR<sub>s</sub> did not change with increasing ABA concentrations at normoxic conditions (Figure 4F), the decrease in elongated LR number during hypoxia was enhanced by ABA in a concentration-dependent manner with a significant effect measured at 0.1  $\mu\text{M}$  ABA. These results suggest a synergistic effect of hypoxia and ABA (Figure 4F). At concentrations of 0.5  $\mu\text{M}$  ABA and higher, inhibition of post-emergence LR growth at hypoxic conditions was saturated in accord with the conclusion that LR elongation is controlled during hypoxia by low levels of ABA. This result indicates that hypoxia-induced inhibition of the elongated LR number is ABA-dependent. To compare the relative effect of ABA on LR growth rate in normoxia and hypoxia, we compared the average length of LR<sub>s</sub> as a percentage of the value measured without ABA at each conditions (Figure 4D). The number of elongated LR<sub>s</sub> was not affected by ABA whereas ABA did inhibit growth in a dose-dependent manner in normoxic conditions (De Smet *et al.*, 2003; Jaffé *et al.*, 2012). Interestingly, in low oxygen, the sensitivity toward ABA with regard to LR growth rate was reduced. In normoxia, LR growth was reduced at 0.01  $\mu\text{M}$  ABA whereas it required 0.1  $\mu\text{M}$  ABA to reduce the average length of LR<sub>s</sub> at hypoxic conditions (Figure 4D). At higher ABA levels, LR elongation was inhibited by ABA to the same degree in normoxic and hypoxic conditions.

### **Hypoxia regulates ABA catabolism by inducing ABA 8'-hydroxylases**

Based on our observation that hypoxia altered the sensitivity of LR<sub>s</sub> to ABA, we analyzed whether hypoxia alters the expression of genes involved in either ABA biosynthesis or degradation by exploring two transcript profiling studies that were carried out on hypoxic roots (van Dongen *et al.*, 2009, Yang *et al.*, 2011). The analysis revealed that the ABA biosynthetic gene *Nine-cis-epoxycarotenoid dioxygenase 3* (*NCED3*) was strongly, and *NCED1* and *NCED9* were weakly downregulated by hypoxia (Figure 5A). Genes encoding the ABA degrading enzymes *ABA 8'-hydroxylase 1* and *3* (*CYP707A1* and *CYP707A3*) were upregulated. Elevated expression of *CYP707A1* and *CYP707A3* by hypoxia was verified in seedlings exposed to hypoxia for up to 4 d using qRT-PCR (Figure 5C). ABA

8'-hydroxylases catalyze the conversion of ABA to (-)-phaseic acid (PA). PA is further converted to (-)-dihydrophaseic acid (DPA) by PA reductase encoded by *ABA hypersensitive 2 (ABH2)* (Weng *et al.*, 2016). To see if hypoxia also controls catabolism of PA, we measured transcript levels of *ABH2*. Expression of *ABH2* transiently increased in hypoxic conditions and, to a lesser degree, in normoxia suggestive of dark induction as has been described previously (Weng *et al.*, 2016) (Figure 5D).

Based on the same microarray data we observed a highly specific up-regulation of the ABA signaling genes *PYL2,5,6* and *SnRK2.6/OST1* and downregulation of *HAB1*, *ABI2* (Figure 5B). Elevated expression of *PYL6* and *OST1* under hypoxic conditions was confirmed by qRT-PCR. Expression of *OST1* was significantly elevated at 8 h of hypoxia and dropped to control level at 24 and 96 h, whereas expression of *PYL6* increased at 8 h and remained elevated under hypoxic conditions (Figure 6B). *pyl6* and *ost1-4* single knock out lines were less sensitive to hypoxia-induced inhibition of LR elongation compared to wild type supporting a role for ABA in LR development during hypoxia (Figure 6A).

As changes in gene expression pointed to a reduction in ABA levels by hypoxia, we next measured ABA content by LC-MS in 6-day-old seedlings exposed to 8 h, 24 h or 96 h of hypoxia or normoxia (Figure 5E). In normoxic roots, ABA content was not significantly altered over time. By contrast, the ABA level significantly decreased in hypoxic roots from 4 pmol/g to 2 pmol/g after 8 h and 24 h. After long-term exposure (96 h) to hypoxia, the ABA level recovered to the control value. In conclusion, hypoxia reduces ABA levels through increased catabolism.

### **ABA inactivation during hypoxia is mediated by ERFVIIs**

Since hypoxia signaling relies in many cases on the activation of group VII ERFs (Gibbs *et al.*, 2011; Licausi *et al.*, 2011; Gasch *et al.*, 2015), we hypothesized that induction of *ABA 8'-hydroxylase 1* by hypoxia might be controlled by ERFVIIs. The expression of *CYP707A1*, *CYP707A2*, and *CYP707A3* was comparable in normoxic wild type and *erfVII* seedling roots. *CYP707A1* and *CYP707A3* were significantly induced by hypoxia in wild type but not or less so in the quintuple *erfVII* mutant (Figure 7A) pointing to a requirement for ERFVIIs in regulating ABA degradation. To identify which ERFVII

regulates the *CYP707A1* and *CYPP707A3* genes, the single gene knock out mutant *rap2.3-2*, *rap2.12-1*, *hre1-1* and *hre2-1* were employed. Except for *hre1-1*, which showed wild type transcript levels of *CYP707A1* under hypoxia (Supplementary Figure S2), the single gene knock out lines showed a reduced induction of *CYP707A1* under hypoxic conditions as compared to wild type (Figure 7B). Transcript levels were, however, still significantly elevated at hypoxic conditions compared to the control in *rap2.3-2*, *rap2.12-1* and *hre2-1* (Figure 7B) revealing a redundant role of RAP2.3, RAP2.12, and HRE2 in regulating *CYP707A1* transcript levels in response to hypoxic stress.

## Discussion

### Hypoxia alters lateral root development

Submergence-induced morphogenic responses of the *Arabidopsis thaliana* shoot, such as hyponastic growth (Millenaar *et al.*, 2005) and petiole elongation (Millenaar *et al.*, 2005; Hwang *et al.*, 2011) have been well-characterized. By contrast, growth and development of roots have received less attention even though roots are the first to encounter oxygen shortage during soil water logging or flooding. Plant roots are characterized by a high degree of plasticity that allows them to respond to their abiotic and biotic environment (Bao *et al.*, 2014). Nitrogen deprivation, for instance, induces primary and LR elongation (Linkohr *et al.*, 2002), whereas drought reduces LR growth but induces primary root elongation (Xiong *et al.*, 2006) indicating that root system development is tailored to each stress condition to increase the chance for plant survival. Recently, the primary root of *Arabidopsis* seedlings was shown to change growth direction in response to hypoxia likely as an escape response to avoid oxygen-deficient soil layers (Eysholdt-Derzsó and Sauter, 2017). The current study shows how plants reprogram LR development to adjust their root system architecture and uncovers the molecular mechanisms that alter the root system architecture in response to low oxygen.

The growth rate of the primary root was reduced by 40% in hypoxia due to reduced mitotic activity as might be predicted from metabolic constraints arising during hypoxia. The shorter primary root length resulted in overall fewer lateral roots, however, with a

slightly reduced LR density. A capacity to generate LRs was revealed by the observation that auxin-induced LR formation about twofold at hypoxic conditions. Under oxygen deprivation, the respiratory metabolism is impaired, and plants trigger metabolic changes to partially balance the lack of oxygen (van Dongen and Licausi, 2015). Anaerobic metabolism then allows plants to maintain essential cellular functions and to improve oxygen supply by inducing aerenchyma or adventitious roots (Yamauchi *et al.*, 2013; Sauter, 2013; Dawood *et al.*, 2014).

However, *Arabidopsis* roots do not form aerenchyma nor have adventitious rooting in response to hypoxia been reported. Rather, *Arabidopsis* seedlings largely alter LR development revealing a dynamic process controlled by a genetic program that governs root system architecture in hypoxic conditions. Within 2 days of hypoxia, LRs arrested at the post-emergence stage. Within 4 days, elongation growth of emerged LRs recovered suggesting that hypoxia activated a rescue program. Depending on the type of abiotic stress, different roots structures develop (Koevoets *et al.*, 2016). Salt stress inhibited LR growth by 84.3% and reduced primary root length by 49.6%. Under hypoxia stress, we observed primary roots that were 40% and LRs that were 59% shorter, indicating that LR growth is more sensitive to salt than to hypoxia (Duan *et al.*, 2013).

Metabolic changes of plants exposed to low oxygen stress are transcriptionally regulated. Expression analysis revealed that plants respond within 1 h by upregulating hypoxia core genes (Licausi *et al.*, 2010). The expression of several hypoxia core genes including *ADH1* or *PCD1,2* declines after 8 h of waterlogged conditions (Hsu *et al.*, 2011). Transcriptional profiling of *Arabidopsis* shoots during short-term, and long-term hypoxia similarly showed a two-step regulation with changes occurring at an early stage (1-8 h) and further changes occurring during late (24-72 h) time points (Hwang *et al.*, 2011). Such biphasic gene regulation is reminiscent of biphasic LR development. In fact, the adjustment of metabolism may be a prerequisite for LR growth recovery after emergence to support the high-energy demand of the LR apical meristem that needs to be activated post-emergence to support LR growth (Thiel *et al.*, 2011).

### **Lateral root development at hypoxic conditions is controlled by ABA**

In *Arabidopsis* roots, ABA levels declined in the early phase of hypoxia. A similar observation was made after flooding up to twelve hours with lowered ABA levels in *Arabidopsis* roots (Hsu *et al.*, 2011) indicating that ABA levels change during flooding in response to a low oxygen signal. After 4 days of hypoxia, ABA returned to control levels suggesting that reduced ABA in the short term supports long-term LR growth possibly by keeping meristematic cells in a cell division-competent state. In rice (Hoffmann-Benning and Kende, 1992; Saika *et al.*, 2007) and *Rumex palustris* (Benschop *et al.*, 2005) ABA declines in submerged shoots, while in *Arabidopsis* ABA reduction was observed in roots indicative of a specific role of ABA regulation in root adaptation. ABA levels declined during hypoxia likely as a result of reduced ABA synthesis via downregulation of *NCED3* (Hsu *et al.*, 2011) and increased degradation through ABA 8'-hydroxylases encoded by *CYP707A1* and 3 genes. Transcript levels of *CYP707A2* were not significantly changed under hypoxic conditions being in accordance with the previous finding that *CYP707A2* functions in germination but not in root development (Kushiro *et al.*, 2004; Okamoto *et al.*, 2006).

Expression analysis of ABA signaling genes revealed that the ABA receptor gene *PYL6* and the SnRK gene *SnRK2.6/OST1* were activated in response to low oxygen stress, a regulation that can be explained in three ways. First, reduced ABA may act as a feedback signal to increase the sensitivity towards ABA by inducing signaling components. Second, ABA signaling is induced through hypoxia signaling in an ABA-independent manner. Or, third, ABA degradation activates an unknown signal pathway that alters ABA signaling. Several PYL receptors, *PYL10*, and to a lesser extend *PYL5* and *PYL6*, inhibit phosphatase activity of PP2C without ligand binding (Hao *et al.*, 2011) which triggers an ABA response. *PYL6* is highly upregulated upon hypoxia, and the *pyl6* knockout mutant is less sensitive to root growth repression by hypoxia. The three SnRKs, *SnRK2.2*, *SnRK2.3*, *SnRK2.6*, are positive regulators of ABA signaling and show functional redundancy in drought stress. *SnRK2.2* and *SnRK2.3* are important in seed dormancy and germination (Fujii *et al.*, 2007), while *SnRK2.6/OST1* plays a major role in stomatal closure upon drought stress (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002). *SnRK2.6/OST1* is the only SnRK that is highly upregulated by hypoxia (van Dongen *et al.*,

2009; Yang *et al.*, 2011) and mediates inhibition of root elongation under hypoxia. Taken together our results indicate tight regulation of ABA levels and ABA signaling under low oxygen conditions that enable the plant to adjust LR development.

### **ERFVIIIs maintain LR development during hypoxia**

ERFVII transcription factors are key mediators of hypoxia signaling in plants (Gibbs *et al.*, 2011; Licausi *et al.*, 2011) and were shown to not only regulate metabolic but also developmental adaptations (Fukao and Bailey-Serres 2008; Hattori *et al.*, 2009; Abbas *et al.*, 2015; Eysholdt-Derzsó and Sauter 2017). Even though roots are the first to encounter flooding stress, it is only now that a role of ERFVIIIs in controlling root system architecture is being studied. It has become clear that ERFVIIIs not only regulate hypoxia core genes which mostly encode for metabolic enzymes (Mustroph *et al.*, 2009; Paul *et al.*, 2016). ERFVIIIs, particularly RAP2.3, RAP2.12 and HRE2, also regulate the plant hormone ABA through redundant regulation of ABA catabolic genes, supporting the notion that ABA regulation is an important feature of flooding adaptation. Redundant roles of RAP2.2, RAP2.3 and RAP2.12 were previously shown for the activation of the transcription LBD41 (Gasch *et al.*, 2015). ERFVIIIs promote a decline in root ABA which may be crucial for recovery of post-emergence LR growth in the long term. Interestingly, while hypoxia inhibits, hypoxia-activated ERFVIIIs promote LR development (Figure 8). We recently demonstrated that hypoxia and ERFVIIIs act antagonistically in primary root slanting (Eysholdt-Derzsó and Sauter 2017) which appears to also be the case in LR development supporting the idea that an ERF-independent low-oxygen signaling pathway exists (Holdsworth, 2017). Primary root slanting and LR development are both balanced by ERF-dependent and ERF-independent signaling pathways pointing to a conserved mechanism in shaping root system architecture in flooded plants. The adjustment of root growth angle and maintenance of an, albeit reduced, LR system under oxygen deprivation could support plant survival by improving oxygen uptake, reducing oxygen consumption and maintaining root function.

## Material and Methods

### Plant material

Experiments were carried out with *Arabidopsis thaliana* Col-0 originally obtained from NASC (Nottingham, UK). All mutants are in the Col-0 background. The *erfVII* line (Abbas *et al.*, 2015) was kindly provided by M.J. Holdsworth (University of Nottingham, Loughborough, UK), the *CyclinB1;1::GUS* marker line (Colón-Carmona *et al.*, 1999) was kindly provided by Dirk Inzé (VIB-UGent Center for Plant Systems Biology, Gent, Belgium). The T-DNA insertion lines *rap2.3-2*, *rap2.12-1*, *hre1-1* and *hre2-1* were described previously (Hess *et al.*, 2011; Marín-de la Rosa *et al.*, 2014; Gibbs *et al.*, 2014). *nced3* (Guo *et al.*, 2009), *abi1-2* (Saez *et al.*, 2006), *pyl6* (Antoni *et al.*, 2013), *ost1-4* (Ding *et al.*, 2015) were received from NASC.

### Growth conditions

Seeds were sterilized, sown on plates and seedlings were grown in long day at 21°C, 60% humidity, and 100  $\mu\text{m m}^{-2} \text{s}^{-1}$  light intensity as described (Eysholdt-Derzsó and Sauter, 2017). For auxin treatment, 5-d-old seedlings were transferred onto new plates supplemented with or without 20 nM  $\alpha$ -naphthalene acetic acid ( $\alpha$ -NAA) (Sigma) for 1 d prior to hypoxic treatment. For exogenous abscisic acid (ABA) treatment, 6-d-old seedlings were transferred onto plates with or without 0.01, 0.1, 0.5, 1 or 5  $\mu\text{M}$  (+)-Cis, trans-abscisic acid (Duchefa). Hypoxic treatment was carried out as described (Eysholdt-Derzsó and Sauter, 2017). Seedlings were exposed to hypoxia for two to four days as indicated. Roots were imaged with an SMZ18 stereo microscope (Nikon) and a DS-U3 camera (Nikon). Primary and lateral root lengths were measured with ImageJ (Schneider *et al.*, 2012). To analyze LR development, LRs were categorized into primordia (stages I-VII), penetrated (VIII) and elongated based on Casimiro *et al.* (2003). For gene expression analysis, 6-d-old wild type seedlings were exposed in the dark to 2 %  $\text{O}_2$  for 0, 8, 24, or 96 h or kept at normoxic conditions in the dark. The complete roots except 5 mm of the root tip were collected for RNA isolation as well as for ABA measurement. For the



expression analysis of *erfVII*, *rap2.3-2*, *rap2.12-1*, *hre1-1* and *hre2-1* seedlings were exposed to the conditions indicated for 8 h.

### Histochemical analysis

To examine promoter activity of *CyclinB1;1::GUS*, seedlings were grown 6 or 10 d and then exposed to hypoxia or normoxia in the dark for the times indicated. Histochemical analysis was performed based as described (Blázquez *et al.*, 1997) with modifications. Seedlings were stained in 50 mM sodium-phosphate, 0.2% (v/v) Triton X-100, 2 mM  $K_4[Fe(CN)_6]$ , 2 mM  $K_3[Fe(CN)_6]$ , and 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) (Duchefa), pH 7.5) for 1 hour at 37°C and subsequently stored in 70% (v/v) ethanol until analysis. For better visualization, samples were cleared in a 6:2:1 (g/ml/ml) solution of chloralhydrate:deionized water:glycerol (Carl Roth), subsequently mounted on a microscope slide and visualized with bright-field illumination using an Olympus BX41 (Olympus) microscope with Color View II camera.

### Generating heat maps

Genevestigator was used to analyze expression of genes involved in ABA metabolism and signaling under hypoxic conditions (Hruz *et al.*, 2008). Two data set with the candidate genes were extracted from the microarray studies GSE11558 (van Dongen *et al.*, 2009) and GSE27475 (Yang *et al.*, 2011) generated by Affymetrix Arabidopsis ATH1 Genome Array on 10-d-old light-grown seedlings grown on plates (GSE11558) or on 14-d-old seedlings that were transferred into a floating platform saturated with 3%  $O_2$  (GSE27475). Raw data in log2 form was used to create heat maps in Microsoft Excel (2013).

### Quantification of abscisic acid content by LC-MS

Six-day-old seedlings were exposed to 2%  $O_2$  for the times indicated. Roots were harvested and snap-frozen in liquid nitrogen. ABA was extracted and quantified according to Balcke *et al.* (2012). Briefly, about 50 mg of frozen material was homogenized and extracted with 500  $\mu$ l of methanol containing 0.1 ng  $\mu$ l<sup>-1</sup> of isotope-labeled internal

standard  $^2\text{H}_6$ -ABA. After centrifugation, supernatant was diluted with nine volumes of water and subjected to solid-phase extraction on HR-XC (Chromabond, Macherey-Nagel, Düren, Germany) column. Fractions containing ABA were eluted with acetonitrile and separated using the ACQUITY UPLC System (Waters, Eschborn, Germany). Detection of ABA and  $^2\text{H}_6$ -ABA by ESI-tandem mass spectrometry (MS/MS) using 3200 Q TRAP® LC/MS/MS mass spectrometer (Waters) was performed as described previously (Balcke *et al.*, 2012). The contents of ABA were calculated using the ratio of analyte and internal standard peak heights.

### Quantitative RT-PCR analysis

qRT-PCR was carried out to measure changes in transcript level of genes involved in ABA metabolism under hypoxic conditions. RNA was isolated using TRI Reagent (Sigma), and cDNA synthesis (Thermo Fisher Scientific) was performed with 1 µg RNA according to manufacturer's protocol. Transcript accumulation of *CYCP707A1* (primers qPCRA<sub>t4g27250</sub>for and qPCRA<sub>t4g27250</sub>rev), *CYP707A2* (primers qPCRA<sub>t4g19230</sub>for and qPCRA<sub>t4g19230</sub>rev), *CYP707A3* (primers qPCRA<sub>t2g29090</sub>for and qPCRA<sub>t2g29090</sub>rev), *ABH2* (primers qPCRA<sub>t5g45340</sub>for and qPCRA<sub>t5g45340</sub>rev) (Supplementary Table S1) was analyzed at different time points of hypoxia. The expression level in wild-type or single knock out of *rap2.3-2*, *rap2.12-1*, *hre1-1*, *hre2-1* seedlings at control conditions was set to 1, and all other values are shown as multiples of that. Target gene expressions were normalized to *ACTIN2* (primers Actin2qF and Actin2qR) and *GAPC1* (primers GAPC1qF and GAPC1qR) (Supplementary Table S1) as reference genes.

### Statistical analysis

Statistical analyses were performed using Minitab14 software ([www.minitab.com](http://www.minitab.com)). A Student's *t*-test (two-sample *t*-test) was used to compare the means of two populations. One-way ANOVA with Tukey's post hoc test was applied for testing the variance of populations when they were normally distributed. The Kruskal-Wallis nonparametric one-way test was used to determine significant differences with Dunn's test for multiple

comparisons among the group. A nonparametric Mann-Whitney U-test implemented the statistical analysis for comparing two groups.

**Accession numbers of genes analyzed**

RAP2.3 - At3g16770  
RAP2.12 - At1g53910  
HRE1 - At1g72360  
HRE2 - At2g47520  
NCED3 - At3g14440  
CYP707A1 - At4g19230  
CYP707A2 - At2g29090  
CYP707A3 - At5g45340  
PYL6 - At2g40330  
ABI1 - At4g26080  
SnRK2.6/OST1 - At4g33950

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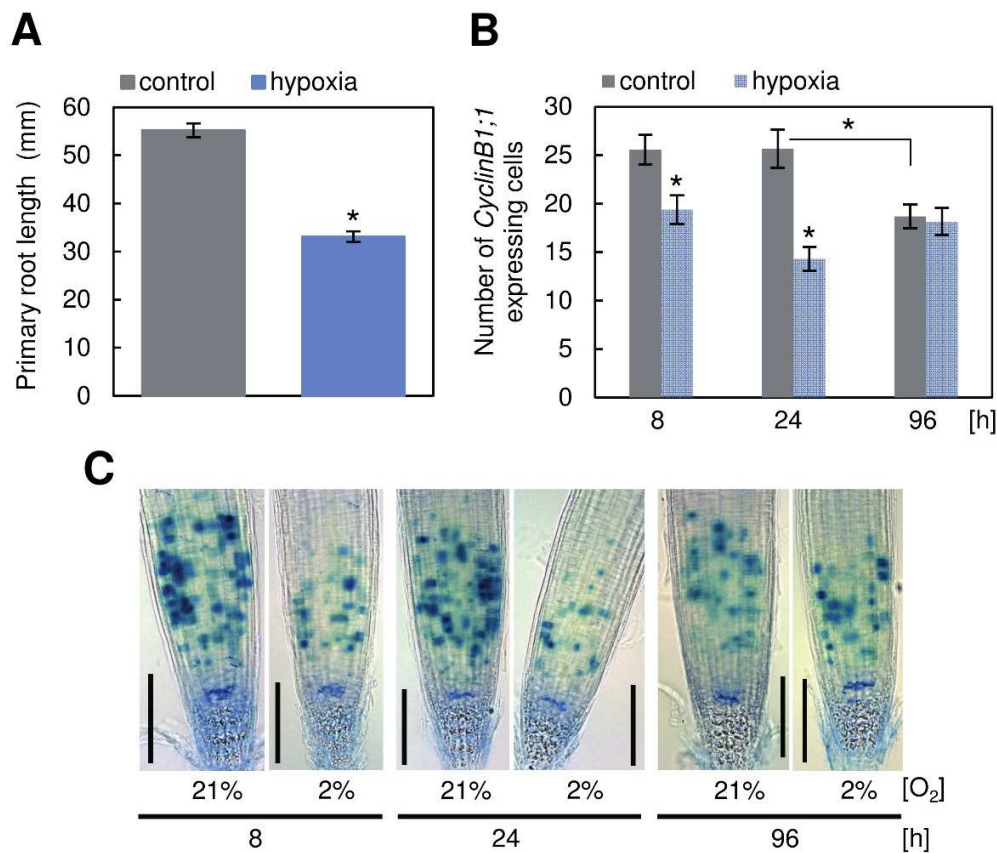
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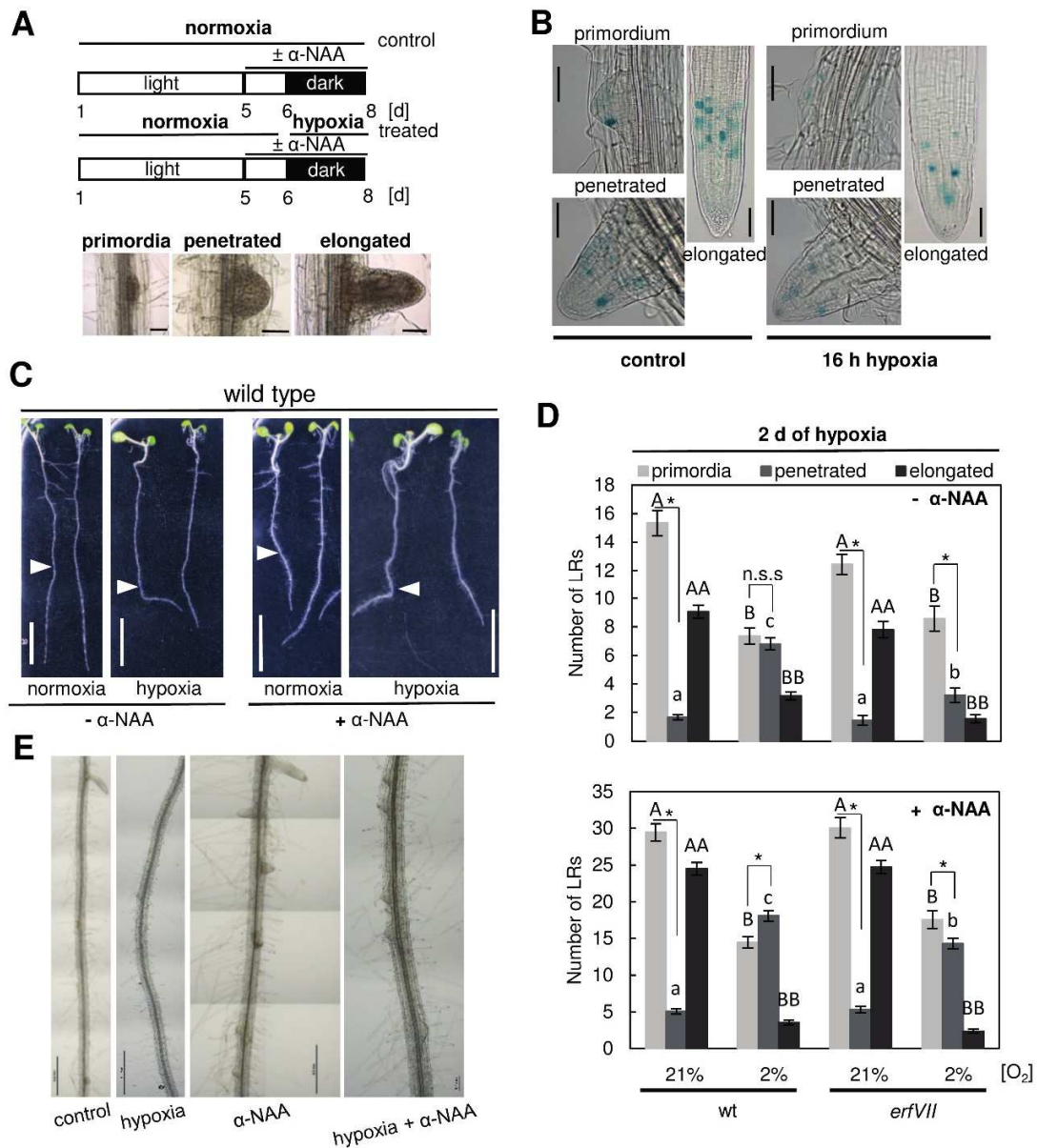


**Figure 1. Hypoxia inhibits meristematic activity and primary root growth.**

**(A)** Primary root length of 6-d-old light-grown wild-type seedlings exposed to control (21% O<sub>2</sub>) or hypoxic (2% O<sub>2</sub>) conditions in the dark for 4 d. Bars represent means ( $\pm$ SE). Asterisks indicate significant differences between control and hypoxia-treated samples (two-sample *t*-test,  $n=21-25$ ,  $P<0.0001$ ).

**(B)** Quantification of *CyclinB1;1::GUS* activity in primary root tips of 6-d-old light-grown seedlings exposed to dark at control or hypoxic conditions for 8, 24 or 96 h. Histochemical staining reveals mitotic cells in primary root tips. Asterisks indicate statistically different means between treatments or as indicated (two-sample *t*-test,  $n=19-23$ ,  $P<0.01$ ).

**(C)** Representative *CyclinB1;1::GUS*-stained primary root tips. Scale bars represent 100 μm.



**Figure 2. Lateral root arrests post emergence at low oxygen conditions.**

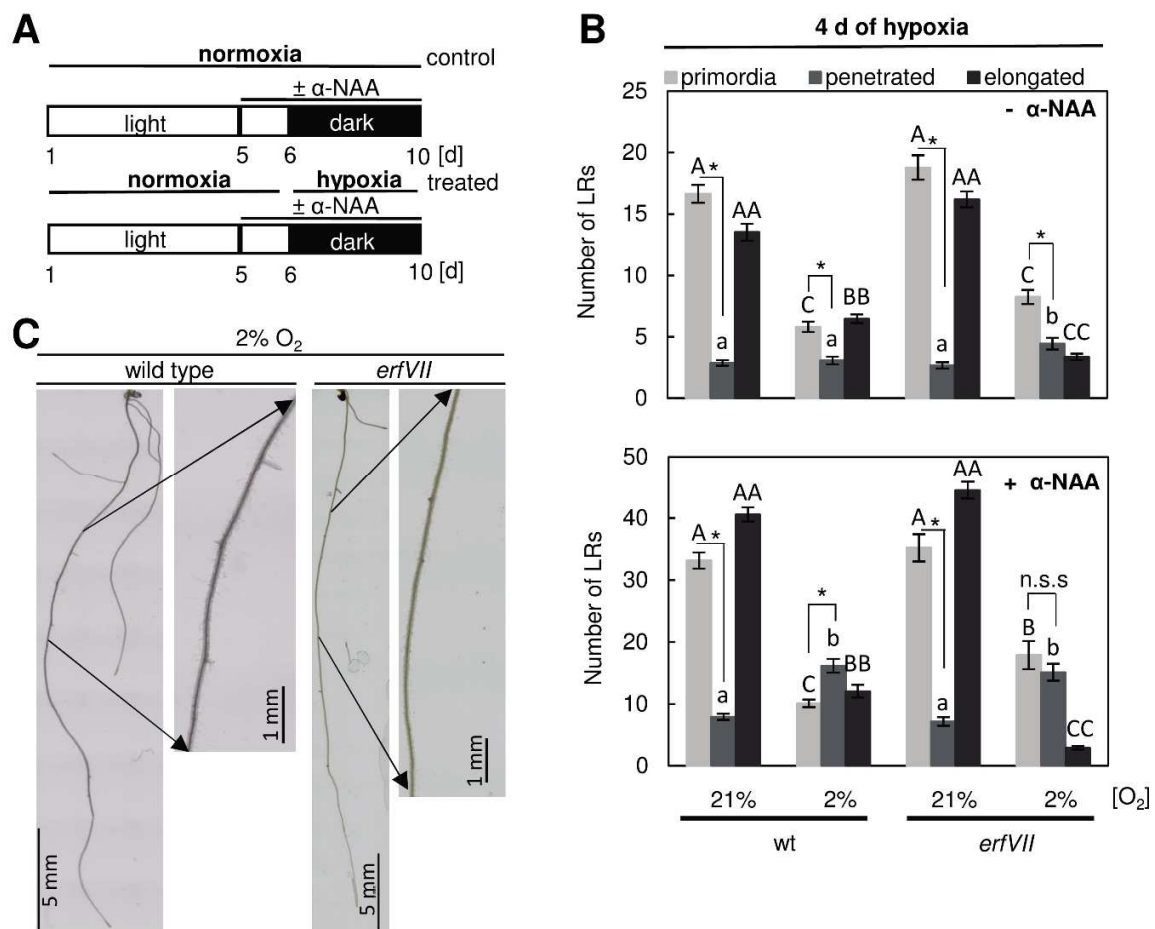
(A) Six-d-old light-grown seedlings were pretreated  $\pm$  20 nM  $\alpha$ -NAA for 1 d and then transferred to the dark at control (21% O<sub>2</sub>) or hypoxic (2% O<sub>2</sub>) conditions for 2 days. Bottom row show representative stages of LR development. Bars = 50  $\mu$ m.

(B) *CyclinB1;1::GUS* expression in developing LRs of 10-d-old light-grown seedlings exposed to dark at control or hypoxic conditions for 16 h. Bars = 50  $\mu$ m.

(C) Root systems of 8-d-old wt seedlings treated as indicated in a. Arrowheads show the position of the root tip at the onset of hypoxia treatment. Bars = 10 mm.

**(D)** Number of LR of 8-d-old seedlings treated (**A** upper) and categorized as indicated in (**A** down) according to Casimiro *et al.* (2003). Bars represent means ( $\pm$ SE). Minor, capital single and capital double letters show significant differences within treatment and asterisks show differences between primordia and penetrated roots (Kruskal-Wallis test,  $n=22-31$ ,  $P<0.05$ , Mann-Whitney test,  $n=22-31$ ,  $P<0.05$ ).

**(E)** Primary roots treated as indicated show arrested LR development after 2 days of hypoxia.

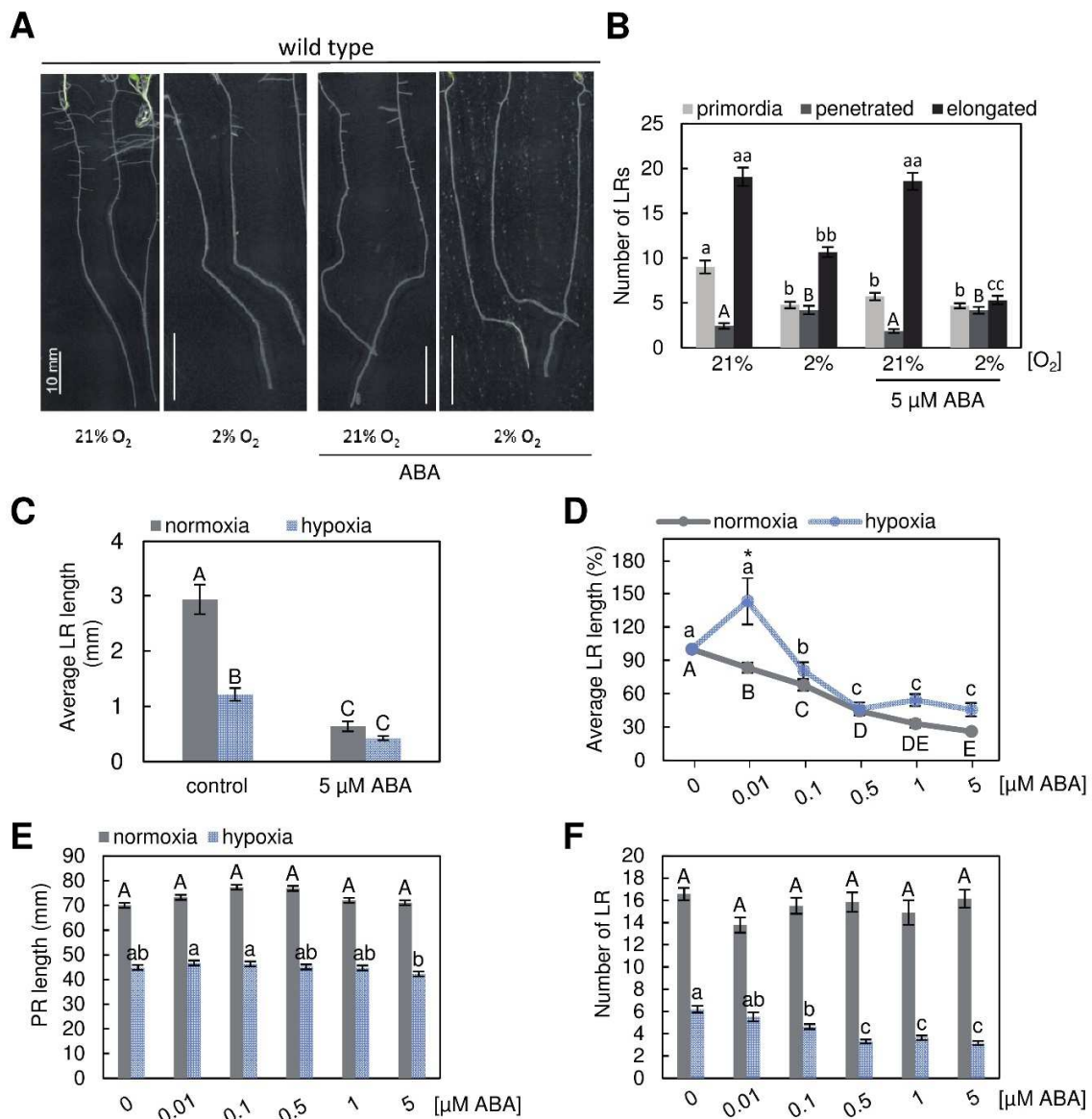


**Figure 3. ERFVIIIs are required to rescue LR during long-term hypoxia.**

**(A)** Six-d-old light-grown seedlings were pretreated  $\pm 20$  nM  $\alpha$ -NAA for 1 d and then transferred to the dark at control (21%  $O_2$ ) or hypoxic (2%  $O_2$ ) conditions for 4 days.

**(B)** LR development in 10-d-old seedlings treated as indicated in **(A)** categorized as indicated in Figure 2A. Bars represent means ( $\pm$ SE). Minor, capital single and capital double letters show significant differences between treatments and asterisks indicate differences between primordia and penetrated roots (Kruskal-Wallis test,  $n=25-33$ ,  $P<0.05$ , Mann-Whitney test,  $n=25-33$ ,  $P<0.05$ ).

**(C)** Representative root morphology of 10-d-old wild-type and *erfVII* seedlings exposed to 2%  $O_2$ .



**Figure 4. ABA inhibits lateral root elongation during hypoxia.**

(A) Representative roots of wild-type treated as indicated. Bar = 10 mm.

(B) The number of primordia, penetrated and elongated LRs of 6-d-old seedlings under indicated treatment for 4 d.

(C) Average LR length of seedlings treated for 4 d as indicated. Minor, capital single and minor double letters indicate significant differences between the treatments (Kruskal-Wallis test,  $n=20-31$ ,  $P<0.05$ ).

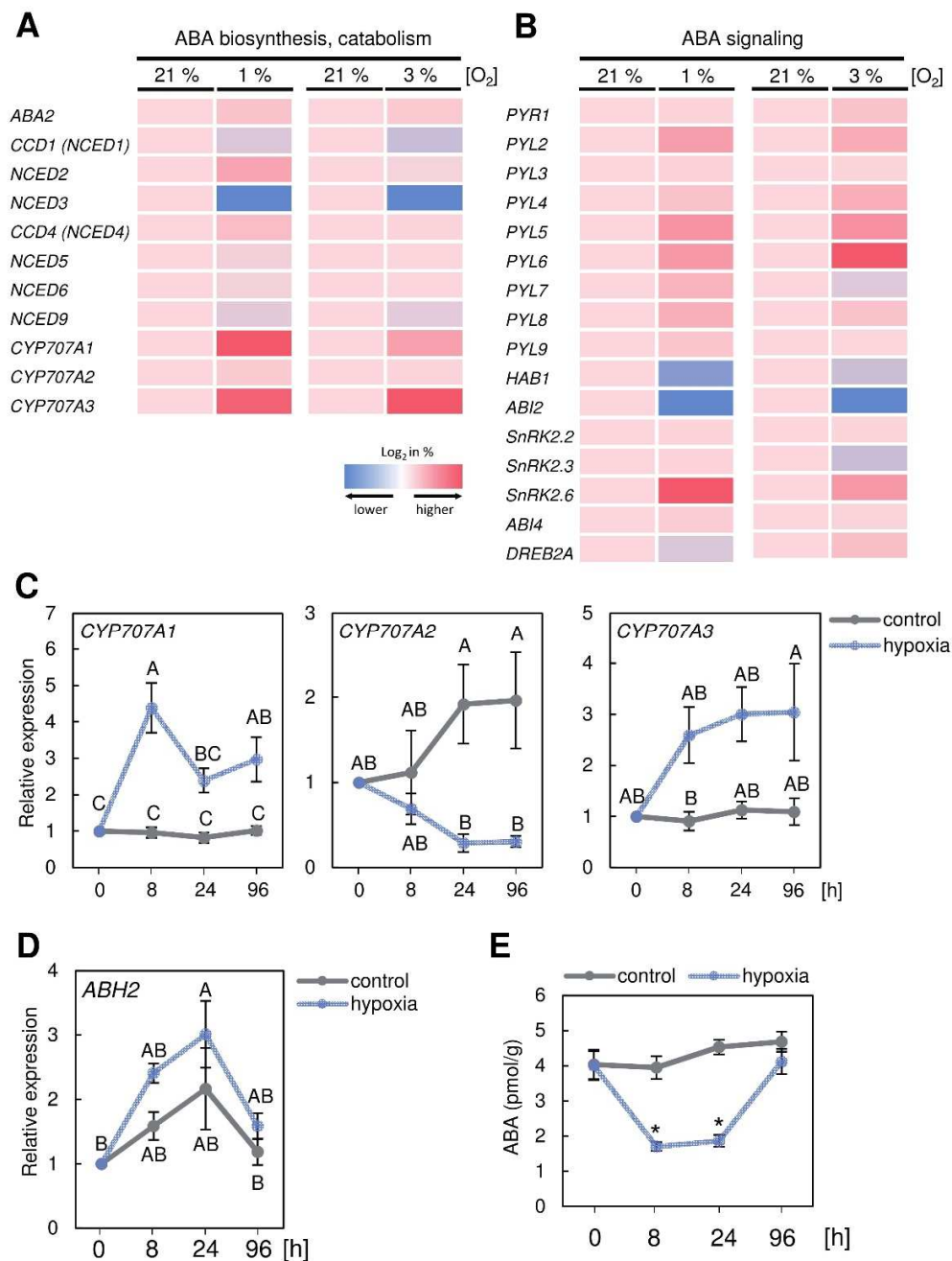
(D) At hypoxic conditions, LR elongation is less sensitive to low levels of ABA. Minor and capital letters show differences within treatments. Asterisks indicate a significant



difference between hypoxia-treated and non-treated samples (Mann-Whitney test,  $n=29-31$ ,  $P<0.05$ ).

**(E)** Primary root length is not altered by exogenous ABA.

**(F)** LR formation is inhibited by ABA under hypoxic but not normoxic conditions. **(E, F)** Bars show averages ( $\pm$ SE) (one-way ANOVA with Tukey's test,  $n=29-31$ ,  $P<0.05$  for primary root length and average LR length, Kruskal-Wallis test,  $n=29-31$ ,  $P<0.05$  for LR numbers).



**Figure 5. Hypoxia transiently decreases ABA levels in roots.**

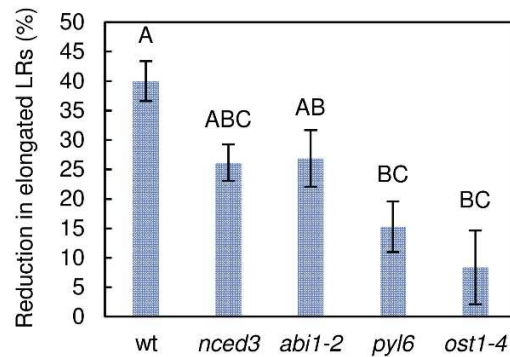
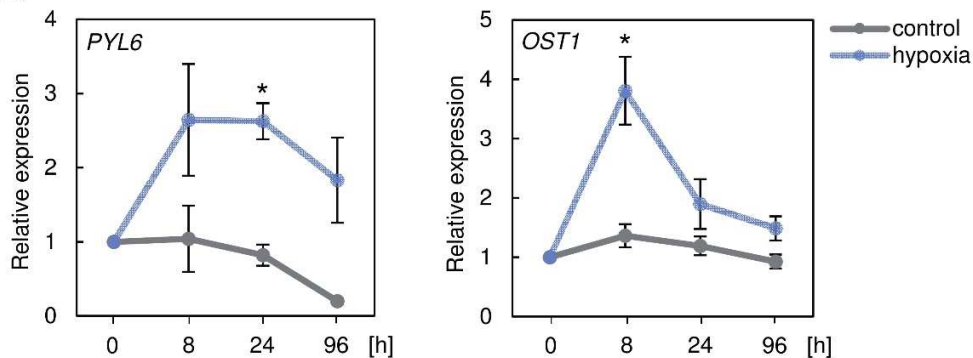
**(A)** Heat maps of ABA synthesis and catabolism genes summarized from previously published transcriptome studies (van Dongen *et al.*, 2009; Yang *et al.*, 2011).

**(B)** Expression of ABA signaling genes obtained as described in **(A)**.

**(C)** qPCR analysis of ABA-8'-hydroxylase genes *CYP707A1*, *CYP707A2* and *CYP707A3* in roots of seedlings exposed to 21% (control) or 2% (hypoxia) O<sub>2</sub> for the times indicated.

**(D)** qPCR analysis of *ABH2*. **(C, D)** Shown are fold changes relative to the 0 h wild type control. *GAPC1* and *ACTIN2* were used for normalization. Averages  $\pm$ SE were calculated from 3 independent experiments with 2 technical replicates each. Different letters indicate significantly different values (one-way ANOVA with Tukey's test,  $P < 0.05$ ).

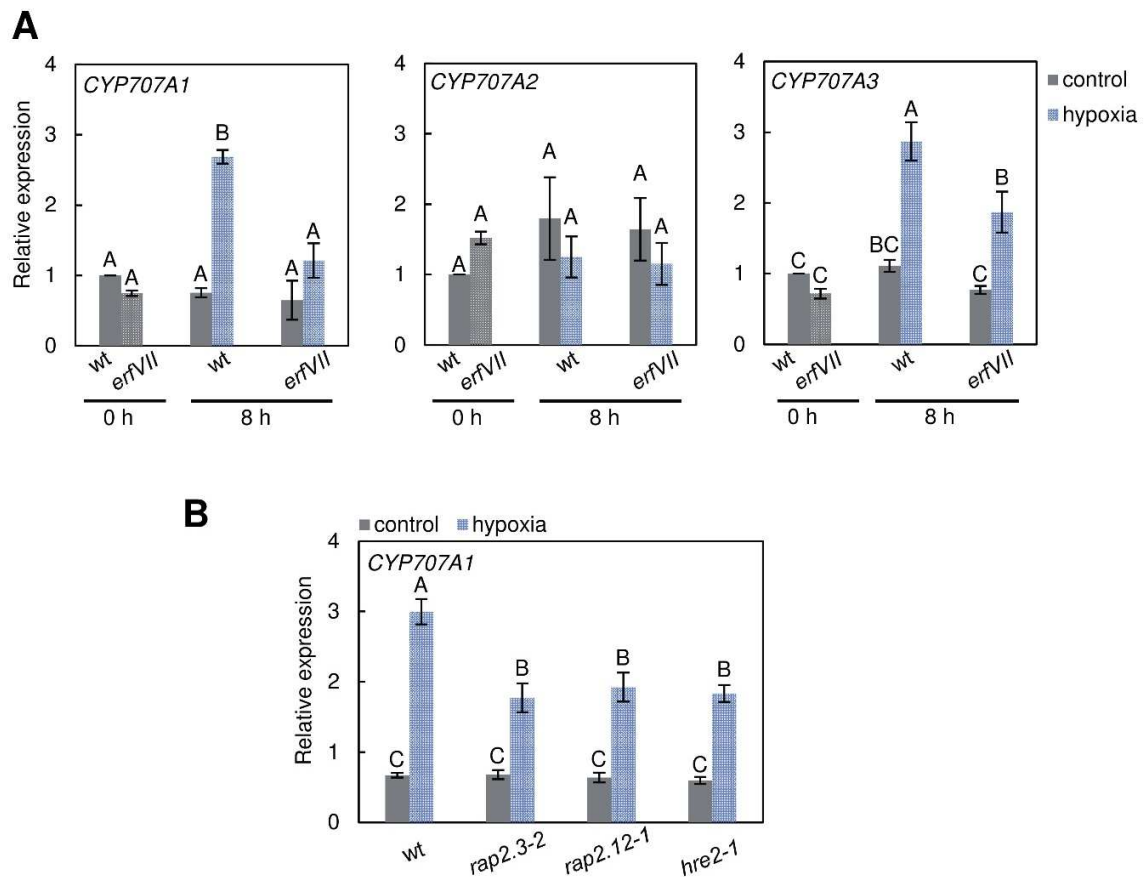
**(E)** ABA content in wild-type roots under normoxic and hypoxic conditions for the times indicated. Asterisks show significantly different means ( $\pm$ SE) between normoxic and hypoxic samples (Mann-Whitney test,  $n=5$ ,  $P < 0.05$ ).

**A****B**

**Figure 6. Knock out of ABA genes relieves lateral root growth inhibition.**

**(A)** Inhibition of LR elongation during 4 d of hypoxia was examined in ABA biosynthesis and signaling mutants. Elongated LRs were counted and averages ( $\pm$ SE) compared relative to wild type at normoxic conditions. Letters indicate significant differences among genotypes (one-way ANOVA with Tukey's test,  $n=22-31$ ,  $P<0.05$ ).

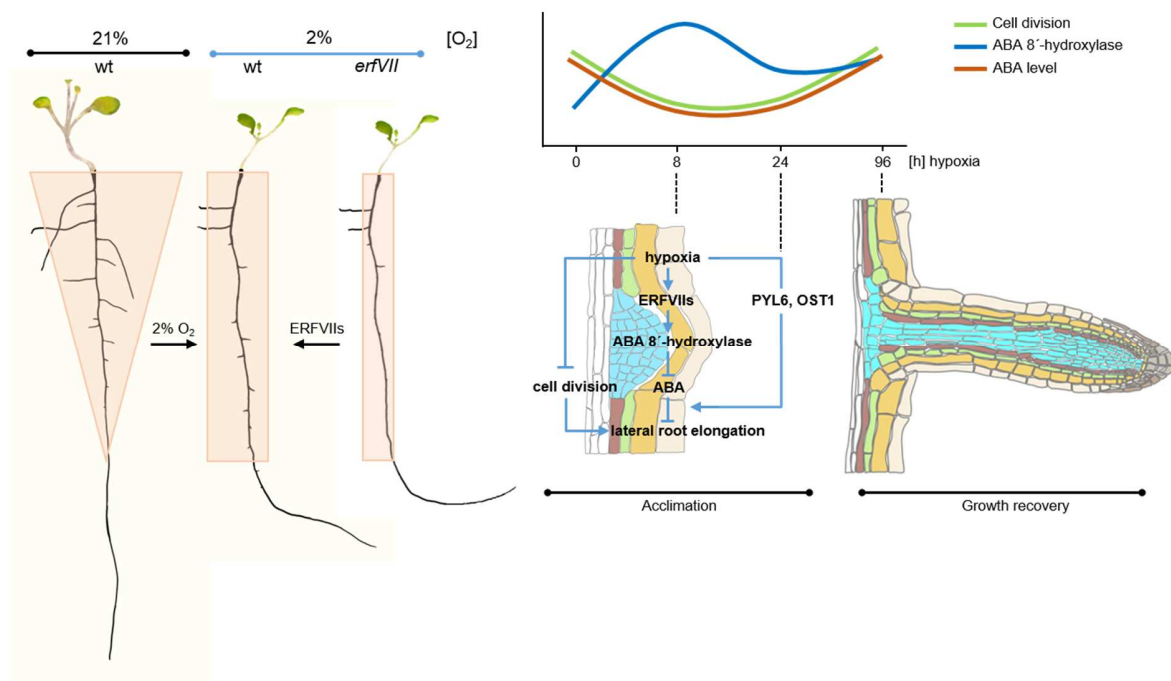
**(B)** Relative transcript levels of *PYL6* and *OST1* after exposure to hypoxia for the times indicated. Values are fold changes of the expression measured at 0 h. *GAPC1* and *ACTIN2* were used for normalization. Results are averages ( $\pm$ SE) of 3 independent biological experiments with 2 technical repeats each. Asterisks indicate significant differences between hypoxia and normoxia samples (two-sample *t*-test,  $P<0.05$ ).



**Figure 7. Hypoxia-induced expression of ABA 8'-hydroxylases is ERFVII-dependent.**

**(A)** Expression of *CYP707A1*, *CYP707A2*, and *CYP707A3* in wild type and *erfVII* seedling roots was analyzed by qPCR. Values were set relative to the 0 h value in wild type.

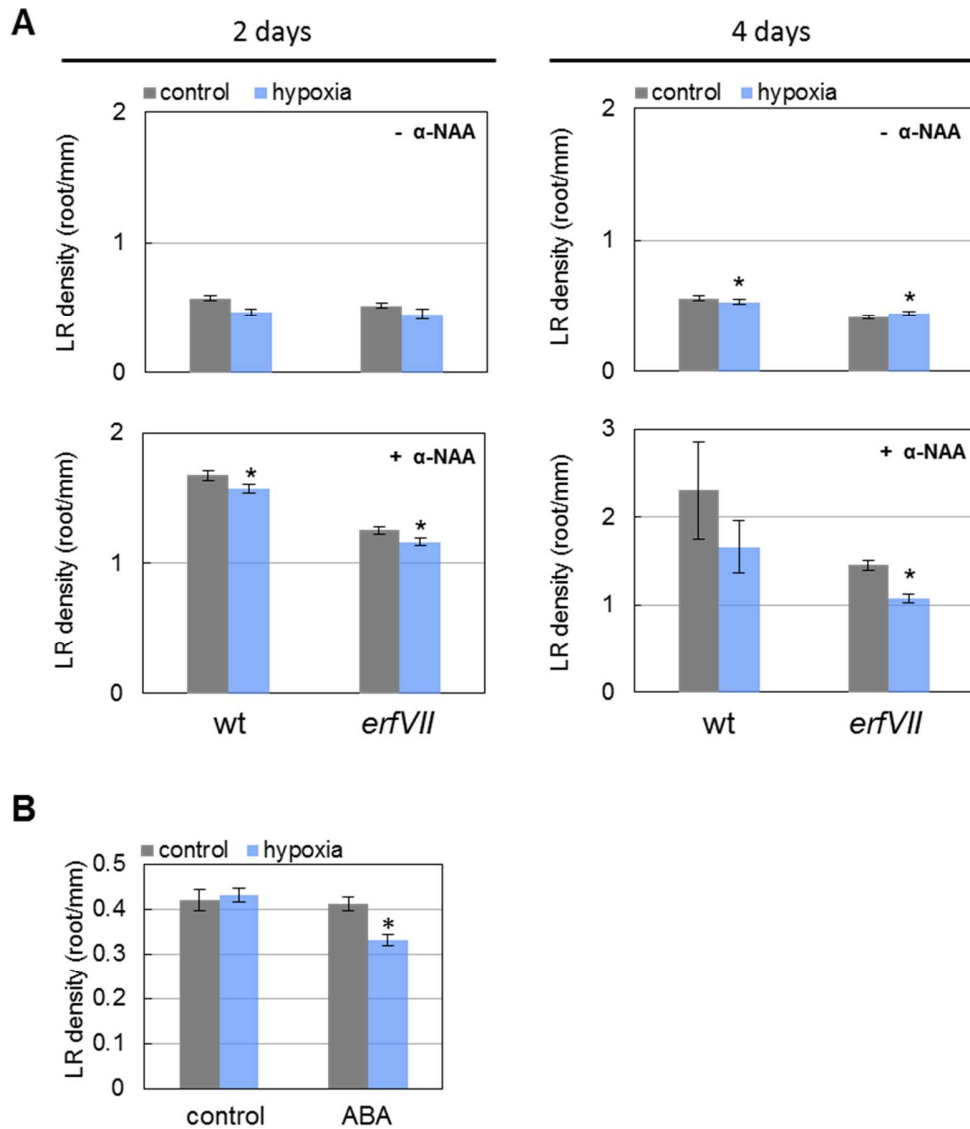
**(B)** Expression of *CYP707A1* under normoxia and hypoxia (2% O<sub>2</sub>) for 8 h analyzed by qPCR. **(A, B)** *GAPC1* and *ACTIN2* were used for normalization. Averages  $\pm$ SE were calculated from 3 independent experiments with 2 technical replicates each. Different letters indicate significantly different values (one-way ANOVA with Tukey's test,  $P < 0.05$ ).



**Figure 8. Hypoxia reshapes the root system of Arabidopsis.**

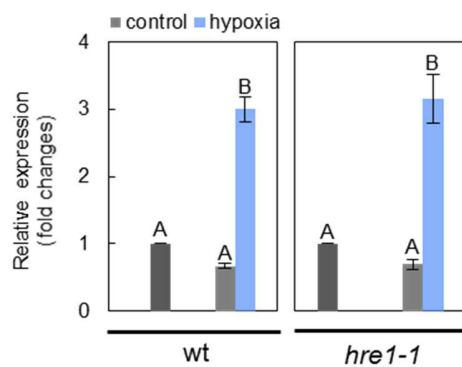
Hypoxia impairs cell division activity resulting in transient arrest of LRs prior to root apical meristem activation post-emergence. At the same time, hypoxia stabilizes ERFVII transcription factors that induce abscisic acid degrading ABA 8'-hydroxylase genes. Reduced ABA levels enable LRs to acclimate to hypoxic conditions and maintain meristematic competence which allows them to resume lateral root elongation. The shading of seedling roots visualizes the shape of root systems of wild type and *erfVII* (right) seedlings at normoxic and hypoxic conditions. Green line: *CyclinB1;1::GUS* expression indicating mitotic activity; blue line: expression of *ABA 8'-hydroxylase 1* (*CYP707A1*); red line: ABA concentration in the LR zone.

## Supplementary Figures



## Supplementary Figure S1. LR density upon hypoxia treatment.

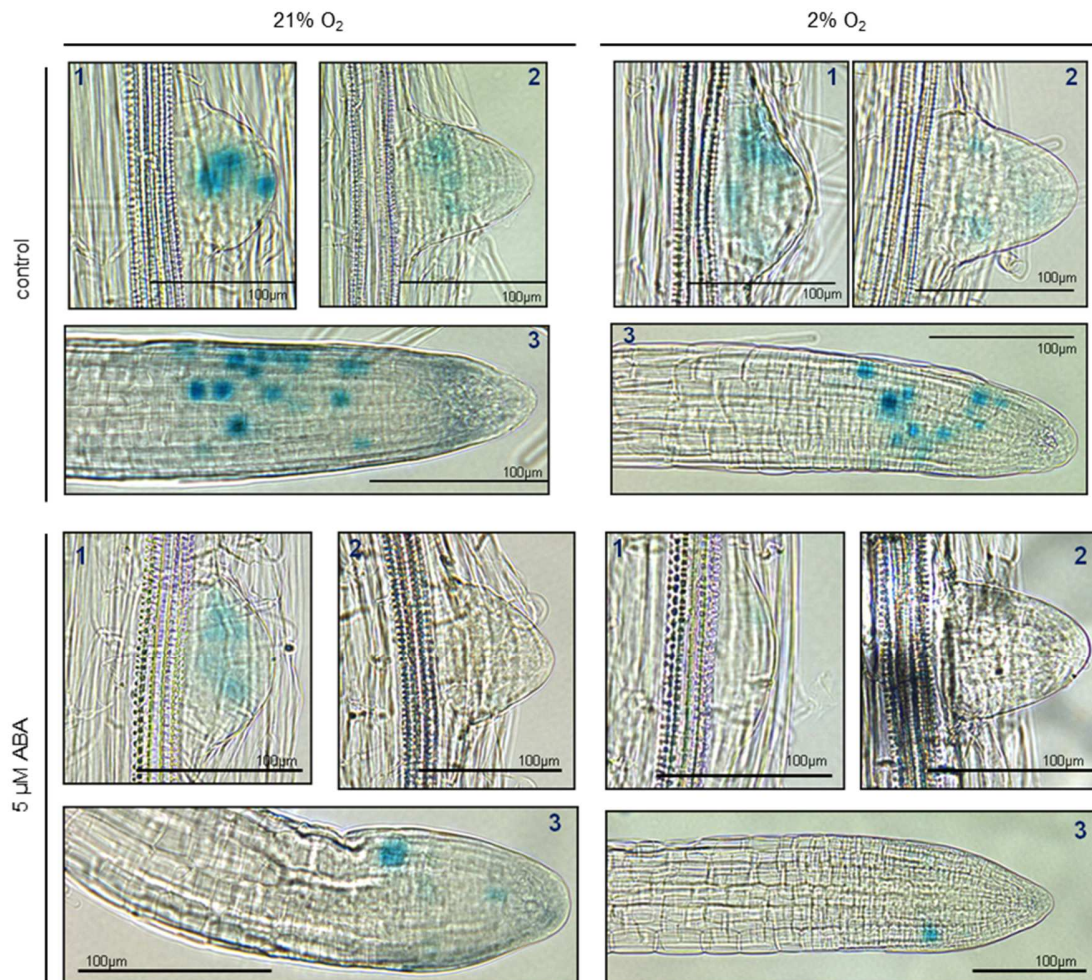
(A-B) show LR density under normoxic and hypoxic conditions with or without 20 nM  $\alpha$ -NAA and 5  $\mu$ M ABA in wild type and *erfVII* (A) or only in wild type (B). Stars show significant difference between control and hypoxia treated samples from 3 biological replicates with  $\pm$ SE (two-sample *t*-test,  $n=21-25$ ,  $P<0.0001$ ).



**Supplementary Figure S2. Hypoxia-induced expression of *ABA 8'-hydroxylase* upon hypoxia.**

qPCR data is shown in single knock-out *hre1-1* mutant for the expression of *CYP707A1* under normoxic and hypoxic conditions at 8 h. Values were set relative to 0 h without treatment wild type or single knock out mutants. *GAPC1* and *ACTIN2* were used for normalization with 2 technical and 3 independent biological replicates. Relative expressions are shown in fold changes with  $\pm$ SE, and different letters indicate significantly different values (one-way ANOVA with Tukey's test,  $n=6$ ,  $P<0.05$ ).





**Supplementary Figure S3. Hypoxia reduces cell division rate lesser extent than ABA.**

Six-d-old *CyclinB1;1::GUS* seedlings treated with 4 days long with or without 5 μM ABA at normoxic (21% O<sub>2</sub>) or at hypoxic (2% O<sub>2</sub>) conditions. Promoter activity of *CyclinB1;1::GUS* was monitored to analyze cell division rate. Left upper picture represents LR primordia stage, right upper represent just emerged LR stage, down picture represents elongated LR stage. Scale bars= 100 μm.

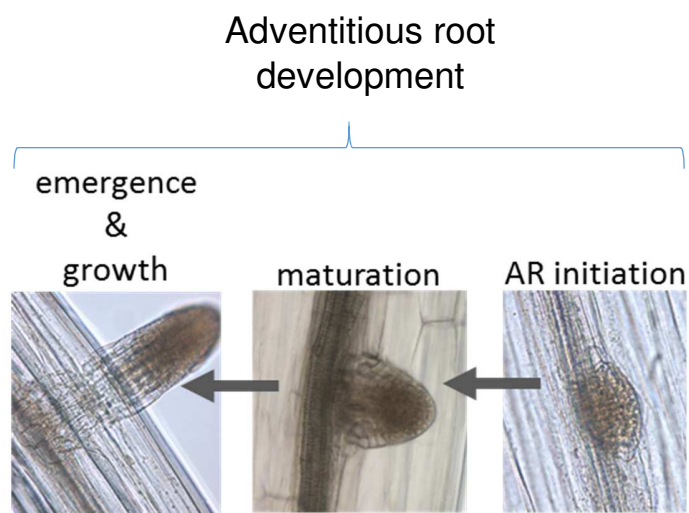
**Supplementary Table**

	Name of primers	Primers 5'-3' sequence
1.	qPCRAt4g27250for	TTCGAGATGACGGCAGCTTT
	qPCRAt4g27250rev	AGCGCCGGTTCTATGACTTT
2.	qPCRAt4g19230for	GAACCCACTCGTGTCTGG
	qPCRAt4g19230rev	GTTTTGGGGAAGCGCGAATG
3.	qPCRAt2g29090for	GGATCAAAAACGCAACGGCT
	qPCRAt2g29090rev	GCTCCCTGGAGACTTCTTGG
4.	qPCRAt5g45340for	CCGTAGCTCCTCCACGAAAC
	qPCRAt5g45340rev	GGGCTCGAGATCATCACACAT
5.	Actin2qF	ACATTCCAGCAGATGTGGATCTC
	Actin2qR	GATCCCATTCAAAAACCCAGC
6.	GAPC1qF	GATTCTACAATGGCTGACAAGAAGA
	GAPC1qR	ATGAAGGGGTCGTTGACAGC

**Supplementary Table S1:** Primer names and sequences used in this study.

## 9 Chapter 3

### 9.1 Hypoxia and the *Arabidopsis* ERFVII transcription factor HRE2 promote adventitious root elongation



Running title: Hypoxia promotes adventitious root elongation

**Hypoxia and the *Arabidopsis* ERFVII transcription factor HRE2 promote adventitious root elongation**

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**Author contributions:**

MS conceived the project; EED and MS designed experiments, EED performed and analyzed experiments, EED and MS wrote the manuscript.

**Keywords:** hypoxia, ethylene, flooding, adventitious root, ERFVII, HRE2, *Arabidopsis thaliana*,

Adventitious root (AR)

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**Abstract**

Soil water logging and flooding are common environmental stress conditions that can impair plant fitness. Roots are the first organs to be confronted with reduced oxygen tension as a result of flooding. While anatomical and morphological adaptations of roots are extensively studied, the root system architecture is only now getting in the focus of flooding research. Adventitious root (AR) formation shifts the root system higher up the plant thereby facilitating supply with oxygen and likely improving root and plant survival. We used the model plant *Arabidopsis* to study AR formation at hypoxic conditions and in response to ethylene.

Results show that ethylene inhibits AR formation. Hypoxia does not alter the rate at which ARs are formed but promotes AR elongation growth. The ERFVII transcription factor HRE2 that is induced by hypoxia induces AR elongation while ethylene inhibits AR initiation and overrides HRE2-induced AR elongation.

In conclusion, HRE2 promotes the establishment of an AR system that is under negative control by ethylene. Inhibition of growth of the main root system and promotion of AR elongation under hypoxia strengthens the root system in upper soil layers where oxygen shortage may last for shorter times.

## Introduction

Flooding as a consequence of weather extremes is a major cause for crop losses (Voeselek and Bailey-Serres, 2015). During flooding, plants are exposed to reduced O<sub>2</sub> as a result of oxygen consumption and limited gas exchange. In addition to oxygen shortage, the gaseous hormone ethylene accumulates due to reduced diffusion rates in water (Grable, 1966). Both, reduced oxygen and elevated ethylene levels serve as signals that induce flooding stress responses. In flooding-tolerant species, several acclimation traits have evolved including morphological and anatomical adaptations that improve oxygen supply, and metabolic adjustments to compensate for limited oxidative ATP synthesis by substrate-level ATP synthesis and NAD<sup>+</sup> recovery in fermentation. To improve gas exchange, flooding-tolerant plants form aerenchyma (Steffens *et al.*, 2011; Yamauchi *et al.*, 2013), adventitious roots (Lorbiecke and Sauter, 1999), and gas films (Herzog *et al.*, 2017), or induce hyponastic growth (Millenaar *et al.*, 2005; Pierik *et al.*, 2005), and stem or petiole elongation (Millenaar *et al.*, 2005, Kende *et al.*, 1998) to raise leaves above the water surface.

Adventitious root (AR) growth is a common adaptive response to flooding e.g. in deepwater rice (*Oryza sativa* L.), tamarack (*Larix laricina*), *Rumex palustris*, and tomato (*Solanum lycopersicum*) (Visser *et al.*, 1995; Lorbiecke and Sauter, 1999; Vidoz *et al.*, 2010; Calvo-Polanco *et al.*, 2012). ARs facilitate gas exchange, uptake of minerals, water, and oxygen, and they anchor the plant during the post-submergence phase. In rice, nodal AR initiation is part of the regular developmental program. However, the emergence of ARs depends on an environmental stimulus such as flooding which traps ethylene that triggers the outgrowth of AR primordia.

*Arabidopsis thaliana* is an intermediate flooding-tolerant species in which ethylene and low oxygen trigger metabolic acclimation (Voeselek and Sasidharan, 2013). Petiole elongation and hyponastic growth improve the supply of submerged tissues with O<sub>2</sub> (Millenaar *et al.*, 2005). The APETALE2/ETHYLENE-RESPONSE FACTOR (AP2/ERF) transcription factors of group VII (ERFVIIIs) are key mediators of low oxygen stress responses in rice and Arabidopsis. Arabidopsis has five ERFVIIIs, RAP2.2, RAP2.3, RAP2.12, HRE1 and HRE2. Expression of ERFVIIIs is differentially controlled by ethylene and low O<sub>2</sub> and differs spatially suggesting that ERFVIIIs play specific roles in hypoxia

adaptation and act in a tissue-specific manner. While ethylene induces the expression of *HRE1* and *RAP2.2*, low oxygen induces the expression of *HRE1* and *HRE2* (Hinz *et al.*, 2010; Hess *et al.*, 2011; Bailey-Serres *et al.*, 2012). ERFVIs regulate metabolic changes and developmental reprogramming under low oxygen conditions (Licausi *et al.*, 2013; Abbas *et al.*, 2015; Paul *et al.*, 2016).

Since roots are the first to encounter hypoxic stress during flooding, it is not surprising that the root system adjusts to these conditions as recently shown for the primary root growth direction in *Arabidopsis* (Eysholdt-Derzsó and Sauter, 2017/ Chapter 1). The root system includes a primary root, lateral roots and possibly ARs (Smith and De Smet, 2012). In this study, we analyzed AR formation in *Arabidopsis* in response to hypoxia and ethylene, two main signals of flooding stress with a particular focus on the involvement of ERFVIs in AR development.

## Results

### Hypoxia promotes adventitious root growth

To study the development of adventitious roots (ARs) in *Arabidopsis* in response to hypoxic conditions, 5-d-old seedlings were transferred to a controlled gas atmosphere with 2% O<sub>2</sub> or kept in air for 6 days in the dark. Darkness induces the formation of ARs from the hypocotyl of *Arabidopsis* seedlings. The AR density was about 0.45 ARs per mm of hypocotyl length in, both, seedlings kept in air and seedlings exposed to hypoxia (Figure 1A). For a more detailed analysis, AR development was studied by distinguishing three categories: (1) young AR primordia of stages I to IV according to the nomenclature described previously for lateral root development (Casimiro *et al.*, 2003), (2) mature primordia prior to emergence at stages V-VII and (3) emerged ARs of stage VII as well as elongated ARs (Figure 1B). In normoxia, most ARs were at the young primordia stage whereas hypoxia promoted AR emergence and growth resulting in a significant increase in category 3 ARs (Figure 1C). Since ethylene is known to control root growth and since ethylene mediates flooding responses (Sasidharan and Voesenek, 2015), we next looked into AR development in the presence of 5 µL/L ethylene at normoxic and hypoxic conditions (Figure 1A, C). Ethylene inhibited the formation of ARs at both oxygen levels

(Figure 1A), and it inhibited AR development at all developmental stages overriding hypoxia-induced AR emergence and growth (Figure 1C).

### **HRE2 promotes adventitious root formation and emergence of adventitious root primordia**

*HRE2* but not its related transcription factor gene *HRE1* was shown to be upregulated by hypoxia in the shoot (Licausi *et al.*, 2010) and *HRE2* overexpression improved anoxia survival (Supplementary Figure S1). To establish a possible link between hypoxia signaling of AR development and ERFVII transcription factors as hypoxia signal mediators, we next analyzed the roles of the ERFVIIs and ethylene in AR development (Figure 2). The *ein3 eil1* mutant is deficient in ethylene signaling. *ein3 eil1* seedlings displayed a significantly higher AR density indicating that basal ethylene signaling takes place in normoxic seedlings that partially inhibits AR formation (Figure 2A). Among the *HRE* single and double knockout and overexpressing mutants analyzed, *HRE2ox1* showed a significantly higher AR density compared to wild type suggesting that *HRE2* and *HRE1* have nonredundant developmental activities. The hypocotyl lengths were not changed in *ein3 eil1* or in *HRE1* and *HRE2* mutants compared to wild type (Supplementary Figure S2) suggesting that AR organogenesis was a highly specific response to *HRE2* overexpression. Knockout and overexpressing mutants of *RAP2.3* and *RAP2.12* (Figure 2A; Supplementary Figure S3) showed a wild-type phenotype. Based on the finding that *HRE2* but not other ERFVIIs control AR formation, we looked in more detail at AR development in *HRE2* mutant seedlings in comparison to *ein3 eil1* and wild type (Figure 2B). Compared to wild type, *ein3 eil1* seedlings had more ARs in all three categories indicating that ethylene inhibits AR initiation but does not alter AR development. By contrast, *HRE2ox1* seedlings had more ARs than wildtype. Hence, ethylene and hypoxia-induced *HRE2* inversely regulate AR formation. Furthermore, *HRE2*, unlike ethylene, controlled the late stages of AR development resulting in more elongated ARs.



### Ethylene overrides HRE2 activity

To test for an interaction of HREs with ethylene signaling, we analyzed HRE mutants exposed to ethylene or 1-MCP, an inhibitor of ethylene perception (Figure 2C, D). AR density was inhibited by 5  $\mu\text{L/L}$  ethylene in wild type but not in the ethylene-insensitive *ein3 eil1* mutant as would be expected (Figure 2C). In *hre1-1*, *HRE1ox1*, *hre2-1*, and *HRE2ox1*, ethylene reduced AR density to wild-type levels even in *HRE2ox1* seedlings that produced more ARs than wild type in the absence of exogenous ethylene. 1-MCP treatment resulted in higher AR densities in wild type, *hre1-1*, *HRE1ox1* and *hre2-1* (Figure 2D). AR density in *HRE2ox1* did not change with 1-MCP and was comparable to 1-MCP-treated wild-type seedlings. The observation that *HRE2ox1* seedlings are insensitive to 1-MCP but sensitive to ethylene is in agreement with the hypothesis that *HRE2ox1* reduces ethylene responsiveness.

### Discussion

AR growth in deepwater rice is a well-known adaptive trait for survival under prolonged flooding that is primarily regulated by ethylene (Lorbiecke and Sauter, 1999). However flooding not only promotes ethylene accumulation but also causes  $\text{O}_2$  deprivation that in turn initiates transcriptional regulation (Mustroph *et al.*, 2014). In rice and *Rumex palustris*, AR primordia are formed as part of the developmental program and emerge upon flooding (Visser *et al.*, 1996; Lorbiecke and Sauter, 1999), while in other plants, AR primordia are initiated only when an environmental change occurs. In tomato and sunflower; flooding, and in Arabidopsis, darkness or wounding promote AR formation (Wample and Reid, 1978; Vidoz *et al.* 2010; da Rocha Correa *et al.*, 2012; Dawood *et al.*, 2014).

In this study, we analyzed AR formation at the hypocotyl of Arabidopsis seedlings. Our results reveal a role for hypoxia signaling in AR growth and repression of hypoxia-induced AR growth by ethylene. While in Arabidopsis growth of ARs is enhanced by hypoxia, growth of the main root system is inhibited (Ellis *et al.*, 1999). As a result, the root system architecture changes with a shift of root mass from the lower ground toward higher ground possibly to invest energy in roots that are closer to the soil surface and hence more likely

to reach aerated zones. Unlike hypoxia, ethylene inhibits AR initiation (Veloccia *et al.*, 2016). When combined, hypoxia and ethylene inhibit AR initiation to the same degree as ethylene alone, indicating that ethylene overrides the hypoxia signal (Supplementary Figure S4). AR growth is inhibited by ethylene in a dose-dependent manner in normoxia (Veloccia *et al.*, 2016). Future work may clarify if the sensitivity to ethylene changes under hypoxia. In contrast to *Arabidopsis*, ethylene has a promotive effect on AR emergence and growth in several flood-tolerant species including rice (Lorbiecke and Sauter, 1999; Lin and Sauter, 2018). Different regulation of ARs by ethylene may reflect different adaptive strategies in flooding-tolerant rice compared to moderately tolerant *Arabidopsis* (Vashisht *et al.*, 2011).

Overexpression of HRE2 promoted AR growth. Inhibition of ethylene perception by 1-MCP did not further increase AR length suggesting that HRE2 may act downstream of ethylene perception. Expression of *HRE2* is highly induced by hypoxia in the shoot and root (Licausi *et al.*, 2010; Hess *et al.*, 2011) and was suggested to regulate root growth by inducing cell expansion (Lee *et al.*, 2015). The *HRE1* expression is induced in roots by hypoxia (Licausi *et al.*, 2010; Eysholdt-Derzsó and Sauter, 2017) and ethylene (Hess *et al.*, 2011) where it reduces ethylene-dependent primary root growth inhibition. The molecular mechanism of HRE1 activity is not known except that it does not alter ethylene production (Yang *et al.*, 2011). While HRE1 regulates primary root growth, it does not control AR development or ethylene signaling of AR growth inhibition supporting the conclusion that AR development under hypoxic conditions is promoted specifically by the ERFVII HRE2 and inhibited by ethylene signaling.

## Materials and Methods

### Plant materials and growth conditions

All experiments were carried out with *Arabidopsis thaliana* Col-0. The *ein3 eil1* knockout line (An *et al.*, 2010) was kindly provided by Hongwei Guo (Peking-Tsinghua Center of Life Sciences, Beijing, China). The *hre1-1* and *hre2-1* single knockout, the *hre1-1*, *hre2-1* double knockout, the *HRE1ox1* and *HRE2ox1* overexpression lines (Hess *et al.*, 2011;

Eysholdt-Derzsó and Sauter, 2017/ Chapter 1) as well as *rap2.3-2* (Marín-de la Rosa *et al.*, 2014) and *rap2.12-1* (Gibbs *et al.*, 2014) were described previously. *HRE1ox1* and *HRE2ox1* were crossed to generate the double overexpressor line *HRE1ox1 x HRE2ox1*.

Seeds were surface-sterilized and plated as described (Eysholdt-Derzsó and Sauter, 2017/ Chapter 1). To induce synchronous germination, seeds were exposed to light for 6 h before they were transferred to the dark for the times indicated. For all treatments, we used 5-d-old dark-grown seedlings that were kept either at 21% O<sub>2</sub> or 2% O<sub>2</sub> as described (Eysholdt-Derzsó and Sauter, 2017/ Chapter 1), or exposed to 5 µL/L (5 ppm) ethylene (Air Liquide) or 2 µL/L (2 ppm) 1-methylcyclopropene (1-MCP) for 6 d in the dark.

### **Cloning and plant transformation**

Gateway technology based on site-specific recombination was used to generate overexpressing lines of RAP2.3 and RAP2.12 by introducing the open reading frames (ORFs) into the pB7WG2 destination vector through ligation into pENTR 1A DS (Thermo Fisher Scientific) (Karimi *et al.*, 2002). The primer pairs Forrap2.3-ox1 and Revrap2.3-ox1, and Forrap2.12-ox1 and Revrap2.12-ox1 (Supplementary Table S1) were used to amplify the ORFs of *RAP2.3* and *RAP2.12* from cDNA generated from leaf RNA. The pB7WG2 expression clones were transformed into *Agrobacterium tumefaciens* strain GV3101 followed by plant transformation of Col-0 with the floral dip method (Clough and Bent, 1998). Plant selection was carried out based on glufosinate resistance from the pB7WG2 vector. Homozygous plants were selected, and expression of *RAP2.3* and *RAP2.12* was analyzed in selected plants by RT-PCR using leaf material for RNA isolation. The gene-specific primers Forrap2.3-ox1 and Revrap2.3-ox1 for *RAP2.3*, RAPForrap2.12-ox1 and Revrap2.12-ox1 for *RAP2.12*, were used to verify overexpression (Supplementary Table S1). *Actin2* was used as a reference gene with primers Actin2F1 and Actin2R1 (Supplementary Table S1)

**Statistics**

Minitab14 software ([www. minitab.com](http://www.minitab.com)) was used for analysis of variance (one-way ANOVA) with Tukey's post hoc test. For non-parametric samples, a Kruskal-Wallis test with Dunn's post hoc test was used. Comparison of two means was performed by Student's *t*-test.

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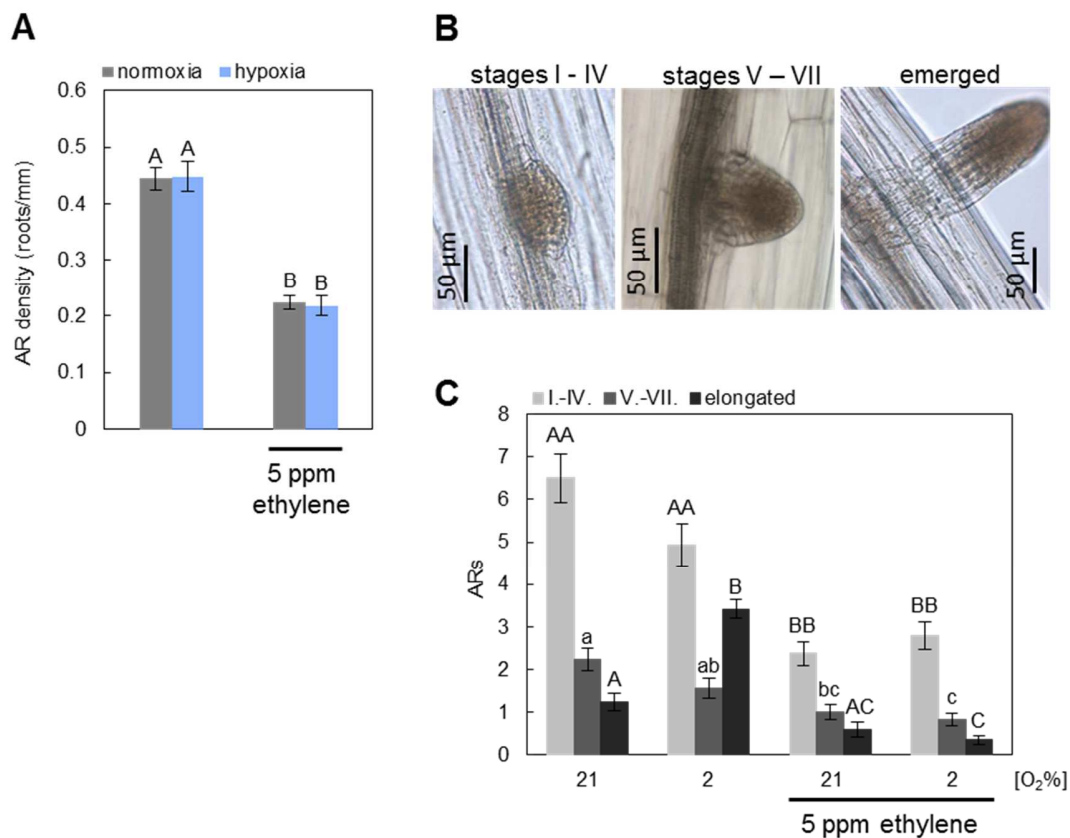
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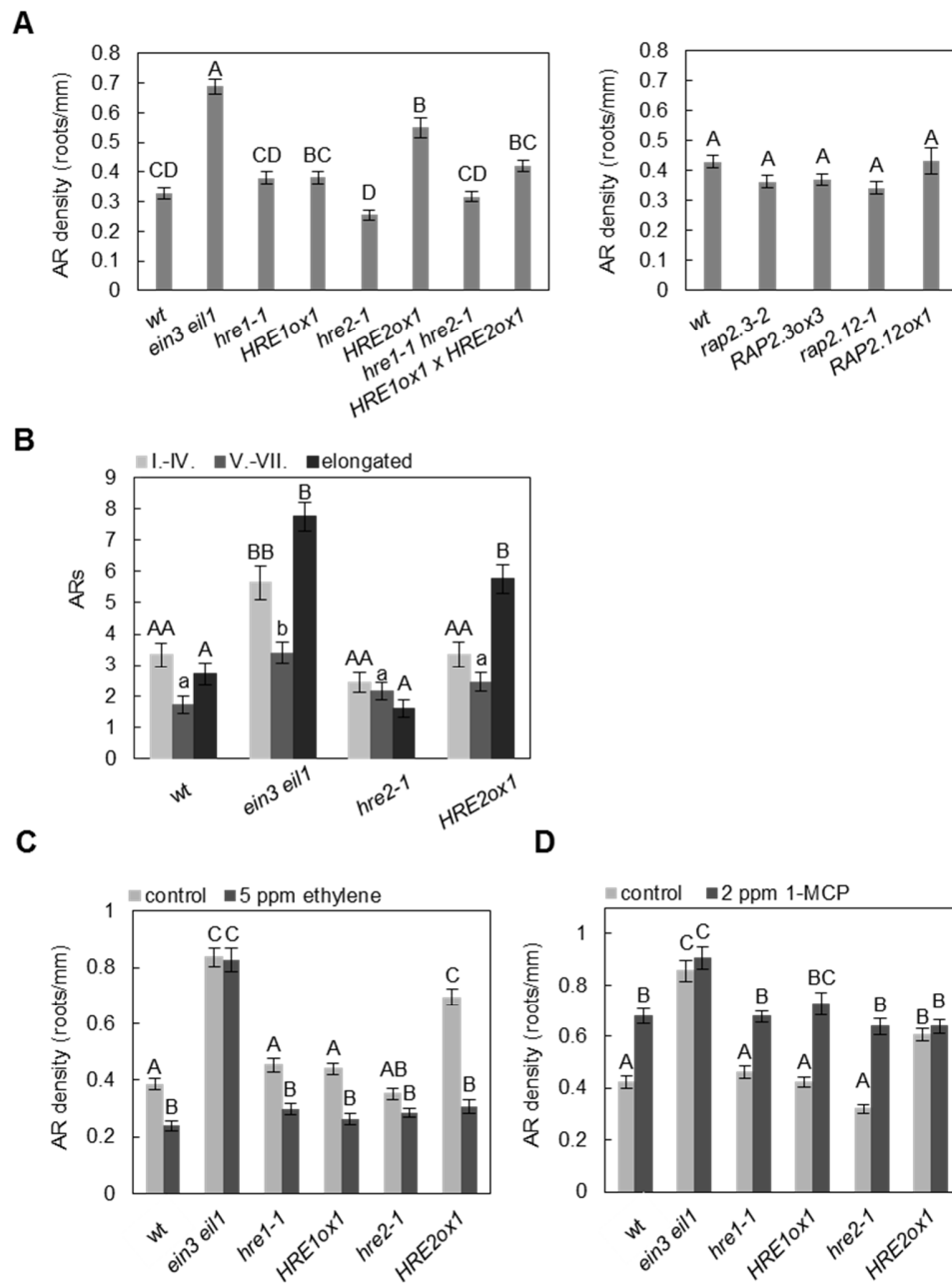
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**Figure 1. Hypoxia promotes adventitious root elongation while ethylene acts as an inhibitor of AR initiation and growth.**

- (A) Average AR densities ( $\pm$ SE) were obtained in three biological replicates. Different letters indicate significantly different values (one-way ANOVA with Tukey's test,  $n=26-29$ ,  $P<0.05$ ).
- (B) ARs were grouped into 3 categories based on the developmental stages described by Casimiro *et al.* (2003) for lateral root development. Representative ARs are shown from each category.
- (C) Five-day-old seedlings were transferred to 21% or 2% O<sub>2</sub> with or without 5  $\mu$ L/L ethylene for 6 d. ARs were categorized as shown in (B). Values are means ( $\pm$ SE) with single minor, capital and double capital letters indicating significant changes within the respective developmental category (Kruskal-Wallis test,  $n=26-29$ ,  $P<0.05$ ).

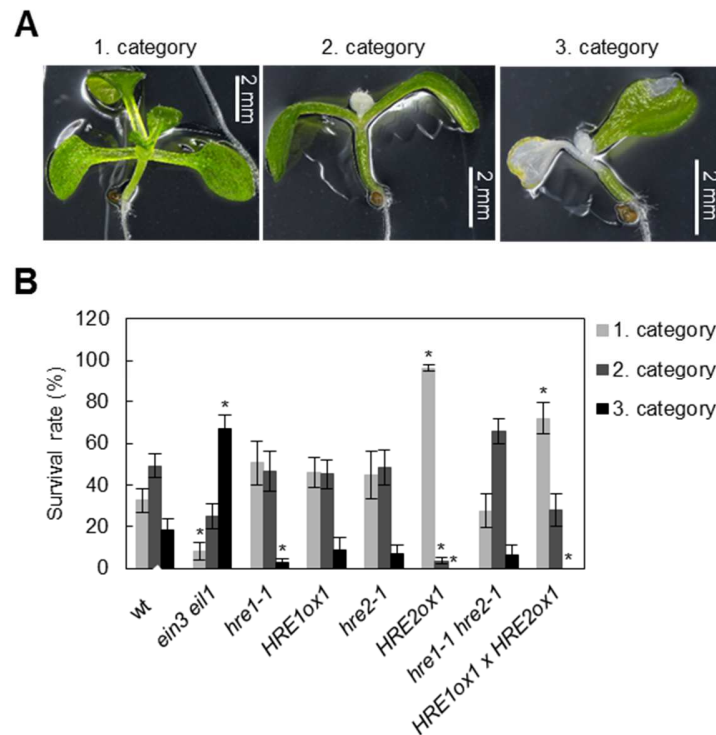


**Figure 2. Ethylene inhibits HRE2-induced AR elongation.**

(A) AR density of 11-d-old wild type and mutant seedlings at normoxia. Error bars represent  $\pm$ SE from 3 biological replicates and letters show statistically different values (one-way ANOVA with Tukey's test,  $n=22-26$ ,  $P<0.05$ ).

- (B) ARs of wild type, *ein3 eil1*, *hre2-1* and *HRE2ox1* seedlings were counted and categorized as described in Figure 1 in 3 biological replicates. Values are means ( $\pm$ SE) with single minor, capital or double capital letters indicating significant differences within each category (Kruskal-Wallis test,  $n=22-26$ ,  $P<0.05$ ).
- (C) AR density of 11-d-old wild-type and mutant seedlings treated with or without 5  $\mu$ L/L ethylene.
- (D) AR density of 11-d-old wild-type and mutant seedlings treated with or without 2  $\mu$ L/L 1-MCP for 6 d. Averages ( $\pm$ SE) in (C) and (D) were obtained in 3 biological replicates. Letters indicate statistically different values (Kruskal-Wallis test,  $n_{\text{C}_2\text{H}_4}=20-31$ ,  $n_{\text{MCP}}=20-28$ ,  $P<0.05$ ).

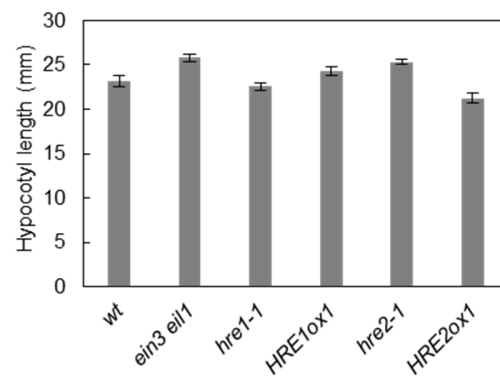
## Supplementary Figures



**Supplementary Figure S1. HRE2 improves and EIN3/EIL1 signaling impairs anoxia survival.**

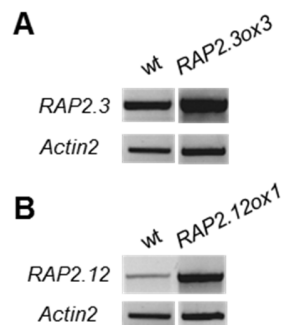
(A) 7-d-old seedlings were exposed to 3 d of 0% O<sub>2</sub> in the dark then transferred to normoxia in the light for a 7 d recovery phase. Seedlings were categorized as being alive (category 1), damaged with bleached true leaves (category 2) or dead (category 3).

(B) Survival rates were scored as shown in (A). Values are means ( $\pm$ SE) of 3 biological and 2 technical replicates in percent. Asterisks indicate significantly different values within each category compared to wild type (two-sample *t*-test,  $n=6$ ,  $P<0.05$ ).



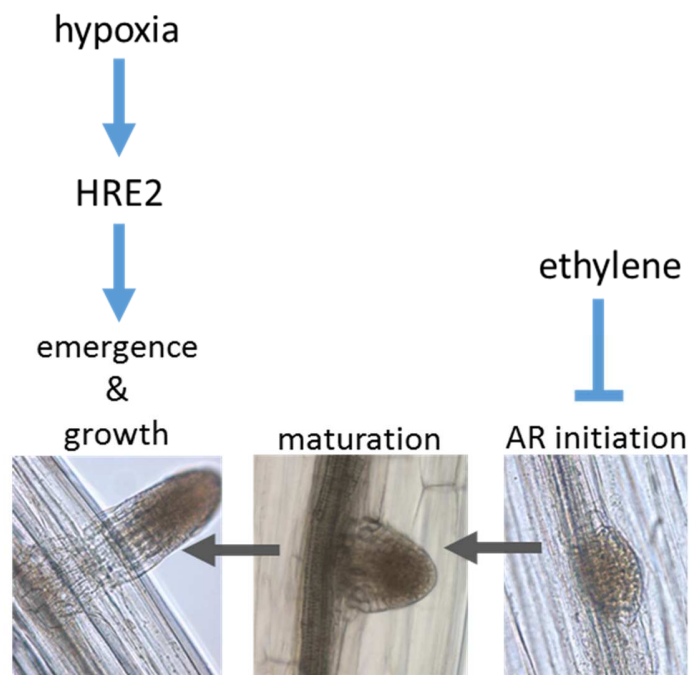
**Supplementary Figure S2. Hypocotyl length is not altered in *ein3 eil1*, and in *HRE1* and *HRE2* mutants.**

Average ( $\pm$ SE) hypocotyl length of 11-d-old seedlings of the indicated genotypes as determined in 3 biological replicates (n=17-31).



**Supplementary Figure S3. Overexpressing lines of *RAP2.3* and *RAP2.12*.**

Verification of expression of *RAP2.3* (A) and *RAP2.12* (B) in overexpressing lines by RT-PCR from leaf tissue. *Actin2* was used as a reference gene for normalization.



**Supplementary Figure S4. Model summarizing the activities of HRE2 and ethylene in adventitious root development.**

HRE2 is induced at hypoxic conditions and promotes, both, initiation and elongation growth of adventitious roots whereas ethylene inhibits AR formation. Hypoxic wild-type seedlings display an intermediary response where neither the promotive effect of HRE2 nor the inhibitory effect of ethylene are saturated.

## 10 Conclusive discussion and perspectives

### 10.1 Changes in root system architecture upon hypoxia

Increased demand on crop production together with the extreme weather conditions force farmers to grow plants under suboptimal conditions. To improve crop productivity, plants need to be adapted to abiotic stress. To be able to do that, we first need to understand the adaptive mechanisms that exist in plants to cope with particular stress. In general, roots play a crucial role for plant growth and productivity (Smith and De Smet, 2012). Root plasticity allows plants to modify the spatial arrangement of the root system by altering the number, length, position and the angle of primary and lateral roots in response to environmental factors to better tolerate changes in the environment (Koevoets *et al.*, 2016). Soil water logging and flooding limit oxygen availability in roots resulting in shortage of oxygenic ATP synthesis in mitochondria which reduces plant vigor and growth (Vashisht *et al.*, 2011; Bailey-Serres and Colmer, 2014). The studies described in this thesis focused on the effect of low oxygen stress on root systems architecture (RSA) and the underlying molecular mechanism that control growth and development of primary, lateral and adventitious roots. Our studies revealed morphological changes of the whole root system of *Arabidopsis* upon oxygen deprivation while previous studies were limited to describe inhibition of root growth upon waterlogging based on the fresh weight of alfalfa root (Castonguay *et al.*, 1993), several wheat genotypes (Huang *et al.*, 1994), reduced primary root elongation rate of *Arabidopsis* (van Dongen *et al.*, 2009) and enhanced adventitious root growth in partially submerged tomato (Vidoz *et al.*, 2010) and rice (Lorbiecke and Sauter, 1999). The reduction in root growth was seen as the consequence of an energy-saving adaptation (van Dongen *et al.*, 2009). This view is in contrast to our finding that the reshaping of the RSA is not only caused by energy limitations but is as well the result of controlled developmental reprogramming. The results suggest that plants integrate metabolic changes with a regulated morphological response to optimize adaptation to low oxygen conditions.

Chapter 1 describes that the primary root shows an agravitropic phenotype at hypoxic conditions with primary root bending. A similar bending phenotype has been observed in the hydrotropic response where roots grow towards the water source

(Takahashi *et al.*, 2002) or during salinity stress as halotropism response that serves to avoid salt exposure (Sun *et al.*, 2008). As a low oxygen response, bending of the primary root might help to escape soil areas with reduced oxygen levels.

Hypoxia also causes the arrest of LR growth in the post-emergence stage as an early ( $\leq 2$  days) response. When prolonged hypoxia ( $>2-4$  days) occurs, lateral root elongation recovers. LR density under hypoxic conditions is comparable with LR density at normoxic conditions indicating that the decreased total number of LRs is the result of primary root growth inhibition that has been reported previously (van Dongen *et al.*, 2009).

Nutrient limitation, drought and salinity stress have been shown to alter RSA (Koevoets *et al.*, 2016). When phosphates accumulate close to the soil surface, the root system is restructured with reduced primary root growth and enhanced LR growth which allows the plant to explore the topsoil area (López-Bucio *et al.*, 2002). Nitrogen as a highly mobile element in the soil tends to accumulate in deeper soil layers. To maintain nitrogen uptake, plants induce primary and lateral root elongation during nitrogen deprivation (Linkohr *et al.*, 2002). Drought causes decreased soil osmotic potential and thus negatively affects plant performance via insufficient water uptake. To reach deep soil water patches, plants enhance primary root growth and strongly inhibit LR emergence and elongation as an adaptive response to drought stress (Xiong *et al.*, 2006). Salinity stress can occur together with flooding stress due to the fact that water can contain NaCl from sea water (Wassmann *et al.*, 2004). Interestingly, among abiotic stresses, salinity and low oxygen conditions cause a highly similar response in *Arabidopsis*. Both, primary and lateral roots are inhibited by salt stress. Furthermore, salt stress causes a temporary arrest in LR emergence due to reduced cell division activity (West *et al.*, 2004) which is also supported by repressed *CyclinB1;1::GUS* expression in root tips after 8 and 24 h of hypoxia indicative of reduced mitotic activity. However, a comparative transcriptome analysis of low oxygen-induced transcript changes did not show a high overlap between drought and salinity stress in *Arabidopsis* indicating different pathways regulating similar root responses (Narsai and Whelan, 2013). Nonetheless, waterlogging of roots alters expression of ABA-related genes that are likewise associated with drought and salt responses (Hsu *et al.*, 2011).

Early phenotypic changes in the root system in response to oxygen deprivation were also observed under salt stress (Duan *et al.*, 2013). When *Arabidopsis* plants were



exposed to salt stress, the repression of primary root growth took approximately 8 h, and the arrest of LR growth was obvious within 2 days (Duan *et al.*, 2013). During hypoxia exposure, redirection of primary root growth occurs approximately after 8 h and its underlying molecular regulation is obvious approximately 3 h after the onset of hypoxia. The LR growth reduction at hypoxic conditions was phenotypically significant compared with normoxia after 2 days, and the inhibition of cell division was observed 8 h after the onset of hypoxia. These changes follow metabolic changes upon hypoxia indicated by upregulation of genes involved in fermentation and starch mobilization starting 1 h after onset of hypoxia (Licausi *et al.*, 2010; Kosmacz *et al.*, 2015) as part of an early response whereas the expression of these fermentative genes are reduced after 8 h of waterlogging (Hsu *et al.*, 2011). It appears that roots undergo an acclimation phase as an early response to hypoxia to readjusts the balance between energy production and consumption, so that roots are able to resume growth at a rate that is adjusted to energy availability. This conclusion is supported by the observations that the primary root starts to grow downward after 1 day and emerged lateral roots elongate after 2 days.

In *Arabidopsis* seedlings, adventitious roots are induced at the hypocotyl by prolonged darkness but not by hypoxia whereas hypoxia enhanced elongation of adventitious roots in the dark. Longer adventitious roots might compensate for the impaired growth of LRs resulting in a root system that is closer to the aerated surface thereby facilitating oxygen exchange. In wetland species such as rice and marsh dock, adventitious root emergence has a positive impact on flooding survival (Visser *et al.*, 1996; Sasidharan and Voesenek, 2015) due to the porous aerenchyma in the adventitious root that facilitates gas exchange (Yamauchi *et al.*, 2017). In *Arabidopsis*, hyponastic growth (Pierik *et al.*, 2005) but not aerenchyma formation in roots (Evans, 2004) contributes to better survival. Furthermore, a negative correlation was shown between submergence tolerance and underwater petiole elongation in 86 accession of *Arabidopsis* (Vashisht *et al.*, 2011) most likely due to the high energy investment in elongation growth. Further studies are necessary to link adventitious root elongation to improved stress tolerance in *Arabidopsis*.

In conclusion, to improve plant survival of hypoxic stress, adaptive traits need to be identified and characterized. In *Arabidopsis*, RSA is altered with specific traits in response to different abiotic stresses. This study shows that the root system adjusts to hypoxic

stress by primary root bending, reduced meristematic activity and transient arrest of lateral roots. Finally, hypoxia promotes adventitious root elongation in the dark. These changes in may be adaptive traits for improved survival of *Arabidopsis* seedlings and possible of dicots in general, but this has yet to be verified. The results described here allow to compare responses to various abiotic stresses and thus to reveal unique and overlapping regulatory pathways. Understanding how plants adapt to multiple stresses will be helpful for targeted crop improvement with plants that can cope better with weather extremes.

## 10.2 Involvement of ERFVIIIs in shaping root system architecture

The developmental reprogramming of the root system in response to oxygen deprivation is controlled by ERFVII transcription factors. Hypoxia-induced root bending is antagonistically regulated by ERFVIIIs, while hypoxia-induced adventitious and lateral root elongation is promoted by ERFVIIIs.

Repression of primary root bending is controlled by RAP2.12. The expression of *RAP2.12* is highest in the cortex cells, stele and epidermis at normoxia (Bui *et al.*, 2015), *HRE1* shows patchy expression in the cortex and epidermis while *HRE2* is specifically induced in the root stele by hypoxia. Asymmetric basipetal auxin transport mediated by PIN2 in the epidermis and lateral root cap is crucial for differential cell elongation that results in root bending (Blilou *et al.*, 2005; Lewis *et al.*, 2007). The expression pattern of *HRE1* and *HRE2* explains why these ERFVIIIs do not play a role in primary root bending while expression of RAP2.12 is compatible with altering root growth direction via altering PIN2 abundance. RAP2.3 shows low expression in the primary root tip even at hypoxic conditions (Licausi *et al.*, 2010; Bui *et al.*, 2015) in accord with the non-significant role in slanting.

LR growth is promoted by ERFVIIIs under hypoxic conditions through the regulation of ABA degradation via ABA 8'-hydroxylases. RAP2.3, RAP2.12 and HRE2 show a redundant role in regulating the expression of *ABA 8'-hydroxylase 1* during LR growth under hypoxic conditions possibly to fine tune the broad function of ABA that also regulates additional abiotic stress responses (Duan *et al.*, 2013; Xu *et al.*, 2013; Janiak *et al.*, 2015; Ondzighi-Assoume *et al.*, 2016). Moreover, Gasch *et al.* (2015) showed a redundant role of RAP2.2, RAP2.3, and RAP2.12 in activating the expression of

developmental and metabolic genes such as *LBD41* and *ADH1* at low oxygen conditions (Pucciariello *et al.*, 2012). This study showed a role of HRE2 in regulating *ABA 8'-hydroxylase 1*, although transactivation studies are necessary to verify the direct binding of ERFVIIIs to the promoter of *ABA 8'-hydroxylase 1*.

The analysis of *erfVII* pentuple knock out seedlings revealed a hypersensitive phenotype at hypoxic conditions with regard to primary root slanting and LR growth revealing an ERFVII-independent oxygen-sensing mechanism. Mitochondrial dysfunction could function as an ERFVII-independent sensor for low oxygen because mitochondria are among the first organelles affected by low oxygen stress due to the uncoupled oxidative phosphorylation. The lack of oxygen as an electron acceptor generates an imbalanced electron flux and causes reactive oxygen species (ROS) accumulation in the intermembrane and matrix spaces. Additional ROS accumulate during hypoxia due to the plasma membrane-integrated NADH oxidases which possibly can serve as a signal for transcriptional changes (Wang *et al.*, 2017; Pucciariello and Perata, 2017). Hence, mitochondrial ROS stimulate plasma membrane-generated ROS accumulation known as ROS-induced ROS-release (RIRR) (Zinkevich and Gutterman 2011; Pucciariello and Perata, 2017). Extracellular H<sub>2</sub>O<sub>2</sub> can actively be transported back to the cytosol through aquaporins (Pucciariello and Perata, 2017) or take part of cell-to-cell systemic signaling (Gilroy *et al.*, 2014). Not only molecular evidence but also physiological observations support the involvement of ROS in low oxygen signaling as shown by a positive effect of H<sub>2</sub>O<sub>2</sub> on anoxia tolerance (Banti *et al.*, 2010). Mitochondria are the main site for ROS production in non-photosynthetic tissues (Petrov *et al.*, 2015) beside NADPH oxidases RbohD and RbohF. However, additional cross-talk is likely necessary to generate a hypoxia-specific signal that is distinct from osmotic and salt stress signaling via ROS (Ben Rejeb *et al.*, 2015).

Today, no evidence exists that indicates an ERFVII-independent regulation of mitochondria-specific enzyme activity by oxygen availability in Arabidopsis. Therefore more explorative approaches are necessary to identify the ERFVII-independent O<sub>2</sub> sensing mechanism.

### 10.3 Hypoxia alters primary root bending and lateral root growth by altering hormonal responses

#### 10.3.1 Hypoxia-induced primary root bending is mediated by auxin

Hypoxia triggers an auxin response in the root tip resulting in bending of the primary root possibly to avoid local oxygen deprivation in the soil. We could correlate the hypoxia-induced bending with spatiotemporal changes in auxin distribution and auxin accumulation in the root tip by using auxin reporters DII-VENUS and DR5:GUS. A gravitropic response is established and maintained in the root tip by generating an auxin gradient mediated mainly by controlled PIN2 relocalization and degradation (Band *et al.*, 2012; Brunoud *et al.*, 2012; Baster *et al.*, 2013). Interestingly, a recent study showed agravitropic primary root growth as a hydrotropic response without auxin redistribution during root bending (Shkolnik *et al.*, 2016) in accord with our results that hypoxia alters root bending independently of auxin asymmetry as well. RAP2.12 represses hypoxia-induced root bending. RAP2.12 positively regulates the protein abundance, but not the transcripts of PIN2 indicating a sufficient auxin flux from the root apex to the elongation zone as counteracting with the auxin accumulation observed in the root tip of hypoxic seedlings. Complex regulation of PIN2 dynamics includes gene expression of *PIN2* (Sun *et al.*, 2009; Willige *et al.*, 2011), posttranslational modification by PIN2 phosphorylation to determine polarity of the protein through PINOID (PID) kinase with the antagonistic action of protein phosphatase 2A (PP2A) (Friml *et al.*, 2004; Michniewicz *et al.*, 2007), clathrin-mediated intracellular endocytosis for recycling of PIN2 protein (Dhonukshe *et al.*, 2007; Kitakura *et al.*, 2011) and altered abundance of PIN2 through targeted protein degradation in the vacuol (Kleine-Vehn *et al.*, 2008; Baster *et al.*, 2013).

Other abiotic stresses also regulate PIN2. Salinity induces agravitropic root growth directing the primary root away from a high salt environment via asymmetric auxin distribution but not abundance via clathrin-mediated endocytosis of PIN2 protein (Sun *et al.*, 2008; Galvan-Ampudia *et al.*, 2013). Furthermore, cold stress reduces PIN2 protein levels in response to gravity stimuli but not the polar localization of PIN2 (Shibasaki *et al.*, 2009). Reduced PIN2 abundance in response to hypoxia suggests that protein degradation is induced. However, it does not exclude the possibility that hypoxia alters PIN2 recycling since we only examined plasma membrane-localized PIN2 abundance.

### 10.3.2 Hypoxia induces ABA catabolism to promote LR growth

Auxin plays a main regulatory role in LR initiation and formation, whereas ABA signaling controls LR emergence via an auxin-independent manner (De Smet *et al.*, 2003). Under hypoxia, auxin increased but not fully restored the number of initiated LRs compared to normoxic conditions indicating some energy-restricted growth. Auxin-induced LRs were transiently arrested at the post-emergence stage during hypoxia. Since ABA is known to inhibit post-emergence LR growth (De Smet *et al.*, 2003), we hypothesized that ABA might play a role in transient hypoxia-induced LR growth arrest. In contrast to our hypothesis, the ABA level was lower during growth arrest at 8 h and 24 h of hypoxia compared to normoxic roots. It has been shown that waterlogging reduced the ABA content in *Arabidopsis* roots within 3 h and up to 12 h (Hsu *et al.*, 2011), but increased it in pea roots within 2 d. Interestingly, the ABA content did not change in waterlogged compared to aerated roots of alfalfa (Castonguay *et al.*, 1993) suggesting a species-specific response to flooding regarding ABA accumulation. It is tempting to speculate that flooding-tolerant species respond with a decline in ABA level as observed in roots of marsh dock and shoots of rice (Benschop *et al.*, 2005; Saika *et al.*, 2007; Khan *et al.*, 2012).

In *Arabidopsis*, to promote LR growth, hypoxia signaling triggers ABA degradation by inducing the expression of *CYP707A1* and *3* in an ERFVII-dependent manner. Nonetheless, LR growth is reduced due to reduced cell division activity indicated by lower expression of *CYCB1;1*, a key regulator of the G2/M phase transition (Xu *et al.*, 2016) possibly due to limited ATP production from decreased respiration (Zabalza *et al.*, 2009). In mammals, the G2/M phase transition is the most energy-demanding phase during the cell cycle. Recently, Wang *et al.* (2014) showed a relocation of the cyclin B1/Cdk1 complex in mammals to the mitochondrial matrix where it was shown to phosphorylate complex I of the mitochondrial electron transport chain resulting in enhanced mitochondrial respiration with higher ATP production. This regulation coordinates energy supply and mitotic activity. The function of cyclin B1;1/Cdk1 complex in mammals might be conserved in plants to maintain energy supply. In plants, ABA inhibits *CYCB1;1* expression (Xu *et al.*, 2016) (Supplementary Figure S3). In hypoxic LRs *CYCB1;1* is less repressed compared with ABA-treated LRs at normoxic conditions. These findings indicate that both,

a root growth limiting and a growth-promoting mechanism are activated under prolonged hypoxic conditions. To achieve a balance between these mechanisms, LR growth seems to go through an acclimation phase with metabolic adjustment in the early hypoxia response (Hsu *et al.*, 2011). After stabilizing energy consumption and production, LR growth accelerates as part of a recovery phase. It has been reported that a low concentration of ABA promotes root growth by maintaining the root apical meristem (Zhang *et al.*, 2010).

In addition to the cell division activity in the meristematic region of the root, it would be interesting to look into the regulation of the stem cell niche hypoxia as a future aspect. It has been shown that ABA maintains quiescence of the quiescent center and suppresses stem cell differentiation through upregulation of WUSCHEL-RELATED HOMEODOMAIN 5 (WOX5) (Zhang *et al.*, 2010; Forzani *et al.*, 2014). Therefore, hypoxia might promote the activity of the stem cell niche by reducing ABA to maintain root growth rate despite of limited energy supply.

ABA, together with ROS, negatively alters the maintenance of stem cell niche (Tripathy and Oelmüller, 2012). Therefore ABA, as well as H<sub>2</sub>O<sub>2</sub>, reduce primary root growth in Arabidopsis (Bai *et al.*, 2007). In the primary root tip, ABA, through elevated mitochondrial ROS production, negatively controls meristematic activity by inhibiting PLETHORA1 (PLT1) and consequently primary root growth (Yang *et al.*, 2014). In LR development RBOH-mediated ROS accumulation in overlying endodermis cells of the emerging primordia that promotes LR emergence through cell wall remodeling (Orman-Ligeza *et al.*, 2016), but information on the effect of ROS in the meristem is yet missing. ABA degradation might help to reduce ROS accumulation and hence maintain stem cell activity.

In summary, hypoxia-induced LR growth arrest at the post-emergence phase may be the result of an energy crisis. The coincident ABA degradation controlled by hypoxia-induced ERFVIs helps resume root growth. Both, primary root slanting and LR growth are antagonistically regulated by hypoxia and ERFVIs resulting in balanced responses. Residual LR growth most likely helps to improve nutrient uptake during oxygen deprivation.

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## 12 Declaration of author contributions

I declare that the thesis “ETHYLENE RESPONSE FACTOR VII transcription factors control the reshaping of the root system architecture in response to hypoxia” has been written by myself with the guidance of my supervisor Prof. Dr. Margret Sauter. This work has not be submitted for any other degree. Additional collaborative contributions have been indicated clearly in the text.

I confirm that the work submitted is my own, except part of the jointly authored publication. My and the other author contribution to this work have been indicated below.

The work presented in Chapter 1 was previously published in Plant Physiology journal cited as follows:

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## 14 Curriculum Vitae

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**Chapter 1**

MS conceived the project and supervised the experiments; EED and MS designed the experiments; EED performed and analyzed the experiments; and MS wrote the manuscript with contributions from EED.

**Chapter 2**

MS and EED conceived the project and designed experiments, EED performed and analyzed experiments, BH performed the LC-MC analysis. MS and EED wrote the manuscript with contributions from BH.

**Chapter 3**

MS conceived the project; EED and MS designed experiments, EED performed and analyzed experiments, EED and MS wrote the manuscript.