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Zeguang Liu

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Ethylene-mediated hypoxia tolerance in *Arabidopsis thaliana*

Ethyleen verhoogt tolerantie van *Arabidopsis thaliana* voor lage zuurstof condities

(met een samenvatting in het Nederlands)

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Zeguang Liu

geboren op 11 november 1988
te Hebei, China

Promotor:

Prof. dr. L.A.C.J. Voesenek

Copromotor:

Dr. R. Sasidharan

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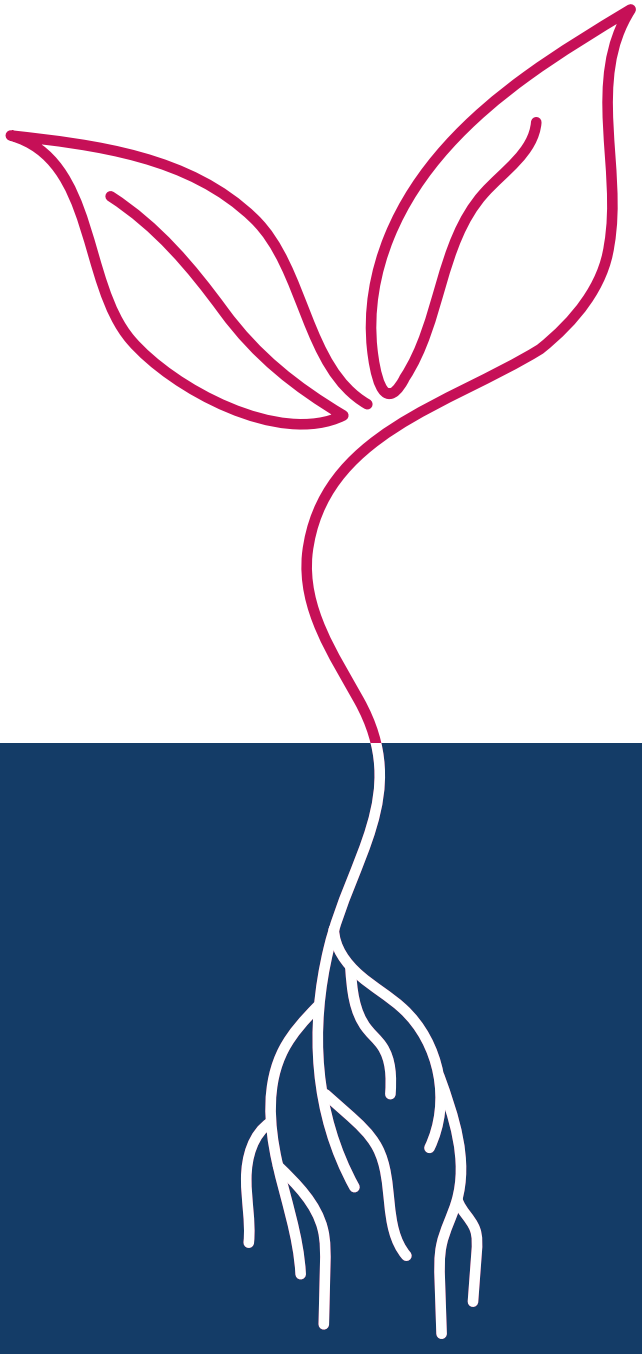
Contents



CHAPTER 1

General introduction

Flooding antagonizes global crop production

Due to global warming, the frequency and severity of extreme climate events such as flooding is expected to increase in the future (Arnell and Liu, 2001; Cassia *et al.*, 2018; Hirabayashi *et al.*, 2013). This could be detrimental to many crop species and thereby greatly restrict crop productivity and global food security (Bailey-Serres *et al.*, 2012a). Plants suffer from flooding stress from the consequences of the 10^4 times slower gas exchange rate underwater compared to the atmosphere (Jackson, 1985). The hampered gas exchange underwater markedly reduces available carbon dioxide (CO_2) and oxygen (O_2), that plants use for photosynthesis and respiration, respectively. Meanwhile, variable light availability further influences the light energy-driven carbon fixation through photosynthesis (Voesenek and Sasidharan, 2013). As a result, a carbohydrate and energy crisis will occur in flooded plants and they will eventually fail to survive (Bailey-Serres and Voesenek, 2008; Licausi and Perata, 2009; Sasidharan *et al.*, 2018).

Flooding can refer to different types of water excess including waterlogging, when only roots of the plant are underwater, and partial or complete submergence, when parts or the entire shoot of a plant is inundated (Colmer and Voesenek, 2009; Sasidharan *et al.*, 2018). During waterlogging, roots have to grow in soil in which gas spaces have been completely replaced by water. Additionally, potentially toxic compounds also develop in waterlogged soil (Ponnamperuma, 1984). Even though not directly flooded, shoots of waterlogged plants are also affected, and leaves can show wilting due to a decrease in hydraulic conductivity (Holbrook and Zwieniecki, 2003). Submergence, particularly when the entire shoot is also completely underwater, directly constrains photosynthesis due to reduced CO_2 influx and low light level in turbid waters.

Plant performance when flooded greatly depends on many aspects of the flooding conditions such as water depth, temperature and clarity of flood water. For instance, light availability differs notably in muddy and static water due to different penetration rates (Mommer and Visser, 2005). Light intensity also decreases gradually with increasing water depth. Light availability therefore becomes a critical factor resulting in different tolerance levels under light and dark submergence depending on whether underwater photosynthesis is possible (Mommer and Visser, 2005; Pedersen *et al.*, 2013). Apart from depth, temperature and clarity, the duration of floods also influences plant survival. Two typical survival responses, based on shoot growth regulation, escape and quiescence, are employed by many terrestrial plant species depending on flooding conditions like depth and

duration (Colmer and Voesenek, 2009). The escape strategy, exemplified in species such as deepwater rice and *Rumex palustris*, involves an acceleration of shoot elongation to escape flood waters. The snorkeling leaves that emerge above the water surface can then re-aerate the plants via tissues rich in gas spaces (aerenchyma) (Benschop *et al.*, 2005; Hattori *et al.*, 2009; Kuroha *et al.*, 2018; van Veen *et al.*, 2013). This strategy is only beneficial if the floods are not too deep since growth is energetically expensive. The quiescence strategy is employed during long term or deep floods where growth and energy consuming processes are limited to conserve reserves. This ensures faster regrowth and recovery when flood waters recede (Fukao and Bailey-Serres, 2008; van Veen *et al.*, 2013; Xu *et al.*, 2006).

After flood waters recede, plants have to cope with yet another set of challenges during the post-submergence period (Tamang and Fukao, 2015). When O_2 is available again upon de-submergence, tissue damage that already occurred during flooding could be further exacerbated due to re-oxygenation stress (Crawford, 1992; Crawford *et al.*, 1994; Vantoai and Bolles, 1991; Yeung *et al.*, 2018). Therefore, flooding tolerance is not only tolerance to the flooding event itself, but includes also the re-oxygenation period following flooding.

Ethylene accumulation and signaling under flooding

As stated above, the underwater environment severely constrains gas exchange. The volatile hormone ethylene is synthesized in all plant cells, except in the cells of a few aquatic species (Voesenek *et al.*, 2015). Additionally, flooding induces the enhanced expression and activity of ethylene biosynthesis rate limiting enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and ACC synthase (ACS) in several plant species (Lee *et al.*, 2011; Van Der Straeten *et al.*, 2001; van Veen *et al.*, 2013). ACS catalyzes the production of ACC from S-adenosyl-L-Methionine. ACC is then oxidized by ACC oxidase (ACO) to ethylene (Yang and Hoffman, 1984). Both ACS and ACO are from large multigene families that are modulated by various external and internal stimuli to affect ethylene biosynthesis (Johnson and Ecker, 1998). Normally, the produced ethylene is vented out of the plant via the stomata. Due to the severely reduced rate of gas exchange during flooding this diffusion process is strongly inhibited. It has been shown that ethylene concentrations rise to elevated levels in flooded shoots and roots of many plant species (Voesenek and Sasidharan, 2013). Within 1 h, ethylene levels could reach 1 $\mu L \cdot L^{-1}$, which is 20-fold higher than non-flooded organs and saturating for many physiological processes like enhanced shoot elongation in *Regnillidium diphyllum* and *Rumex palustris* (Musgrave and Waiters, 1974; Voesenek and Sasidharan, 2013; Voesenek *et al.*, 1997). However, ethylene levels in roots can be variable depending on the species and

their natural habitats. Roots of wetland species accumulate relatively little ethylene due to ventilation of ethylene via aerenchymatous tissue to the atmosphere. Roots of non-wetland species, on the other hand, accumulate more ethylene since they have a low porosity (Visser *et al*., 1997; Visser and Pierik, 2007).

In *Arabidopsis* the ethylene signaling pathway is deeply studied and described (Ju and Chang, 2015; Stepanova and Alonso, 2005). Endogenous ethylene represses the function of the five ethylene receptor genes, *ETHYLENE RESPONSE 1* (*ETR1*), *ETHYLENE RESPONSE SENSOR 1* (*ERS1*), *ETR2*, *ETHYLENE INSENSITIVE 4* (*EIN4*) and *ERS2*, which otherwise activate the negative regulator of ethylene response *CONSTITUTIVE RESPONSE 1* (*CTR1*). Thereafter, rather than being phosphorylated in a CTR1-dependent manner, the positive regulator ETHYLENE INSENSITIVE 2 (EIN2) translocates from endoplasmic reticulum to the nucleus and activates the key transcription factors (TFs) EIN3 and EIN3 LIKE 1 (EIL1), thereby initiating downstream primary and secondary responses (Ju and Chang, 2015). EIN3 and EIL1 stabilization is modulated by two EIN3 BINDING F BOX proteins, EBF1 and EBF2, through ubiquitin-mediated turnover, which appears to be blocked by ethylene (Binder *et al*., 2007).

As an important flooding signal, ethylene has been shown to regulate many flood-adaptive traits (Sasidharan and Voesenek, 2015; Voesenek and Bailey-serres, 2015). For example, aerenchyma formation and adventitious root development during flooding were reported to be regulated by ethylene together with many other factors such as auxin and reactive oxygen species (ROS) (Evans, 2004; Rajhi *et al*., 2011; Steffens and Sauter, 2009; Tavares *et al*., 2018; Vidoz *et al*., 2010; Visser *et al*., 1996). The well-established "escape" and "quiescence" strategy deployed by several plant varieties and species were also shown to be differentially regulated by ethylene (Fukao and Bailey-Serres, 2008; Hattori *et al*., 2009; Kuroha *et al*., 2018; Xu *et al*., 2006; Yin *et al*., 2017). Ethylene initiates submergence-induced shoot elongation of deepwater rice, by activating the ethylene response factor (ERF) TF genes *SNORKEL1* (*SK1*) and *SK2*, facilitating successful escape from flood water. On the other hand, it also represses shoot elongation in lowland rice, via activation of another ERF gene *SUBMERGENCE 1A* (*SUB1A*), to ultimately conserve energy until the flood waters recede.

Hypoxia sensing and adaptation

Low O_2 (hypoxia) due to reduced gas exchange directly affects the energy status of flooded plants (Bailey-Serres and Chang, 2005). Indeed, hypoxia and flooding share strong transcriptome similarities in plants (Bailey-Serres *et al*., 2012b). However, hypoxia severity

greatly depends on not only flooding conditions like light availability, but also tissues. Shoots and roots can differ strongly in O_2 levels during flooding (Sasidharan and Voesenek, 2015). Roots can more easily suffer from hypoxia since flooded soils are rapidly depleted of O_2 due to microbial and root respiration (Vashisht *et al.*, 2011). Roots are strongly dependent on shoot derived O_2 and therefore O_2 status in roots is also largely influenced by potential shoot-to-root transport. O_2 levels of submerged shoots however, is determined by many more factors. Light availability allows photosynthetic O_2 production in green tissue underwater. In some species, O_2 status is enhanced due to certain adaptive traits like thinner leaf cuticle/cell wall and leaf gas films. These traits facilitate better gas exchange and enhance underwater photosynthesis (Mommer *et al.*, 2004; Mommer and Visser, 2005; Pedersen *et al.*, 2009). Studies have shown that O_2 levels of submerged shoots stayed around 21% (normoxia) when light was available (van Veen *et al.*, 2013; Vashisht *et al.*, 2011).

O_2 sensing in plants, is mediated by the N-end rule pathway of protein degradation (Gibbs *et al.*, 2011, 2015; Kosmacz *et al.*, 2015; Licausi *et al.*, 2011). The N-end rule pathway determines the stability of proteins with a conserved sequence of amino acids at the N-terminus (Graciet and Wellmer, 2010). There are two well studied branches of the N-end rule pathway: the Ac/N-end rule and Arg/N-end rule, which drive acetylated (Ac) and unacetylated N-terminal residues, respectively (Gibbs *et al.*, 2014a; Hwang *et al.*, 2010; Tasaki *et al.*, 2012; Varshavsky 2011). In the Arg/N-end rule pathway, following cleavage of the N-terminal amino acid methionine (Met) by Methionine amino peptidases (MAPs), exposure of the second residue cysteine (Cys) makes it eligible for oxidation by plant cysteine oxidases (PCOs). After Cys oxidation, the N-terminus will react with an arginine residue via arginyl tRNA transferases (ATEs) before being recognized by an E3 ligase like PROTELYSIS 6 (PRT6) leading to ubiquitin-driven degradation of the protein (Graciet and Wellmer, 2010). The oxidation of the Cys residue requires atmospheric O_2 and as shown recently also nitric oxide availability (Gibbs *et al.*, 2014b).

The five group VII ERFs proteins (ERFVIIs) in *Arabidopsis*, possess a conserved N-terminus and are subject to the Arg/N-end rule -driven proteolysis. Those ERFVIIs, including HYPOXIA RESPONSE ERF 1 (HRE1), HRE2, RELATED TO AP2 2 (RAP2.2), RAP2.12 and RAP2.3, belong to APETALA2-ETHYLENE RESPONSIVE ELEMENT BINDING PROTEINS (AP2-EREBP) TF family (Nakano *et al.*, 2006). Functional studies have shown a major role of these ERFVIIs in regulating hypoxia responses (Gasch *et al.*, 2015; Gibbs *et al.*, 2011; Hinz *et al.*, 2010; Kosmacz *et al.*, 2015; Licausi *et al.*, 2010, 2011). During normoxic conditions the degradation of these TFs via the N-end rule ensures a block on hypoxia responses. When hypoxic conditions

develop, ERFVIIs can be stabilized and subsequently translocate to the nucleus to initiate transcription of hypoxia responsive genes, such as *ALCOHOL DEHYDROGENASE 1* (*ADH1*) and *SUCROSE SYNTHASE 4* (*SUS4*). For example, RAP2.2 and RAP2.12 were shown to bind directly to the promoter regions of hypoxia responsive genes in *Arabidopsis* (Gasch *et al.*, 2015). The existence of hypoxia responsive gene expression in single or double mutants of ERFVIIs suggests functional redundancy among the ERFVIIs (Hinz *et al.*, 2010; Licausi *et al.*, 2010). Recently published work suggests that the constitutively expressed RAP2.2 and RAP2.12 are primary activators of core hypoxia gene expression (Gasch *et al.*, 2015).

Ethylene and hypoxia interaction

Both ethylene and hypoxia act as stress signals that trigger downstream adaptations during flooding. In fact, ethylene and hypoxia signaling interact with each other in many ways. Flooding-induced ethylene accumulation leads to multiple hypoxia responses like adventitious root emergence and aerenchyma formation (Fukao and Bailey-Serres, 2008; Rajhi *et al.*, 2011; Vidoz *et al.*, 2010; Visser *et al.*, 1996; Yamauchi *et al.*, 2014). The interaction of ethylene and hypoxia quite often involves many other players, such as multiple hormones and ROS. Adventitious root development in flooded *Rumex* and tomato plants require ethylene and auxin interplay (Vidoz *et al.*, 2010; Visser *et al.*, 1996). Hypoxia inducible *HRE1* and *ADH1* transcription was suggested to be influenced by hydrogen peroxide via interaction with ethylene (Yang, 2014).

The key regulators of flooding stress, ERFVIIs have been shown to be regulated by both ethylene and hypoxia signals. *HRE1*, being inducible by hypoxia stress, was also up-regulated by addition of the ethylene precursor ACC (Yang *et al.*, 2011). Transcript levels of both *RAP2.2* and *RAP2.3* could be enhanced by ethylene as well (Buttner and Singh, 1997; Hinz *et al.*, 2010). Similarly, ERFVIIs in rice, SUB1A and SKs (SK1 and SK2), are also regulated by ethylene to facilitate downstream flooding adaptations (Fukao and Bailey-Serres, 2008; Hattori *et al.*, 2009; Kuroha *et al.*, 2018; Xu *et al.*, 2006; Yin *et al.*, 2017). However, studies on N-terminal features of both SUB1A and SKs revealed a mismatch with the conserved ERFVIIs N-terminal residues in *Arabidopsis* (Bailey-Serres *et al.*, 2012b; Gibbs *et al.*, 2011). These imply that likely not all ERFVIIs are subject to the N-end rule -driven protein destruction, which indeed was proved by the *in vitro* study on SUB1A (Bailey-Serres *et al.*, 2012b; Gibbs *et al.*, 2011).

As stated earlier, O_2 levels in submerged plants vary greatly depending on not only flooding conditions, but also the types of tissues and organs involved. Ethylene, on the other hand,

invariably accumulates to high concentrations since it is not metabolized in the plant (Sasidharan and Voesenek, 2015; Voesenek and Sasidharan, 2013). This makes ethylene an early and consistent flooding signal due to its pervasiveness and reliability (Sasidharan *et al.*, 2018). Indeed, a study (van Veen *et al.*, 2013) on two *Rumex* species (*Rumex palustris* and *Rumex acetosa*) found that under light submergence, O_2 levels in petioles of both species did not decline below 17%. However, several hypoxia responsive gene orthologs displayed enhanced transcript abundance in *R. palustris*, irrespective of the high O_2 levels. Since ethylene entrapment occurs inevitably under submergence, it was therefore proposed that accumulated ethylene might have mediated increased expression of those hypoxia responsive genes. Interestingly, *R. palustris* survival was significantly improved under hypoxia if an ethylene pre-treatment preceded hypoxia, while *R. acetosa* did not benefit from the early ethylene exposure. In accordance with survival, none of the tested hypoxia responsive genes were enhanced by ethylene pre-treatment in *R. acetosa*. In contrast, 6 out of 8 genes tested in *R. palustris* were significantly up-regulated by ethylene pre-treatment. This study suggested that the fast accumulation of ethylene, at least in some plant species, facilitates changes that prepare plants for upcoming hypoxia stress (van Veen *et al.*, 2013). But how ethylene could enable this improvement of hypoxia tolerance was not elucidated.

Thesis outline

As a next step, here we used the model plant *Arabidopsis thaliana* to uncover the molecular mechanism of ethylene-mediated enhancement of hypoxia tolerance. In **Chapter 2**, we tested the suitability of *Arabidopsis* to identify this mechanism. We investigated whether *Arabidopsis* is also able to benefit from the early ethylene pre-treatment for subsequent hypoxia stress using a root tip survival assay. With this assay, the levels and duration of ethylene pre-treatment needed for the improvement were studied. It was found that several variables influenced hypoxia survival such as light conditions, developmental stage of seedlings and re-oxygenation stress. These investigations of the impact of ethylene pre-treatment on hypoxia and post-hypoxia survival allowed us to develop a robust system to study the underlying molecular regulation.

In **Chapter 3**, a transcriptome analysis using microarrays was used to characterize ethylene-mediated hypoxia and re-oxygenation responses. Data analysis using hierarchical clustering and gene ontology enrichment approaches revealed the involvement of multiple hormones and growth regulatory processes, epigenetic modifications, and particularly oxidative stress responses upon re-oxygenation.

The results of Chapter 3, led to the hypothesis that ethylene facilitates changes allowing the induction of genes benefiting seedlings survival under re-oxygenation. To investigate this, in **Chapter 4**, we first confirmed the induction of oxidative stress related genes using qRT-PCR. Then we investigated the existence of ROS over-accumulation upon re-oxygenation and its negative impact on survival. Then ethylene pre-treatment was applied to study the effect of ethylene on ROS accumulation during re-oxygenation. Genetic and chemical manipulations of ROS production were used to show the beneficial effect of limiting ROS on hypoxia survival. Subsequently the potential involvement of N-end rule protein degradation in ROS regulation was investigated.

In chapter 2, an age-dependent ethylene-mediated hypoxia sensitivity was identified. The underlying molecular mechanism was further investigated by a transcriptome comparison between 4- and 7-day-old seedlings in **Chapter 5**. Similar bioinformatics tools as chapter 3 were used to discover biological processes and regulators involved in the age-specific responses. Transcriptome analysis suggested multiple age-specific responses including differences in redox balance maintenance, energy coordination, stress sensitivity and hormone regulation. Additionally, basal developmental differences of the two age groups were also found to partially contribute to the differential hypoxia sensitivity.

In **Chapter 6**, the findings of previous chapters are summarized and discussed. The findings in this thesis contribute to the knowledge of how hypoxia responses are regulated by ethylene. It confirmed the potential of ethylene function as an early signal to benefit hypoxia responses in certain plant species. The molecular mechanisms underlying this ethylene-mediated increase in hypoxia tolerance provide useful inputs for further studies towards ethylene-hypoxia interactions, hypoxia survival mechanisms and ultimately, the application of fundamental knowledge into improving stress resilience of agricultural crops.

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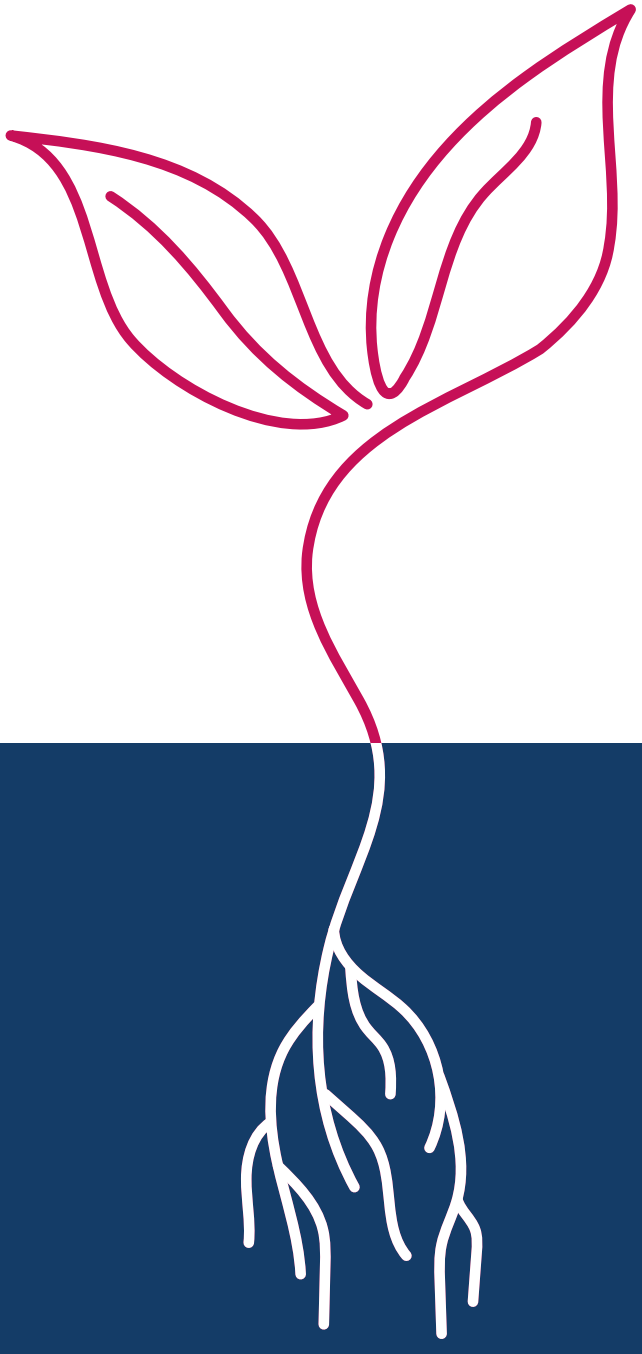


CHAPTER 2

Ethylene prolongs primary root tip hypoxia survival in *Arabidopsis thaliana*

Zeguang Liu
Shanice Martopawiro
Sjon Hartman
Laurentius ACJ Voesenek*
Rashmi Sasidharan*

*Shared senior authors

Plant Ecophysiology, Institute of Environmental Biology,
Utrecht University, Utrecht, The Netherlands

Abstract

When flooded, plants have to deal with the 10^4 times slower gas exchange rate underwater than in the air. The fast accumulation of the gaseous plant hormone ethylene within submerged plant tissues due to physical entrapment and the lack of metabolic degradation makes it a reliable and early flooding signal mediating adaptive responses to this stress in the plant kingdom. Pre-exposure of *Arabidopsis* seedlings to levels of ethylene that mimic underwater tissue concentrations improved root tip survival in subsequent hypoxic conditions. Furthermore, root tip hypoxia survival was influenced not only by light conditions and developmental stage of seedlings, but also by the ability to adapt to post-hypoxia conditions. Ethylene pre-treatment prevented cell death during both hypoxic and post-hypoxic conditions in root tips, suggesting that ethylene is important to facilitate changes to adapt to not only hypoxia, but also re-oxygenation.

Introduction

Flooding is one of the many abiotic stresses terrestrial plants can face worldwide. The severity and number of flooding events is reported to have increased during the last few decades (Hirabayashi *et al.*, 2013). Gas diffusion underwater is around 10^4 times lower than in the air, leading to greatly reduced concentrations of carbon dioxide (CO_2) and oxygen (O_2) in flooded plants (Colmer and Voesenek, 2009). Furthermore, light availability could be limited if the flood waters are muddy and turbid (Vervuren *et al.*, 2003). The limited availability of O_2, CO_2 and light reduces photosynthesis and the mitochondrial respiration rate in plant cells leading to carbohydrate starvation and an energy crisis (Perata and Alpi, 1993; Voesenek and Bailey-serres, 2015).

Plants utilize multiple signals to sense and adapt to conditions of flooding stress (Sasidharan *et al.*, 2018). O_2 availability in submerged plants is highly dependent on the flooding conditions and therefore highly variable. For instance, in clear water underwater photosynthesis is possible resulting in internal O_2 levels close to ambient. Complete lack of light, such as in muddy waters, hampers photosynthesis and results in much lower O_2 levels. Moreover, O_2 content also varies between different plant tissues (Sasidharan and Voesenek, 2015). Under light submergence, O_2 content in petioles of five *Arabidopsis* accessions tested were between 18.7 kPa and 25.8 kPa. On the other hand, that of roots were below 10 kPa. Similarly, O_2 concentration in petioles were significantly higher than in roots upon dark submergence (Vashisht *et al.*, 2011). Unlike O_2, ethylene levels are high and stable in cells of flooded organs due to physical entrapment underwater and absence of metabolism, respectively. This makes ethylene an early and reliable signal for flooding that operates upstream of many flood-adaptive processes (Sasidharan and Voesenek, 2015). The plant hormone ethylene has been shown to be crucial for plants in mediating growth, development and stress responses throughout their entire life cycle (Dubois *et al.*, 2018; Iqbal *et al.*, 2017; Schaller, 2012).

Ethylene regulates several signal transduction pathways leading to flood-adaptive traits. Adventitious roots emergence and aerenchyma formation are induced by ethylene in many plant species upon flooding (Sasidharan and Voesenek, 2015; Visser *et al.*, 1996; Voesenek and Bailey-serres, 2015). Both deepwater and lowland rice benefit from ethylene-mediated processes to survive floods (Fukao and Bailey-Serres, 2008; Hattori *et al.*, 2009; Kuroha *et al.*, 2018; Xu *et al.*, 2006; Yin *et al.*, 2017). Enhanced shoot elongation in deepwater rice facilitates escape from rising flood waters to re-establish aerial contact, while staying quiescent via shoot growth inhibition preserves energy for recovery until flood recedes.

Being an important component of flooding stress, low O_2 (hypoxia) shares strong similarities with flooding regarding transcriptomic responses in plants (Bailey-Serres *et al.*, 2012b). A major role of group VII Ethylene Response Factors (ERFVIIs) in regulating flooding and hypoxia tolerance has been described in recent years (Gasch *et al.*, 2015; Gibbs *et al.*, 2011; Hinz *et al.*, 2010; Kosmacz *et al.*, 2015; Licausi *et al.*, 2010, 2011). Possessing conserved N-terminal residues, ERFVIIs are subject to degradation via the N-end rule pathway dependent on O_2 and nitric oxide (Gibbs *et al.*, 2015). Thus these ERFVIIs transcription factors are destroyed under normoxia while constitutively stabilized under hypoxia and translocate to the nucleus to initiate transcription of core hypoxia responsive genes, which prolong stress survival. Many of these core hypoxia genes such as *ALCOHOL DEHYDROGENASE 1 (ADH1)* and *SUCROSE SYNTHASE 4 (SUS4)* are important for energy homeostasis under O_2 deprivation (Gasch *et al.*, 2015; Gibbs *et al.*, 2011; Licausi *et al.*, 2010, 2011).

A recent study of two *Rumex* species (*Rumex palustris* and *Rumex acetosa*) showing contrasting flooding survival strategies suggested a novel role for ethylene (van Veen *et al.*, 2013). When submerged in the light, the O_2 content within petioles was above 17% in both *Rumex* species. Despite this high O_2 content, several core hypoxia gene orthologs were up-regulated only in *R. palustris*. Based on this, it was hypothesized that in the submerged shoots of this species, early ethylene accumulation was responsible for the up-regulation of hypoxia response genes before the onset of hypoxia. In keeping with the hypothesis, when pre-treated by ethylene, *R. palustris* performed better upon hypoxia than those without an ethylene pre-treatment, while *R. acetosa* was not able to benefit from this ethylene pre-treatment. This work suggested that ethylene, at least in some plant species, enables plants to be prepared for subsequent O_2 crisis (van Veen *et al.*, 2013). However, the molecular mechanism underlying the improvement of this ethylene-mediated hypoxia tolerance is still unclear.

As a first step towards investigating the mechanisms underlying the beneficial effect of ethylene on hypoxia tolerance, we investigated whether ethylene can also improve hypoxia tolerance in *Arabidopsis thaliana*. Hypoxia tolerance was assessed using seedling root tip re-growth as a proxy. After establishing the effect of ethylene on hypoxia tolerance we studied the levels and duration of ethylene required for this effect. The timing and location of cell death was also investigated and indicated that post-hypoxia tolerance is an important aspect of hypoxia survival in *Arabidopsis* root tips. These experiments revealed that an ethylene pre-treatment confers enhanced tolerance to hypoxia in *Arabidopsis* seedlings.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana wild type Col-0 and mutants *ETHYLENE INSENSITIVE 2* (*EIN2*, *ein2-5*), *EIN3/EIN3-LIKE-1* (*EIN3/EIL1*, *ein3eil1*) and *EIN3-BINDING F BOX PROTEIN 1* (*EBF1*) and *EBF2* (*EBF1/EBF2*, *ebf1ebf2*) were sterilized for 3 h in a mixture of bleach (30 mL) and concentrated hydrochloric acid (1.5 mL, Sigma-Aldrich). In *ein2-5*, there is a 7-base-pair deletion at nt939-945, causing a frameshift mutation (Alonso *et al.*, 1999). For the *ein3eil1*, a G-to-A substitution exists at nucleotide 1598 in *ein3-1* and an En-I transposon insertion exists at nucleotide 697 in *eil1-1* (Alonso *et al.*, 2003). The mutations in *ebf1ebf2* are *ebf1-3* (SALK_020997) and *ebf2-3* (SALK_092571) (Binder *et al.*, 2007). Seeds were sown on sterile, square petri dishes (120×120×17 mm, Greiner Bio One) on 1% plant agar (25 mL, Duchefa Biochemie B.V., P1001) supplemented with ¼ Murashige & Skoog (MS) medium (Duchefa Biochemie B.V., M0245). Forty-six seeds were sown in two rows per plate (Figure 2.1A) and plates were sealed with Leucopore tape (12.5 mm, Duchefa Biochemie B.V., L3302) after sowing. Sown plates were kept at 4°C for 4 days in the dark for stratification. Seedlings were grown vertically in climate-controlled growth chambers with short day conditions (daytime: 9-17 h, light intensity: ~120 $\mu mol \cdot m^{-2} \cdot S^{-2}$, humidity: 70%, temperature: 21°C). For recovery after hypoxia, plates were kept in the dark and then moved back to the same short day growth conditions when the light was switched off in the climate chamber at the end of the day.

Ethylene treatment and measurement

For ethylene treatments, seedlings grown on square plates with lids removed were placed vertically into closed glass desiccators injected with specific ethylene concentrations (light intensity within desiccators: ~5 $\mu mol \cdot m^{-2} \cdot s^{-2}$, temperature: 21°C). Control seedlings were placed in glass desiccators with the lids removed for a similar duration. Gaseous ethylene was purchased condensed within a cylinder and diluted to designated concentration before injecting into glass desiccators with plastic syringes. After 30 minutes, gas samples taken from the glass desiccators were measured with gas chromatography (Syntech Spectras GC955) to confirm the concentration of ethylene applied during the treatment.

Hypoxia treatment

For hypoxia treatments, seedlings grown on square plates with lids removed were placed vertically into closed glass desiccators. Hypoxia was imposed by flushing the desiccators with gaseous nitrogen at a rate of 2 $L \cdot min^{-1}$. The decline of the O_2 concentration in a desiccator is shown in supplemental data (Figure S2.1). Control seedlings were placed in desiccators in which air was flushed at a similar rate. Hypoxia treatments were carried out in the dark to avoid effects of photosynthetically derived O_2.

Enzymatic browning detection of cell damage

Cell damage was assessed by visualizing enzymatic browning. This is an oxidation process during which phenols are oxidized to quinones and eventually brown pigments, catalyzed by enzymes such as polyphenol oxidase. In intact cells, the compartmentalization of polyphenol oxidase in vacuoles prevents this process from occurring. Upon cell disintegration during death, phenols are oxidized, which is visualized by the occurrence of brown pigments. To visualize browning, for each treatment per replicate, around 20 seedlings were randomly taken to check the presence of enzymatic browning at the primary root tips either directly following hypoxia treatments or at designated time points during post-hypoxia phase.

Evans blue staining detection of cell viability

Seedlings were taken for cell viability analysis by Evans blue staining upon both hypoxic and post-hypoxic stress, respectively. For each treatment per replicate, around 20 seedlings were randomly taken from the plates for Evans blue staining either directly following hypoxic treatment or at the designated time points during post-hypoxia phase. Seedlings were incubated in 0.25% aqueous Evans blue staining solution for 15 minutes in the dark (temperature: 21°C), then washed three times with Milli-Q (MQ) water to remove excess dye.

Microscopy

OLYMPUS BX50WI was used to image enzymatic browning at the primary root tips of Col-0 seedlings, as well as the cell viability of root tips with Evans blue staining. Images were taken under 10× objective lens and saved in a suitable format.

Survival scoring and data analysis

At the end of the hypoxia treatment, the position of root tips on agar was marked at the back of the square petri dishes with a permanent marker. Plates were kept in the dark after hypoxia and then moved back to short day growth conditions (light intensity: ~120 $\mu mol \cdot m^{-2} \cdot s^{-2}$, humidity: 70%, temperature: 21°C) when the light was switched off in the climate chamber

at the end of the day for recovery. After 3 days of recovery, seedlings were scored as either alive or dead based on root tip re-growth beyond the line on the back of the plate (Figure 2.1A). Primary root tip survival was calculated as the percentage of seedlings that showed root tip re-growth out of 46 seedlings per plate, which was considered as one biological replicate. Plates were scanned with Epson Perfection V300 scanner and saved as JPEG images. Two-way ANOVA with Tukey's Post-hoc test ($p<0.05$) was used to analyze the data.

Results

Ethylene pre-treatment improves primary root tip survival in *Arabidopsis* Col-0 seedlings upon hypoxia

To assess the effect of an ethylene pre-treatment on hypoxia survival in *Arabidopsis*, seedlings of the wild-type accession Col-0 were exposed to 4 h of either air or ethylene (approximately 5 $\mu L \cdot L^{-1}$) after which they were immediately transferred to hypoxic conditions for 3-5 h (Figure 2.1B). Thereafter seedlings were allowed to recover and re-growth capacity of primary root tips was used as a proxy for hypoxia survival (Figure 2.1A). Pre-exposure of 4-day-old *Arabidopsis* seedlings to 5 $\mu L \cdot L^{-1}$ of ethylene significantly improved primary root tip survival upon subsequent hypoxia treatments (Figure 2.1C). From 3.5 h to 4.5 h hypoxia, primary root tip survival in air exposed seedlings, dropped from nearly 90% to below 20% with the increase of the hypoxia duration. Only a slight decrease in survival was observed in ethylene pre-treated seedlings. The positive effect of ethylene on hypoxia survival was most significant during 4 and 4.5 h hypoxia treatment. After 3.5 h hypoxia no difference in survival between air and ethylene pre-treated plants was observed. A very similar response was found in 5-day-old seedlings upon 3 to 5 h hypoxia treatment (Figure 2.1D). The increased root tip survival in ethylene pre-treated seedlings was absent in the ethylene insensitive mutant *ein2-5* and *ein3eil1* upon exposure to hypoxia. Although *ebf1ebf2*, a mutant that constitutively signals ethylene (Ju and Chang, 2015), still responded to ethylene, it showed significantly increased hypoxia survival compare to wild-type Col-0 when exposed to air (Figure 2.1E). This mutant analysis indicated the requirement for a functional ethylene signaling pathway in ethylene-induced hypoxia tolerance.

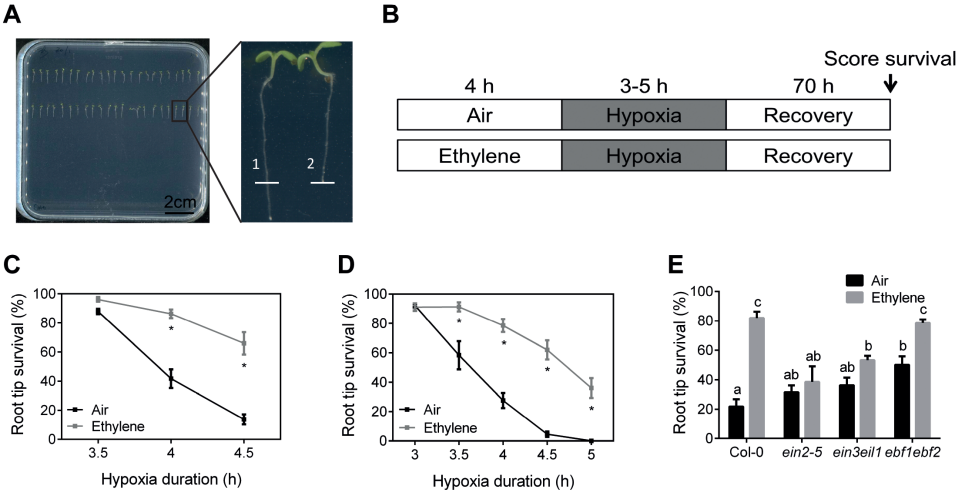


Figure 2.1 Ethylene-induced hypoxia tolerance in *Arabidopsis* seedlings.
(**A**) Image showing seedlings grown on agar plates and root tips scored as alive (1) or dead (2) based on re-growth at the end of recovery phase. (**B**) Scheme indicating experimental design. Seedlings were given 4 h of air or ethylene (5 $\mu L \cdot L^{-1}$) pre-treatment starting at 9:00 (light intensity: ~5 $\mu mol \cdot m^{-2} \cdot S^{-2}$, temperature: 21°C), followed by 3-5 h hypoxia treatment (in the darkness, temperature: 21°C) starting at 13:00 h. Root tips were marked at the end of hypoxia and kept in the dark before being moved back to climate chamber after lights were switched off. Root tip survival was scored at the end of recovery phase (daytime: 9:00-17:00 h, light intensity: ~120 $\mu mol \cdot m^{-2} \cdot S^{-2}$, humidity: 70%, temperature: 21°C). (**C&D**) Root tip survival of 4-day-old (C) and 5-day-old (D) *Arabidopsis* Col-0 upon 3-5 h hypoxia following air or ethylene pre-treatment. Data shown is mean ± SE, (C) n=8, (D) n=4 (Biological replicates). Asterisks indicate statistically significant differences between air and ethylene pre-treatment. (**E**) Root tip survival of 4-day-old *Arabidopsis* Col-0 and ethylene related mutants upon 4.5 h hypoxia. Data shown is mean ± SE, n=6 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Different letters indicate statistically significant differences. Statistical significance was calculated using a two-way ANOVA with Tukey's Post-hoc test, $p<0.05$.

Saturation of the ethylene response

Next we investigated the influence of varying ethylene concentrations and ethylene pre-treatment durations on hypoxia root tip survival (Figure 2.2A&B). Improved root tip survival upon hypoxia (4 and 4.5 h) in 4-day-old seedlings saturated already at an ethylene concentration of ~0.12 $\mu L \cdot L^{-1}$ (Figure 2.2C). At higher concentrations, there was no negative effect on root tip survival.

To test the influence of the pre-treatment duration, ~5 $\mu L \cdot L^{-1}$ of ethylene was given to 4-day-old seedlings from 1 to 4 h (Figure 2.2B) followed by hypoxia treatment (4.5 and 5 h). Root tip survival of seedlings upon 4.5 h hypoxia exposure increased steadily for up to 3 h ethylene

pre-treatment, after which a plateau was reached (Figure 2.2D). In response to a 5 h hypoxia treatment, a similar trend was observed. Root tip survival increased up to 3 h after which there was no significant change. Taken together, ethylene improved hypoxia survival already at slightly enhanced concentrations and an application of 3 h was enough to saturate the response.

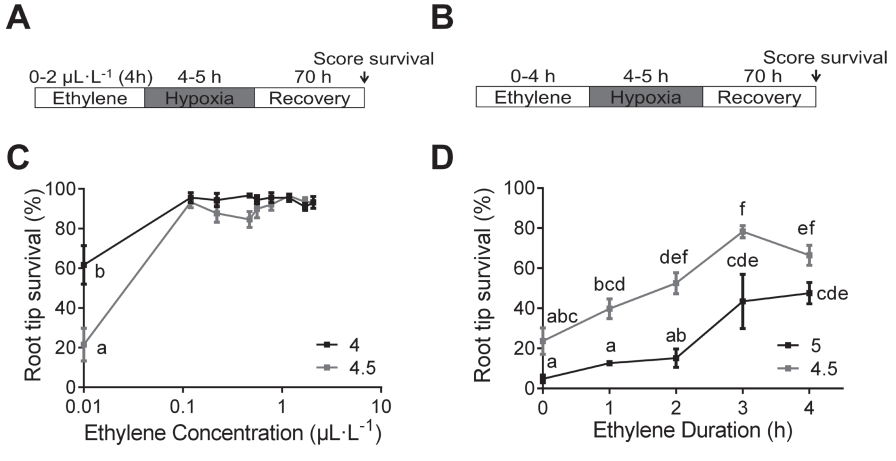


Figure 2.2 Saturation of ethylene-induced root tip survival in 4-day-old *Arabidopsis* Col-0 seedlings.
(**A&B**) Experimental scheme to study ethylene pre-treatment conditions. Either 4 h of ethylene (0-2 µL·L^{-1}) or 5 µL·L^{-1} of ethylene (0-4 h) pre-treatment was given followed by 4-5 h hypoxia. (**C&D**) Root tip survival of 4-day-old Col-0 at the end of recovery phase with corresponding conditions. Data shown is mean ± SE, (C) n=4, (D) n=6 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Different letters in C and D indicate statistically significant differences. Letters in C indicate statistically significant difference with other data points without letters. Statistical significance was calculated using a two-way ANOVA with Tukey's Post-hoc test, $p<0.05$.

Developmental stage affects hypoxia root tip survival

As shown in Figure 2.1 (C&D), both 4- and 5-day-old seedlings benefited from the early ethylene exposure in hypoxia root tip survival. To investigate whether hypoxia survival was influenced by a broader range of developmental ages, root tip survival of 2-, 4-, 7- and 10-day-old seedlings were evaluated after 4 h of hypoxia. Root tip survival of both air and ethylene pre-treated seedlings declined with age (Figure 2.3). Only during the 2- and 4- day stages ethylene pre-treatment increased survival. The older stages seemed to be insensitive to ethylene-driven improvement of root tip survival. However, when a sublethal stress was given (3 h hypoxia), survival of older age groups (7- and 10-day-old) could also be improved by ethylene pre-treatment (Figure S2.2).

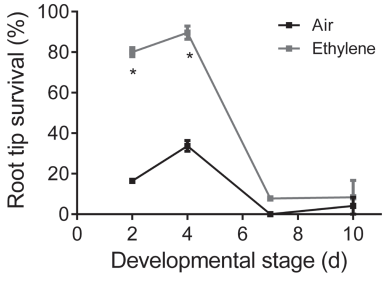


Figure 2.3 Age-dependent sensitivity to hypoxia stress in *Arabidopsis* Col-0 seedlings. Root tip survival of 2-, 4-, 7- and 10-day-old Col-0 seedlings after 4 h hypoxia treatment. Root tip survival was scored at the end of recovery phase as described in Figure 2.1. Data shown is mean ± SE, n=2 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Experiments in this figure were repeated 3 times with similar results. Asterisks indicate statistically significant differences between air and ethylene pre-treatment. Statistical significance was calculated using a two-way ANOVA with Tukey's Post-hoc test, $p<0.05$.

Light antagonizes ethylene-mediated increase in hypoxia tolerance

To evaluate how long the ethylene effect persists, seedlings were placed under normoxic conditions in either light or darkness for varying durations (0 to 4 h) directly following the pre-treatment but before exposure to hypoxic conditions (Figure 2.4A). As expected, ethylene improved root tip survival significantly when the interval was absent. In air as well as in the ethylene pre-treated seedlings, survival gradually dropped in the light with increased gap duration. However, root tip survival of ethylene pre-treated seedlings remained higher than air pre-treated controls at a gap duration of up to 2 h (Figure 2.4B). However, when the gap was in darkness, following pre-treatment and prior to hypoxia exposure, root tip survival did not decline with increasing gap duration. Root tip survival of ethylene pre-treated seedlings was higher than air pre-treated group at 0 h and 1 h gap durations. However, with increasing gap durations in the dark, the survival of air pre-treated seedlings increased and was similar to ethylene pre-treated seedlings at 2, 3 and 4 h gap durations (Figure 2.4C). Overall, these results suggested that the effect of ethylene on hypoxia survival was strongly influenced by light conditions directly after the pre-treatment.

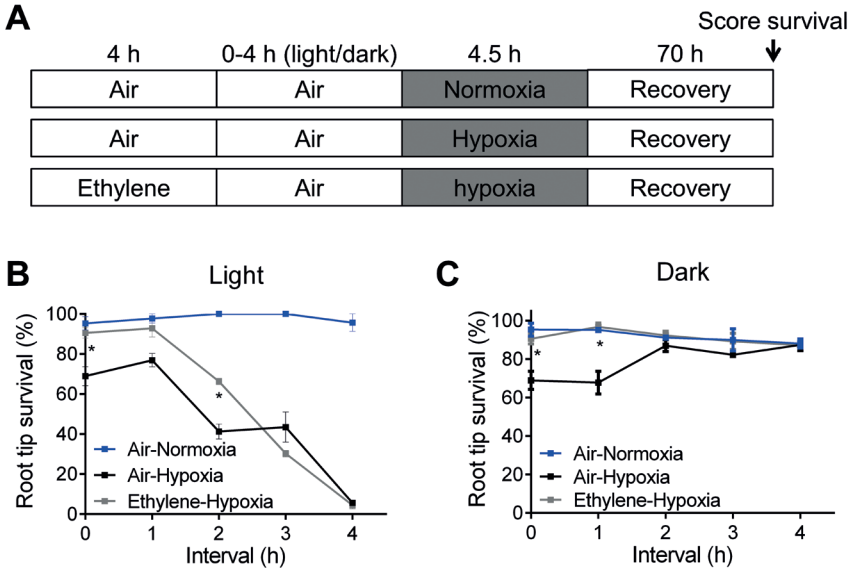


Figure 2.4 Light antagonizes ethylene effect on root tip survival in 4-day-old *Arabidopsis* Col-0 seedlings.
(**A**) Experimental scheme to study the influence of light on ethylene-induced hypoxia tolerance. Four-day-old Col-0 seedlings were given 4 h of air or ethylene (5 $\mu L \cdot L^{-1}$) pre-treatment followed by 0-4 h in the air under light (light intensity: ~120 $\mu mol \cdot m^{-2} \cdot S^{-2}$, humidity: 70%, temperature: 21°C) or darkness (humidity: 70%, temperature: 21°C) before 4.5 h hypoxia treatment in the dark. Root tip survival was scored at the end of recovery phase. (**B&C**) Root tip survival with a series of intervals between pre-treatment and hypoxia in the light (B) or darkness (C). Data shown is mean ± SE, n=4 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Asterisks indicate statistically significant differences between air and ethylene pre-treatment. Statistical significance was calculated using a two-way ANOVA with Tukey's Post-hoc test, $p<0.05$.

Both hypoxia and re-oxygenation cause cell death in *Arabidopsis* root tips

Root tip survival is based on evaluation of root tip re-growth following a recovery period, and can be determined by effects of both hypoxia and re-oxygenation upon recovery. To evaluate the influence of both these phases on root tip survival we evaluated cell death using histochemical detection techniques.

Enzymatic browning is a process occurring when cell integrity is affected, resulting in dark brown pigment accumulation in affected tissues. Here we used it as a proxy to evaluate cell damage by hypoxia and recovery on root tips. Air and ethylene pre-treated seedlings were exposed to different hypoxia durations from 3 to 8 h (Figure 2.5A) and root tips were imaged

immediately after removal from hypoxia to visualize browning. Hypoxia clearly increased the accumulation of brown pigments on the root tips with increased hypoxia durations (Figure 2.5C). To evaluate the effect of recovery, seedlings exposed to 4 h of hypoxia were allowed to recover under normoxic conditions and browning was evaluated at 0, 2, and 4 h of recovery (Figure 2.5B). The brown staining intensified (Figure 2.5D), when seedlings were placed back under normoxic conditions following a 4 h hypoxia treatment. During hypoxia and following recovery (Figure 2.5C&D), the ethylene pre-treated seedlings had little to no brown staining.

Cell death was also evaluated using Evans blue staining. This dye can only penetrate a cell when the cell membrane is not intact anymore. With increasing hypoxia durations (2-7 h), Evans blue staining increased in air pre-treated seedlings (Figure 2.5A) from almost all alive after 2 h hypoxia, to nearly completely dead with 7 h hypoxia (Figure 2.5E). Transfer to normoxic conditions after 4 h of hypoxia (Figure 2.5B), caused an increase in cell death during recovery till around 4 h post-hypoxia (Figure 2.5F). When an ethylene pre-treatment was present, cell death increased as well but at a much slower rate than air pre-treated ones (Figure 2.5E). A similar effect was observed during the recovery stage. Cell damage barely increased during re-oxygenation in ethylene pre-treated seedlings before the 4 h hypoxia (Figure 2.5F).

On the other hand, the distribution of cell damage seems to expand on root tips over time as well. With the increase of hypoxia duration, Evans blue staining of air pre-treated roots started showing at the conjunction of root apex meristem (RAM) and elongation zone, followed by spreading to both directions of the root over time (Figure 2.5E). A similar pattern was found during the recovery stage for Evans blue staining, occurring all over the root tip with greater intensity in RAM (Figure 2.5F). Even though ethylene limits cell death increase of root tips, it was not able to contain the spread of cell death over the root tip. The pattern of enzymatic browning was slightly different. Probably due to the fact that the enzymatic browning reaction occurs quite fast, it was observed in the RAM and a restricted part of elongation zone and did not expand significantly over time, from 5 h hypoxia onwards (Figure 2.5C), or during re-oxygenation (Figure 2.5D). In conclusion, ethylene pre-treatment limited the extent of cell death in root tips which occurred both during hypoxia and re-oxygenation.

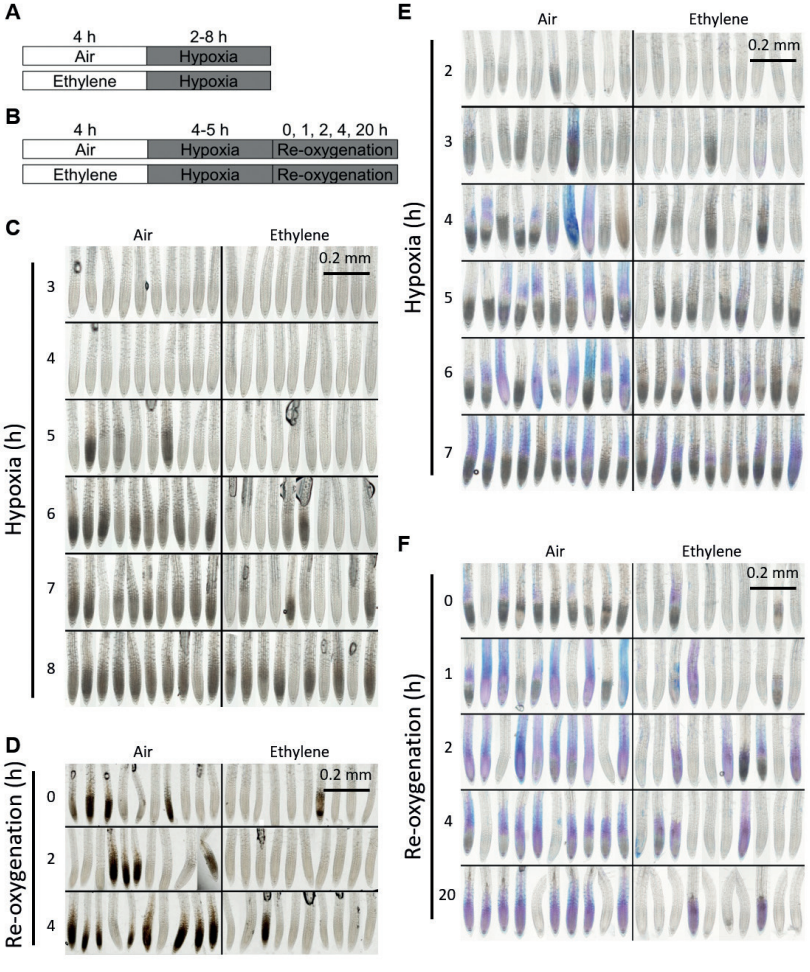


Figure 2.5 Histochemical detection of cell damage in 4-day-old *Arabidopsis* Col-0 root tips.

(**A**) Experimental scheme to study cell damage upon hypoxia. Four-day-old Col-0 seedlings were given 4 h of air or ethylene (5 µL·L^{-1}) pre-treatment followed by 2-8 h hypoxia. Seedlings were taken for enzymatic browning reaction or Evans blue staining detection at the end of each hypoxia treatment. **(B)** Experimental scheme to study cell damage post-hypoxia. Four-day-old Col-0 seedlings were given 4 h of air or ethylene (5 µL·L^{-1}) pre-treatment followed by 4-5 h hypoxia. Seedlings were taken for enzymatic browning reaction and Evans blue staining detection at several post-hypoxia time points (0, 2, 4 h for enzymatic browning detection and 0, 1, 2, 4, 20 h for Evans blue detection). (**C**) Enzymatic browning activity of air or ethylene pre-treated seedlings upon 3-8 h hypoxia. (**D**) Enzymatic browning activity of air or ethylene pre-treated seedlings upon 0, 2, 4 h post-hypoxia following 4.5 h hypoxia. (**E**) Evans blue staining of air or ethylene pre-treated seedlings upon 2-7 h hypoxia. **(F)** Evans blue staining of air or ethylene pre-treated seedlings upon 0, 1, 2, 4, 20 h post-hypoxia following 4 h hypoxia.

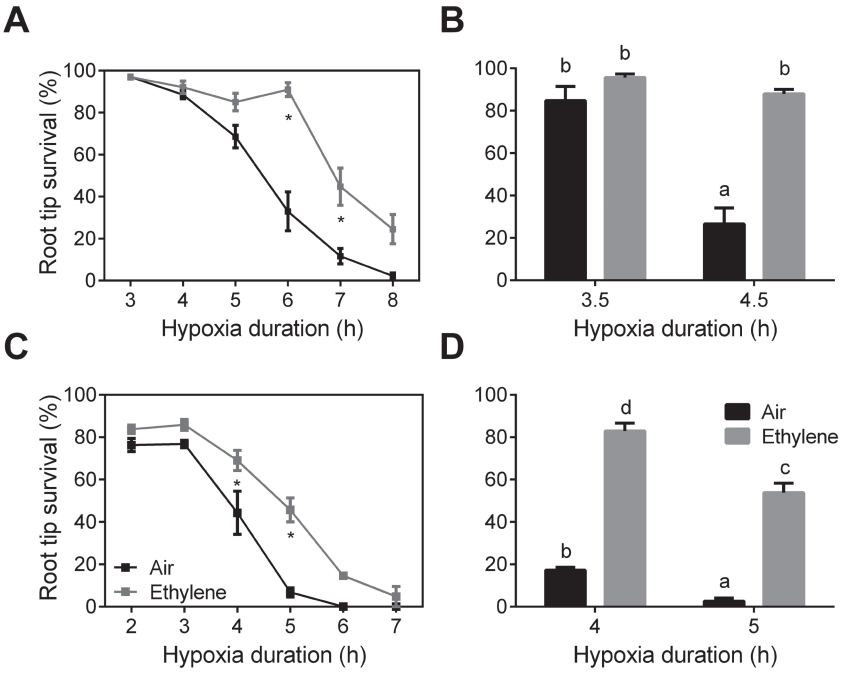


Figure 2.6 Root tip survival of 4-day-old *Arabidopsis* Col-0 corresponding to histochemical study on cell damage.

(**A**) Root tip survival corresponding to enzymatic browning shown in Figure 2.5C. Data shown is mean ± SE, n=6 (Biological replicates). (**B**) Root tip survival corresponding to enzymatic browning shown in Figure 2.5D. Data shown is mean ± SE, n=4 (Biological replicates). (**C**) Root tip survival corresponding to Evans blue staining shown in Figure 2.5E. Data shown is mean ± SE, n=2 (Biological replicates). (**D**) Root tip survival corresponding to Evans blue staining shown in Figure 2.5F. Data shown is mean ± SE, n=3 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Asterisks in A and C indicate statistically significant differences between air and ethylene pre-treatment. Different letters in B and D indicate statistically significant differences. Statistical significance was calculated using a two-way ANOVA with Tukey's Post-hoc test, $p<0.05$.

Discussion

Ethylene concentrations in flooded plant tissues can rise up to around 20-fold higher (Sasidharan *et al.*, 2018; Voesenek and Sasidharan, 2013). As an early and reliable signal, ethylene has proved to be a pivotal regulator mediating many flooding adaptive responses such as adventitious root growth, aerenchyma formation, shoot elongation and hyponasty growth (Kuroha *et al.*, 2018; Polko *et al.*, 2011; Visser *et al.*, 1996; Yamauchi *et al.*, 2014). For instance, ethylene is a primary regulator of the two contrasting shoot growth strategies,

escape and quiescence, in rice cultivars (Fukao and Bailey-Serres, 2008; Hattori *et al.*, 2009; Kuroha *et al.*, 2018; Xu *et al.*, 2006; Yin *et al.*, 2017). In a previous study (van Veen *et al.*, 2013), a novel mechanism was identified in which this gas had the capacity to improve shoot tolerance to hypoxia. Here we investigated whether a similar ethylene-mediated hypoxia tolerance exists in the model *Arabidopsis*.

A short ethylene pre-treatment improved hypoxia tolerance of root tips of *Arabidopsis* Col-0 seedlings (Figure 2.1C&D). A significant positive effect of ethylene on hypoxia survival required a treatment of at least 3 h and the response was saturated already at slightly elevated ethylene concentrations. An increase to 0.12 $\mu L \cdot L^{-1}$, a level 20-fold higher than in the atmosphere, was enough to saturate the response (Figure 2.2C). In flooded plants, ethylene levels have been known to reach 1 $\mu L \cdot L^{-1}$ already within 1 h of submergence (Voesenek and Sasidharan, 2013). To test whether the well-established ethylene signaling pathway operates during ethylene-mediated hypoxia tolerance we studied ethylene-induced hypoxia tolerance in knock out mutants of several key regulators of ethylene signaling. The ethylene insensitive mutants *ein2-5* and *ein3eil1* did not show increased root tip survival when they received an ethylene pre-treatment prior to hypoxia. Moreover, mutation of EBF1 and EBF2, both of which target EIN3 for degradation, showed significant increased root tip survival already without ethylene pre-treatment. We conclude that ethylene-mediated hypoxia tolerance operates via the established ethylene signaling route.

Following ethylene pre-treatment, the beneficial effect of ethylene was lost if seedlings were subsequently placed in the light without ethylene (Figure 2.4B). This implied that firstly, a persistent ethylene signal is required for improved hypoxia tolerance, and secondly, light exposure is detrimental to hypoxia survival. Furthermore, darkness clearly improved hypoxia survival of both air and ethylene pre-treated seedlings (Figure 2.4C).

Light is not only indispensable for photosynthesis but also acts as a critical environmental cue interacting with other regulators in plant development and growth (Franklin and Whitelam, 2004). There have been several reports showing an interaction between the light and ethylene signaling pathways. In *Arabidopsis* ethylene accelerates hypocotyl growth by enhancing cell elongation in the light whilst impeding growth in the dark (Zhong *et al.*, 2012). In this case stabilization of two transcription factors, PHYTOCHROME INTERACTING FACTOR 3 (PIF3) and ETHYLENE RESPONSE FACTOR 1 (ERF1), under different conditions are fine-tuned to regulate different responses. Ethylene affects *PIF3* transcription by allowing EIN3 binding directly to the EIN3 binding sites (EBS) of *PIF3* promoter both in light and dark.

ERF1 was recently shown to be stabilized and functionally redundant in the light. In the dark, function of PIFs are saturated due to their constitutive stabilization and ERF1 turns to be the limiting factor inhibiting hypocotyl elongation. If and how these signaling pathways could interact to influence hypoxia tolerance is unclear. During flooding, light can be highly variable and the combination of ethylene and light variability could be critical cues to plants depending on the conditions of flooding. These interacting pathways could also influence the stability of the ERFVIIs, primary regulators of the plant hypoxia response (Gasch *et al.*, 2015; Gibbs *et al.*, 2011; Hinz *et al.*, 2010; Kosmacz *et al.*, 2015; Licausi *et al.*, 2010, 2011), and therefore influence hypoxia survival.

Environmental cues, both light and hypoxia, were proved to be critical for the transition of seedlings from skotomorphogenesis to photomorphogenesis (Abbas *et al.*, 2015). It was suggested that hypoxia, which is sensed by the destabilization of ERFVIIs via N-end rule protein degradation in plants, enhances seedling survival in the darkness leading to successful transition to photomorphogenesis. Moreover, the observation that ERFVII could be degraded even without the conserved N-terminal suggests a potential light-driven regulation of ERFVII stability independent of the N-end rule pathway. This could be a way for plants to integrate various cues indicating the type of flooded conditions.

Apart from light conditions, many other aspects, such as developmental stage, also affect plant performance under hypoxia. Developmental age clearly impacted hypoxia survival in both ethylene and air pre-treated plants (Figure 2.3). Interestingly, root tip survival of younger seedlings was higher than that of older seedlings. In a comparison of young seedlings (4-day-old) with adult plants (4-week-old rosettes), developmental stage was found to clearly affect the ERFVII RELATED TO AP2 12 (RAP2.12) -mediated responses (Giuntoli *et al.*, 2017). In the N-end rule mutants *prt6* and *ate1ate2* seedlings, the induction of RAP2.12 target genes were comparable to the effect of overexpressing *RAP2.12*. In adult *prt6* and *ate1ate2* plants, the enhancement of hypoxic gene expression was much less than that of 35S::13*RAP2.12*. This suggests that there might be an additional regulation of ERFVIIs stability other than N-end rule in adult plants. Here, the variable hypoxia sensitivity of seedlings dependent on age suggests an important role of development in survival under stress.

After surviving submergence, re-aeration becomes the next challenge for plants. When flood water recedes, re-exposure to normoxia, known as re-oxygenation, could result in massive production of reactive oxygen species, inducing oxidative stress and cellular damage in plants (Pavelic *et al.*, 2000). Recovery effects can be very dependent on the

duration and severity of the submergence stress (Tamang and Fukao, 2015). This is also evident from our data where a 5 h hypoxia duration killed most root tip cells (Figure S2.3). Re-aeration effects were only visible following a more sub-lethal hypoxia stress duration of 4 h. Cell death increased progressively post-hypoxia as indicated by enhanced Evans blue staining intensity in root tips (Figure 2.5F) demonstrating re-oxygenation injury. This cell death occurred later in the RAM compared to the differentiation and elongation zone. This suggests that roots prioritize the survival of the RAM above other cells during hypoxia stress. When ethylene pre-treatment was present, cell death was significantly decreased but still cells in the differentiation and elongation zone were the first to die.

Many studies have shown an induction of ethylene biosynthesis upon re-aeration. In *Arabidopsis*, enhanced activity of 1-aminocyclopropane-1-carboxylic acid (ACC) enzymes could contribute to the increase of ethylene biosynthesis during re-oxygenation (Tsai *et al*., 2014, 2016). Similarly, up-regulation of *ACC SYNTHASE* (*ACS*) and *ACC OXIDASE* (*ACO*) activity resulted in ethylene elevation in *R. palustris* (Voesenek *et al*., 2003). In the Tsai *et al*. study (2014), comparison of wild type and ethylene insensitive mutant *Arabidopsis* seedlings revealed a set of genes relating to oxidative stress in re-oxygenation, suggesting a function of ethylene in coping with this challenge. In addition, it was also indicated in this study that ethylene might be involved in regulating other hormonal pathways like jasmonate and abscisic acid signaling. However, studies in *Arabidopsis* rosettes, revealed a negative function for post-submergence ethylene associated with accelerated senescence and water loss (Yeung *et al*., 2018). In the current study, the benefits of ethylene are likely to be molecular changes imposed during the pre-treatment that confer advantage in root tip survival of both hypoxia and re-oxygenation.

The results presented here clearly show the ability of ethylene to prolong hypoxia and post-hypoxia survival of *Arabidopsis* root tips and the *Arabidopsis* root tip survival assay established in this chapter is a reliable system to further study the mechanisms associated with ethylene mediated-priming of hypoxia recovery in *Arabidopsis* seedlings.

Acknowledgement

Kristy Parhiala helped with the enzymatic browning detection experiments. Peng Su helped with the Evans blue staining assay. We thank Rob Welschen for building the experimental facility and managing the growth rooms.

Supplemental data

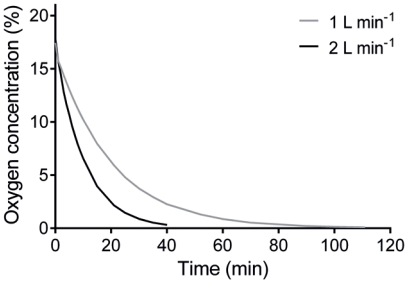


Figure S2.1 O_2 dynamics in the desiccators with different flow rates of nitrogen.
The flow rate used in this study was 2 L·min^{-1}. O_2 concentration in the desiccator reaches 0% after ~40-50 minutes.

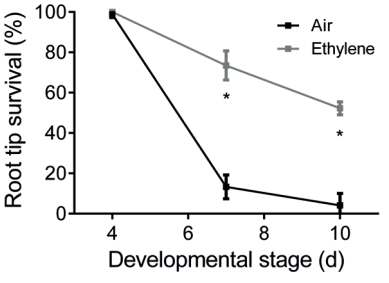


Figure S2.2 Age-dependent sensitivity to hypoxia stress in *Arabidopsis* Col-0 seedlings.
Root tip survival of 4-, 7- and 10-day-old Col-0 seedlings after 3 h hypoxia following air or ethylene (5 µL·L^{-1}) pre-treatment. Root tip survival was scored at the end of recovery phase as described in Figure 2.1. Data shown is mean ± SE, n=2 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Experiments in this figure were repeated 3 times with similar results. Asterisks indicate statistically significant differences between air and ethylene pre-treatment. Statistical significance was calculated using a two-way ANOVA with Tukey's Post-hoc test, $p<0.05$.

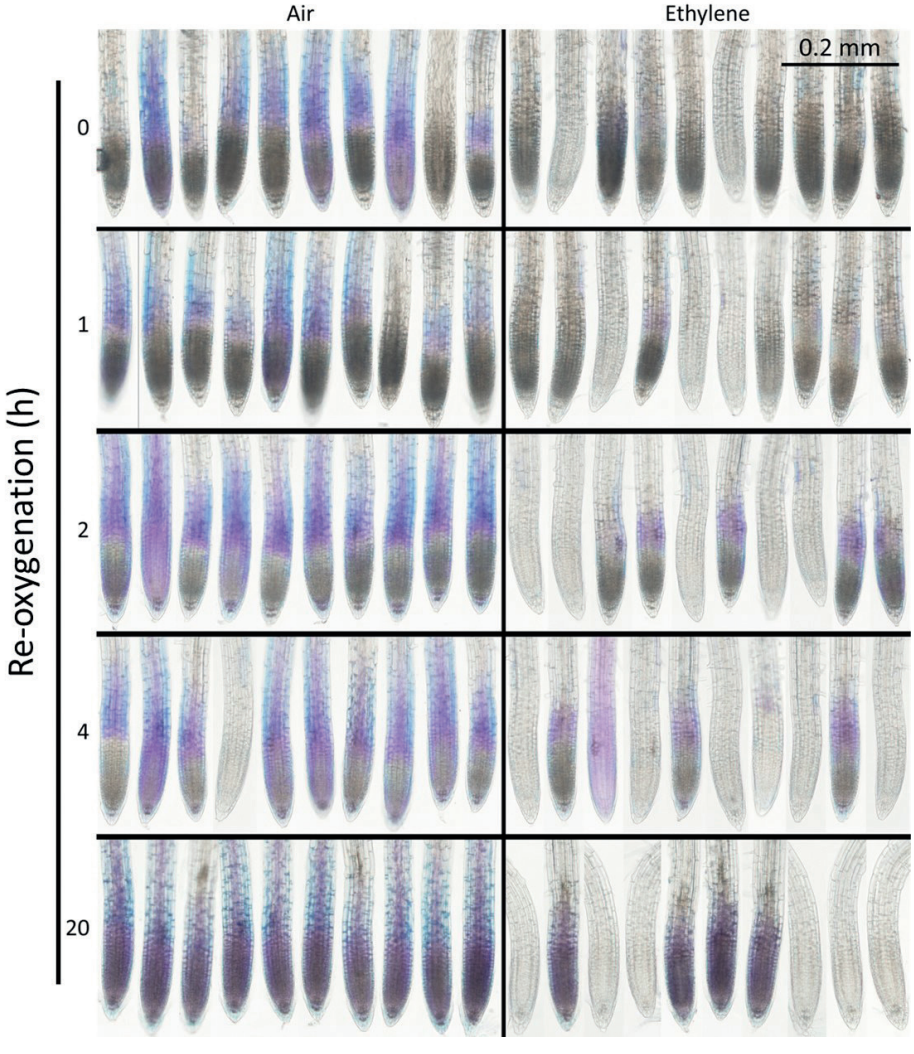
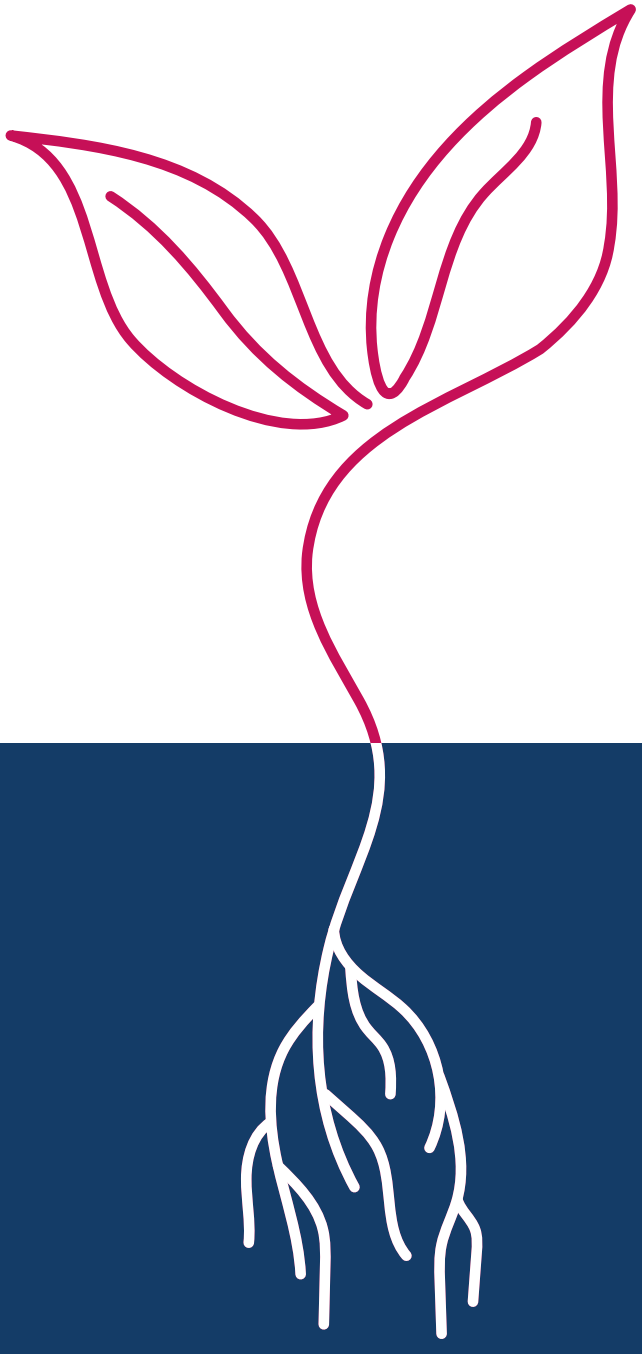


Figure S2.3 Evans blue staining of air or ethylene pre-treated 4-day-old Col-0 seedlings upon post-hypoxia following 5 h hypoxia.
Left panel: air pre-treated samples. Right panel: ethylene (5 $\mu L \cdot L^{-1}$) pre-treated samples. Post-hypoxia time points shown are 0, 1, 2, 4 and 20 h.



CHAPTER 3

Transcriptomic analysis of the ethylene-mediated hypoxia and re-oxygenation responses in *Arabidopsis* roots

Zeguang Liu[1]
Hans van Veen[1]
Shanice Martopawiro[1]
Maureen Hummel[2]
Julia Bailey-Serres[2]
Rashmi Sasidharan[1]*
Laurentius ACJ Voesenek[1]*

*Shared senior authors

[1] Plant Ecophysiology, Institute of Environmental Biology, Utrecht University, Utrecht, The Netherlands

[2] Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, USA

Abstract

Both hypoxia and re-aeration after hypoxia determine the final performance of plants after flooding stress. The ability of roots to sense hypoxia and subsequent re-aeration is critical to plants since roots are the first parts to contact water when flooding events happen. *Arabidopsis* roots were able to benefit from an ethylene pre-treatment and were able to survive subsequent hypoxia and re-oxygenation better. Transcriptome analysis revealed that the early ethylene exposure enhanced regulation of synthesis and signaling pathways for multiple hormones, down-regulation of growth related processes, various epigenetic modification processes and reactive oxygen species homeostasis. We suggest that the ethylene pre-treatment improves hypoxia and re-oxygenation survival partially due to energy conservation from down-regulated growth and the ability to maintain redox balance post-hypoxia.

Introduction

Plants need water to survive throughout their lifecycle. However, excessive water can be detrimental to plants because it decreases gas diffusion underwater thus limiting carbon dioxide (CO_2) and oxygen (O_2) supply to the plant. This restricted gas exchange limits photosynthesis in the shoot and respiration throughout the plant. Ultimately this results in a severe shortage of carbohydrates and energy and eventually plant death (Voesenek *et al.*, 2006). In addition, the depth, temperature and clarity of flood waters can strongly influence plant survival (Mommer and Visser, 2005).

Restricted gas diffusion under water also causes accumulation of the volatile hormone ethylene due to continuous production and physical entrapment (Sasidharan *et al.*, 2018; Voesenek and Sasidharan, 2013). In contrast to other flooding signals such as O_2, levels of which greatly depend on many other factors, ethylene consistently accumulates quickly regardless of plant tissue and flooding conditions. Therefore, it is considered as an early and reliable signal of flooding stress (Sasidharan *et al.*, 2018).

Ethylene is an established regulator of several flood adaptive traits. These include the escape or quiescent growth strategies that allow flooded plants to either escape or cope with the consequences of limited gas diffusion (quiescence). Species employing the quiescence strategy down-regulate metabolism and shoot growth when flooded to conserve energy and carbohydrates and they can therefore recover faster when the flood water recedes (Bailey-Serres and Voesenek, 2008; Luo *et al.*, 2012; Mackill *et al.*, 2012; van Veen *et al.*, 2013). This quiescence strategy in lowland rice involves the interaction between ethylene and the transcription factor (TF) SUBMERGENCE 1A (SUB1A) (Fukao and Bailey-Serres, 2008; Xu *et al.*, 2006). Ethylene is also an important regulator of root adaptive traits like adventitious root formation and aerenchyma development (Drew *et al.*, 2000; Visser *et al.*, 1996; Yamauchi *et al.*, 2014). Soil hypoxia also causes changes in root architecture and particularly primary root growth can be seriously restrained when roots encounter hypoxia (Mira *et al.*, 2016; Webb and Armstrong, 1983). This arrested root growth could be partially attributed to accumulation of ethylene (Armstrong and Drew 2002). However, ethylene is also known to either promote or reduce root growth in a concentration and species-specific manner (Konings and Jackson, 1979; Pierik et al., 2007; Visser and Pierik, 2007). Ethylene has also been implicated in adaptations that promote re-aeration such as enhanced shoot elongation and in reactive oxygen species (ROS) amelioration and dehydration modulation (Tsai *et al.*, 2014; Voesenek *et al.*, 2003; Yeung *et al.*, 2018).

In the previous chapter of this study, we found that root tips of *Arabidopsis* seedlings exposed to high concentrations of ethylene had improved hypoxia survival. We hypothesized that the short ethylene exposure triggers molecular changes that facilitate faster adaptive responses to subsequent hypoxia therefore prolonging survival. Although variable in magnitude, ethylene was able to improve hypoxia survival across several developmental ages in *Arabidopsis* seedlings.

To understand the molecular mechanisms underlying the ethylene-mediated enhancement of hypoxia survival, we used a transcriptomics approach. This allowed us to define the impact of an ethylene pre-treatment on hypoxia and subsequent re-aeration effects and thereby identify molecular processes and players potentially related to improved root tip survival. Our transcriptome survey suggests that (i) ethylene considerably reconfigured the hypoxia and re-oxygenation transcriptomes (ii) the ethylene effect was not unique but rather an enhancement of the response to hypoxia (iii) ethylene impacted several other hormonal responses and caused a down-regulation of energy consuming processes (iv) ethylene might limit post hypoxic injury by improving oxidative stress response and (v) ethylene effects include several epigenetic changes.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana wild type Col-0 was sterilized, sown and grown as described in chapter 2. For all experiments 4-day-old seedlings were used.

Sample harvesting for microarray analysis

The experimental setup for ethylene and hypoxia treatment was as described in Chapter 2. Briefly, four-day-old Col-0 seedlings were pre-treated with air or ethylene (~5 $\mu L \cdot L^{-1}$) for 4 h from 9:00 to 13:00 h (light intensity within desiccators: ~5 $\mu mol \cdot m^{-2} \cdot s^{-2}$, temperature: 21°C), after which hypoxia was applied by 2 $L \cdot min^{-1}$ nitrogen (N_2) flow in the dark. Only root tips were harvested (1/2 cm from root tip). Samples were harvested at 13:00 h (end of pre-treatment; 0 h hypoxia), 15:00 h (2 h hypoxia), 17:00 h (4 h hypoxia) and 18:00 h (1 h re-oxygenation) in the dark. For the re-oxygenation time point harvest, seedlings were taken out of desiccators (air or N_2), and kept vertically in the dark for 1 h before being harvested at 18:00 h for 1 h re-oxygenation. Seedlings pre-treated with air in dim light followed by air treatment in the dark were taken as a control group. The air control sample at 13:00 h (0 h) was the same as air pre-treated sample at 13:00 h (0 h). A schematic explanation of

the treatments and harvest time points are summarized in Figure 3.1. For each sample, a total of approximately 184 root tips pooled from 4 different plates (from 2 desiccators) were considered as one biological replicate. For each time point per treatment, 3 biological replicates were harvested. Harvested tissues were frozen in liquid N_2 immediately and then stored at -80°C.

RNA isolation and sample preparation

Total RNA was isolated from the harvested root tips using the RNeasy mini kit (Qiagen, Germany). A total of 35 µL volume containing 1 µg RNA was mixed gently with 3.5 µL 3M sodium acetate (pH 5.2) and 77 µL 100% ethanol. Samples were analyzed using the Agilent SurePrint HD *Arabidopsis* GE 4X44K Microarrays (Agilent®).

Quality check, sample labeling and purification, hybridization and scan, and raw data preparation

Quality check, sample labeling and purification, hybridization and scan, and raw data preparation were done commercially by Macrogen (Seoul, South Korea) as described below. RNA purity and integrity were evaluated by ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA), Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). RNA labeling and hybridization were performed by using the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). Briefly, 100ng of total RNA from each sample was linearly amplified and labeled with Cy3-dCTP. The labeled cRNAs were purified by RNAeasy Mini Kit (Qiagen, Germany). The concentration and specific activity of the labeled cRNAs (pmol Cy3/µg cRNA) were measured by NanoDrop ND-1000(NanoDrop, Wilmington, USA). 1650ng of each labeled cRNA was fragmented by adding 11 µL 10 x blocking agent and 2.2 µL of 25 x fragmentation buffer, and then heated at 60°C for 30 min. Finally, 55 µL 2 x GE hybridization buffer was added to dilute the labeled cRNA. 100µL of hybridization solution was dispensed into the gasket slide and assembled to the Agilent SurePrint HD *Arabidopsis* GE 4X44K Microarrays (Agilent®). The slides were incubated for 17 h at 65°C in an Agilent hybridization oven. Then washed at room temperature by using the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). The hybridized array was immediately scanned with an Agilent Microarray Scanner D (Agilent Technologies, Inc.) Results were extracted using Agilent Feature Extraction software v11.0 (Agilent Technologies).

Data processing, DEG calculating, Hierarchical cluster and Gene Ontology (GO) enrichment analysis

Raw data were background corrected and normalized using Bioconductor R packages "limma". Probesets were re-annotated by a blast (Basic Local Alignment Search Tool, Altschul *et al.*, 1990) of the probesets against the transcriptome of the most recent *Arabidopsis* annotation (Araport11). Probesets were assigned only to highly similar transcripts, in case of multiple hits, both genes are considered in the analysis. All matches had an identity higher than 90 %, and 93 % of the probes had 100 % identity to the aligned transcript. R language and the "limma" package was used to calculate Log_2 Fold Changes (Log_2FC), P values and the number of differentially expressed genes (DEGs). Log_2FC values of DEGs were scaled prior to hierarchical clustering with the R function "hclust()", to identify gene clusters that showed similar pattern of differences between air and ethylene pre-treated samples after pre-treatment, hypoxia and re-oxygenation. DEG groups were analyzed using Bioconductor R packages "GOstats" and "org.AT.tair.db" to discover enriched GO terms.

TF analysis

Arabidopsis Locus IDs of ethylene specific DEGs were uploaded to the *Arabidopsis* TF database (AtTFDB, https://agris-knowledgebase.org/AtTFDB/), which identifies genes if they were annotated as one of the 50 TF families. DEGs with Log_2FC <-1 or >1, $P < 0.001$ were taken for TF analysis. Log_2FC of the identified TFs were shown on a heatmap generated using R.

Results

The transcriptome response to hypoxia and re-oxygenation is significantly modified by an ethylene pre-treatment

In chapter 2, we found that an ethylene pre-treatment benefits *Arabidopsis* for subsequent hypoxia and re-oxygenation survival. Post-hypoxic injury in the root tips was particularly evident at longer hypoxia durations (4 h) (Figure 2.5). To assess the influence of an ethylene pre-treatment on hypoxia and re-oxygenation responses in *Arabidopsis* root tips, a transcriptomics approach was used. Root tips were sampled from air or ethylene pre-treated seedlings at the end of the pre-treatment, during hypoxia (2 and 4 h) and after re-oxygenation (1 h) following 4 h of hypoxia (Figure 3.1). Samples were also harvested at corresponding time points from seedlings that remained in air (Air control) (Figure 3.1). This experimental design facilitated several transcriptome comparisons (Table 3.1). Hypoxia treated samples (2 and 4 h) from air or ethylene pre-treated samples were compared with the air controls at the same time point to determine the hypoxia effect (Air_2 h, Air_4 h) and ethylene+hypoxia effect (Eth_2 h, Eth_4 h), respectively (Figure 3.1, Table 3.1). To identify re-oxygenation effects, air and ethylene pre-treated root tips harvested

after 1 h re-oxygenation (following 4 h hypoxia) were compared to the preceding 4 h hypoxia samples, thus within the same pre-treatment groups (Air_Reox, Eth_Reox) (Figure 3.1, Table 3.1).

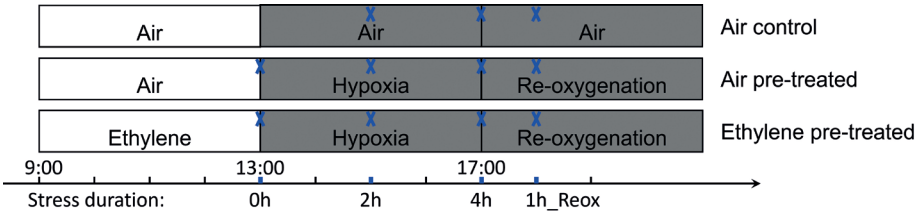


Figure 3.1 Schematic showing the experimental design and sampling time points for transcriptomic survey of ethylene-mediated hypoxia and re-oxygenation responses in *Arabidopsis*.
Four-day-old Col-0 seedlings were pre-treated with air or ethylene (5 $\mu L \cdot L^{-1}$), as indicated in the scheme from 9:00 h (ZT0) to 13:00 h (ZT4) in dim light (~5 $\mu mol \cdot m^{2} \cdot S^{-2}$). Subsequently seedlings were treated to 4 h hypoxia (13:00 h to 17:00 h (ZT8)) in the dark. Root tips from both air and ethylene pre-treated seedlings were harvested at 0 h (end of pre-treatment), 2 h, 4 h hypoxia and 1 h re-oxygenation (in the dark). Blue crosses in the scheme indicate the 11 sampling time points. Seedlings in the air were harvested at corresponding time points as air control. Air control at 0 h was the same sample as air pre-treated sample at 0 h. A total of approximately 184 root tips from 4 plates (from 2 desiccators) were pooled together as one biological replicate.

Table 3.1 List of defined responses and corresponding comparisons made using the microarray data

Responses	Comparison	Abbreviation
Hypoxia effect	Air pre-treated samples compared to air control	Air_2h, Air_4h
Ethylene +Hypoxia effect	Ethylene pre-treated samples compared to air control	Eth_2h, Eth_4h
Re-oxygenation effect	1 h re-oxygenated samples compared to 4 h hypoxic samples	Air_Reox, Eth_Reox
Ethylene effect	Ethylene pre-treated samples compared to air pre-treated samples	EvA_0h, EvA_2h, EvA_4h, EvA_1h_Reox

The hypoxia response was significantly influenced by an ethylene pre-treatment. In both pre-treatment groups, the number of DEGs increased with increasing hypoxia duration. However, the number of up- and down-regulated DEGs were much higher for the ethylene pre-treated samples at both hypoxia time points. Briefly, a total of 1992 and 3094 genes were up- and down-regulated, respectively, after two hours of hypoxia following ethylene exposure (Eth_2 h). In comparison, only 827 and 1392 genes were modulated by two hours of hypoxia after the air pre-treatment

(Air_2h). After 4 h hypoxia, the ethylene effect was even more pronounced. In total, for an ethylene pre-treatment, 3184 and 3806 up- and down-regulated DEGs (Eth_4h) were identified compared to 2350 up- and 3186 down-regulated DEGs for air pre-treatment (Air_4h) (Table 3.1, Figure 3.2). Upon re-oxygenation, the ethylene pre-treatment seemed to have less influence (with respect to number of DEGs), compared to the air pre-treated samples. A total of 3657 and 5208 up- and down-regulated DEGs were identified during re-oxygenation in the air pre-treated group (Air_Reox). In comparison, fewer DEGs (3295 up and 3943 down) were identified in the ethylene pre-treated group (Eth_Reox) (Table 3.1, Figure 3.2).

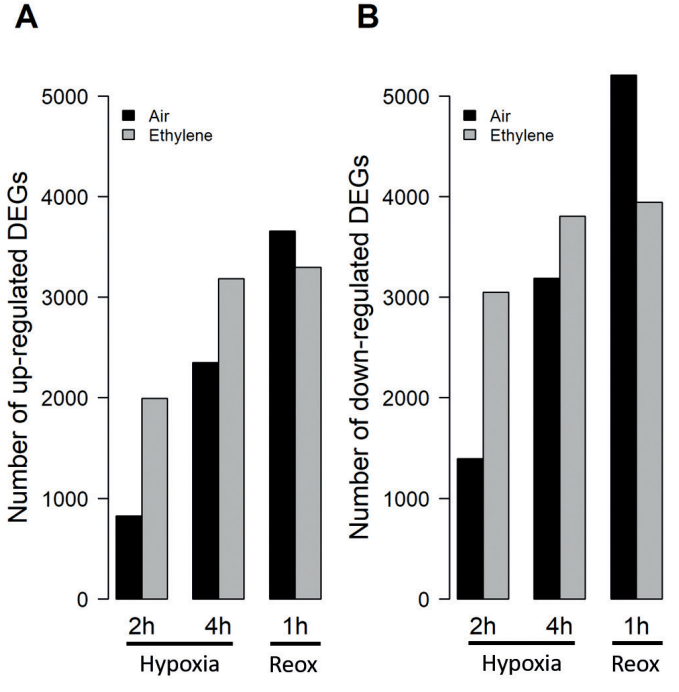


Figure 3.2 Ethylene pre-treatment modulates the hypoxia and re-oxygenation transcriptome response.
The number of (**A**) up- and (**B**) down-regulated DEGs ($P < 0.001$) at 2 h and 4 h hypoxia (Air_2h, Air_4h, Eth_2h, Eth_4h; Table 3.1) and 1 h of re-oxygenation (Air_Reox, Eth_Reox; Table 3.1) in air (black bars) or ethylene (gray bars) pre-treated root tips. Reox refer to re-oxygenation response at 1 h post-hypoxia following 4 h hypoxia as indicated in Table 3.1. Differential expression was calculated as indicated in Table 3.1.

Hypoxia and re-oxygenation attenuate ethylene specific responses

The direct comparison between ethylene and air pre-treated samples at the different sampling time points (0h, 2h, 4h, 1h_Reox; Figure 3.1) allowed identification of the ethylene effect (EvA_0h, EvA_2h, EvA_4h, EvA_1h_Reox) (Table 3.1). As expected, the ethylene pre-treatment itself had a considerable influence on the transcriptome. Following a 4 h pre-treatment (EvA_0h), there was a significant change in the transcript abundance of over a thousand genes (1202 up and 1366 down) (Table3.1, Figure 3.3). However, following transfer to hypoxia and upon re-oxygenation the responses were attenuated. Specifically, only 731 (up) and 1035 (down) DEGs displayed ethylene specificity at 2 h hypoxia (EvA_2h) and even less (512 up and 581 down DEGs) at 4 h hypoxia (EvA_4h) (Table3.1, Figure 3.3). At 1 h re-oxygenation (EvA_1h_Reox), only 516 up-regulated and 793 down-regulated DEGs were identified as ethylene specific (Figure 3.3). The ethylene effect (Figure 3.4, black dots) during hypoxia and re-oxygenation was visualized with scatter plot comparisons between the air and ethylene pre-treated DEGs. The linearity of the scatter plots indicated a general similarity between air and ethylene pre-treated samples both during hypoxia and re-oxygenation. Ethylene-specific hypoxia DEGs clearly deviated from the mean fold change response. This suggested that ethylene effect is more quantitative rather than qualitative.

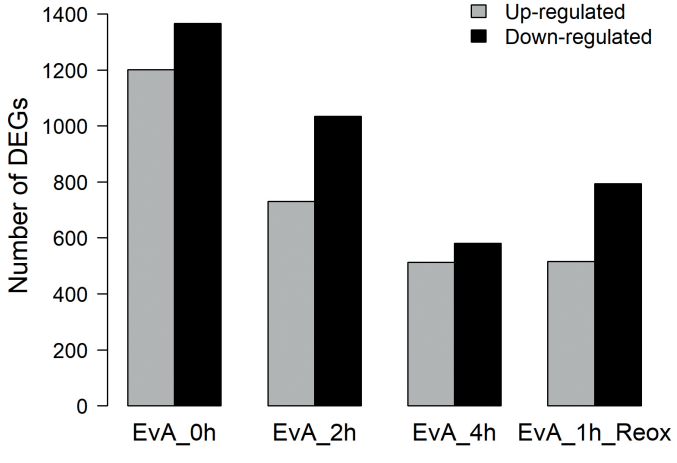


Figure 3.3 Ethylene induces transcriptome changes after pre-treatment, hypoxia and re-oxygenation.

The number of ethylene responsive up- (gray bars) or down- (black bars) regulated DEGs ($P < 0.001$). The comparisons were made between ethylene and air pre-treated samples (Ethylene effect in Table 3.1, EvA_0h, EvA_2h, EvA_4h, EvA_1h_Reox).

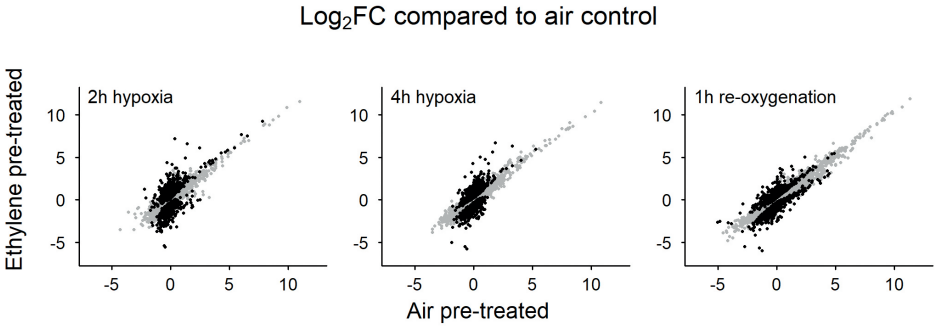


Figure 3.4 Ethylene modulates specific responses after both hypoxia and re-oxygenation. Scatter plots showing comparisons of air pre-treated and ethylene pre-treated Log_2FC values (compared to air control at corresponding time point). In the first two plots from the left, gray dots represent either air (Air_2h, Air_4h; Table 3.1) or ethylene (Eth_2h, Eth_4h; Table 3.1) pre-treatment DEGs and black dots represent ethylene specific (EvA_2h, EvA_4h; Table 3.1) responsive DEGs. In first plot from the right, gray dots represent the total re-oxygenation effect (combined effect pre-treatment+hypoxia+re-oxygenation) (air or ethylene pre-treated sample at 1 h re-oxygenation compared to air control sample at the corresponding time point). Black dots indicate ethylene specific responsive DEGs (EvA_1h_Reox). Genes with $P < 0.001$ are defined as DEGs.

Hypoxia initiates limited novel ethylene specific responses

To investigate whether the ethylene specific responses (EvA_0h) during the pre-treatment, are also modulated during hypoxia, ethylene specific pre-treatment DEGs (Log_2FC) were plotted against ethylene specific hypoxia DEGs (EvA_2h, EvA_4h). The majority of ethylene specific hypoxia DEGs (EvA_2h, EvA_4h) were already differentially regulated after pre-treatment (EvA_0h) (Figure 3.5, gray dots). The ethylene specific DEGs that were differently regulated between pre-treatment and hypoxia were limited (Figure 3.5, black dots). In addition, most of these DEGs (black dots) were actually only differentially regulated after pre-treatment, but not hypoxia. In general, hypoxia only initiated very limited novel ethylene specific responses.

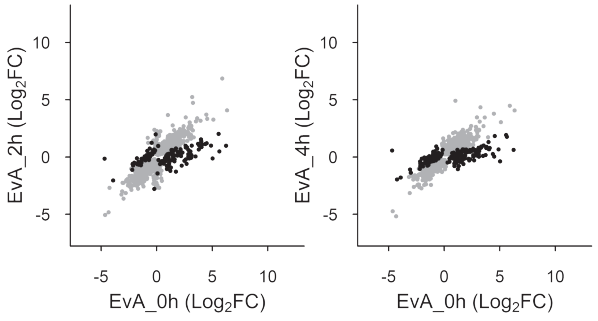


Figure 3.5 Similar ethylene responses after pre-treatment and hypoxia.
Scatter plots showing comparisons of ethylene specific pre-treatment DEGs (EvA_0h) and hypoxia DEGs (EvA_2h and EvA_4h) Log_2FC values. Gray dots represent ethylene specific DEGs that were similarly regulated after pre-treatment and hypoxia. Black dots represent ethylene specific DEGs that were differently regulated between pre-treatment and hypoxia. Genes with $P < 0.001$ are defined as DEGs.

Hierarchical clustering and GO enrichment analysis reveal ethylene specific responses after pre-treatment, hypoxia and re-oxygenation

To visualize regulatory patterns over time, hierarchical clustering was performed on the ethylene specific DEG groups identified ($P < 0.001$) for the pre-treatment (EvA_0h), hypoxia (EvA_2h, EvA_4h), and re-oxygenation (EvA_1h_Reox) time points. (Table 3.1, Figure 3.3, Figure 3.4). This allowed us to i) identify how the large effect of the ethylene pre-treatment carried over to later time points, ii) investigate when the effect of pre-treatment on the hypoxia becomes apparent, iii) reveal how the re-oxygenation response is related to the transcriptome behavior during the pre-treatment and hypoxia. In addition, a GO analysis on these genes also helped identifying functional gene categories enriched at each of these time points.

For each treatment group (pre-treatment, hypoxia, re-oxygenation), hierarchical clustering ordered ethylene specific DEGs into several clusters (Up=U; Down=D). Genes displaying similar dynamics of expression levels (scaled Log_2FC) across the different time points from pre-treatment until end of re-aeration were clustered into the same group.

In total, 2568 genes responded to ethylene after pre-treatment (Figure 3.3, EvA_0h). Those genes were clustered into 9 groups (5 down-regulated: D1-D5, 4 up-regulated: U1-U4) (Figure 3.6). Across all the down-regulated groups, particularly in D2 and D5, GO terms

involved in growth (GO:0040008) and development (GO:0048589) were strongly enriched. In cluster D4 and D5, several GO terms related to brassinosteroid (BR) metabolism (GO:0016132; GO:0016128) were enriched. Cluster D1 contained several GO terms related to epigenetic modifications (histone methylation, GO:0016571; histone H3-K9 methylation, GO:0051567) and in cluster D3 particularly a starvation related GO term (GO:0042594) was identified. In the up-regulated groups U1, U2 and U4, there was a strong enrichment of GO terms related to hormone regulation, such as ethylene (GO:0009723), auxin (GO:0009733), abscisic acid (ABA) (GO:0009738) and jasmonic acid (JA) (GO:0009753) responses. In cluster U4, genes involved in programmed cell death (GO:0043069) and epidermis development (GO:0090558) were also significantly enriched (Table 3.2, Figure S3.1). In most of the clusters, such as D2, U2 and U3, the ethylene-mediated up- or down-regulation at the end of pre-treatment were maintained during subsequent hypoxia and re-oxygenation. However, in a few clusters like D3 and U1, the ethylene effect was somewhat lost during later time points (Figure 3.6).

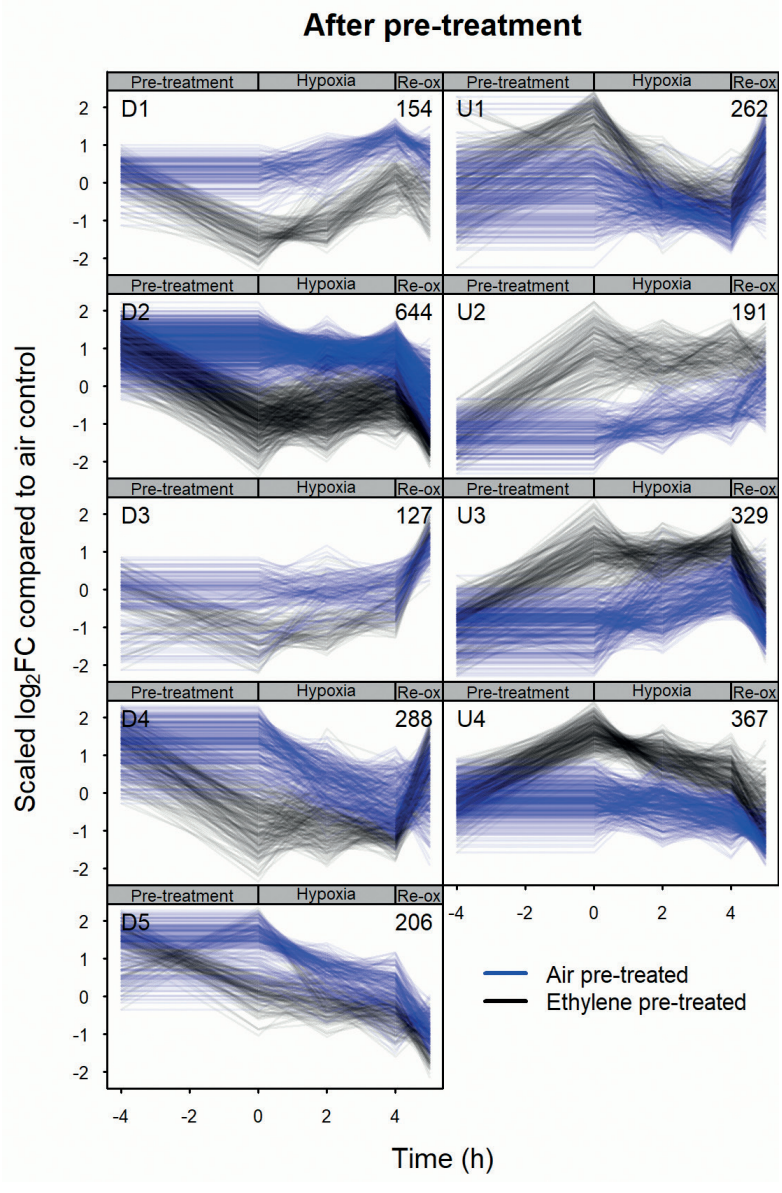


Figure 3.6 Transcript dynamics of ethylene responsive pre-treatment DEGs.
Hierarchical cluster analysis was carried out based on the list of ethylene specific DEGs after pre-treatment (EvA_0h). Genes with $P < 0.001$ are defined as DEGs. Log_2FC of DEGs at the beginning of both air and ethylene pre-treatment were 0 and scaled Log_2FC values were calculated. DEGs clustered into 5 down-regulated (D1-D5) and 4 up-regulated (U1-U4) groups based on similar transcript abundance patterns during pre-treatment, hypoxia and re-oxygenation, and plotted using scaled Log_2FC. Number of DEGs for each cluster is indicated at the top right of each plot.

Table 3.2 Key GO terms enriched from ethylene responsive pre-treatment DEGs

Clusters	Biological processes	GO terms
D1	Histone modification	GO:0016575
	Histone H3-K9 methylation	GO:0051567
D2, D5	Growth	GO:004008
	Development	GO:0048589
D3	Starvation	GO:0042594
D2, D4, D5	BR	GO:0016132; GO:0016128
U1, U2, U4	Ethylene	GO:0009723
	Auxin	GO:0009733
	ABA	GO:0009738
	JA	GO:0009753
U4	Programmed cell death	GO:0043069; GO:0010941
	Plant epidermis	GO:0090558, GO:0090627

For the hypoxia treatment (2 and 4 h), the 2014 ethylene responsive DEGs (Figure 3.3, EvA_2h and EvA_4h), were clustered into 9 groups (5 down-regulated: D1-D5, 4 up-regulated: U1-U4) (Figure 3.7). In general, the ethylene responsive genes being either down- or up-regulated during hypoxia, were already down- or up-regulated by ethylene at the end of pre-treatment, and maintained during re-oxygenation (Figure 3.7). In cluster D1, a clear enrichment of genes with molecular function in light (GO:0009644), hydrogen peroxide (H_2O_2) (GO:0042542) and heat (GO:0009408) responses were identified. In clusters D4 and D5, GO terms related to multiple molecular pathways were enriched. For instance, meristem growth (GO:0010075), cell growth (GO:0016049), hormones (ABA, GO:0009737; BRs, GO:0016132; auxin, GO:0009926) and epigenetic modification gene groups (histone phosphorylation, GO:0016572; chromatin silencing, GO:0006342) were identified. In the up-regulated cluster U1, most genes were related to stress responses such as heat acclimation (GO:0010286) and water deprivation (GO:0009414). In the other 3 clusters, U2, U3 and U4, GO terms involved in epidermis (GO:0090558, GO:0090627) and root hair growth (GO:0048767) were enriched (Table 3.3, Figure S3.2).

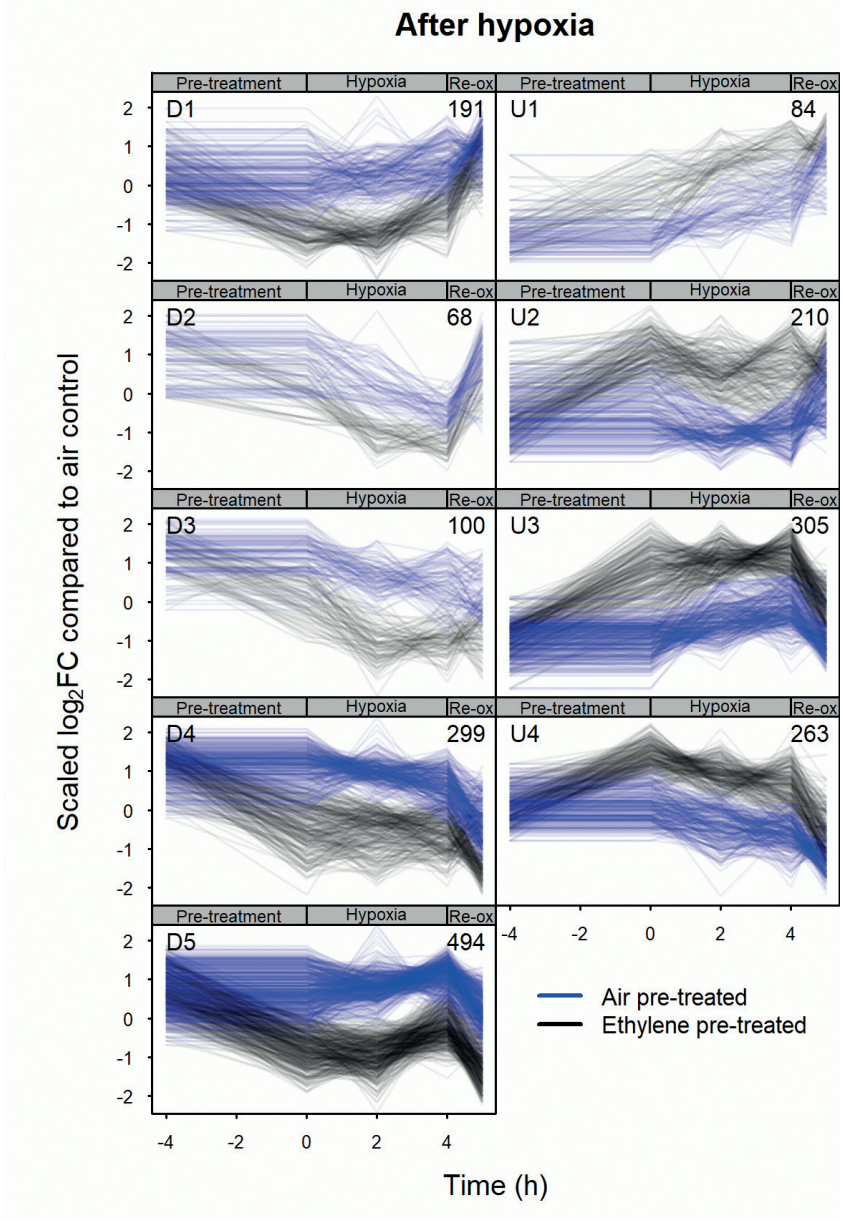


Figure 3.7 Transcript dynamics of ethylene responsive hypoxia DEGs.
Hierarchical cluster analysis was carried out based on the list of ethylene specific DEGs after hypoxia (EvA_2h, EvA_4h). Genes with $P < 0.001$ are defined as DEGs. Log_2FC of DEGs at the beginning of both air and ethylene pre-treatment were 0 and scaled Log_2FC values were calculated. DEGs clustered into 5 down-regulated (D1-D5) and 4 up-regulated (U1-U4) groups based on similar transcript abundance patterns during pre-treatment, hypoxia and re-oxygenation, and plotted using scaled Log_2FC. Number of DEGs for each cluster is indicated at the top right of each plot.

Table 3.3 Key GO terms enriched from ethylene responsive hypoxia DEGs

Clusters	Biological processes	GO terms
D1	High light intensity	GO:0009644
	H_2O_2	GO:0042542
	Heat	GO:0009408
D4, D5	Meristem growth	GO:0010075
	Cell growth	GO:0016049
	BR	GO:0016132
	Hormone transport	GO:0009914
	Auxin transport	GO:0009926
	Histone modification	GO:0016575
	Histone H3-K9 methylation	GO:0051567
	Chromatin silencing	GO:0006342
U1	Heat	GO:0010286
	Water deprivation	GO:0009414
U2, U3, U4	Plant epidermis	GO:0090558, GO:0090627
	Root hair growth	GO:0048767

The re-oxygenation treatment group had the lowest number (1309) of ethylene responsive DEGs (Figure 3.3, EvA_1h_Reox). These genes were clustered into 7 groups (4 down-regulated: D1-D4, 3 up-regulated: U1-U3) (Figure 3.8). Modulation of many ethylene responsive DEGs were a carryover effect of pre-treatment and hypoxia (D1, D2, D3, U2). DEGs from other groups (D4, U1, U3) were not clearly modulated by ethylene during pre-treatment and hypoxia, but only induced after re-oxygenation (Figure 3.8). Several growth-related GO terms, such as cell division (GO:0051301), cell proliferation (GO:0008283) and mitotic cytokinesis (GO:0000281), were significantly enriched in the down-regulated clusters D1, D2 and D3. Moreover, strongly enriched epigenetic GO terms were also identified after re-aeration. Root hair (GO:0048767) and epidermis development (GO:0090558) related GO terms were enriched in both down- and up-regulated groups (D2 and U2). Genes from cluster U3 were not clearly separated between air and ethylene pre-treated groups during pre-treatment and hypoxia. They were significantly enhanced by ethylene at 1 h re-oxygenation, with a clear enrichment of GO terms response to oxidative stress (GO:0006979), heat (GO:0009408), high light intensity (GO:0009644) and H_2O_2 (GO:0042542) (Table 3.4, Figure S3.3).

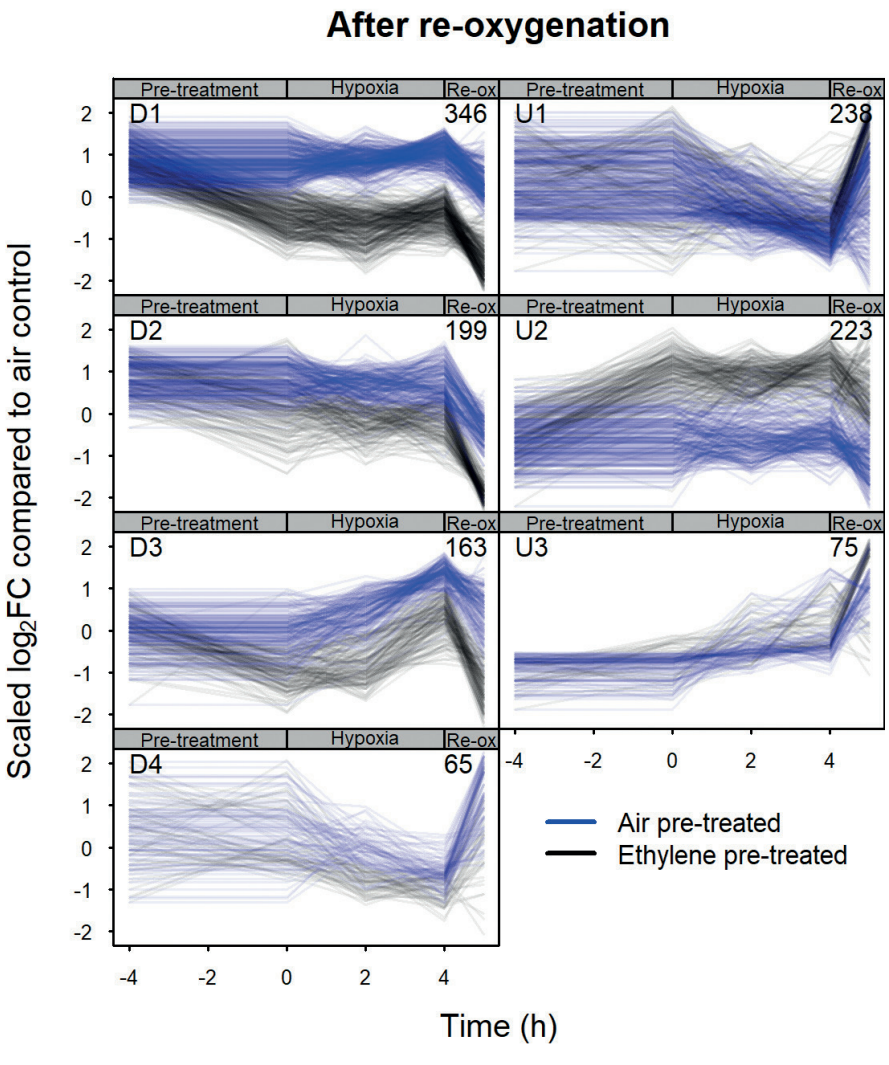


Figure 3.8 Transcript dynamics of ethylene responsive re-oxygenation DEGs.
Hierarchical cluster analysis was carried out based on the list of ethylene specific DEGs after re-oxygenation (EvA_1h_Reox). Genes with $P < 0.001$ are defined as DEGs. Log_2FC of DEGs at the beginning of both air and ethylene pre-treatment were 0 and scaled Log_2FC values were calculated. DEGs clustered into 4 down-regulated (D1-D4) and 3 up-regulated (U1-U3) groups based on similar transcript abundance patterns during pre-treatment, hypoxia and re-oxygenation, and plotted using scaled Log_2FC. Number of DEGs for each cluster is indicated at the top right of each plot.

Table 3.4 Key GO terms enriched from ethylene responsive re-oxygenation DEGs

Clusters	Biological processes	GO terms
D1, D2, D3	Cell division	GO:0051301
	Cell proliferation	GO:0008283
	Mitotic cytokinesis	GO:0000281
	histone modification	GO:0016570
	Histone H3-K9 methylation	GO:0051567
	Histone phosphorylation	GO:0016572
D4	JA	GO:0009753; GO:0009867
	ABA	GO:0009738; GO:0009737
	Ethylene	GO:0009693
D2, U2	Root hair elongation	GO:0048767
	Plant epidermis	GO:0090558; GO:0090627
U3	Oxidative stress	GO:0006979
	High light intensity	GO:0009644
	H_2O_2	GO:0042542
	Heat	GO:0009408

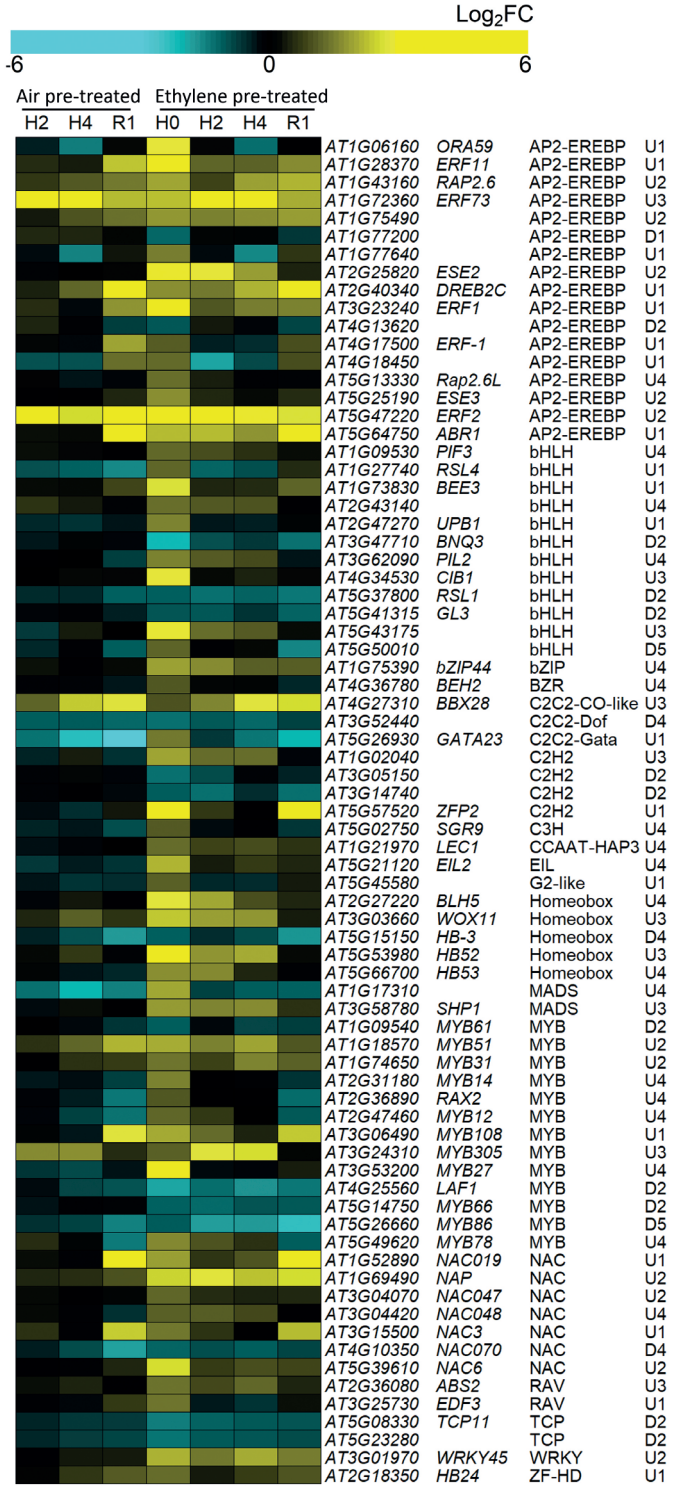
TFs regulate ethylene-induced hypoxia and re-oxygenation responses

The ethylene responsive DEGs for each treatment group (pre-treatment EvA_0h, hypoxia EvA_2h, EvA_4h and re-oxygenation EvA_1h_Reox; Table 3.1), were further analyzed to identify TFs. Only DEGs with $\log_2$FC <-1 or >1, and $P < 0.001$ were considered in the TF analysis. Ethylene specific DEGs were recognized as TFs if they correlated to the 50 annotated TF families listed from AtTFDB (https://agris-knowledgebase.org/AtTFDB/).

In total, 20, 13 and 12 TF families were identified from the ethylene specific DEGs after pre-treatment (EvA_0h), hypoxia (EvA_2h, EvA_4h) and re-oxygenation (EvA_1h_Reox) separately (Table 3.1, Figure 3.9, Figure 3.10, Figure 3.11). After pre-treatment, the majority of the identified TFs were from clusters where DEGs were either down- or up-regulated by ethylene and maintained during later time points (Figure 3.6, Figure 3.9). TFs identified from hypoxia and re-oxygenation treatments were relatively spread over all clusters except for D2 (hypoxia) and U3 (re-oxygenation) (Figure 3.7, Figure 3.8, Figure 3.10, Figure 3.11).

In general, the major TF families identified after pre-treatment and hypoxia were quite similar. These TF families included AP2-EREBP (APETALA2 and Ethylene-Responsive Element Binding Proteins), bHLH (basic helix-loop-helix), C2H2 (cysteine2/histidine2), homeobox,

MYB (myeloblast), NAC (no apical meristems (NAM), ATAF1/2, cup-shaped cotyledon (CUC2)). All the six families listed above were also identified in the re-oxygenation treatment group, but only bHLH and C2H2 were major groups. Most of the AP2-ERFBP TFs identified here were up-regulated by ethylene but displayed distinctive expression patterns at later time points (Figure 3.9, Figure 3.10, Figure 3.11). For example, *ETHYLENE RESPONSE FACTOR 73* (*ERF73*, U3*)* and *RELATED TO AP2 6L* (*RAP2.6L*, U4) were induced after pre-treatment and maintained high during hypoxia and re-oxygenation. Whereas the induction of *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2C* (*DREB2C*, U1) was somewhat gradually lost during hypoxia (Figure 3.9). Two other AP2-EREBP TFs, *ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN* (*EBP*) and *ERF2*, induced by ethylene after hypoxia and re-oxygenation, respectively, displayed similar expression pattern as *ERF73* and *RAP2.6L* (Figure 3.10, Figure 3.11). Two TFs (SHOOT GRAVITROPISM 9 (SGR9) and AT4G38140) from the same C3H (Cys3His) family were found to be pre-treatment and re-oxygenation specific, respectively (Figure 3.9, Figure 3.11). *SGR9*, belonging to U4, was clearly up-regulated by ethylene after pre-treatment (Figure 3.6, U4). The other C3H TF, *AT4G38140* from U1 was induced only upon re-oxygenation in ethylene pre-treated samples (Figure 3.8, U1). There were also 8 TF families responsive only to the pre-treatment, including *ETHYLENE INSENSITIVE 3-LIKE* (*EIL*) (Figure 3.9). Some other TF families were identified only after either hypoxia or re-oxygenation. These included the hypoxia specific (Trihelix: LEUCINE-RICH REPEAT/EXTENSIN 1 (LRX1), *AT1G12040*; PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE 12 (PERK12), *AT1G23540*) and re-oxygenation specific (GROWTH REGULATING FACTOR (GRF): *GRF6*, *AT2G06200*) TFs families (Figure 3.10, Figure 3.11). All these 3 identified TFs induced by ethylene during hypoxia (*LRX1*, U4; *PERK12*, U2; Figure 3.10) and re-oxygenation (*GRF6*, D1; Figure 3.11), were already up-regulated after pre-treatment.



AT1G06160	ORA59	AP2-EREBP	U1
AT1G28370	ERF11	AP2-EREBP	U1
AT1G43160	RAP2.6	AP2-EREBP	U2
AT1G72360	ERF73	AP2-EREBP	U3
AT1G75490		AP2-EREBP	U2
AT1G77200		AP2-EREBP	D1
AT1G77640		AP2-EREBP	U1
AT2G25820	ESE2	AP2-EREBP	U2
AT2G40340	DREB2C	AP2-EREBP	U1
AT3G23240	ERF1	AP2-EREBP	U1
AT4G13620		AP2-EREBP	D2
AT4G17500	ERF-1	AP2-EREBP	U1
AT4G18450		AP2-EREBP	U1
AT5G13330	Rap2.6L	AP2-EREBP	U4
AT5G25190	ESE3	AP2-EREBP	U2
AT5G47220	ERF2	AP2-EREBP	U2
AT5G64750	ABR1	AP2-EREBP	U1
AT1G09530	PIF3	bHLH	U4
AT1G27740	RSL4	bHLH	U1
AT1G73830	BEE3	bHLH	U1
AT2G43140		bHLH	U4
AT2G47270	UPB1	bHLH	U1
AT3G47710	BNQ3	bHLH	D2
AT3G62090	PIL2	bHLH	U4
AT4G34530	CIB1	bHLH	U3
AT5G37800	RSL1	bHLH	D2
AT5G41315	GL3	bHLH	D2
AT5G43175		bHLH	U3
AT5G50010		bHLH	D5
AT1G75390	bZIP44	bZIP	U4
AT4G36780	BEH2	BZR	U4
AT4G27310	BBX28	C2C2-CO-like	U3
AT3G52440		C2C2-Dof	D4
AT5G26930	GATA23	C2C2-Gata	U1
AT1G02040		C2H2	U3
AT3G05150		C2H2	D2
AT3G14740		C2H2	D2
AT5G57520	ZFP2	C2H2	U1
AT5G02750	SGR9	C3H	U4
AT1G21970	LEC1	CCAAT-HAP3	U4
AT5G21120	EIL2	EIL	U4
AT5G45580		G2-like	U1
AT2G27220	BLH5	Homeobox	U4
AT3G03660	WOX11	Homeobox	U3
AT5G15150	HB-3	Homeobox	D4
AT5G53980	HB52	Homeobox	U3
AT5G66700	HB53	Homeobox	U4
AT1G17310		MADS	U4
AT3G58780	SHP1	MADS	U3
AT1G09540	MYB61	MYB	D2
AT1G18570	MYB51	MYB	U2
AT1G74650	MYB31	MYB	U2
AT2G31180	MYB14	MYB	U4
AT2G36890	RAX2	MYB	U4
AT2G47460	MYB12	MYB	U4
AT3G06490	MYB108	MYB	U1
AT3G24310	MYB305	MYB	U3
AT3G53200	MYB27	MYB	U4
AT4G25560	LAF1	MYB	D2
AT5G14750	MYB66	MYB	D2
AT5G26660	MYB86	MYB	D5
AT5G49620	MYB78	MYB	U4
AT1G52890	NAC019	NAC	U1
AT1G69490	NAP	NAC	U2
AT3G04070	NAC047	NAC	U2
AT3G04420	NAC048	NAC	U4
AT3G15500	NAC3	NAC	U1
AT4G10350	NAC070	NAC	D4
AT5G39610	NAC6	NAC	U2
AT2G36080	ABS2	RAV	U3
AT3G25730	EDF3	RAV	U1
AT5G08330	TCP11	TCP	D2
AT5G23280		TCP	D2
AT3G01970	WRKY45	WRKY	U2
AT2G18350	HB24	ZF-HD	U1

Figure 3.9 TFs identified in the ethylene responsive pre-treatment DEGs.
TF identification was performed on the list of ethylene specific DEGs after pre-treatment (EvA_0h). Genes with Log_2FC >1 or Log_2FC <-1, and P < 0.001 are defined as DEGs. Log_2FC values of air and ethylene pre-treated samples compared to corresponding air control are indicated by intensity of the color scale from -6 (cyan) to 6 (yellow). On the right side of the heatmap, are listed the *Arabidopsis* gene locus ID in the first column, gene abbreviation in the second column if available, the TF family in the third column and the clusters (Figure 3.6) they belong to.

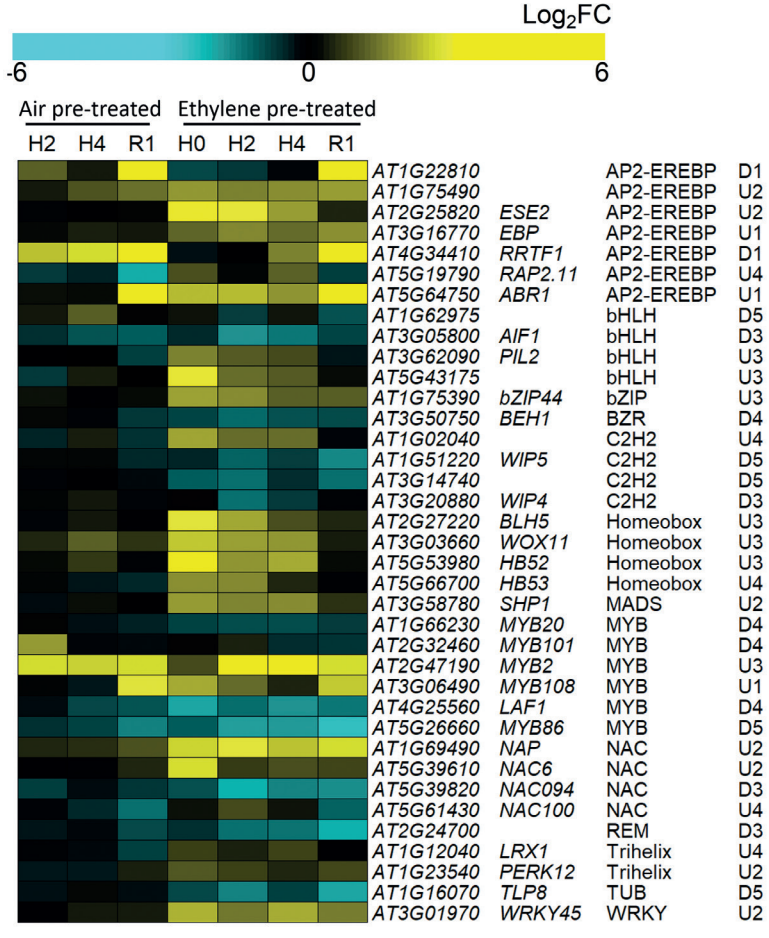


Figure 3.10 TFs identified in the ethylene responsive hypoxia DEGs.
TF identification was performed on the list of ethylene specific DEGs after hypoxia (EvA_2h, EvA_4h). Genes with Log_2FC >1 or Log_2FC <-1, and P < 0.001 are defined as DEGs. Log_2FC values of air and ethylene pre-treated samples compared to corresponding air control are indicated by intensity of the color scale from -6 (cyan) to 6 (yellow). On the right side of the heatmap, are listed the *Arabidopsis* gene locus ID in the first column, gene abbreviation in the second column if available, and the TF family in the third column and the clusters (Figure 3.7) they belong to.

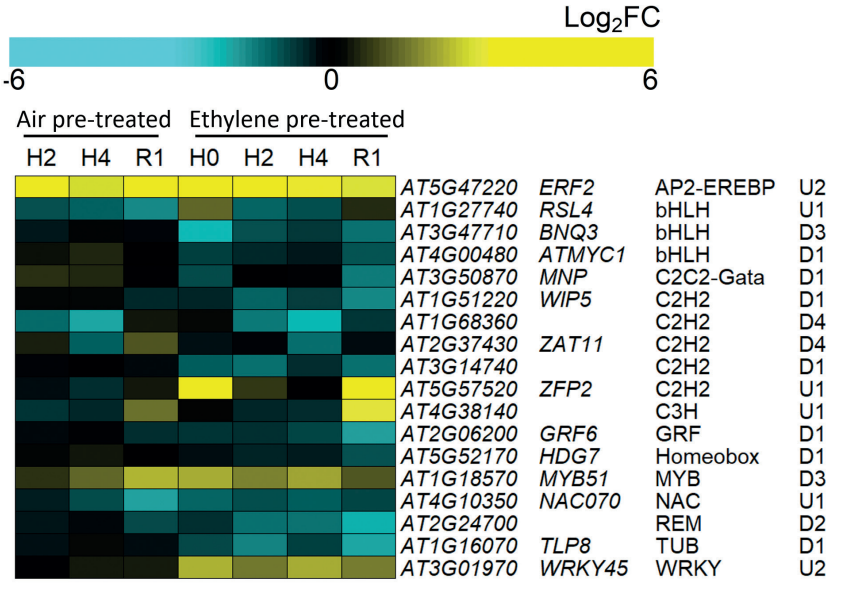


Figure 3.11 TFs identified in the ethylene responsive re-oxygenation DEGs.
TF identification was performed on the list of ethylene specific DEGs after re-oxygenation (EvA_1h_Reox). Genes with Log_2FC >1 or Log_2FC <-1, $P < 0.001$ are defined as DEGs. Log_2FC values of air and ethylene pre-treated samples compared to corresponding air control are indicated by intensity of the color scale from -6 (cyan) to 6 (yellow). On the right side of the heatmap, are listed the *Arabidopsis* gene locus ID in the first column, gene abbreviation in the second column if available, and the TF family in the third column and the clusters (Figure 3.8) they belong to.

Discussion

The main challenge for plants underwater is O_2 deficiency due to greatly reduced gas diffusion (Colmer and Voesenek, 2009). Hypoxia is an important flooding signal, triggering various morphological and metabolic responses in flooded plants. However, ethylene, which accumulates to very high concentrations in flooded plants is also an important signal. Ethylene is an established regulator of several flood adaptive traits including shoot elongation, adventitious root and aerenchyma formation (Fukao and Bailey-Serres, 2008; Hattori *et al.*, 2009; Kuroha *et al.*, 2018; Visser *et al.*, 1996; Xu *et al.*, 2006; Yamauchi *et al.*, 2014). Many recent studies also reported a regulatory role of ethylene in response to re-aeration in *Arabidopsis* (Tsai *et al.*, 2014, 2016; Voesenek *et al.*, 2003; Yeung *et al.*, 2018). The pervasive buildup of ethylene in flooded tissues is presumed to precede the decline in O_2 upon flooding. Consistent with this assumption, an ethylene pre-treatment was found to improve root tip survival to subsequent hypoxia and re-oxygenation (Chapter 2, Figure 2.5E&F). It is hypothesized that the ethylene pre-treatment induces molecular changes

that benefit hypoxia survival. Here we characterized genome wide transcriptome changes occurring in ethylene pre-treated root tips during hypoxia and re-oxygenation. These ethylene responsive genes and molecular processes potentially allow seedling roots to survive not only during hypoxia, but also the following re-oxygenation period better.

Since ethylene is a universal stress hormone in plants it was perhaps not surprising that the largest number of ethylene responsive DEGs were identified at the end of the pre-treatment. While not all these genes are likely to be involved in hypoxia responses, a majority of these genes were also regulated during subsequent hypoxia stress (Figure 3.5). The majority of the ethylene effect during hypoxia and re-oxygenation were due to the carryover effect from the pre-treatment. There were only a limited number of genes that were not regulated after pre-treatment, but displayed expression changes at later time points. In general, ethylene did not qualitatively change the hypoxia responses (Figure 3.4), instead dampening or exaggerating it (Figure 3.6, Figure 3.7, Figure 3.8).

TFs function as master molecular switches controlling downstream gene expression in response to environmental stresses (Khan *et al.*, 2018). Here many TFs were identified displaying distinct regulation patterns. The AP2-EREBP TF *ERF73*, also known as *HYPOXIA RESPONSE ERF 1* (*HRE1*), was significantly induced after the ethylene pre-treatment (Figure 3.9). *HRE1* functions redundantly with *HRE2*, in improving hypoxia survival through enhancement of anaerobic metabolism (Licausi *et al.*, 2010). Both belong to the group VII ERFs (ERFVIIs) that are established regulators of the hypoxia response in *Arabidopsis* (Gasch *et al.*, 2015; Gibbs *et al.*, 2011; Hinz *et al.*, 2010; Kosmacz *et al.*, 2015; Licausi *et al.*, 2010, 2011). The increased abundance of these TFs already during the pre-treatment might trigger a faster downstream response during subsequent hypoxia. Among the ethylene responsive AP2-EREBP members identified in the hypoxia treatment group, was *EBP*, also known as *RAP2.3* (Figure 3.10). *RAP2.3*, even though playing a minor role, together with *RAP2.2* and *RAP2.12* were shown to be the main regulators of hypoxic responses (Bui *et al.*, 2015; Gasch *et al.*, 2015). Two TFs from trihelix family, *LRX1* and *PERK12*, were found as hypoxia-induced ethylene specific targets (Figure 3.10). Some TFs from the trihelix family, such as *HYPOXIA RESPONSE ATTENUATOR 1* (*HRA1*) in *Arabidopsis*, are inducible in different plant species under hypoxia conditions (Giuntoli *et al.*, 2014), and serves to curtail the activity of RAP2.12. The induction of those two members potentially could be beneficial to performance of seedlings under hypoxia.

Ethylene interacts with several other hormones to regulate flooding responses. For instance, enhanced shoot elongation underwater requires the interplay of at least ethylene, ABA and GA, coordinating molecular processes that benefit the escape response (Bailey-Serres and Voesenek, 2008). Here the GO enrichment analysis of ethylene responsive DEGs, identified many hormone-related groups, including genes encoding auxin polar transport (GO:0009926), response to ABA (GO:0009737) and hormone transport (GO:009914), suggesting the potential involvement of these hormones in the ethylene-mediated hypoxia and re-oxygenation responses (Table 3.2, Table 3.3, Table 3.4; Figure S3.1, Figure S3.2, Figure S3.3). A few homeobox TFs, *Homeobox protein 52* (*HB52*), for instance were identified after pre-treatment and hypoxia (Figure 3.9, Figure 3.10). *HB52* was recently proposed to bridge the interaction between ethylene and auxin to facilitate root growth inhibition (Miao *et al.*, 2018). The massive growth regulation discovered in the GO enrichment analysis could partly be attributed to the ethylene induced *HB52*. Two other AP2-EREBP members identified, *DREB2C* and *RAP2.6L*, were reported to interact with ABA to regulate stress responses (Lee *et al.*, 2010; Liu *et al.*, 2012). *RAP2.6L* improved waterlogging survival by enhancing antioxidant activity and by inducing stomatal closure to avoid dehydration.

DEGs relating to another growth regulating hormone, BRs, were also enriched (Table 3.2, Table 3.3, Table 3.4; Figure S3.1, Figure S3.2, Figure S3.3). BRs are a group of polyhydroxylated steroidal compounds regulating multiple aspects of plant growth and development (Clouse and Sasse, 1998). BRs promote root growth at low concentrations and inhibit at high concentrations (Wei and Li, 2016). Therefore, plants can coordinate the level of BRs to adapt to environmental conditions and respond accordingly. BR-mediated regulation of root growth was shown to be linked to ethylene synthesis via the direct interaction of BR signaling TFs, BRASSINOSTEROID INSENSITIVE 1 (BRI1)-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLE-RESISTANT 1 (BZR1), with promoters of ethylene synthesis genes (*1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 7* (*ACS7), ACS9, ACS11*) (Lv *et al.*, 2018). The enriched BRs related genes could potentially interact with ethylene, and possibly also with other hormones that were regulated to fine-tune molecular processes modulating growth.

A typical consequence of flooding stress is a carbohydrate and energy crisis due to reduced photosynthesis and respiration (Sasidharan *et al.*, 2018). To maintain energy status, plants generally down-regulate certain molecular processes to prioritize energy distribution under flooding conditions (Branco-Price *et al.*, 2008; Edwards *et al.*, 2012; Sorenson and Bailey-Serres, 2014). Interestingly, there were already starvation-related genes enriched as ethylene specific responses after pre-treatment (EvA_0h). This starvation signature could possibly

benefit seedlings by already preparing cells for conservative energy expenditure and adjusting metabolism during subsequent hypoxia conditions (Figure S3.1). Multiple studies have shown that starvation triggered energy prioritization is crucial for plants to survive flooding (Branco-Price *et al*., 2008; Edwards *et al*., 2012; Sorenson and Bailey-Serres, 2014). However, it is also not always considered as beneficial. For instance, starch degradation could be greatly inhibited, thereby limiting germination in rice accessions perceiving starvation underwater (Loreti *et al*., 2016; Magneschi and Perata, 2009). Meanwhile, as stated above, many growth metabolic processes were down-regulated by the ethylene pre-treatment. It could be that ethylene initiates the starvation signal that allows seedlings to re-allocate energy to adapt to subsequent hypoxic and re-aeration stress.

The ethylene related hormonal and growth gene regulation signature was not only significantly enriched after pre-treatment and hypoxia, but also upon re-oxygenation (Figure S3.3). The ability to adapt to re-aeration is another crucial aspect of plants surviving flooding. Genes related to oxidative stress alleviation were significantly up-regulated at 1 h re-oxygenation in the ethylene pre-treated samples. It can be speculated that the higher expression of oxidative stress related genes due to early ethylene exposure together with the interplay of multiple hormone pathways could be beneficial for hypoxia re-aeration. Both JA and ABA were previously proved to be important for post-submergence recovery in *Arabidopsis* (Yeung *et al*., 2018; Yuan *et al*., 2017). JA positively regulated antioxidant accumulation, which enhances the capacity to alleviate oxidative damage upon de-submergence. Oxidative stress is also a major component of re-oxygenation stress and is likely to be a major contributor to the post-hypoxic injury experienced by seedling root tips in our system.

The bHLH TF *UPBEAT1* (*UPB1*) was induced strongly only after pre-treatment, with relatively less separation between air and ethylene pre-treated samples at later stages (Figure 3.9). Genetic modification of *UPB1* expression was shown to affect primary root growth via modulation of H_2O_2 levels (Tsukagoshi *et al*., 2010). Therefore, the initiation of *UPB1* after ethylene pre-treatment could be not only crucial to root growth regulation, but also ROS homeostasis, which is important under both hypoxia and re-oxygenation. The only TF from AP2-EREBP family identified after re-oxygenation was *ERF2*, that was previously shown to be induced in response to re-aeration in *Arabidopsis* seedlings (Figure 3.11, Tsai *et al*., 2014). In this study, *ERF2* was highly expressed under hypoxia and re-oxygenation, confirming its role in re-oxygenation adaptation. However, in our system, transcript abundance declined during re-oxygenation in the ethylene pre-treated samples.

Across all the three treatment groups of pre-treatment, hypoxia and re-oxygenation, genes linked to epigenetic modification were strongly enriched (Figure S3.1, Figure S3.2, Figure S3.3). Epigenetic modulation of gene activity plays an important role in plant growth and responses to environmental changes (Baulcombe and Dean, 2014; Pikaard and Scheid, 2014). TFs from C2H2 family, were clearly induced through all three treatment groups (Figure 3.9, Figure 3.10, Figure 3.11), and were previously suggested to be involved in chromatin-remodeling (Englbrecht *et al.*, 2004). Therefore, the epigenetic modification initiated by the early ethylene signal could be responsible for subsequent modulation of hypoxia and re-aeration responses. For example, the expression of genes from oxidative stress related GO terms enriched at 1 h re-oxygenation was not clearly separated during pre-treatment and hypoxia between air and ethylene pre-treated groups, but only upon re-aeration (Figure 3.8, U3; Figure S3.3, Table 3.4). This might be linked to the beneficial effect of epigenetic changes that already happened during pre-treatment or hypoxia.

In conclusion, the ethylene effect identified was not a novel regulation process but rather amplification or counteraction of the existing responses. Ethylene pre-treatment initiated multiple hormonal regulation, together with putatively beneficial TFs, which likely fine-tunes growth processes under hypoxia and re-aeration. Epigenetic modifications were broadly triggered, potentially to facilitate molecular changes later on. The ethylene pre-treatment also could improve oxidative stress adaptability post-hypoxia by enhancing ROS alleviation genes. In conclusion, ethylene pre-treatment induced transcriptome changes linked to processes that improve hypoxia and re-oxygenation adaptability.

Acknowledgement

We thank members of the Plant Ecophysiology group for help with tissue harvesting for gene expression analysis.

Supplemental data

Figure S3.1 GO terms enriched from clustered ethylene specific pre-treatment DEGs.

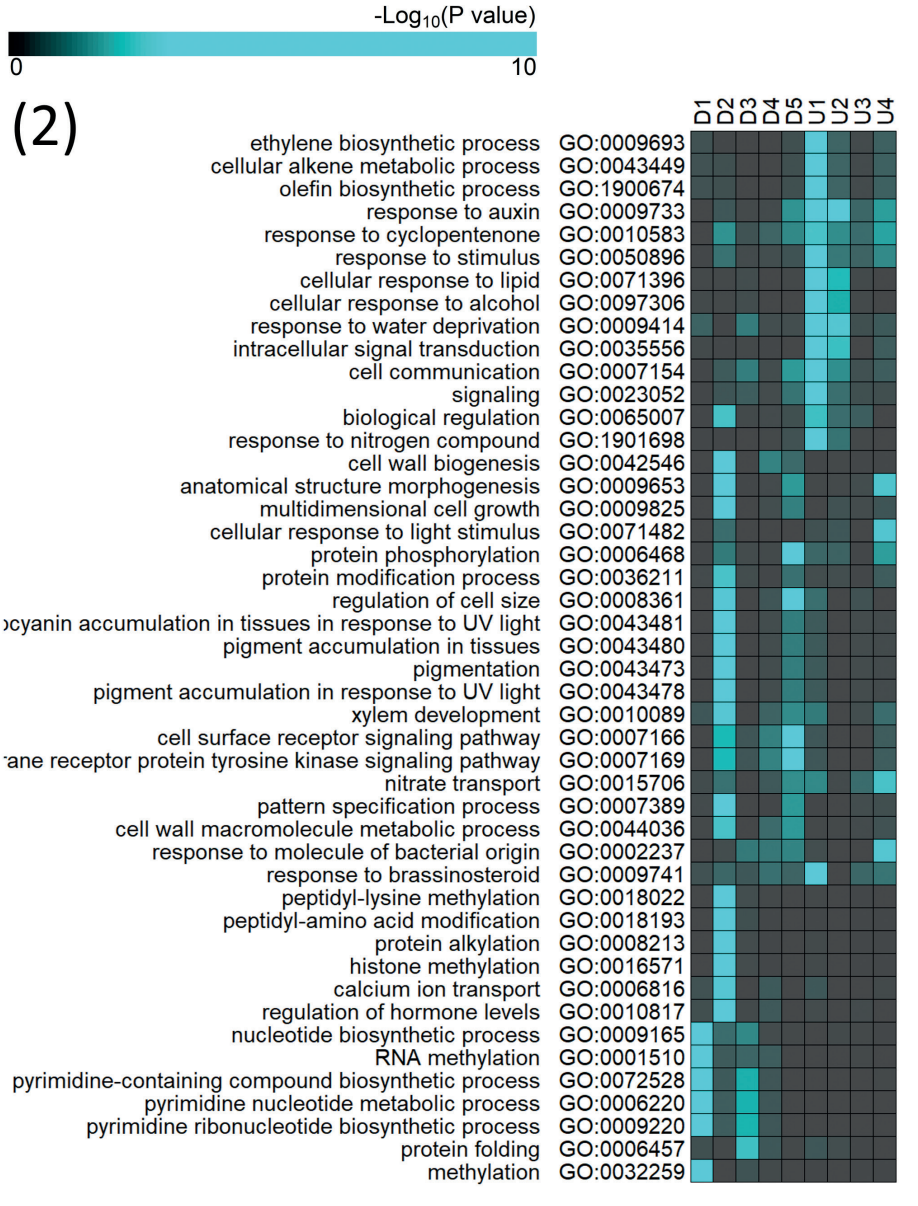


Figure S3.1 GO terms enriched from clustered ethylene specific pre-treatment DEGs.

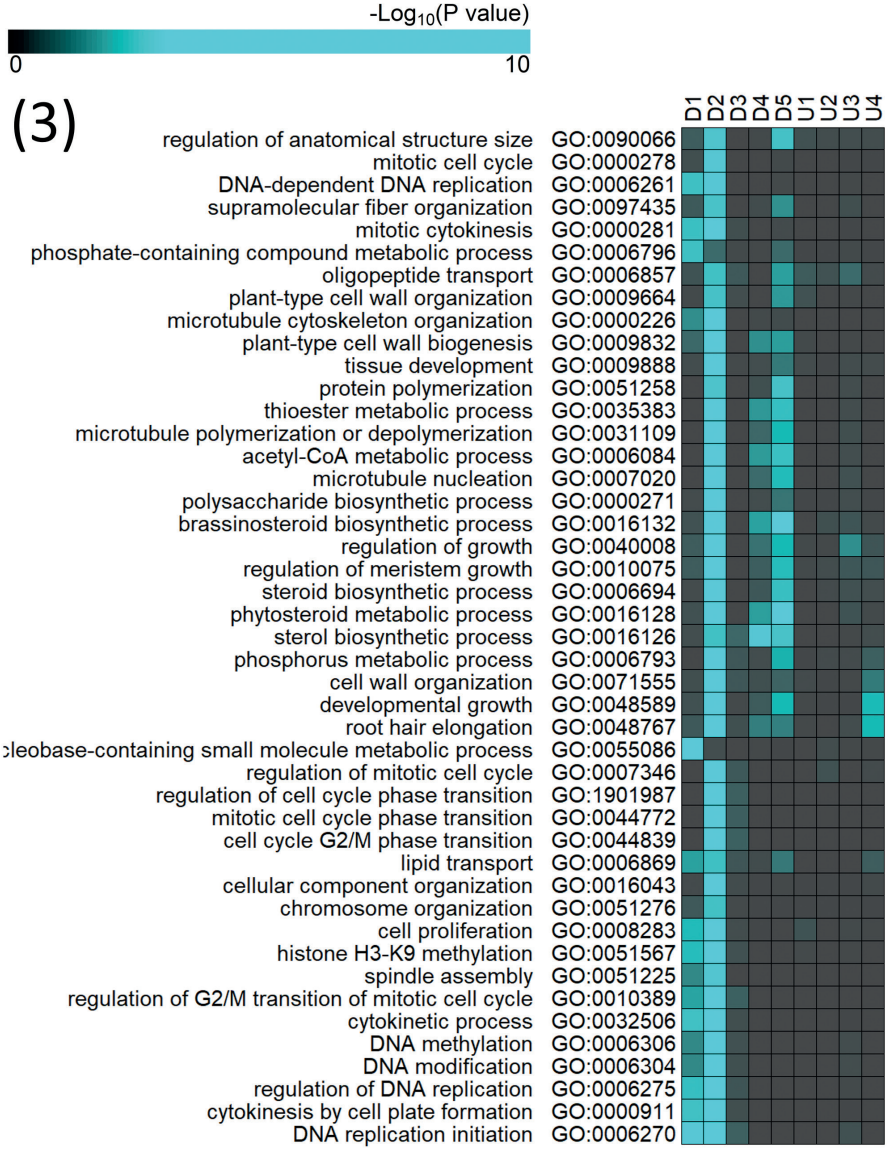


Figure S3.1 GO terms enriched from clustered ethylene specific pre-treatment DEGs.
GO enrichment analysis was carried out based on the clustered ethylene specific DEGs after pre-treatment (EvA_0h). GO terms with $P < 0.001$ and GO_{counts} (count of genes) > 6 were displayed in the heatmap, where a higher cyan intensity represents a strong enrichment of relevant GO biological processes.

Figure S3.2 GO terms enriched from clustered ethylene specific hypoxia DEGs.

Figure S3.2 GO terms enriched from clustered ethylene specific hypoxia DEGs.

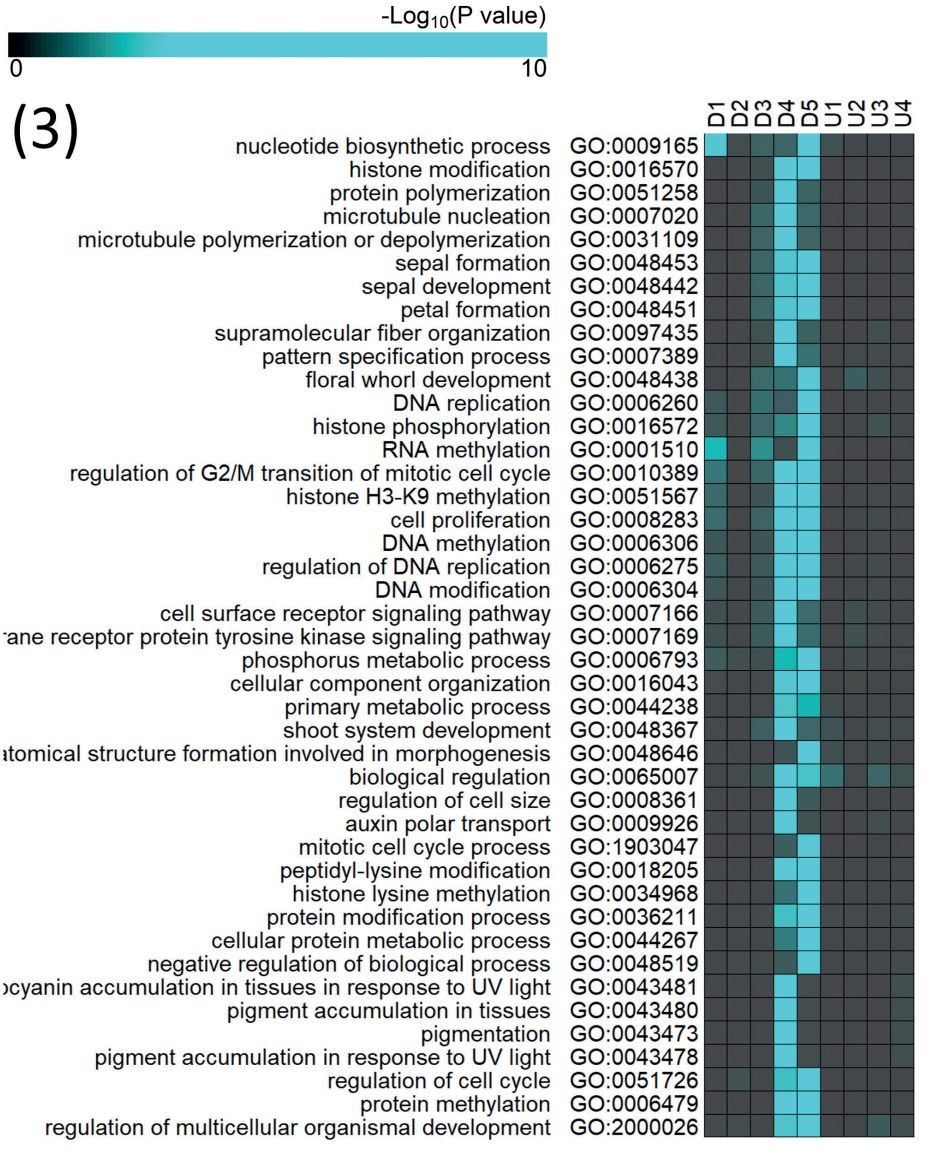


Figure S3.2 GO terms enriched from clustered ethylene specific hypoxia DEGs.
GO enrichment analysis was carried out based on the clustered ethylene specific DEGs after hypoxia (EvA_2h, EvA_4h). GO terms with $P < 0.001$ and GO_{counts} (count of genes) > 6 were displayed in the heatmap, where a higher cyan intensity represents a strong enrichment of relevant GO biological processes.

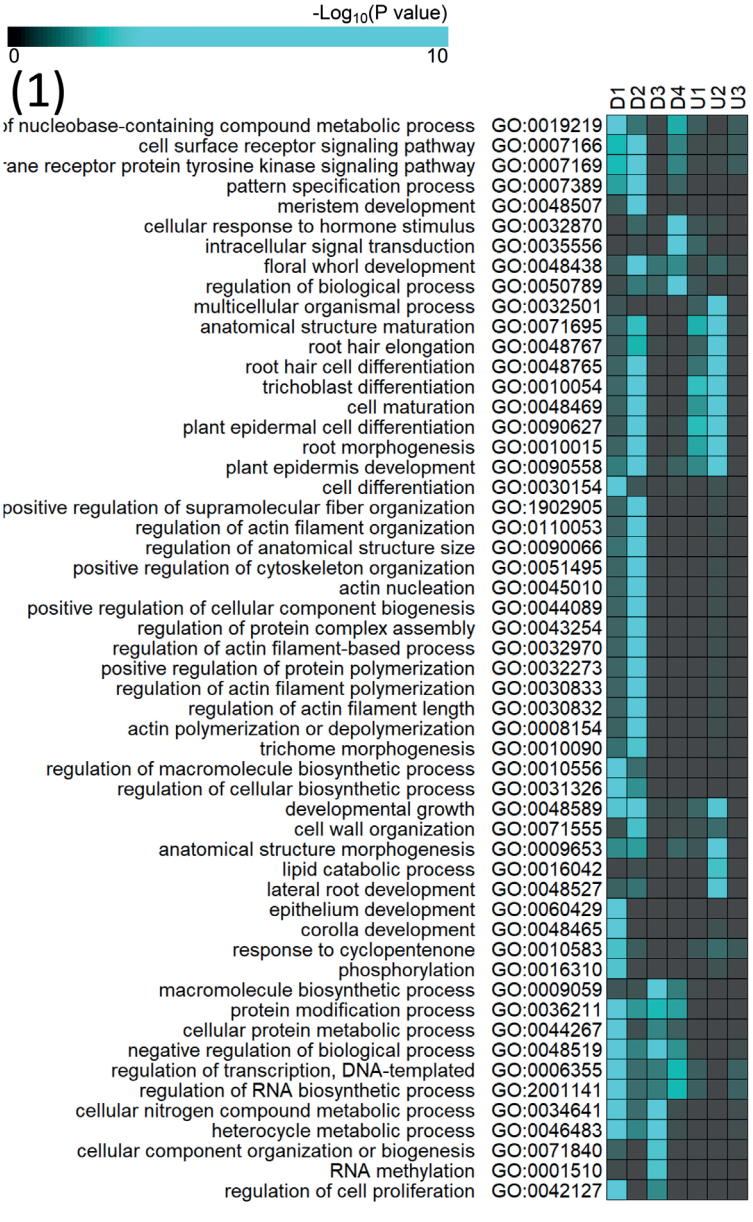


Figure S3.3 GO terms enriched from clustered ethylene specific re-oxygenation DEGs.

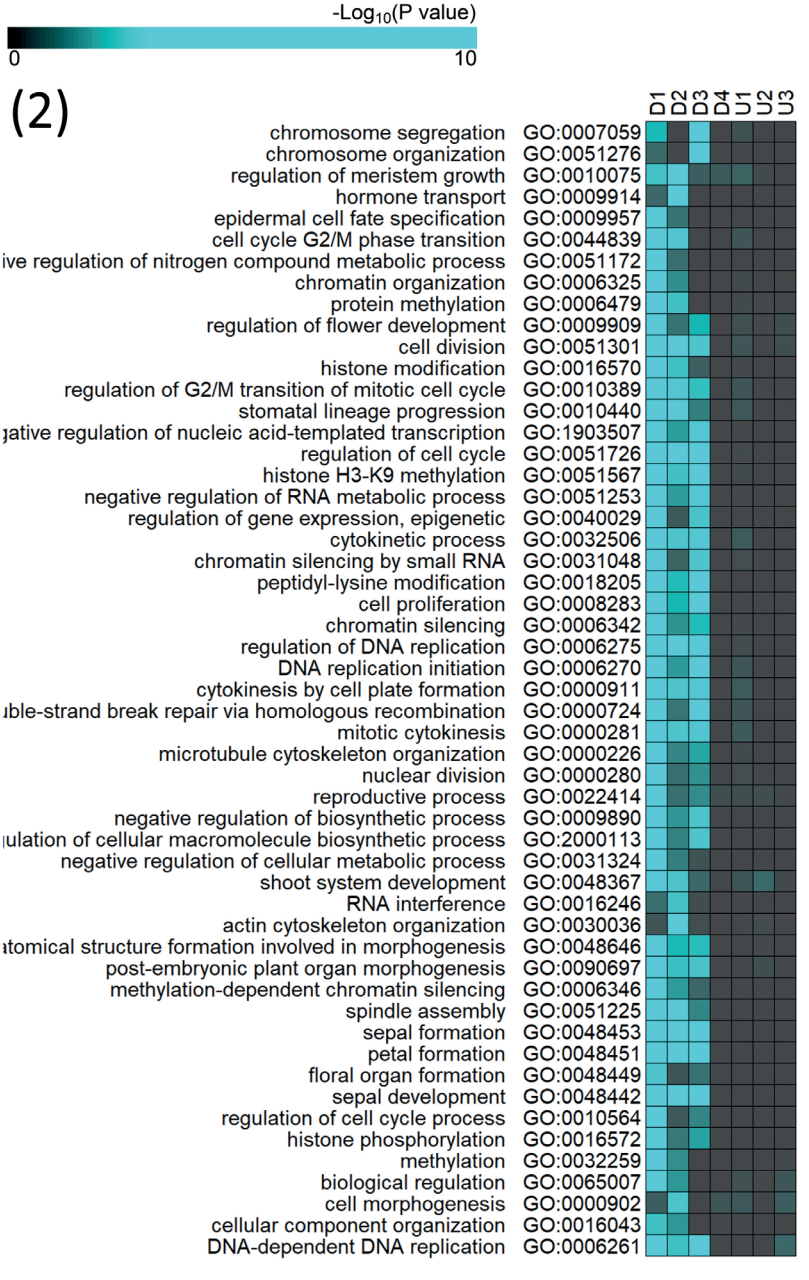
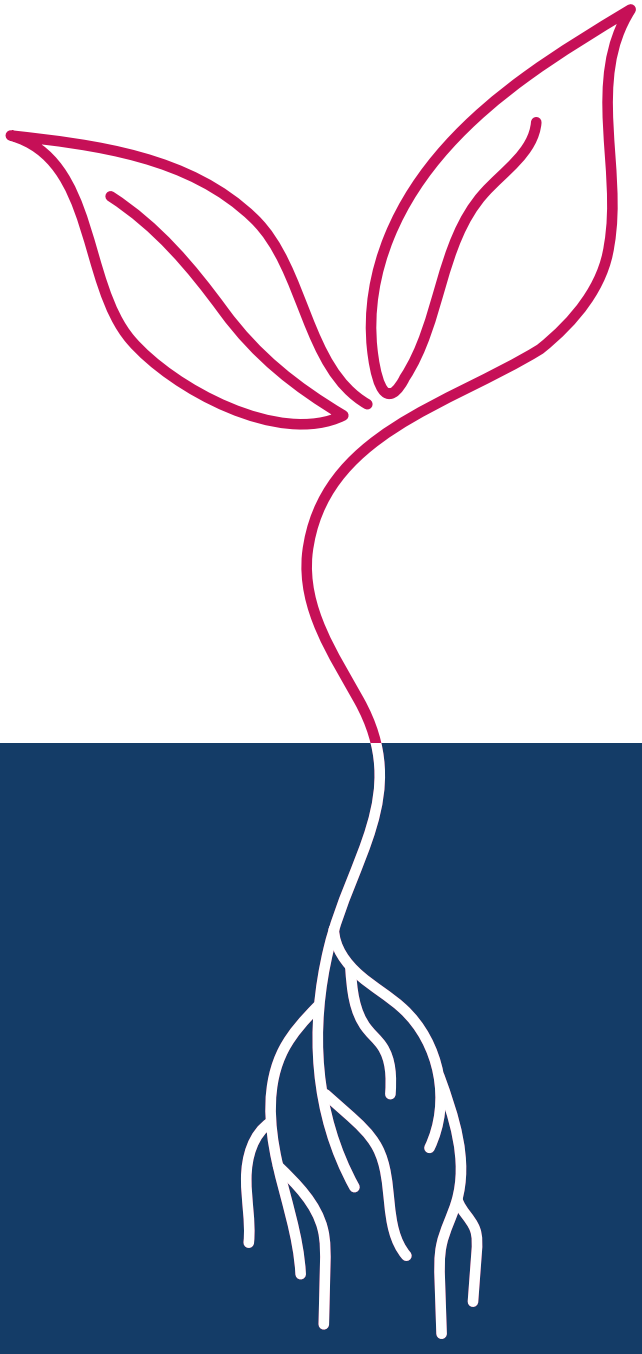


Figure S3.3 GO terms enriched from clustered ethylene specific re-oxygenation DEGs.

Figure S3.3 GO terms enriched from clustered ethylene specific re-oxygenation DEGs. GO enrichment analysis was carried out based on the clustered ethylene specific DEGs after re-oxygenation (EvA_1h_Reox). GO terms with $P < 0.001$ and GO_{counts} (count of genes) > 6 were displayed in the heatmap, where a higher cyan intensity represents a strong enrichment of relevant GO biological processes.



CHAPTER 4

Ethylene primes *Arabidopsis* root tips for coping with post-hypoxic injury during re-oxygenation

Zeguang Liu
Emilie Reinen
Laurentius ACJ Voesenek*
Rashmi Sasidharan*

*Shared senior authors

Plant Ecophysiology, Institute of Environmental Biology,
Utrecht University, Utrecht, The Netherlands

Abstract

Re-aeration following hypoxia can be stressful for plants and can affect the ability to recover. Re-oxygenation post-hypoxia is associated with production of reactive oxygen species (ROS), over-accumulation of which could result in extensive damage to plant cells. Indeed, *Arabidopsis* root tips accumulated high levels of ROS post-hypoxia corroborating induction of several re-oxygenation specific oxidative stress related gene clusters in our microarray survey. The excessive production of ROS correlated with reduced root tip survival. Accordingly, both ROS inhibition and scavenging rescued this decrease. Ethylene pre-treatment enabled the strong induction of oxidative stress related genes and root tips from ethylene pre-treated seedlings had significantly less ROS accumulation post-hypoxia. Improved hypoxia survival in ethylene pre-treated seedlings might thus be attributed to higher oxidative stress tolerance conferred by ethylene. The N-end rule mutant *prt6-1* was less sensitive to both hypoxia and oxidative stress. We speculate that in the re-oxygenation phase, N-end rule targets could potentially mediate post-hypoxia tolerance via ROS regulation.

Introduction

Plant tolerance to hypoxia depends not only on the capacity to survive oxygen (O_2) deficiency, but also on the ability to deal with the challenge of re-oxygenation during the post-hypoxia period. Several studies have shown that re-exposure to air following prolonged hypoxia further exacerbates tissue damage (Crawford, 1992; Crawford *et al.*, 1994; Vantoai and Bolles, 1991; Yeung *et al.*, 2018). Hypoxia leads to cell death, mainly as a result of changes in energy metabolism, while re-aeration of hypoxic tissues could be even more detrimental due to enhanced generation of radical species when O_2 is available again. The generation of these radicals, mostly reactive oxygen/nitrogen species, is responsible for DNA and protein damage and lipid peroxidation ultimately leading to cell death and post-anoxic injury (Crawford *et al.*, 1994; Pfister-Sieber and Brändle, 1994).

Reactive oxygen species (ROS), partially reduced or excited forms of atmospheric O_2, are thought to play dual roles in plant growth, development and responses to environmental cues (Halliwell and Gutteridge, 2015; Mittler, 2017; Yang *et al.*, 2018). On the one hand, ROS can function as signaling molecules to affect biological processes like cell elongation, meristem activity, and plant responses to biotic and abiotic stresses (Choudhury *et al.*, 2016; Huang *et al.*, 2018; Mabuchi *et al.*, 2018; Zhang *et al.*, 2017; Zhang *et al.*, 2018a). On the other hand, they are also considered as toxic by-products of aerobic metabolism responsible for tissue damage (Xiong *et al.*, 2018; Yeung *et al.*, 2018). ROS levels are normally under strict control but under certain environmental perturbations, high quantities of ROS can accumulate and lead to oxidative stress, and subsequent damage in plants (Mittler, 2017). Therefore, maintenance of ROS homeostasis is extremely crucial throughout the lifecycle of plants.

ROS homeostasis is precisely regulated by multiple production and scavenging systems in plant cells (Noctor *et al.*, 2017). As a powerful electron acceptor, O_2 could easily receive electrons from the electron transport systems (ETCs) and then transform into active radicals. In photosynthetic tissue, ROS, specifically superoxide ($O_2^{\cdot-}$) is produced mainly in chloroplasts through the photosynthetic ETC (Pospíšil, 2012). In non-photosynthetic tissues such as roots, majority of ROS production is attributed to the ETC reaction during mitochondrial respiration (Huang *et al.*, 2016). Besides, peroxisomes are also shown to be one of the main contributors for cellular ROS production in plants (Del Río and López-Huertas, 2016; Sandalio *et al.*, 2013). The most extensively studied source of ROS generation in plants is plant Nicotinamide adenine dinucleotide phosphate oxidases (NADPH oxidase), which is a membrane-bound

enzyme complex that contributes to hydrogen peroxide (H_2O_2) production by catalyzing $O_2^{\cdot-}$ formation (Kaur *et al.*, 2014; Marino *et al.*, 2012). In *Arabidopsis*, a total of 10 NADPH oxidase homologs (*RESPIRATION BURST OXIDASE HOMOLOGS*, *RBOHA-J*) with distinctive and cooperative biological functions have been identified (Sagi and Fluhr, 2006).

ROS are also continuously scavenged via multiple mechanisms and plants have evolved quite a complicated but efficient scavenging and antioxidant system to achieve balance. Enzymatic scavengers, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR) and glutathione peroxidase (GPX), together with non-enzymatic antioxidants like ascorbic acid (ASA) and glutathione (GSH) function cooperatively to maintain redox balance in plants (Foyer and Noctor, 2013; Mittler *et al.*, 2004; Noctor *et al.*, 2017).

As mentioned earlier, the emerging role of ROS as a signaling molecule has acquired much attention in the last decade. For instance, ROS was identified as a key regulator of proliferation via redox reactions in cancer cells (Cairns *et al.*, 2011; Schieber and Chandel, 2014). In plants as well, ROS were proved to be beneficial to many biological processes, and are implicated in the coordination of plant growth, development and response to environmental cues (Mhamdi and Breusegem, 2018). Since ROS production and scavenging occurs simultaneously, a strong trend in either direction would generate a signal caused by the rapid alteration of ROS levels. However, ROS overproduction and/or inefficient scavenging could lead to oxidative stress, thereby damaging DNA, proteins and lipids (Kapoor *et al.*, 2015; Karuppanapandian *et al.*, 2011).

Since ROS are generated from molecular O_2, it is plausible to speculate a limited ROS production under flooding-driven hypoxic/anoxic environments. However, several studies have shown a ROS burst under hypoxia conditions, serving as a signal to regulate downstream adaptive responses (Baxter-Burrell *et al.*, 2002; Gonzali *et al.*, 2015; Pucciariello *et al.*, 2012). For example, RHO-like small G protein of plants (Rop), guanosine triphosphatase (GTPase)-dependent H_2O_2 production through NADPH oxidase is essential for expression of *ROP GTPase ACTIVATING PROTEIN 4* (*ROPGAP4*). *ROPGAP4* is in turn essential for *ALCOHOL DEHYDROGENASE 1* (*ADH1*) induction and hypoxia tolerance (Baxter-Burrell *et al.*, 2002). Another study identified submergence and hypoxia inducible HYPOXIA RESPONSIVE UNIVERSAL STRESS PROTEIN (HRU1) that regulates H_2O_2 production during these stresses. Mutation of *HRU1* altered H_2O_2 production and reduced tolerance to submergence and hypoxia (Gonzali *et al.*, 2015).

The perfect conditions to generate O_2 radicals are low energy charge values, high reducing equivalent levels and saturated electron transport components, all of which could exist in anoxic/hypoxic plant tissues (Vantoai and Bolles, 1991). Therefore, when O_2 becomes available again upon re-oxygenation, a huge amount of ROS can be generated, benefiting from the perfect conditions created by prior hypoxia. The most susceptible molecules for radical attack are polyunsaturated fatty acid (PUFA) (Ursini *et al.*, 1991). Lipid peroxidation of PUFA is one of the major injuries occurring post-hypoxia since PUFA content is high in a lot of organelles containing biomembranes. This may eventually result in damage and death of plant cells.

Studies on ROS biology are crucial not only due to its critical role in plant growth and development, but also related to its complexity and diversity. ROS has been shown to interplay with many signaling pathways such as the well-studied mitogen-activated protein kinase cascade, and multiple plant hormones, particularly the flooding signal ethylene (Mittler *et al.*, 2011; Sasidharan *et al.*, 2018). Lysigenous aerenchyma formation under hypoxia is mediated by ethylene-driven ROS production in multiple crop species like rice, wheat and maize (Takahashi *et al.*, 2015; Yamauchi *et al.*, 2014, 2017). For example, ethylene-driven ROS accumulation was identified to be responsible for the parenchymal cell death giving rise to stem aerenchyma formation in deepwater rice (Steffens *et al.*, 2012). Furthermore, a few recent studies reported that interplay between ethylene and ROS contribute to *Arabidopsis* seedling tolerance to re-oxygenation post-anoxia (Tsai *et al.*, 2014). Mutation of *ETHYLENE INSENSITIVE 2* (*EIN2*, *ein2-5*) AND *EIN3/EIN3-LIKE 1* (*EIL1*) (*ein3eil1*) displayed enhanced post-anoxic injury compared to wild type Col-0. Meanwhile, induction of ROS scavenging genes suggested the potential role of ethylene in ROS elimination.

In chapter 3 of this study, a hierarchical cluster analysis of transcript data generated from ethylene pre-treated and control roots revealed a strong enrichment of oxidative stress related gene ontology (GO) terms in response to ethylene. Particularly after re-oxygenation, genes encoding heat shock proteins, and those responsive to H_2O_2 and high light were induced significantly more pronounced when seedlings were pre-treated with ethylene prior to hypoxia than those that were not. This suggested that the higher survival of ethylene pre-treated seedlings could partly be attributed to higher tolerance to oxidative stress that is beneficial upon re-aeration following hypoxia.

To study the potential function of ethylene on oxidative stress alleviation post-hypoxia, we first investigated whether: (i) there is ROS accumulation post-hypoxia (ii) post-hypoxic ROS

is detrimental to root tip survival and (iii) post-hypoxic ROS accumulation was reduced in ethylene pre-treated seedlings. Finally, we also investigated whether this ethylene-mediated regulation of ROS amelioration was dependent on the N-end rule pathway. Our results demonstrate that ethylene pre-treatment increases root tip survival partially by regulating ROS dynamics during post-hypoxic conditions.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana wild type Col-0 and mutants *rbohD-3* (NASC, N9555), *PROTEOLYSIS 6* defect *prt6-1* (SAIL 1278_H11) were sterilized, sown and grown as described in chapter 2. For all experiments 4-day-old seedlings were used.

Heatmap of GO clusters

Scaled Log_2 Fold Change (Log_2FC) values from the transcript data calculated in Chapter 3 were displayed on a heatmap using R.

Quantitative real-time qPCR

Half centimeter of 4-day-old Col-0 roots were harvested and approximately 184 roots from 4 vertical agar plates (from 2 desiccators) were pooled together as one biological replicate for RNA extraction. Complementary DNA (cDNA) was synthesized from RNA in a two-step reaction by Reverse transcriptase. For the first step, 50 µM random hexamer primers (Thermo Fisher Scientific) was added together with RNA to start the 5-minutes initial denaturation at 65°C. In the second step, a mix including 5× buffer, 10 mM dNTPs (Thermo Fisher Scientific), 40 units·µL^{-1} RNase inhibitor (Thermo Fisher Scientific) and 200 units·µL^{-1} RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific) was added to continue with the following processes: annealing at 25°C for 10 minutes, cDNA synthesis at 42°C for 60 minutes and inactivation at 70°C for 10 minutes. Synthesized cDNA was diluted into 5 ng·µL^{-1} and stored at -20°C. Applied Biosystems ViiA 7 Real-Time PCR system (Thermo Fisher Scientific) was used to perform qRT-PCR. The reaction solution of qRT-PCR contains 2.5 µL 2× SYBR Green MasterMix (Bio-Rad), 0.25 µL of both forward and reverse primer (10 µM) and 2 µL of 5ng·µL^{-1} cDNA. Relative transcript abundance was calculated by the comparative C_T method (Livak and Schmittgen, 2001). Three genes (*ADENINE PHOSPHORIBOSYL TRANSFERASE 1* (*APT1*), *AT5G25760* and *AT1G13320*) were used as reference genes to calculate relative expression.

3,3′-Diaminobenzidine (DAB) staining for H_2O_2 visualization

DAB is oxidized by H_2O_2 in the presence of peroxidases to generate a dark brown precipitate (Thordal-Christensen *et al.*, 1997). Seedlings were incubated with 1 mg·mL^{-1} DAB (Sigma-Aldrich) in 20 mM 2-ethanesulfonic acid (MES, Sigma-Aldrich) buffer (pH 6.2) supplemented by 10 units·mL^{-1} peroxidase from horseradish (Sigma-Aldrich) for 1 h. Then seedlings were rinsed with MES buffer for 1 minute twice (Dubreuil-Maurizi *et al.*, 2011).

H_2O_2 treatment

H_2O_2 (30% w/w, Merck KGaA) was diluted into ¼ MS to achieve desired concentrations. For application, 5 µL of H_2O_2 solution was added to each root tip in the dark. Hereafter, agar plates were kept horizontal for 15 minutes to allow the roots to absorb the solution.

2′,7′-Dichlorofluorescin diacetate (H_2DCFDA) staining for ROS visualization

H_2DCFDA could be deacetylated by cellular esterase and then subject to oxidization by ROS to 2′,7′-dichlorofluorescein (DCF), which is highly fluorescent and could be detected under excitation and emission spectra of 492-495 nm and 517-527 mm, respectively. H_2DCFDA (Sigma-Aldrich) was dissolved into dimethyl sulfoxide (DMSO, VWR Life Science) to make a 60 mM stock, stored at -20°C (up to 2-3 weeks) in the dark before use. We used always freshly made H_2DCFDA. Seedlings were incubated in 50 µM H_2DCFDA (0.08% DMSO) dissolved in 20 µM potassium phosphate buffer (pH 6.0) for 15 minutes. After incubation, seedlings were rinsed with potassium phosphate buffer 3 times, 1 minute each time, before mounted for microscopy (Yang *et al.*, 2014).

Confocal microscopy and fluorescence quantification

Seedlings were cut to remove shoots before being mounted onto slides for confocal observation. Samples from different treatments were mounted onto the same slide to avoid background variation. Samples were imaged with Zeiss LSM700 confocal microscope (excitation 488 mm, emission 500-550 mm). Z-stack images were saved as CZI files and analyzed with Icy image software (http://icy.bioimageanalysis.org/).

Potassium iodide (KI) treatment

KI acts as a catalyst in the decomposition of H_2O_2 into H_2O and O_2. KI (Merck KGaA) was dissolved into ¼ MS to achieve desired concentration. For application, 5 µL of KI solution was added to each root tip in the dark before start of pre-treatment. Agar plates were kept horizontal for 15 minutes in the dark to allow the roots to absorb the solution after which they were transferred to the light (5 µmol·m^{-2}·S^{-2}) and kept vertically where pre-treatment was done.

Diphenyleneiodonium (DPI) treatment

DPI (Sigma-Aldrich), known to inhibit NADPH oxidase, was dissolved into ¼ MS to achieve desired concentration. For application, 5 µL of DPI solution was added to each root tip in the dark before start of the pre-treatment. Agar plates were kept horizontal for 15 minutes in the dark to allow the roots to absorb the solution after which they were transferred to the light (5 $\mu mol \cdot m^{-2} \cdot S^{-2}$) and kept vertically where pre-treatment was done.

Survival scoring and data analysis

Root tip survival was scored and analyzed as described in chapter 2. Statistics used in this chapter were student's t-test, one-way ANOVA and two-way ANOVA with Tukey's Post-hoc test ($p<0.05$).

Results

Hierarchical cluster analysis identifies ROS related GO terms post-hypoxia

In the transcriptomic study of ethylene-mediated hypoxia tolerance described in chapter 3, several GO clusters were identified for the re-oxygenation phase. These were significantly enriched post-hypoxia in both air and ethylene pre-treated seedlings. Interestingly, a total of 19 genes from these GO clusters, including oxidative stress (GO:0006979), heat stress (GO:0009408), high light intensity (GO:0009644) and H_2O_2 (GO:0042542), were not clearly induced and separated between the air and the ethylene pre-treated roots during the pre-treatment and the hypoxia period of the experiment. It was only at the 1 h re-oxygenation time point that they were highly induced in air pre-treated seedlings and even more pronounced in ethylene pre-treated ones (Figure 4.1). Based on the presence of several oxidative stress related clusters (Chapter 3, Table 3.4, Figure S3.3), and the clear increase in cell death upon re-oxygenation (Chapter 2, Figure 2.5F), we hypothesized that (i) root tips of hypoxia treated seedlings experience oxidative stress upon re-oxygenation and (ii) increased hypoxia survival of ethylene pre-treated seedling root tips is partly due to improved oxidative stress tolerance during re-oxygenation.

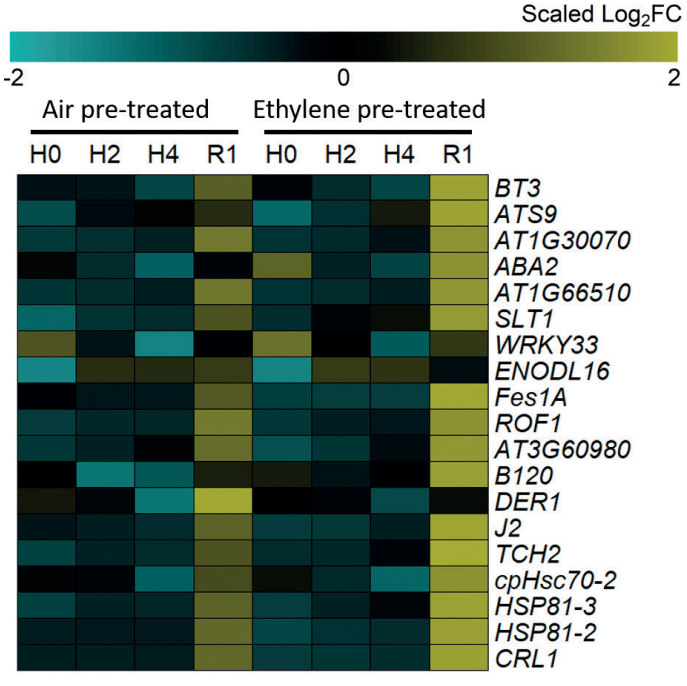


Figure 4.1 Heatmap of re-oxygenation specific oxidative stress related genes.
Genes from oxidative stress related clusters that were induced upon re-oxygenation in the transcriptomic analyses from Chapter 3 are shown. Genes belong to the following GO terms: response to oxidative stress (GO:0006979), response to heat stress (GO:0009408), response to high light intensity (GO:0009644) and response to H_2O_2 (GO:0042542). For each gene scaled Log_2FC at 0 h, 2 h, 4 h hypoxia, and 1 h re-oxygenation in air or ethylene pre-treated root tips is shown and is indicated by color scale from -2 (cyan) to 2 (yellow).

To further confirm the enrichment of oxidative stress related clusters, 15 genes were selected based on strongest fold change from the 19 identified genes stated above for qRT-PCR validation. As expected, of the 15 genes studied, 14 were not differentially expressed at the end of 4 h hypoxia period (0 h post-hypoxia) in both air and ethylene pre-treated root tips (Figure 4.2). Only *TOUCH 2* (*TCH2*) was significantly induced by the early ethylene exposure compared to the air control. At 1 h post-hypoxia, and corresponding with the microarray sampling point, 11 genes showed post-hypoxic up-regulation. Of these 5 genes were already induced with air pre-treatment and 9 genes had a higher induction in ethylene pre-treated samples. At 2 h post-hypoxia most genes showed a reduction in transcript abundance relative to the 1 h time point and the ethylene effect was lost. However, the transcript abundance for 4 genes was still higher than the 4 h hypoxia (0 h post-hypoxia) time point (Table 4.1).

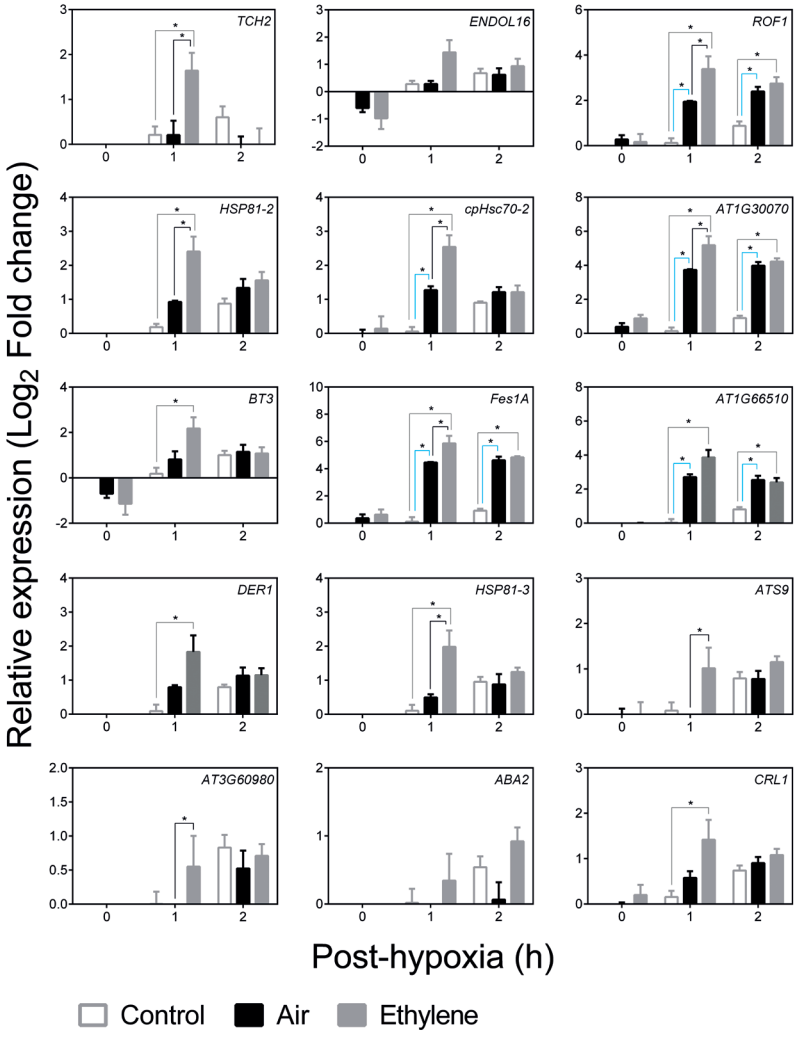


Figure 4.2 Relative expression of ROS related genes post-hypoxia.
The expression of selected re-oxygenation genes was validated using qRT-PCR. These genes were significantly ethylene-induced upon re-oxygenation in the hierarchical cluster analysis. Data shown is mean ± SE, n=3 (Biological replicates). For each biological replicate, a total of approximately 184 roots from 4 plates (from 2 desiccators) with each containing 46 seedlings were pooled together. Asterisks indicate statistically significant differences. Blue lines and gray lines indicate genes that showed a significant induction upon re-oxygenation in air or ethylene pre-treated samples, respectively, relative to control samples. Black lines indicate significant differences between air and ethylene pre-treated samples. Statistical significance was calculated using a two-way ANOVA with tukey's Post-hoc test, p<0,05.

Table 4.1 List of post-hypoxia responsive genes

Post-hypoxia (h)	Effect	NO.	List of genes
0	Re-oxygenation	0	
	Ethylene+Re-oxygenation	1	*TCH2*
	Ethylene	0	
1	Re-oxygenation	5	*ROF1, cpHsc70-2, AT1G30070, Fes1A, AT1G66510*
	Ethylene+Re-oxygenation	11	*TCH2, ROF1, HSP81-2, cpHsc70-2, AT1G30070, BT3, Fes1A, AT1G66510, DER1, HSP81-3, CRL1*
	Ethylene	9	*TCH2, ROF1, HSP81-2, cpHsc70-2, AT1G30070, Fes1A, HSP81-3, ATS9, AT3G60980*
2	Re-oxygenation	4	*ROF1, AT1G30070, Fes1A, AT1G66510*
	Ethylene+Re-oxygenation	4	*ROF1, AT1G30070, Fes1A, AT1G66510*
	Ethylene	0	

Ethylene pre-treatment prevents ROS accumulation occurring in primary root tips post-hypoxia

We investigated whether there is ROS accumulation in root tips of hypoxia treated seedlings upon re-aeration and if this is influenced by an ethylene pre-treatment. For this, 4-day-old air or ethylene pre-treated *Arabidopsis* seedlings were stained with DAB to detect H_2O_2 upon re-oxygenation following 4 h hypoxia (Figure 4.3A). At 0 h post-hypoxia, air pre-treated root tips already showed intensive brown staining likely indicating hypoxia induced ROS. In contrast, very few ethylene pre-treated root tips showed this ROS staining. At later re-oxygenation time points this trend persisted. Ethylene pre-treated root tips showed barely any staining suggesting low levels of ROS. Air pre-treated seedlings in contrast showed relatively higher ROS levels at both 2 h and 4 h post-hypoxia (Figure 4.3B). It can be concluded that the ethylene pre-treatment induces changes that prevent excessive ROS accumulation both during hypoxia and upon re-oxygenation.

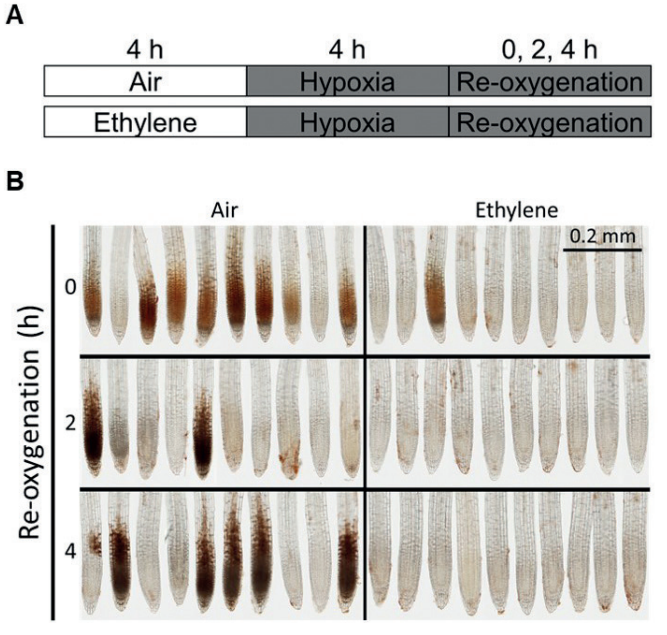


Figure 4.3 Ethylene pre-treatment limits re-oxygenation induced H_2O_2 accumulation in *Arabidopsis* Col-0 seedlings.
(**A**) Experimental scheme to monitor ROS accumulation through DAB staining upon re-oxygenation. Four-day-old seedlings were given 4 h of air or ethylene pre-treatment followed by 4 h hypoxia. Seedlings were stained for H_2O_2 using DAB at 0, 2 and 4 h of re-oxygenation. (**B**) DAB staining of air or ethylene pre-treated seedling root tips upon 0, 2, 4 h re-oxygenation following 4 h hypoxia. Experiments were repeated 3 times with similar results.

Ethylene pre-treatment improves oxidative stress tolerance in root tips

The reduction in ROS production upon re-oxygenation suggests a potential function of ethylene in alleviating oxidative stress during post-hypoxic conditions. We next investigated whether ethylene is beneficial for coping with oxidative stress. For this, 4-day-old *Arabidopsis* Col-0 seedlings were given 6 mM H_2O_2 following an air or ethylene pre-treatment (Figure 4.4A), to induce oxidative stress. Root tip survival was analyzed based on method described in chapter 2 (Figure 2.1A). As expected, ethylene pre-treated root tips had a significantly higher survival than air pre-treated ones (Figure 4.4B). Evans blue staining after 1 day of H_2O_2 treatment further confirmed the increased root tip survival in ethylene pre-treated seedlings (Figure 4.4D). Reduced ROS levels in ethylene pre-treated seedlings were confirmed using both histochemical (DAB) and fluorescent (H_2DCFDA) staining to visualize H_2O_2. Staining in both cases was significantly intensified when ethylene pre-treatment was absent (Figure 4.4C&E&F), confirming the beneficial function of ethylene in plant responses to oxidative stress.

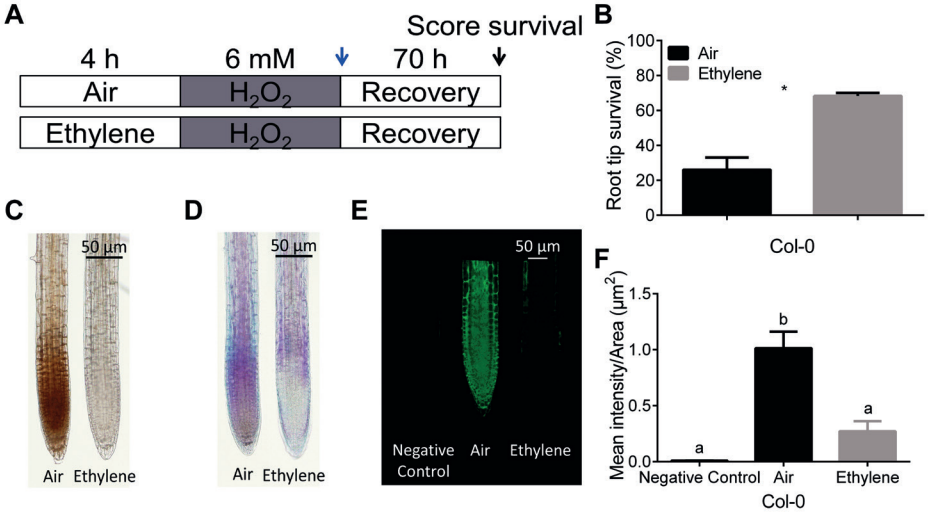


Figure 4.4 Ethylene pre-treatment improves oxidative stress tolerance in *Arabidopsis* Col-0 seedlings.

(**A**) Experimental scheme to study the effect of ethylene on oxidative stress tolerance. Four-day-old *Arabidopsis* Col-0 were given 4 h of air or ethylene pre-treatment followed by 6 mM of H_2O_2. Root tip survival was scored after 70 h recovery. Blue arrow indicates the harvesting time (2 h after application of H_2O_2 root tips) of samples for histochemical and fluorescent detection of H_2O_2. (**B**) Root tip survival of Col-0 upon 6 mM H_2O_2. Data shown is mean ± SE, n=3 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Asterisk indicates statistically significant differences. Statistical significance was calculated using a student's t-test, p<0,05. (**C**) DAB staining of 4-day-old air or ethylene pre-treated Col-0 roots after 2 h of 6 mM H_2O_2 treatment. Experiment was repeated 3 times with similar results. (**D**) Evans blue staining of 4-day-old Col-0 roots upon 6 mM H_2O_2 treatment after air or ethylene pre-treatment. Staining was done after 1 day of H_2O_2 application. Experiment was repeated 3 times with similar results. (**E**) Fluorescent dye detection of ROS in 4-day-old Col-0 root tips after 2 h of 6 mM H_2O_2 treatment after air or ethylene pre-treatment. Experiment was repeated 4 times with similar results. (**F**) Quantification of fluorescent signal from (**E**). Data shown is mean ± SE, n=12 (Biological replicate). Each replicate represents quantitative fluorescent of one root. Different letters indicate statistically significant differences. Statistical significance was calculated using a one-way ANOVA with tukey's Post-hoc test, p<0,05.

Excessive ROS accumulation is detrimental to hypoxia root tip survival

The above experiments suggested that ethylene pre-treatment enhanced root tip survival partially via alleviation of oxidative stress from ROS overproduction upon re-oxygenation. To investigate whether post-hypoxic ROS production is detrimental to seedling survival, genetic and chemical tools were used to manipulate ROS production and scavenging.

In *Arabidopsis*, the plasma membrane NADPH oxidase, or RBOHs family, is one of the main contributors to ROS production (Kaur *et al.*, 2014; Marino *et al.*, 2012). Together with RBOHF, RBOHD mediates the spatial control of reactive oxygen intermediates production and hypersensitive responses. The *rbohD-3* mutant has severely reduced ROS production (Torres *et al.*, 2002). The hypoxia tolerance of *rbohD-3* mutant was tested using the root tip survival assay described in chapter 2 (Figure 2.1A, Figure 4.5A). Clearly root tip survival of *rbohD-3* upon 4 h hypoxia was significantly higher than air-pretreated wild type Col-0 seedlings. Furthermore, air pre-treated *rbohD-3* seedlings root tips had survival rates comparable to ethylene pre-treated wild type Col-0 seedlings (Figure 4.5B). To check whether indeed ROS production was reduced in *rbohD-3* mutant, seedlings were stained with DAB (Figure 4.5C). As expected, ROS accumulation in *rbohD-3* was much lower upon hypoxia and the 4 h re-oxygenation time point than wild type Col-0 (Figure 4.5D).

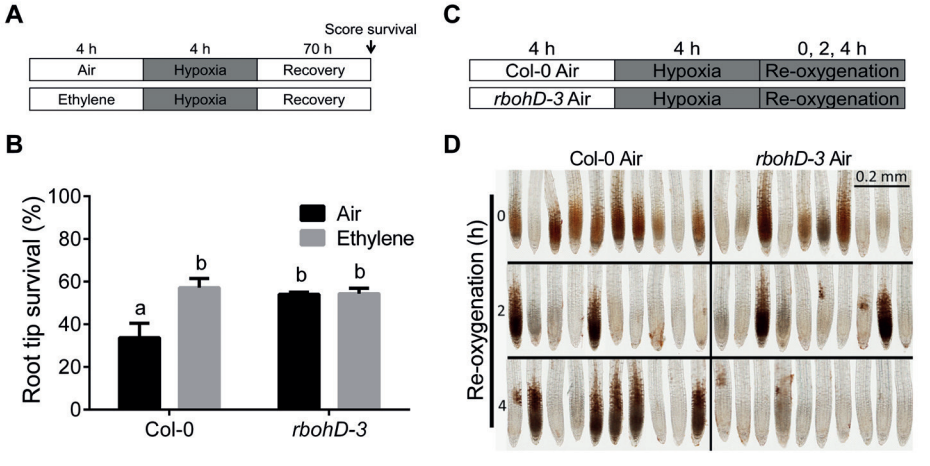


Figure 4.5 An NADPH oxidase mutant with impaired ROS production has improved root tip survival.
(**A**) Experimental scheme to study root tip survival in Col-0 and NADPH oxidase mutant *rbohD-3* upon hypoxia. (**B**) Root tip survival of Col-0 and *rbohD-3* upon 4 h hypoxia. Data shown is mean ± SE, n=3 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Different letters indicate statistically significant differences. Statistics significance was calculated using a two-way ANOVA with tukey's Post-hoc test, $p<0,05$. (**C**) Experimental scheme to study ROS accumulation upon re-oxygenation. Four-day-old Col-0 and *rbohD-3* seedlings were given 4 h of air pre-treatment followed by 4 h hypoxia. Seedlings were taken for DAB staining detection of ROS accumulation at 0, 2 and 4 h of re-oxygenation. (**D**) DAB staining of air pretreated Col-0 and *rbohD-3* seedlings upon 0, 2, 4 h re-oxygenation following 4 h hypoxia. Experiment was repeated 3 times with similar results.

Chemical inhibition of NADPH oxidase activity by DPI (Cross and Jones, 1986) showed increased root tip survival as well (Figure S4.2A&B). Root tips of DPI treated seedlings had higher hypoxia survival rates than mock treated controls. The beneficial effect of DPI was comparable to that of root tip survival rates of ethylene pre-treated seedlings. ROS production in DPI treated Col-0 root tips was clearly lower than air pre-treated roots at the first moment seedlings returned to normoxia as well (Figure S4.2C&D).

Next we manipulated ROS levels by applying a ROS scavenger. For this KI, a common ROS scavenger (Liszkay *et al.*, 2004), was used to study root tip survival upon hypoxic stress (Figure 4.6A). When 4-day-old Col-0 seedlings were pre-treated with different concentrations of KI (1 mM, 5 mM), root tip survival was significantly higher than seedlings without KI upon 4 h hypoxia. Root tips of seedlings pre-treated with KI had equal survival rates as those of ethylene pre-treated ones (Figure 4.6B). The visualization of ROS production upon re-oxygenation in KI treated seedlings showed no differences at 0 h and 2 h post hypoxia (Figure 4.6C&D). However, at the 4 h re-oxygenation time point, ROS accumulation in KI pre-treated plants were lower than air pre-treated ones.

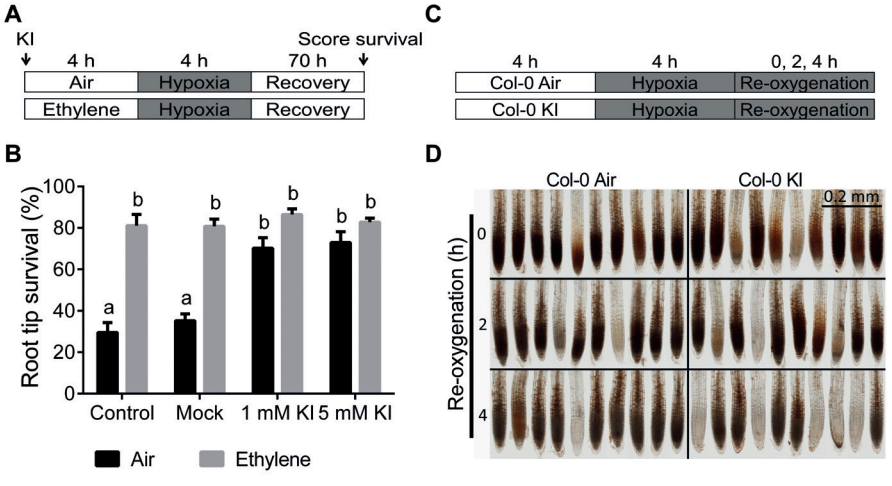


Figure 4.6 Chemical scavenging of ROS improves root tip survival.
(**A**) Experimental scheme to study effect of KI on root tip survival in 4-day-old Col-0 seedlings. (**B**) Root tip survival upon 4 h hypoxia following KI treatment. Data shown is mean ± SE, n=5 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Different letters indicate statistically significant differences. Statistical significance was calculated using a two-way ANOVA with tukey's Post-hoc test, p<0,05. (**C**) Experimental scheme to study ROS accumulation upon re-oxygenation. Four-day-old Col-0 seedlings were given 4 h of air or KI pre-treatment followed by 4 h hypoxia. Seedlings were taken for DAB staining detection of ROS accumulation at 0, 2 and 4 h of re-oxygenation. (**D**) DAB staining of air or KI pre-treated Col-0 seedlings upon 0, 2, 4 h re-oxygenation following 4 h hypoxia. Experiment was repeated 3 times with similar results.

In conclusion, reduction of ROS levels either through chemical or genetic means improved hypoxia survival of root tips. This suggests that limiting ROS levels upon re-aeration is beneficial for root tip survival post-hypoxia.

Potential regulation of ROS homeostasis via N-end rule pathway

As discussed in chapter 3, N-end rule regulation of protein degradation and group VII ethylene response factors (ERFVIIs) may play a role in ethylene-mediated hypoxia tolerance. To investigate the relevance of the N-end rule pathway, we tested hypoxia root tip survival of *prt6-1*. *Prt6-1* contains a mutation in the E3 ubiquitin protein ligase PRT6, and thereby has increased stabilization of N-end rule substrates including ERFVIIs (Garzón *et al.*, 2007; Gibbs *et al.*, 2011; Licausi *et al.*, 2011). As expected, root tips of *prt6-1* seedlings had higher hypoxia (4 h) tolerance than wild type Col-0. Meanwhile, root tip survival of both air and ethylene pre-treated *prt6-1* was comparable to ethylene pre-treated Col-0 seedlings, suggesting the potential involvement of N-end rule pathway in ethylene-enhanced hypoxia tolerance (Figure 4.7A).

We hypothesized that enhanced survival observed in *prt6-1* seedlings might be at least partly be due to improved tolerance to oxidative stress during re-oxygenation. To test this, oxidative stress was imposed by treating *prt6-1* seedlings with H_2O_2. Root tips survival of seedlings treated with H_2O_2 was significantly higher in *prt6-1* than Col-0 (Figure 4.7B&C). These suggested that the ethylene regulation of ROS levels could be partially dependent of N-end rule pathway.

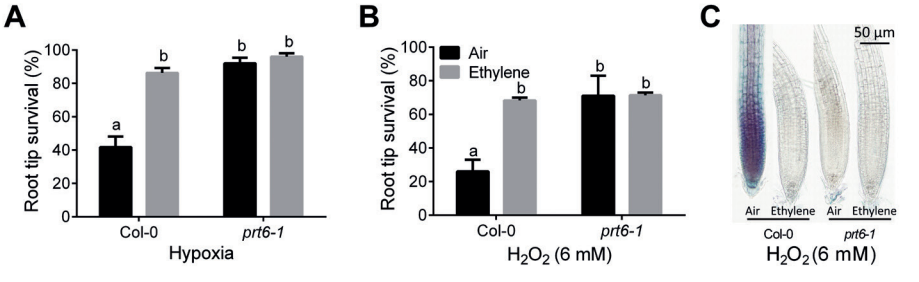


Figure 4.7 N-end-rule dependent regulation of root tip survival.
(**A**) Root tip survival of 4-day-old Col-0 and E3 ubiquitin-protein ligase PRT6 defective mutant *prt6-1* upon 4 h hypoxia. Data shown is mean ± SE, n=8 (Biological replicates). (**B**) Root tip survival of 4-day-old Col-0 and *prt6-1* mutant upon 6 mM H_2O_2 stress. After 4 h air/ethylene pre-treatment, 6 mM H_2O_2 was applied to root tips in the dark. Survival was scored after 3 days recovery. Data shown is mean ± SE, n=3 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Different letters in A&B indicate statistically significant differences. Statistical significance was calculated using a two-way ANOVA with tukey's Post-hoc test, $p<0,05$. (**C**) Evans blue staining of 4-day-old *Arabidopsis* Col-0 and *prt6-1* after 1 day of 6 mM H_2O_2 stress.

Discussion

As evidenced by increased cell death (Chapter 2, Figure 2.5), hypoxia survival is determined by acclimation to both hypoxia and re-oxygenation. Post-hypoxia, plants typically suffer from oxidative stress caused by re-oxygenation and respond accordingly (Bailey-Serres and Chang, 2005; Irfan *et al.*, 2010). The ability to maintain ROS homeostasis during hypoxia and post-hypoxia greatly determines overall tolerance (Fukao *et al.*, 2012; Paradiso *et al.*, 2016). In the seedling survival assay used here, a clear increase in ROS was detected in root tips upon re-aeration following hypoxia stress (Figure 4.3B). ROS accumulation already occurred at the end of the hypoxia treatment and increased during re-oxygenation. This ROS over-accumulation is damaging for cells and correlated with cell death in root tips. When ROS levels were reduced by either genetic or chemical means, this clearly benefitted survival (Figure 4.5B, Figure 4.6B, Figure S4.2B). The regulation of ROS production is an important aspect of hypoxia survival. ROS has been demonstrated to be essential for stress signaling during hypoxia (Baxter-Burrell *et al.*, 2002; Gonzali *et al.*, 2015; Pucciariello *et al.*, 2012). A burst in H_2O_2 production occurred in *Arabidopsis* seedlings during anoxia, and was shown to be essential for anoxia survival (Pucciariello *et al.*, 2012). In the system used here, the *rbohD-3* mutant had improved hypoxia survival (Figure 4.5). Interestingly, other allelic mutants of *RBOHD* demonstrate decreased survival during hypoxia (Pucciariello *et al.*, 2012). Evidently, the relationship between ROS and hypoxia survival is complex and depends on the tissue affected, ROS spatial and temporal dynamics and the overall effect of hypoxia and re-oxygenation. Although limiting ROS over-accumulation is essential to avoid oxidative stress, ROS maintenance at a certain level is also required towards its role as a signaling molecule modulating growth and development (Yang *et al.*, 2018). A recent study suggested distributional regulation of ROS levels contribute to the maintenance of root stem cell niche through *Root Meristem Growth Factor 1* (*RGF1*) (Yamada *et al.*, 2018). As a critical aspect of development, the balance between cell proliferation and differentiation was shown to be controlled by a transcription factor, UPBEAT1 (UPB1), in *Arabidopsis* (Tsukagoshi *et al.*, 2010). This regulation was achieved by transcriptional fine-tuning of ROS homeostasis in the root, failure of which disrupted the initiation of differentiation.

The ROS over-accumulation observed in root tips after hypoxia, was significantly decreased in ethylene pre-treated seedlings. This suggested that the ethylene treatment triggers changes that facilitate maintenance of ROS homeostasis and oxidative stress adaptability (Figure 4.3B). Hierarchical cluster analysis based on transcriptional differences between air and ethylene pre-treated seedlings uncovered GO terms like oxidative stress, H_2O_2 and

heat stress responses during the re-oxygenation phase (Figure 4.1; Chapter 3, Table 3.4, Figure S3.3). Additionally, the qRT-PCR verification further confirmed that a broad range of oxidative stress related genes responded to not only re-oxygenation but more importantly, to ethylene (Figure 4.2, Table 4.1). Specifically, 5 genes responded to re-oxygenation at 1 h post-hypoxia and 4 of them stayed highly expressed at 2 h post-hypoxia. These genes are likely part of molecular processes to adapt to oxidative stress when seedlings return to normoxia (Figure 4.2, Table 4.1). When seedlings were pre-exposed to ethylene prior to hypoxia, expression changes of tested genes to re-oxygenation were much more enhanced. A total of 11 genes were induced at 1 h post-hypoxia compared to air control. Furthermore, 9 genes were highly induced by ethylene compared to air pre-treated seedlings. These included typical oxidative stress and heat stress responsive genes, which share some common responses like the induction of Heat Shock Proteins (HSPs) (Dat *et al.*, 1998; Lee and Vierling, 2000). Induction of heat shock genes under multiple stresses were proposed to be attributed to ROS generation (Swindell *et al.*, 2007). H_2O_2 was produced under anoxia as well (Blokhina *et al.*, 2001; Gonzali *et al.*, 2015). *HSP81-2* and *HSP81-3*, belonging to *HSP90* gene family, were both induced at 1 h post-hypoxia in ethylene pre-treated root tips and could play redundant roles in plants response to re-oxygenation. *HSP90* genes have been shown to play multiple roles in development and response processes in plants (Koning *et al.*, 1992; Marrs *et al.*, 1993). HSP90 was reported to interact directly with and affect heat shock factor activity in tomato (Hahn *et al.*, 2011). Therefore, the induction of the two *HSP90* genes could potentially benefit plants by interacting with other regulators to adapt to re-oxygenation. The genes *Fes1A* and *ROTAMASE FKBP 1* (*ROF1*) were induced by ethylene, but also responded to re-oxygenation. *Fes1A* is the ortholog of human HSP70-binding protein in *Arabidopsis* and is required for heat stress responses. *ROF1* has been shown to interact with *HSP90* gene family in response to heat (Meiri and Breiman, 2009).

The higher induction of oxidative stress related genes in ethylene pre-treated seedlings upon re-oxygenation correlated with the significantly reduced ROS accumulation post-hypoxia and the corresponding increase in root tip survival (Figure 4.3B). Thus the beneficial effect of early ethylene exposure is at least partly attributed to the ability to adapt to re-oxygenation stress post-hypoxia. Some previous studies have shown the critical roles of ethylene in re-aeration. Ethylene signaling *Arabidopsis* mutants have a compromised hypoxia and recovery response. A comparison of the transcriptome responses of wild-type and ethylene insensitive mutants *ein2-5* and *ein3eil1* revealed an essential role of ethylene in regulation of gene expression profiling in response to re-aeration (Tsai *et al.*, 2014). Genes regulated by ethylene in the recovery phase included those responsive to high light, H_2O_2

and high temperature, and potentially linked to managing ROS increase during re-aeration. In the current study, the application of ethylene prior to hypoxia conferred an advantage in coping with re-oxygenation stress post-hypoxia. This suggests that during the ethylene treatment already molecular or possibly epigenetic changes occur that prime the cells for upcoming hypoxic and post-hypoxic stress. For example, a higher antioxidant status already during hypoxia could mean that hypoxic tissues are ready for recovery conditions (Figure 3.5, Figure S3.3).

Ethylene pre-treatments have also been shown to improve tolerance to salt stress. A gain- and loss-of-function study of the ethylene activated transcription factors *EIN3* and *EIL1* demonstrated how either ethylene pre-treatment or EIN3 activation enhanced salinity tolerance in *Arabidopsis* (Peng *et al.*, 2014). After growing on 1-aminocyclopropane-1-carboxylic acid supplemented MS medium for 4 days, plants displayed increased salinity tolerance dependent on the induction of Salt-Induced and *EIN3/EIL1*-Dependent genes, including ROS metabolism genes. *EIN3* also directly enhanced peroxidase activity transcriptionally. Accordingly, an early ethylene signal was beneficial for salinity tolerance via alleviation of oxidative stress.

In this study as well, it seems the early ethylene signal contributed to improved root tip survival via oxidative stress alleviation. It maintained the viability of apical root meristem to allow plants to recover from the stress. However, the mechanism via which ethylene does so requires further investigation. For example, it is unclear what the upstream regulators of the re-oxygenation response are and whether changes occurring already during hypoxia strongly influence post-hypoxic responses. In *Arabidopsis*, RELATED TO AP2 12 (RAP2.12), belonging to the ERFVIIs family, initiates transcriptional induction of downstream targets dependent on a ROS burst mediated by *RBOHD* (Gonzali *et al.*, 2015). H_2O_2 induction is required for the expression of *ADH1*, direct targets of RAP2.12, in a ROP-dependent manner (Baxter-Burrell *et al.*, 2002; Gasch *et al.*, 2015). However, mediation of ROS homeostasis and its relevance during re-oxygenation is still largely unclear. The mutation of PRT6, a key enzyme of the N-end rule proteolysis pathway, improved hypoxia root tip survival, suggesting the potential involvement of N-end rule substrates in ethylene-mediated hypoxia tolerance (Figure 4.7A). When 2 h re-oxygenated *prt6-1* seedlings were stained for ROS, staining was clearly reduced compared to Col-0 (Figure S4.3). However, *HSP81-2*, which displayed decreased N-terminal peptides abundance in *prt6* (Zhang *et al.*, 2018b), was shown to be induced in re-oxygenation in this study. It could be that the redundancy of many HSPs masked the decrease of HSP81-2. In addition, *prt6-1* also showed higher oxidative stress

tolerance than wild type Col-0 (Figure 4.7B&C). This could mean that N-end rule proteolysis targets are part of the regulatory pathway to adapt to oxidative stress upon re-aeration. However, it is important to note that, in principle, all N-end rule targets are stabilized under hypoxia and subject to degradation upon re-aeration when O_2 is available again. Since ERFVIIs are known to be key regulators of hypoxia adaptation (Gasch *et al.*, 2015; Gibbs *et al.*, 2011, 2015; Kosmacz *et al.*, 2015; Licausi *et al.*, 2011), it is interesting to know whether the early ethylene signal affect the stability of ERFVIIs, and the stabilized ERFVIIs during hypoxia could have some residual effect upon recovery.

Acknowledgement

Peng Su helped with the ROS staining and manipulation experiments. We thank members of the Plant Ecophysiology group for help with tissue harvesting for gene expression analysis.

Supplemental data

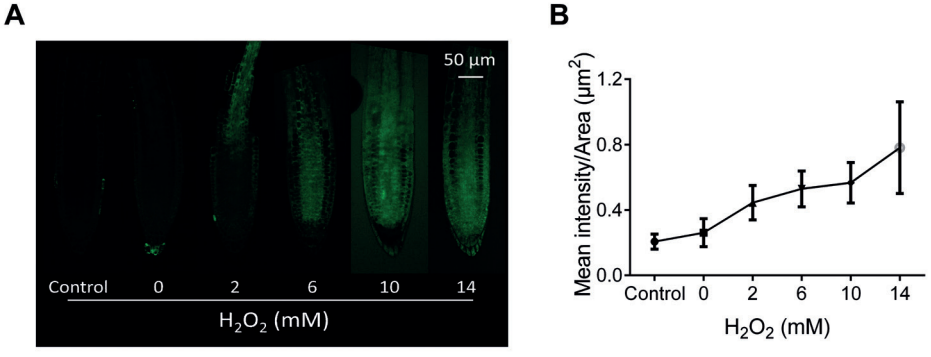


Figure S4.1 Fluorescent staining of H_2O_2 in *Arabidopsis* roots.
(**A**) Fluorescent signal of 4-day-old Col-0 upon 0, 2, 6, 10, 14 mM H_2O_2 stress. Seedlings were treated with H_2O_2 at 13:00 when they were 4-day-old. Staining was done after 2 h of H_2O_2 stress at 15:00, after which imaging was done. Control indicate seedlings without any treatment and mock treatment was represented by 0 mM with ¼ MS. (**B**) Quantification of fluorescent signal from (A). Data shown is mean ± SE, n=5 (Biological replicate). Each replicate represents quantitative fluorescent of one root.

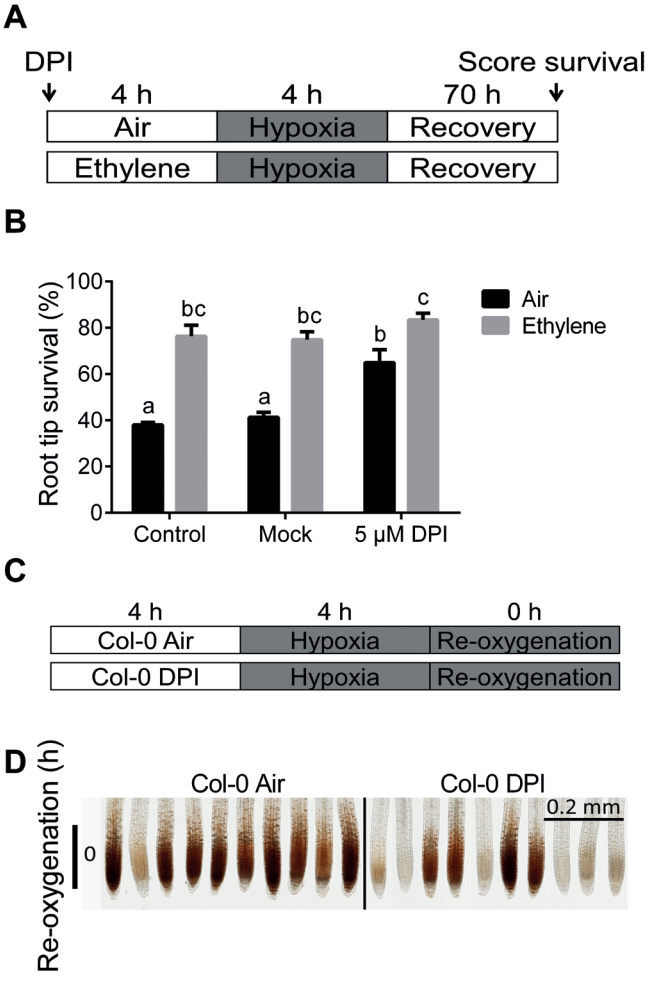


Figure S4.2 Application of *RBOHD* inhibitor DPI on root tip survival and H_2O_2 accumulation during re-oxygenation.

(**A**) Experimental scheme to study effect of DPI on root tip survival in 4-day-old Col-0 seedlings. (**B**) Root tip survival upon 4 h hypoxia following DPI treatment. Data shown is mean ± SE, n=4 (Biological replicate). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Different letters indicate statistically significant differences. Statistical significance was calculated using a two-way ANOVA with tukey's Post-hoc test, $p<0,05$. (**C**) Experimental scheme to study ROS accumulation upon re-oxygenation. Four-day-old Col-0 seedlings were given 4 h of air or DPI pre-treatment followed by 4 h hypoxia. Seedlings were taken for DAB staining detection of ROS accumulation at 0 h of re-oxygenation. (**D**) DAB staining of air or DPI pre-treated Col-0 seedling root tips upon 0 h re-oxygenation following 4 h hypoxia.

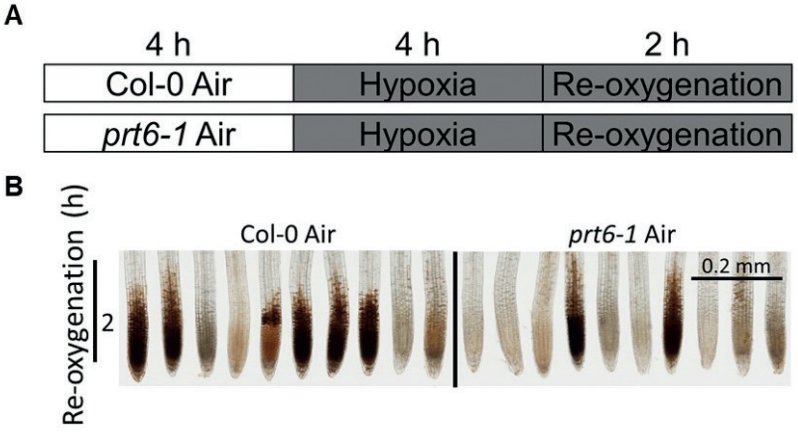
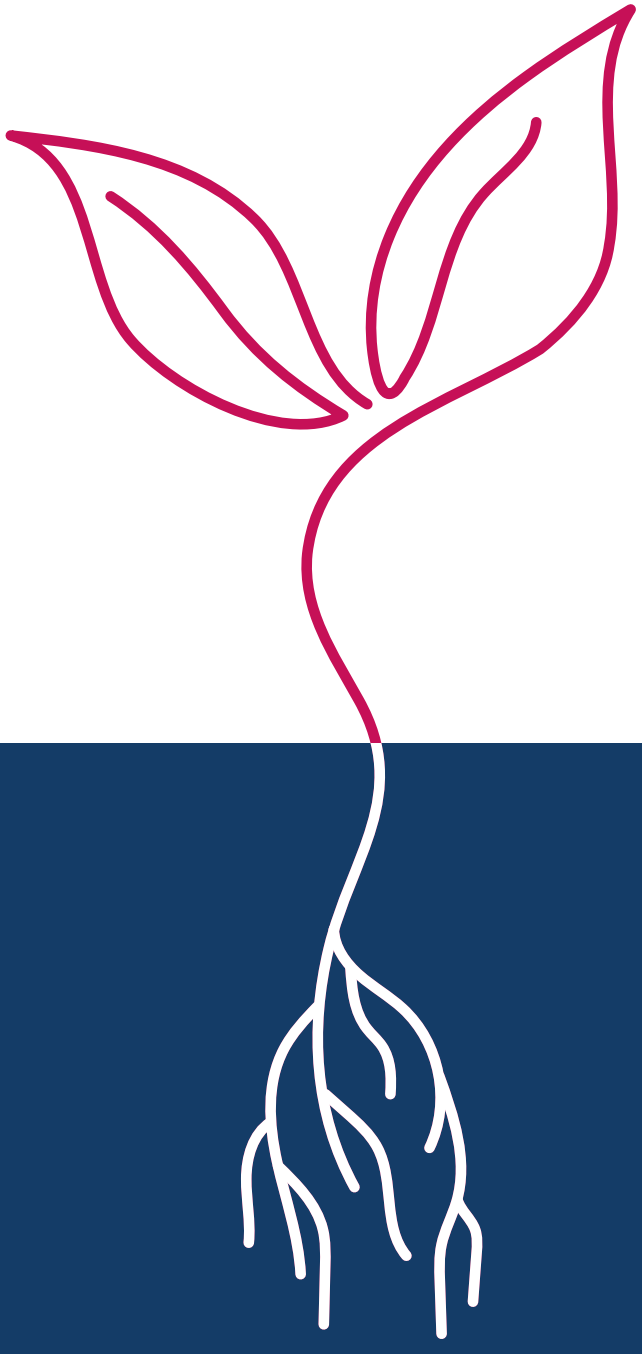


Figure S4.3 DAB staining of H_2O_2 accumulation in 4-day-old *Arabidopsis* Col-0 and *prt6-1* seedlings during re-oxygenation.
(**A**) Experimental scheme to study ROS accumulation upon re-oxygenation. Four-day-old *Arabidopsis* Col-0 and *prt6-1* seedlings were given 4 h of air pre-treatment followed by 4 h hypoxia. Seedlings were taken for DAB staining of ROS accumulation at 2 h of re-oxygenation. (**B**) DAB staining of air pre-treated Col-0 and *prt6-1* seedlings upon 2 h re-oxygenation following 4 h hypoxia.

Table S4.1 List of primers used for qRT-PCR

AGI	Description	Forward	Reverse
AT5G37770	*TCH2*	TCTCTCACCAACAGCATCAC	CGTTTCGATTGTTACCTCCT
AT3G01070	*ENODL16*	CTATACCATTTGGGCTCAGG	TTGGGATTGCAGCTTATGTA
AT3G25230	*ROF1*	GGATATGAGCGTCCTAACGA	CCACTTCACCCTTTTTCATC
AT5G56030	*HSP81-2*	CTTGACGGACAAGAGCAAG	CATGCTAACATCAGCTCCAG
AT5G49910	*cpHsc70-2*	AGGCAGCTGAAAAAGCTAAA	GAATTCTCCACAGGTGTCCT
AT1G30070		TTCTTCTTCGGCATTGAACT	ATGGCACATCGGTAATTTTT
AT1G05690	*BT3*	CTTTCCTGCCCATTCAAGT	GAACCAATAAATGGAGCACA
AT3G09350	*Fes1A*	TGTAGTCAAACGCATGAAGG	TTTTGCCCGAATATTAGCAT
AT1G66510		AAAGCACAAGGGAATGTCTG	GTGCAGCCCAGTAAAAGACT
AT4G29330	*DER1*	CAGCAGACTTTTTGTGGATG	ATAGGCTGATGTTGGCATTT
AT5G56010	*HSP81-3*	TCCTTGACAGACAAGAGCAA	CATGCTAACATCAGCTCCAG
AT1G29150	*ATS9*	GAAACTTACCCAAGGCAAAA	GGACTCAAAGGCTTCAAAGA
AT3G60980		CCTTTCTTAGAGGCAGGACA	AGGAATGCGACTCTGTTTTC
AT1G52340	*ABA2*	CAACGAACACTGAATCTTCTTC	CAGACGAACAATGCTCTCAC
AT2G33590	*CRL1*	GGCTTTCATGAACCCTATGT	GCTGCAGTATAGGTCCCAAA
*AT1G27450**	*APT1*	AATGGCGACTGAAGATGTGC	TCAGTGTCGAGAAGAAGCGT
*AT5G25760**		TGCAACCTCCTCAAGTTCGA	TGAGTCGCAGTTAAGAGGACT
*AT1G13320**		GTAGGACCGGAGCCAACTAG	ACAGGGAAGAATGTGCTGGA

*: used as a reference gene



CHAPTER 5

Developmental stage affects hypoxia sensitivity in *Arabidopsis* roots

Zeguang Liu[1]
Hans van Veen[1]
Shanice Martopawiro[1]
Maureen Hummel[2]
Julia Bailey-Serres[2]
Rashmi Sasidharan[1]*
Laurentius ACJ Voesenek[1]*

*Shared senior authors

[1] Plant Ecophysiology, Institute of Environmental Biology, Utrecht University, Utrecht, The Netherlands

[2] Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, USA

Abstract

Flooding can occur at any moment in the life cycle of a plant. The vulnerability to flooding stress can be strongly dependent on developmental stage. *Arabidopsis* seedling root tips showed a strong age-dependent hypoxia survival even when they received an ethylene pre-treatment. This age-dependent hypoxia sensitivity could provide clues regarding hypoxia tolerance traits and responses. Here we used 4- and 7-day-old seedling root tips with different sensitivities to hypoxia to probe underlying mechanisms using a transcriptomic approach. The transcriptome response of young and older seedlings to hypoxia was very similar, though there were some age-specific effects. Various hormone signaling and response genes, including auxin, jasmonic acid, salicylic acid, gibberellin and cytokinin, were found to be age-specific in response to hypoxia regardless of the pre-treatment conditions. Basal differences between the two developmental stages were identified as well. The capacity to maintain redox homeostasis, coordinate energy metabolism and stress sensitivity also displayed age-specific responses under hypoxia. We suggest that the age-specific responses, initiated by multiple hormone interplay and basal developmental differences, give rise to the developmentally distinct hypoxia sensitivity.

Introduction

Over the past few decades, flooding has been one of the main abiotic stresses constraining agricultural production and its severity is expected to increase further this century (Hirabayashi *et al.*, 2013). Plant performance under flooding stress is determined by several factors such as the plant species and specific flooding conditions. For instance, deepwater rice displays shoot elongation to avoid submergence while lowland rice varieties restrict growth to stay quiescent under water until flood waters recede (Hattori *et al.*, 2009; Kuroha *et al.*, 2018; Xu *et al.*, 2006). Strong variation in submergence tolerance has also been reported among *Arabidopsis* accessions (Vashisht *et al.*, 2011). Flooding conditions can also strongly determine plant survival. Plants can survive longer under submergence in the light than in the darkness due to the possibility of underwater photosynthesis when light is available (Mommer and Visser, 2005; Pedersen *et al.*, 2013).

Another factor that can strongly determine flooding response and survival is developmental stage. Flooding events can occur at any moment of the plants life cycle, from germination to flowering. The vulnerability to flooding and the survival response is strongly determined by the developmental stage at which flooding occurs. The ability to trigger a certain adaptive response might also be strongly determined by developmental stage. Older, mature plants with more carbohydrate reserves thus potentially have more fermentable substrates to sustain growth, metabolism and express adaptive features underwater. At the same time, younger plants/tissues might have better basal protective mechanisms which might also confer stress resilience. Developmental stage-dependent differential sensitivity to flooding has been reported in several species. Younger seedlings of rice cultivars displayed higher sensitivity to submergence and recovery than older seedlings, due to dramatically reduced ability to maintain carbohydrates (Gautam *et al.*, 2017). Comparison of adult and seedling stage plants in response to hypoxia and oxidative stress revealed age-specific molecular regulations by the group VII ethylene response factors (ERFVIIs) (Giuntoli *et al.*, 2017), which were reported to regulate hypoxia responses (Gasch *et al.*, 2015; Gibbs *et al.*, 2011; Kosmacz *et al.*, 2015; Licausi *et al.*, 2011). ERFVIIs were found to induce oxidative stress responsive genes at seedling stage, while the induction of these genes required an additional component in adult plants. In *Solanum dulcamara*, the ability to form adventitious roots (AR) was strongly dependent on developmental stage and flooding depth (Zhang *et al.*, 2015). Under shallow flooding AR formation was markedly inhibited in younger plants compared to older ones.

The developmental effects on hypoxia sensitivity also applies to the age of specific tissues of a plant. Old, intermediate and young leaves of two *Arabidopsis* accessions showed differential

submergence damage after recovery (Yeung *et al.*, 2018). While old and intermediate leaves showed the most damage, young leaves and the shoot meristem hardly showed any visible symptoms of submergence stress. The developmental effects on stress sensitivity have been shown to be related to hormone regulation. In *Arabidopsis,* an interplay between abscisic acid (ABA) and ethylene was proposed to determine the rate of senescence and dehydration during submergence and influenced the ability to develop new leaves by sacrificing older leaves (Yeung *et al.*, 2018). Despite the obvious variation in hypoxia and flooding survival across developmental stages, information on the causal mechanisms are scarce.

In chapter 2 of this thesis, we found that the hypoxia sensitivity of *Arabidopsis* Col-0 seedlings increased with developmental age. Four-day-old seedlings had significantly higher root tip hypoxia survival than 7- and 10-day-old ones (Chapter 2, Figure 2.3; Figure 5.1). Additionally, survival of 4-day-old seedling root tips after 4 h hypoxia could be enhanced by a short ethylene pre-treatment. However, in the older seedlings (7- and 10-day-old) even an ethylene pre-treatment only marginally boosted hypoxia survival. This suggested that ethylene-mediated enhancement of hypoxia tolerance is also age-dependent.

To further investigate the developmental effect on hypoxia survival, we analyzed the transcriptome response to hypoxia in 4- and 7-day-old air and ethylene pre-treated root tips. This allowed us to identify potential age-dependent hypoxia responses. The results revealed that (i) although there were some age-specific responses, the transcriptome responses of 4- and 7-day-old seedlings to hypoxia and ethylene were very similar (ii) 4- and 7-day-old seedlings had a variable capacity to maintain redox balance leading to differential hypoxia sensitivity (iii) hormonal interactions, including auxin, jasmonic acid (JA), salicylic acid (SA), gibberellins (GA) and cytokinin, could be playing an important role in the age-dependent hypoxia sensitivity in *Arabidopsis* seedlings and (iv) the basal developmental differences between 4- and 7-day-old seedlings likely also contribute to the age-specific hypoxia sensitivity.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana wild type Col-0 seeds were sterilized, sown and grown as described in chapter 2. For all experiments, 7- and 4-day old seedlings were used simultaneously. For this, the 7-day-old seedlings were sown and started growth 3 days earlier than 4-day-old seedlings.

Sample harvesting for microarray analysis

The experimental setup for ethylene and hypoxia treatment was as described in Chapter 2. Briefly, 4- and 7-day-old Col-0 seedlings were pre-treated with air or ethylene (5 $\mu L \cdot L^{-1}$) for 4 h from 9:00 to 13:00 h (light intensity within desiccators: ~5 $\mu mol \cdot m^{-2} \cdot s^{-2}$, temperature: 21°C), after which hypoxia was applied with 2 $L \cdot min^{-1}$ of nitrogen flow (Figure S2.1) in the dark. Only root tips were harvested (½ cm from root tip). Samples were harvested at 15:00 h (2 h hypoxia) in the dark. Seedlings pre-treated with air in dim light (~5 $\mu mol \cdot m^{-2} \cdot s^{-2}$) followed by air treatment in the dark were taken as a control group. A schematic explanation of the treatments and harvest time points are summarized in Figure 5.2. For each sample, a total of approximately 184 root tips pooled from 4 plates (from 2 desiccators) were considered as one biological replicate. For each treatment, 3 biological replicates were harvested. Harvested tissues were frozen in liquid nitrogen immediately and then stored at -80°C.

RNA isolation and sample preparation

Total RNA isolation and sample preparation for microarray analyses was done as described in chapter 3.

Quality check, sample labeling and purification, hybridization and scan, and raw data preparation

Quality check, sample labeling and purification, hybridization and scan, raw data preparation were done commercially by Macrogen (http://www.macrogen.com/en/, Seoul, South Korea) and was as described in chapter 3.

Data processing, DEG calculating, Hierarchical cluster and Gene Ontology (GO) enrichment analysis

Raw data were background corrected and normalized using Bioconductor R packages "limma". Probe sets were re-annotated by a blast (Basic Local Alignment Search Tool, Altschul *et al*., 1990) of the probesets against the transcriptome of the most recent *Arabidopsis* annotation (Araport11). Probesets were assigned only to highly similar transcripts, in case of multiple hits, both genes are considered in the analysis. All matches had an identity higher than 90 %, and 93 % of the probes had 100 % identity to the aligned transcript. R language and the "limma" package was used to calculate Log_2 Fold Changes (Log_2FC), P values and the number of differentially expressed genes (DEGs). Log_2FC values of DEGs were scaled prior to hierarchical clustering with the R function "hclust()", to identify gene clusters that showed similar patterns of difference between 4- and 7-day-old samples after hypoxia. DEG groups were analyzed using Bioconductor R packages "GOstats" and "org.AT.tair.db" to discover enriched GO terms.

Results

Transcriptome responses to hypoxia, ethylene, and ethylene+hypoxia in 4- and 7-day-old *Arabidopsis* Col-0 seedlings

Hypoxia survival of *Arabidopsis* root tips gradually decreased as seedlings grew older. The effect of an ethylene pre-treatment was also found to be age-dependent (Figure 5.1). While 4-day-old seedlings could benefit from the early ethylene exposure before 4 h hypoxia, 7-day-old seedlings could not (Figure 5.1). To study the developmental effects on ethylene and hypoxia responses, a transcriptomics approach was used. Root tips of both 4- and 7-day-old seedlings were sampled after air or ethylene pre-treatment followed by 2 h hypoxia. The 2 h time point was chosen based on the rationale that the transcriptome changes mediating the observed age-dependent differences in 4 h hypoxia root survival occur earlier. Samples were also harvested at corresponding time points from 4- and 7-day-old seedlings that remained in air (Air control) (Figure 5.2). The experimental design allowed multiple transcriptome comparisons (Table 5.1).

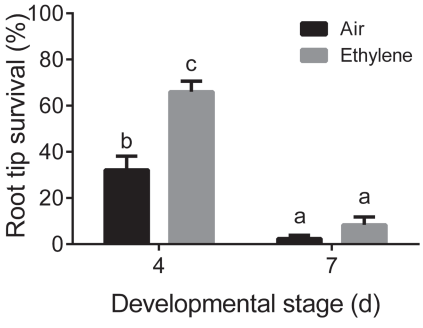


Figure 5.1 Age-dependent sensitivity to hypoxia in *Arabidopsis* Col-0 seedlings.

Root tip survival of 4- and 7-day-old Col-0 seedlings after 4 h hypoxia following 4 h of air (black) and ethylene (gray) (5 µL·L^{-1}) pre-treatment. Root tip survival was scored at the end of recovery phase as described in Figure 2.1. Data shown is mean ± SE, n=10 (Biological replicates). For each biological replicate, one plate containing 46 seedlings was used to calculate root tip survival. Different letters indicate statistically significant differences. Statistical significance was calculated using a two-way ANOVA with Tukey's Post-hoc test, $p<0.05$.

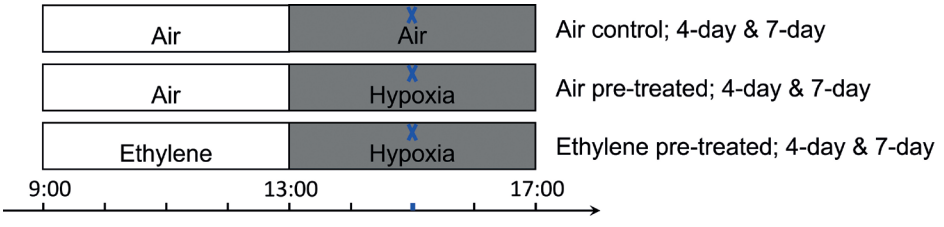


Figure 5.2 Schematic showing the experimental design and sampling time points for transcriptomic survey of age-dependent ethylene-mediated hypoxia responses in *Arabidopsis*.
Four- and seven-day-old Col-0 seedlings were pre-treated with air or ethylene (5 µL·L^{-1}), as indicated in the scheme, from 9:00 (ZT0) to 13:00 h (ZT4) in dim light (~5 µmol·m^{-2}·S^{-2}). Subsequently seedlings were treated to 2 h hypoxia (13:00 to 17:00 h) in the dark. Root tips from both air and ethylene pre-treated seedlings were harvested at 2 h hypoxia in the dark. Blue crosses in the scheme indicate the harvest time points. Root tips from seedlings in the air were harvested at corresponding time points as air control. A total of approximately 184 roots from 4 plates (from 2 desiccators) were pooled together as one biological replicate.

The transcriptomic response of 2 h hypoxia treated 4-day-old and 7-day old root tips to their respective air controls was defined as the "hypoxia effect" (Table 5.1, Hyp4, Hyp7). For the 4-day-old root tips the analysis resulted 1761 and 2764 up- and down-regulated DEGs, respectively. Less DEGs were identified in 7-day-old samples (Table 5.1, Hyp7) with just 1457 up- and 2428 down-regulated genes (Figure 5.3A&B). The "ethylene+hypoxia effect" was determined by comparison of the responses of 2 h hypoxia treated root tips following 4 h of ethylene pre-treatment to their respective air controls (Table 5.1, Ethhyp4, Ethhyp7). In this comparison the number of DEGs almost doubled in both age groups compared to hypoxia response (Hyp4, Hyp7). We found 3546 up- and 4810 down-regulated DEGs in 4-day-old root tips for "ethylene+hypoxia" (Ethhyp4). In 7-day-old root tips this was 3302 up- and 4342 down-regulated DEGs (Ethhyp7) (Figure 5.3A&B). The "ethylene effect" was identified by comparison between ethylene pre-treated samples of both ages and their respective air pre-treated samples after 2 h hypoxia (Table 5.1, Eth4, Eth7). This also resulted in more DEGs in 4-day-old root tips (Eth4, 1915 up and 2369 down) than 7-day-old ones (Eth7, 1511 up and 1828 down) (Figure 5.3A&B). The basal transcriptional differences between the two age groups was determined by comparing 7-day-old samples to 4-day-old ones under control conditions (Table 5.1, BD). There were a similar number of DEGs that were either up- (567) or down-regulated (562) in in the basal difference comparison (Figure 5.3C). Irrespective of age, the "ethylene+hypoxia" treatment resulted in the most extensive transcriptome reconfiguration in *Arabidopsis* root tips.

Table 5.1 List of defined responses and corresponding comparisons made using microarray data

Responses	Comparison	Abbreviation
Hypoxia effect	Air pre-treated 4-day compared to Air control 4-day	Hyp4
	Air pre-treated 7-day compared to Air control 7-day	Hyp7
Ethylene+hypoxia effect	Ethylene pre-treated 4-day compared to Air control 4-day	Ethhyp4
	Ethylene pre-treated 7-day compared to Air control 7-day	Ethhyp7
Ethylene effect	Ethylene pre-treated 4-day compared to Air pre-treated 4-day	Eth4
	Ethylene pre-treated 7-day compared to Air pre-treated 7-day	Eth7
Basal developmental differences of 7 and 4 day	Air control 7-day compared to Air control 4-day	BD
Age-specific responses (corrected for BD)	Hyp7 compared to Hyp4	Hyp7v4
	Ethhyp7 compared to Ethhyp4	Ethhyp7v4
	Eth7 compared to Eth4	Eth7v4

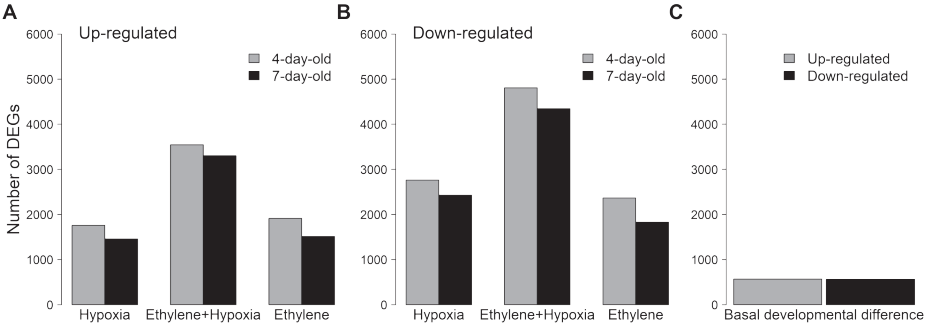


Figure 5.3 Transcriptome responses to hypoxia in air and ethylene pre-treated 4- and 7-day-old seedlings.

(**A&B**) Number of DEGs that were up- (A) or down- (B) regulated in 4-day (gray) and 7-day (black) seedlings. DEGs are shown for the "hypoxia effect" (Hyp4, Hyp7), "ethylene+hypoxia effect" (Ethhyp4, Ethhyp7) and "ethylene effect" (Eth4, Eth7). (**C**) Basal developmental differences of 4- and 7-day-old seedlings under control conditions (Table 5.1, BD) are shown with the number of up- (gray) and down- (black) regulated DEGs. Genes with $P < 0.05$ were defined as DEGs.

Common and age-specific responses in 4- and 7-day-old seedlings

To compare the degree of overlap between the hypoxia, ethylene+hypoxia and ethylene transcriptomes of 4- and 7-day-old seedlings, the responses of 7-day-old samples (Table 5.1; Hyp7, Ethhyp7, Eth7) were compared to those of 4-day-old samples (Table 5.1; Hyp4, Ethhyp4, Eth4) and visualized with scatter plots (Figure 5.4). This revealed the common (gray dots) and age-specific (black and blue dots) responses of the two developmental groups (Figure 5.4). As seen from the massive number of gray dots (2805 for hypoxia, 5790 for ethylene+hypoxia, 2398 for ethylene) in the scatter plot, the majority of the transcriptome responses were common in 4- and 7-day-old samples (Figure 5.4). However, there were some age-specific DEGs (Figure 5.4, blue and black dots) identified among 4- or 7-day-old responses ($Log_2FC < -1.6$ or > 1.6 for Hyp7v4, Ethhyp7v4 and Eth7v4). Briefly, 44, 126 and 71 DEGs were identified as age-specific responses for hypoxia, ethylene+hypoxia and ethylene effects, separately. There were equal number, both 22, of 4- and 7-day-old specific hypoxia DEGs. Ethylene+hypoxia effect again acquired the most obvious age-specific response. There were 86 and 40 age-specific responsive DEGs for 4- and 7-day-old samples, respectively. As for age-specific ethylene response, 41 DEGs were found to be 4-day-old specific and 30 were 7-day-old specific (Figure 5.4).

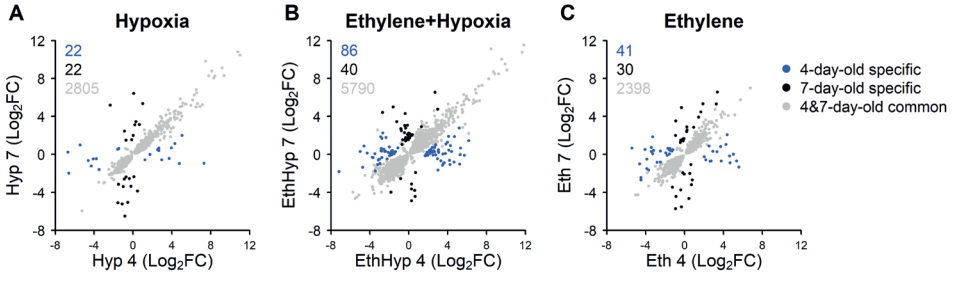


Figure 5.4 Four- and 7-day-old seedlings show largely overlapping transcriptome responses. The scatter plots display the Log_2FC values of (**A**) "hypoxia effect" (Hyp4, Hyp7) (**B**) "ethylene+hypoxia effect" (Ethhyp4, Ethhyp7) and (**C**) "ethylene effect" (Eth4, Eth7) in both 4- and 7-day-old seedlings. Blue and black dots represent 4- and 7-day-specific DEGs, respectively. Gray dots represent 4- and 7-day-old common DEGs. DEGs were defined as age-specific when either one of the 4- or 7-day-old DEGs (Hyp4 or Hyp7, Ethhyp4 or Ethhyp7, Eth4 or Eth7) had $P < 0.05$, and for Hyp7v4, Ethhyp7v4, Eth7v4, Log2FC < -1.6 or > 1.6. DEGs are defined as common responses when same responses of both 4- and 7-day-old (Hyp4 and Hyp7, Ethhyp4 and Ethhyp7, Eth4 and Eth7) have $P < 0.05$. Number of age-specific and common DEGs were listed at the top left of each plot with blue, black and gray for 4-, 7-day-specific and common, separately.

Hierarchical clustering and GO enrichment analysis reveal age-specific hypoxia responses

Since there is a considerable difference in hypoxia root tip survival of 4 and 7-day-old seedlings in both air and ethylene pre-treated seedlings (Figure 5.1), the age-specific hypoxia and ethylene+hypoxia DEGs (blue and black dots, Figure 5.4), could potentially be linked to hypoxia tolerance. Hierarchical clustering was performed on these age-specific hypoxia and ethylene+hypoxia responsive DEGs (Figure 5.4A&B, blue and black dots, 161 DEGs), to cluster similarly responding genes. These 161 DEGs were clustered into 6 groups (Cluster 1-6), and Cluster 1 and Cluster 2, with exactly opposite expression patterns between the two age groups, were the two biggest clusters (Figure 5.5A). The 70 DEGs in Cluster 1 clearly showed higher transcript abundance in 7-day-old root tip samples than the 4-day-old ones in both the "hypoxia" and "ethylene+hypoxia" comparisons. The 57 genes in Cluster 2, on the contrary, displayed higher abundance in 4-day-old samples than the 7-day-old ones, irrespective of pre-treatment conditions. Limited number of age-specific DEGs were clustered into the 4 other groups (Cluster 3-6) containing only 34 genes in total.

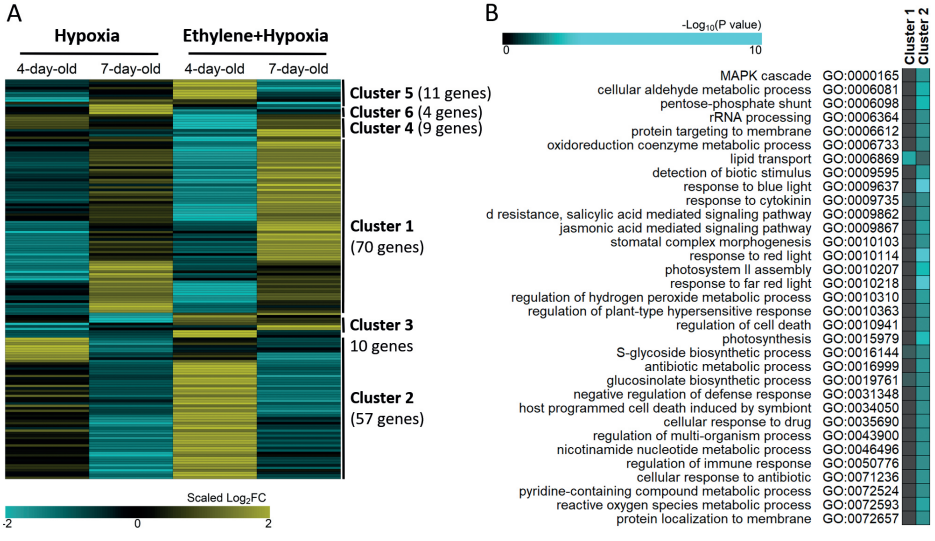


Figure 5.5 Hierarchical cluster analysis of age-specific hypoxia responses.
(**A**) Hierarchical clustering was carried out on the "hypoxia" and "ethylene+hypoxia" age-specific DEGs (Figure 5.4A&B, blue and black dots). Scaled Log_2FC are displayed in the heatmap. A total of 6 clusters (161 genes) were identified and 2 clusters (70 genes in "Cluster 1" and 57 genes in "Cluster 2") were the major groups. Genes from "Cluster 1" and "Cluster 2" were categorized into functional groups and are listed in Table 5.2 and Table 5.3. (**B**) GO enrichment analysis of the DEGs in the two major age-specific clusters (Cluster 1 and 2). GO terms with $P < 0.001$ and GO_{counts} (count of genes) > 3 are displayed in the heatmap, where a higher cyan intensity represents a strong enrichment of relevant GO terms under biological processes.

DEGs in Cluster 1 and 2 clearly displayed age-specificity regardless of pre-treatment conditions. Therefore, a GO enrichment analysis was carried out to identify relevant biological processes responsible for the age-specific sensitivity. Most of the enriched biological processes were found for Cluster 2, where DEGs showed a stronger induction in younger seedling root tips. The significantly enriched GO terms here included light responses (GO:0009637, GO:0010114, GO:0010218), regulation of hydrogen peroxide (H_2O_2) metabolic process (GO:0010310), reactive oxygen species (ROS) metabolic process (GO:0072593), cytokinin (GO:0009735), SA (GO:0009862) and JA (GO:0009867) (Figure 5.5B). In addition, a few biological processes, such as cytokinin (GO:0009735), lipid transport (GO:0006869), S-glycoside and glucosinolate metabolism (GO:0016144, GO:0019761) were found to be enriched in Cluster 1, which comprised DEGs that were down-regulated in the younger seedlings (Figure 5.5).

Next we investigated which genes belonged to Cluster 1 and 2 and what their biological function was based on previously published studies (https://www.arabidopsis.org/) (Table 5.2, Table 5.3). Among the 70 genes in Cluster 1, with higher abundance in 7-day-old seedlings, 7 genes were related to redox homeostasis and 8 were involved in growth and energy metabolism (Table 5.2). Numerous genes involved in stress responses (13), and a few hormones related genes (3 auxin and 4 cytokinin related) were found as well. Of interest was the gene *GLYCINE-RICH PROTEIN 5* (*GRP5*) identified in Cluster 1, which was shown to be submergence inducible in adult *Arabidopsis* plants (de Oliverira *et al*., 1990) (Table 5.2). In addition, Cluster 1 also included 7 development related genes. In Cluster 2, containing DEGs down-regulated in 7-day-old seedlings, genes related to redox balance (11), carbohydrate and energy metabolism (15), stress responses (11), cytokinin (5), and development (5) were found (Table 5.3). Furthermore, several light responsive DEGs were also identified, including *RUBISCO SMALL SUBUNIT 2B (RBCS2B)*, *RBCS3B*, and *LIGHT HARVESTING CHLOROPHYLL B-BINDING 2* (*LHCB2.1 and LHCB2.2*), which are involved in photosynthesis-associated carbohydrate metabolism.

Table 5.2 Genes from "Cluster 1" and related categories

Category	No.	Gene
unknown	21	
Redox balance	7	*AT5G44440, CYP78A9, AT1G62190, LSU2, LSU1, AT5G38000, AT5G37940*
Growth/energy	8	*CYP78A9, GRP5, GSM1, DL1D, ACA2, CCHA1, AT4G19730, AT1G73110*
Stress	13	*AT5G44440, GSM1, AT4G22214, GRP5, LTP6, LTP2, MLO5, AT1G42700, LSU2, LSU1, AT5G38000, AT5G37940, At2G43550, AT1G56540*
Auxin	3	*AT5G51470, AT1G23160, SAUR73*
Cytokinin	4	*ST4B, IPT4, ARR15, AT5G57980*
Development	7	*LSH, CYP78A9, SNZ, LTP6, ARPN, RGF5, NAC025*

Table 5.3 Genes from "Cluster 2" and related categories

Category	No.	Gene
unknown	16	
Redox balance	11	*SKS9, AT2G29170, RBCS2B, RBCS3B, ROXY10, CYP706A1, GOX1, RBCS1A, FNR2, CYP79C1, PMDH2*
Energy/carbohydrate	15	*RBCS2B, RBCS3B, CA2, PMDH2, SBPASE, GOX1, LHB1B1, LHCB2.1, LHCB2.2, RBCS1A, AB165, FNR2, CA1, FBA1, AT5G40000*
Light	8	*PRSL1, RBCS2B, RBCS3B, LHB1B1, LHCB2.1, LHCB2.2, RBCS1A, AB165*
Stress	11	*CA2, GOX1, LHCB2.1, LHCB2.2, FNR2, TPS21, LEA4-5, AZI3, HSFA6B, CA1, LEA7*
Cytokinin	5	*PMDH2, GOX1, FNR2, CSP41A, HB-3*
Development	5	*AT5G53270, LEA4-5, CA1, HB-3, LEA7*

Functional categorization of age-specific ethylene responses

To reveal what is causing the different ethylene sensitivity of 4- and7-day-old samples under hypoxia, we also looked at the functional categories of age-specific ethylene DEGs (blue and black dots, 71 genes, Figure 5.4C), based on previously published studies (https://www.arabidopsis.org/). Similar categories were found as identified from the age-specific hypoxia responses (Table 5.4). Briefly, redox balance (7), stresses (9) and hormones (11) related genes were identified. Besides, some growth and energy metabolic genes were also found to be ethylene specific in either age group.

Table 5.4. Functional categories of age-specific ethylene DEGs

Category		No.	Gene
unknown		20	
Redox balance		7	*AG1G59950, CYP79C1, CYP78A9, SKS9, GA20OX3, ROXY10, AT5G45180*
Growth/development		6	*AT1G60783, CYP78A9, AT3G61970, CPL3, GH9B15, GA20OX3*
Energy		3	*AT3G59620, AT5G6090, AT5G60740*
Lipid/fatty acid metabolism		4	*OLE3, AT4G22440, AT4G22490, FAE1*
Hormones	Cytokinin	4	*ST4B, PUP3, AT3G22640; ARR18*
	Auxin	1	*AT5G51470*
	JA	2	*TAT, CPL3*
	ABA	2	*CPL3, AT4G25580*
	SA	1	*CPL3*
	GA	1	*GA20OX3*
Stress		9	*AT1G42700, AT1G63350, TAT, AT3G22640, OLE3, CPL3, RBCX1, AT4G19630, ADR1-L3*

Basal developmental differences facilitate age-specific hypoxia responses

As stated above, there is already a basal developmental difference of transcript abundance between the two age groups (Table 5.1, BD; Figure 5.3C). From the hierarchical clustering, GO enrichment and functional categorization analysis above (Figure 5.5, Table 5.2, Table 5.3), development related processes were identified as age-specific hypoxia responses. Therefore, we hypothesized that the basal developmental differences of 4- and 7-day-old seedlings could be partly facilitating the age-specific hypoxia responses and sensitivity. A GO enrichment analysis was carried out on the up- and down-regulated basal developmental DEGs (Table 5.1, BD; Figure 5.3C). This allowed us to investigate the potential involvement of development dependent biological processes in the age-specific hypoxia responses.

Most of the identified biological processes from up-regulated DEGs were related to stress responses, hormones and sugar metabolism (Figure 5.6). For example, responses to oxidative stress (GO:0006979), wounding (GO:0009611), SA (GO:0009751), sucrose (GO:0009744), fructose (GO:0009750) were all significantly enriched processes in up-regulated DEGs in 7-day-old root tips. Most of the biological GO terms enriched from DEGs with higher abundance in 4-day-old root tips were growth and development related, such as morphogenesis processes (GO:0009653, GO:0010015), root and epidermis development (GO:0022622, GO:0090558), cell differentiation and growth (GO:0048765 GO:0009826) (Figure 5.6).

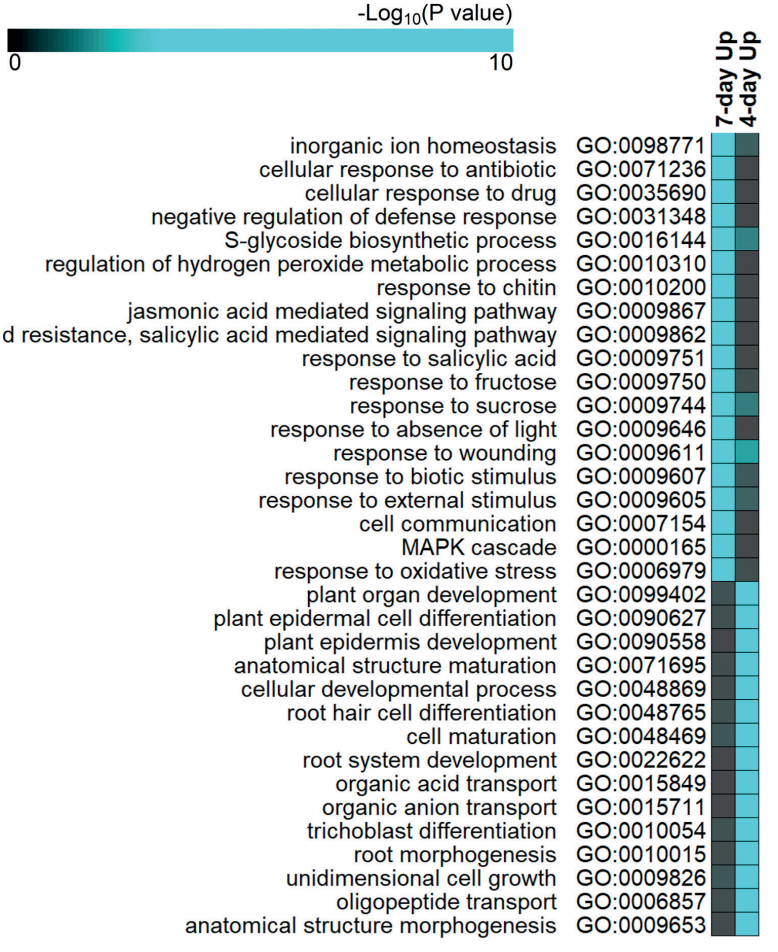


Figure 5.6 GO enrichment analysis of DEGs contributing to basal developmental differences. GO enrichment analysis was carried out on DEGs showing the basal developmental differences (Table 5.1, BD; Figure 5.3C). GO terms with $P < 0.001$ and GO_{counts} (count of genes) > 6 are displayed in the heatmap, where a higher cyan intensity represents a strong enrichment of relevant GO terms under biological processes.

Discussion

Age-dependent stress sensitivity has been commonly described in many eukaryotic species (Colinet *et al.*, 2013, 2016; Hellweger *et al.*, 2014; Miazek *et al.*, 2017). In plants, stress responses were also shown to be affected by developmental stage in many species (Gautam *et al.*, 2017; Giuntoli *et al.*, 2017; González-Villagra *et al.*, 2018; Miazek *et al.*, 2017). In chapter 2 of this study, we found that hypoxia root tip survival decreased when seedlings grow older irrespective of the ethylene pre-treatment (Figure 2.3). While 4-day-old seedling root tips were able to benefit from an early ethylene pre-treatment for survival of 4 h hypoxia, 7-day-old seedling root tips could only benefit under a milder hypoxia stress (3 h) (Figure S2.2).

The transcriptome response of ethylene and air pre-treated 4- and 7-day-old Col-0 seedlings provided a means to identify genes and processes potentially linked to an age-related decline in hypoxia survival. Analyses of the transcriptomes revealed both common and age-specific responses of the two age groups towards hypoxia, ethylene+hypoxia and ethylene (Figure 5.4). While the majority of the responses in the two age groups were quite similar, several genes were identified in each comparison that displayed an age-specific regulation. This differential hypoxia and ethylene sensitivity between 4- and 7-day-old seedlings could potentially be attributed to the activity of these age-specific DEGs identified here (Figure 5.4, blue and black dots, 161 DEGs). The age-specific hypoxia and ethylene+hypoxia DEGs, clustered into two major groups, Cluster 1 and Cluster 2, which displayed higher transcript abundance in 7- and 4-day-old samples, respectively (Figure 5.5A). GO enrichment analysis and functional categorization identified certain biological processes that could contribute to the age-specific responses.

In most of the identified biological GO terms younger root tips under hypoxia had a higher gene abundance (Cluster 2) (Figure 5.5). Genes from both clusters were categorized into similar functional groups based on the biological processes they were linked to (Table 5.2, Table 5.3), while quite similar functional groups were identified among the age-specific ethylene DEGs (Table 5.4). Putatively, the differential regulation of those relevant processes and genes could contribute to the different hypoxia and ethylene sensitivity between the two age groups.

Interestingly, 7 and 11 genes involved in redox balance were found in Cluster 1 and Cluster 2, respectively. Of particular interest was *PEROXISOMAL NAD MALATE DEHYDROGENASE 2* (*PMDH2*) with higher abundance in 4-day-old samples (Cluster 2). This gene was previously shown to be darkness and submergence inducible in *Arabidopsis* (van Veen *et al.*, 2016), and is crucial to maintain redox balance during peroxisomal fatty acid beta-oxidation (Pracharoenwattana *et al.*, 2007). Furthermore, the GO enrichment analysis on the basal

developmental differences also revealed biological processes like oxidative stress responses (GO:0006979), ROS metabolic process (GO:0072593) and H_2O_2 metabolism (GO:0010310), which could affect internal redox homeostasis (Figure 5.6).

Amongst other redox related genes with higher abundance in 4-day-old samples (Figure 5.5A, Cluster2), were several genes encoding Rubisco subunits *RBCS2B*, *RBCS3B* and *RBCS1A* (Table 5.3). It is interesting that these genes, which are closely linked to photosynthesis, were identified in the transcriptome of root samples. The detection of these genes suggests the presence of root greening, which is known to occur in the presence of a light signal (Dinneny *et al.*, 2008). The induction of photosynthetic genes in roots was previously reported in flooded/hypoxic *Arabidopsis* and *Rorippa spp* (Chang *et al.*, 2012; Sasidharan *et al.*, 2013; van Veen *et al.*, 2016). While the roots in our studies were exposed to light, photosynthetic gene induction in roots has also been reported for dark grown roots. In *Arabidopsis,* root specific photosynthetic genes were identified in plants submerged in the dark (van Veen *et al.*, 2016). Photosynthetic genes were also shown to be induced in roots by sugar starvation and salt stress (Baena-González *et al.*, 2007; Dinneny *et al.*, 2008; Sheen 1990). It was proposed that these genes could be ROS induced and might play a role in ROS regulation as well (Dinneny *et al.*, 2008; van Veen *et al.*, 2016). Here the presence of light stimuli, together with potential starvation under hypoxia, likely contributed to the induction of photosynthetic genes. The higher abundance of those genes in 4-day-old seedlings (Figure 5.5A, Cluster 2, Table 5.3) might also be important to maintain redox balance.

Additionally, genes and processes related to multiple hormones, that might contribute to redox balance maintenance under stress conditions, were identified in age-specific DEGs and basal developmental differences (Figure 5.5B, Figure 5.6; Table 5.2, Table 5.3, Table 5.4). For example, JA (GO:0009867), also identified in chapter 3 of this study as ethylene specific pre-treatment response (Table 3.2, Figure S3.1), was shown to manipulate antioxidant status of *Arabidopsis* to adapt to post-submergence re-oxygenation (Yuan *et al.*, 2017). Another hormone SA (GO:0009751, GO:000986), was previously reported to interact with *β-CARBONIC ANHYDRASE 1* (*CA1*) to benefit the antioxidant system during viral infection in *Arabidopsis* (Slaymaker *et al.*, 2002). The hormone cytokinin (GO:0009735), together with nitrogen, affected antioxidant activity and thereby promoted plant growth and limited lipid peroxidation upon de-submergence in *Agrostis stolonifera* (Liu and Jiang, 2016). Many redox homeostasis related genes (Table 5.2, Table 5.3, Table 5.4, Figure 5.5, Figure 5.6) and lipid metabolic process (Figure 5.5B, GO:0006733; Table 5.3, *LATE EMBRYOGENESIS ABUNDANT* (*LEA*), *LEA4-5*, *LEA7*; Table 5.4) were differentially regulated in 4- and 7-day-old seedlings. The initiation of cytokinin metabolism and signaling could be attributed to the altered antioxidant system and lipid metabolism determining re-oxygenation survival.

In chapter 3 of this study, enrichment of ROS related biological processes was found to be a significant signature of ethylene pre-treatment in response to hypoxia and re-oxygenation (Table3.3, Table 3.4; Figure S3.2, Figure S3.3). Moreover, as shown in chapter 4 many oxidative stress responsive genes were induced irrespective of pre-treatment conditions (Figure 4.1, Figure 4.2). Both genetic and chemical inhibition of ROS production significantly improved hypoxia survival, further suggesting the importance of maintaining redox balance during hypoxia and re-oxygenation (Figure 4.5, Figure 4.6, Figure S4.2). Given that a number of redox balance associated processes and genes were differentially regulated between the two age groups, we speculate that differential ability of young and old seedlings to maintain redox balance could partly determine the distinctive hypoxia sensitivity.

The highlighted hormones are not only linked to redox balance maintenance, but also multiple stress responses and growth regulation, both of which were also identified from GO enrichment and functional categorization analysis (Figure 5.5B, Figure 5.6, Table 5.2, Table 5.3, Table 5.4). For instance, cytokinin interacts with many other hormones such as ethylene, auxin and ABA, to coordinate responses to the changing environment (Ha *et al.*, 2012; Liu *et al.*, 2017). The cytokinin responsive type-A *Arabidopsis RESPONSE REGULATOR* (*ARR*) *ARR15*, displayed higher transcript abundance in older seedlings (Figure 5.5A "Cluster 1", Table 5.2) and is negatively regulated by ETHYLENE INSENSITIVE 3 (EIN3), the key transcription factor modulating ethylene signaling, in response to freezing (Shi *et al.*, 2012). Potentially, differential stress sensitivity could also contribute to the age-specific responses.

GRP5, with higher abundance in older seedlings (Cluster 1), positively regulates cell and organ growth, is flooding inducible and is regulated by ABA (de Oliverira *et al.*, 1990; Mangeon *et al.*, 2009). The enhanced abundance of *GRP5* in 7-day-old seedling roots (Figure 5.5A, Table 5.2) could possibly modulate root growth and affect energy expenditure, which is crucial for hypoxia survival as stated in chapter 3. In rice, older seedlings were shown to have significantly higher submergence survival and re-generation ability because they could maintain sugar and starch at a relatively higher levels (Gautam *et al.*, 2017). Similarly, transplanting of older rice seedlings led to higher plant survival and improved grain yield (Bhowmick *et al.*, 2014).

Many enriched GO terms from basal developmental differences could probably explain the distinct capacity in managing energy expenditure between the two age groups. The 7-day-old root tips seemed unable to induce genes that coordinate cell and organ growth. For instance, root morphogenesis (GO:0010015) and multiple differentiation (GO:0048765, GO:0090627) regulation genes were markedly up-regulated only in 4-day-old root tips (Figure 5.6). On the

other hand, sucrose (GO:0009744) and fructose (GO:0009750) responsive genes were significantly up-regulated in 7-day-old seedlings (Figure 5.6). In both of the clusters identified (Figure 5.5A), genes that are important in energy metabolic processes were found to be age-specific as well (Table 5.2, Table 5.3). *FRUCOTOSE BIPHOSPHATE ALDOLASE 1* (*FBA1*), a sugar responsive gene that was underrepresented in 7-day-old samples (Cluster 2, Table 5.3), was previously shown to be darkness and submergence inducible in *Arabidopsis* (Lu *et al.*, 2012; van Veen *et al.*, 2016). Here also several photosynthetic genes, *LHB1B1*, *LHCB2.1* and *LHCB2.2* displayed increased abundance in 4-day-old seedlings. Likely, the induction of those genes, similar to *RBCS2B*, *RBCS3B* and *RBCS1A*, was due to the light stimuli. Yet it remained unclear whether and to what extent they are responsible for energy and carbohydrate status of seedlings. *CA1* and *CA2*, showed less abundance in 7-day-old samples as well (Cluster 2, Table 5.3). These genes not only play a role in carbohydrate related processes like photosynthesis, but are also involved in many other aspects such as stress responses as mentioned earlier (DiMario *et al.*, 2017). Taken together, likely the ability to manage energy expenditure could determine the ability to cope with hypoxia and might underlie the age-dependent differential stress sensitivity.

Amongst the age-specific DEGs (Figure 5.4, blue and black dots), were also many development-related genes (Figure 5.5, Table 5.2, Table 5.3, Table 5.4). This suggested that the basal developmental difference between the two age groups, could already determine the ability to cope with hypoxia stress. For instance, the cytokinin responsive gene *ARR15* was also shown to be influenced by auxin signaling in establishing the root stem cell niche during early embryogenesis (Müller and Sheen, 2008). The down regulation of auxin responses in 7-day-old seedlings (Figure 5.5A, Table 5.2) could therefore interplay with other metabolic processes and impact hypoxia sensitivity.

In summary, the transcriptome analysis suggested that hormonal interactions, including auxin, JA, SA, GA and cytokinin, probably coordinates the age-specific regulation of processes in 4- and 7-day-old root tips, such as redox metabolism, stress adaptation and growth regulation. Ultimately, this can lead to differential hypoxia sensitivity between the two age groups.

Acknowledgement

We thank members of the Plant Ecophysiology group for help with tissue harvesting for gene expression analysis.

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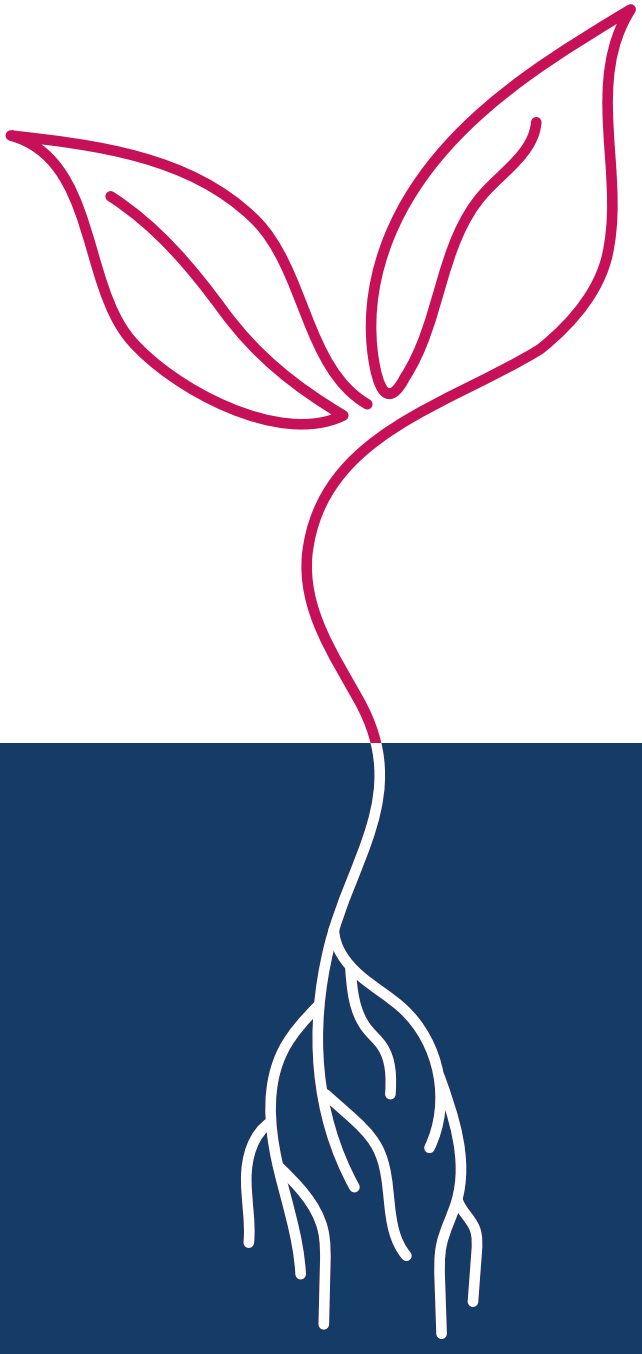

CHAPTER 6

General discussion

Extreme climate conditions like rainfall-induced flooding are expected to increase in the future and thereby impose challenges on global crop production (Arnell and Liu, 2001; Bailey-Serres *et al.*, 2012a; Cassia *et al.*, 2018). Over the last few decades, studies on flooding tolerance in many species unraveled crucial molecular mechanisms underlying various traits benefiting plant performance upon flooding. These studies have unraveled complex signaling pathways involving various flooding signals, including oxygen (O_2), ethylene, nitric oxide (NO) and reactive oxygen species (ROS). Particularly, the conserved N-end rule protein degradation pathway was identified as playing a vital role in O_2 and NO sensing in plants (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). Studies in *Arabidopsis* reported that the primary regulators of hypoxia responses, the group VII ethylene response factors (ERFVIIs) transcription factors (TFs), were N-end rule targets (Gasch *et al.*, 2015; Gibbs *et al.*, 2011; Hinz *et al.*, 2010; Kosmacz *et al.*, 2015; Licausi *et al.*, 2010, 2011). Despite the considerable progress in understanding the regulatory roles of various flooding signals and interactions between them, the dynamics and hierarchy between these signals under various flooded conditions remains unclear. For example, ethylene has an established role as an important cue during and after flooding, whereas the dynamics of ethylene production and signaling during long term flooding, and its interaction with other flooding cues such as light and O_2 remain unestablished.

The early flooding signal ethylene improves subsequent hypoxia and re-oxygenation survival

Ethylene, being a volatile hormone, quickly accumulates in plants underwater irrespective of flooding conditions and types of plant tissue (Sasidharan and Voesenek, 2015; Voesenek and Sasidharan, 2013). Thus it is considered an important flooding signal for plant performance underwater (Sasidharan *et al.*, 2018). Ethylene is a primary regulator not only in many flooding adaptive traits such as adventitious root formation (AR) (Steffens and Sauter, 2009; Vidoz *et al.*, 2010; Visser *et al.*, 1996), aerenchyma development (Evans 2004; Rajhi *et al.*, 2011; Tavares *et al.*, 2018), escape and quiescence responses (Fukao and Bailey-Serres, 2008; Hattori *et al.*, 2009; Kuroha *et al.*, 2018; Xu *et al.*, 2006; Yin *et al.*, 2017), but also in plant responses to re-aeration (Tsai *et al.*, 2014; Voesenek *et al.*, 2003; Yeung *et al.*, 2018). In a recent study of two *Rumex* species (*Rumex palustris* and *Rumex acetosa*), the induction of hypoxia responsive genes in *R. palustris* during the onset of hypoxia was thought to have resulted from the early accumulation of the gaseous hormone ethylene upon submergence (van Veen *et al.*, 2013). The induction of hypoxia responsive genes in *R. palustris*, attributable to ethylene, also correlated with better hypoxia survival in this species.

This beneficial effect of ethylene was also observed in *Arabidopsis* wild type Col-0 shoots. In this study, we report that *Arabidopsis* seedling root tips are also able to benefit from the early ethylene exposure for subsequent hypoxia stress in a root tip survival assay (Figure 2.1). Clearly, even though not likely observed in all plant species, there is the potential for plants, including crop species, to utilize the accumulation of ethylene as a beneficial early warning signal to adapt to flooding stress. The robust system developed in chapter 2 gave us the possibility to assess the mechanism by which ethylene improved hypoxia tolerance. However, since we focused in this study on the root tip only, there is still the necessity to include other relevant traits such as shoot meristem viability, leaf chlorosis or senescence when looking at adult plant sensitivity. Moreover, considering that this early ethylene signal is not only beneficial to seedlings (*Arabidopsis* Col-0, 4-, 5- and 7-day-old; Figure 2.1, Figure 2.3, Figure S2.2), but also adult plants (*R. palustris*, van Veen *et al.*, 2013), the molecular mechanism might be distinct due to potential developmental effects. However, this also further emphasized the crucial function of ethylene in early flooding signaling throughout the entire plant life cycle. Additionally, the system also allowed us to uncover the importance of the ethylene signal also in limiting damage upon re-aeration, which is a further challenge for plants after flood waters recede.

Ethylene modulates transcriptome changes facilitating subsequent hypoxia and re-oxygenation adaptations

Many established flood adaptive responses regulated by ethylene involves interplay with other plant hormones such as auxin (Vidoz *et al.*, 2010; Visser *et al.*, 1996), abscisic acid (ABA) and gibberellin (GA) (Fukao and Bailey-Serres, 2008; Hattori *et al.*, 2009; Kuroha *et al.*, 2018; Xu *et al.*, 2006; Yin *et al.*, 2017). Many of these adaptive responses help plants survive the stress either by increasing aeration of flooded plants, for instance emergence of AR or enhanced shoot elongation to escape above the flood water, or by conserving energy for recovery until re-aeration like the quiescence strategy employed by lowland rice and *R. acetosa* (Sasidharan and Voesenek, 2015). Clearly, managing energy metabolism is extremely crucial for plants to survive flooding stress because it is important to not spend all existing energy reserves.

Acclimation responses to re-aeration are mostly those attempting to avoid dehydration and oxidative stress resulting from accumulation of ROS (Voesenek and Bailey-Serres, 2015). Given the dual role of ROS in plant growth, development and responses to environmental cues (Halliwell and Gutteridge, 2015; Mittler, 2017; Yang *et al.*, 2018), particularly its regulatory role in hypoxia gene expression (Baxter-Burrell *et al.*, 2002; Gonzali *et al.*, 2015; Pucciariello

et al., 2012), it is to be expected that maintenance of redox balance during hypoxia and subsequent re-aeration are important (Fukao *et al.*, 2012; Paradiso *et al.*, 2016).

The transcriptome analysis in this thesis, both the ethylene-mediated hypoxia and re-oxygenation responses of 4-day-old Col-0 seedlings (Chapter 3) and the comparison between 4- and 7-day-old seedlings (Chapter 5), allowed us to unravel the molecular changes responsible for the survival differences. Among those biological processes identified, metabolic reprogramming of hormones biosynthesis and signaling, energy metabolism, redox homeostasis and epigenetic modifications were highlighted.

Energy distribution determines hypoxia and re-oxygenation responses

When flooded, it is very likely that plants suffer from a carbohydrate and energy crisis since photosynthesis and respiration are both markedly restricted due to limiting CO_2 and O_2 availability (Sasidharan *et al.*, 2018). Therefore, the ability to maintain and prioritize energy expenditure is crucial to survive the stress. The submergence survival responses, escape and quiescence, observed in rice cultivars and wild plant *Rumex* species, demonstrates the energy prioritization strategies used by plants based on flooding conditions. In both responses, the interplay of ethylene with two other hormones ABA and GA facilitates the adjustment of energy metabolism (Bailey-Serres *et al.*, 2012a). *Arabidopsis* is a relatively flood intolerant species that does not display morphological adaptations to facilitate aeration and relies instead on metabolic acclimation strategies(Branco-Price *et al.*, 2005, 2008; Loreti *et al.*, 2005; Mustroph *et al.*, 2009; van Dongen *et al.*, 2009; van Veen *et al.*, 2016; Yeung *et al.*, 2018). Microarray based characterization of the hypoxia response in *Arabidopsis* root tips revealed a substantial down-regulation of genes linked to growth-associated processes, and the modulation of multiple growth regulating hormone responses, like auxin and brassinosteroids (BRs) (Chapter 3, Table 3.2, Table 3.3). This suggests an energy conserving behavior via dampening of expensive growth responses. Particularly, when an ethylene pre-treatment was deployed, this down-regulation was even more enhanced. Apparently, the ethylene pre-treatment allows more spare energy to be spent on coping with other challenges such as re-oxygenation stress under re-aeration.

Moreover, the differential hypoxia sensitivity of young and old seedlings could also be partially attributed to regulation of energy metabolism (Chapter 5, Figure 5.5, Figure 5.6, Table 5.2, Table 5.3, Table 5.4). That could possibly also be the reason that 4- and 7-day-old seedlings displayed different ethylene sensitivity. For longer hypoxia durations, an ethylene pre-treatment could not boost survival of older seedlings as much as younger ones. Likely, the higher ability to prioritize energy of younger seedlings permitted the ethylene-enhanced hypoxia survival

under severe stress (4 h, Chapter 2, Figure 2.3), while older seedlings could do so only under a sublethal stress (3 h, Chapter 2, Figure S2.2). However, our results are contrary to some previous reports of age-dependent flooding sensitivity. Comparison of young and old seedlings of rice cultivars revealed that the latter had a significantly higher submergence tolerance and recovery capacity. This was partly due to the fact the older seedlings possessed more sugar and starch than the younger ones (Gautam *et al.*, 2017). However, in this rice study the rice seedlings (15-40 days) had much more photosynthetic tissue. Here, both the 4- and 7-day-old *Arabidopsis* seedlings used were still very young and not reliant on photosynthesis that much yet. As true leaves form, the greater photosynthetic capacity of older seedlings might be beneficial in terms of sugar and starch reserves. Therefore, *Arabidopsis* seedlings might still rely mostly on energy sources from the seeds and cotyledons rather than photosynthesis-driven sources.

Redox balance is critical to survival success

A universal transcriptome modulation of redox balance associated genes was identified not only as an ethylene specific adaption in hypoxia and re-oxygenation, but also as an age-specific response (Chapter 3 and Chapter 5). Obviously, this stresses the importance of maintaining redox balance for stress survival. As molecules that greatly influence redox balance, ROS are critical for growth and development as well as adaptation to stresses like flooding. This is due to the fact that ROS have dual roles dependent on concentration and location. They can be extremely toxic in case of over accumulation, but when produced in controlled amounts they function as signaling molecules (Halliwell and Gutteridge, 2015; Mittler, 2017; Yang *et al.*, 2018). Indeed, enzymatically produced ROS was found to be essential for adaptive responses such as aerenchyma formation (Takahashi *et al.*, 2015; Yamauchi *et al.*, 2014, 2017) and hypoxia signaling (Baxter-Burrell *et al.*, 2002; Gonzali *et al.*, 2015; Pucciariello *et al.*, 2012).

The importance of ethylene in maintaining redox balance has been previously established for abiotic stresses such as salinity and post-anaerobiosis re-oxygenation (Peng *et al.*, 2014; Tsai *et al.*, 2014). Here we found that re-aeration after hypoxia triggered excessive ROS accumulation resulting in oxidative stress (Chapter 4, Figure 4.3B). In contrast, ethylene pre-treated seedlings had limited ROS accumulation and could withstand oxidative stress better. This was in accordance with the up-regulation of oxidative stress and redox-related genes in root tips of ethylene pre-treated seedlings. The genetic and chemical manipulation of ROS production in this study verified that limiting ROS accumulation clearly is beneficial for hypoxia survival (Chapter 4, Figure 4.5, Figure 4.6, Figure S4.2). The better survival of the knock out mutant of *Respiratory Burst Oxidase Homolog Protein D* (*RBOHD*) *rbohD-3* compared to Col-0 observed in our system suggested that limited ROS production benefits hypoxia survival. However,

mutation of *RBOHD* was previously reported to result in decreased anoxia and de-submergence survival (Pucciariello *et al*., 2012; Yeung *et al*., 2018). In both of these studies, on whole seedlings and adult shoot tissues, respectively, a transient ROS production was suggested to be crucial for the initiation of signal transduction routes beneficial for hypoxia responses. Our study focused on root tips of much younger seedlings, which could be more sensitive to a ROS burst. Thus, the mutation of *RBOHD* and subsequent reduction in ROS, probably brought more benefits than disadvantages. In addition, the production of ROS for signaling functions might be taken over by homologs of *RBOHD* or other ROS generation pathways.

The beneficial effect of ethylene for oxidative stress tolerance during and after hypoxia might be manifold. In addition to influencing ROS metabolism and signaling genes and processes, it also influenced hormones such as JA and cytokinin that are also linked to redox balance maintenance (Chapter 3 and Chapter 5). Both these hormones were previously reported to affect antioxidant activity in plant acclimation to de-submergence(Liu and Jiang, 2016; Yuan *et al*., 2017). Putatively, the tight regulation of JA and cytokinin are important to maintain the redox balance and limit oxidative stress-mediated damage.

Epigenetic modifications facilitate potential changes

Another significant ethylene specific response across pre-treatment, hypoxia and re-oxygenation were epigenetic modifications (Chapter 3). Particularly, the proposed function of C2H2 (cysteine2/histidine2) family members in chromatin-remodeling (Englbrecht *et al*., 2004) emphasized the importance of identified TFs of this family (Chapter 3, Figure 3.9, Figure 3.10, Figure 3.11). Additionally, a very recent study on chromatin accessibility revealed the importance of cytokinin-mediated processes involving the type-B *Arabidopsis Response Regulators* (*ARRs*) in both shoot and roots (Potter *et al*., 2018). In chapter 5, we identified many cytokinin associated genes, particularly the type-B *ARR18* and the type-A *ARR15* as age-specific responses. It is possible that these cytokinin associated processes might be linked to epigenetic regulations like chromatin accessibility, which probably contributes to the specific responses of the two age groups.

Concluding remarks and future perspectives

In conclusion, ethylene appears to boost hypoxia survival by influencing several aspects of the hypoxia response. The transcriptome analysis revealed considerable ethylene specific responses that could be responsible for the enhanced hypoxia survival. These include the interplay of ethylene with multiple hormones, energy metabolism, redox balance maintenance, and epigenetic modifications (Figure 6.1). Our results support the importance

of ethylene as an early flooding signal preceding the onset of hypoxia. However, during flooding, many other variables are likely to influence the interplay between hypoxia and ethylene signaling. In the root-based hypoxia assay used here, several variables such as stress duration, light variability and developmental stage influenced the efficacy of the ethylene pre-treatment in boosting hypoxia survival. During a real flooding event, the final hypoxia response likely involves a complex interplay of these factors. Complete darkness significantly improved hypoxia survival (Chapter 2, Figure 2.4) even without an ethylene pre-treatment. This is not surprising since darkness is known to initiate starvation, which could be an important signal for hypoxia survival. Furthermore, decreasing light intensity could be an important cue conveying the depth of flooding. Interestingly, the ethylene pre-treatment effects were clearly antagonized under high light conditions. It is interesting to know whether high light intensity affects ethylene signaling or the hypoxia response.

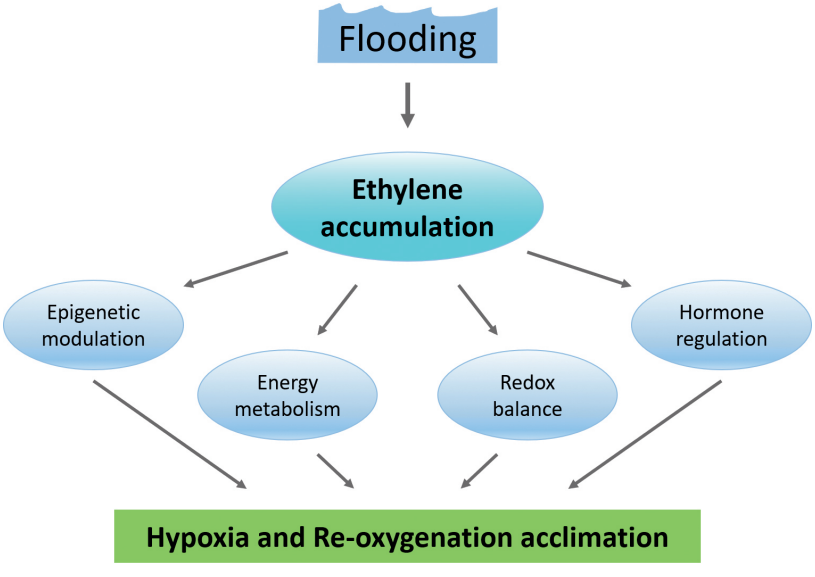

Figure 6.1 Molecular mechanisms of ethylene-mediated hypoxia and re-oxygenation acclimation. Suggested model for ethylene-mediated enhancement of hypoxia survival based on data from *Arabidopsis* seedling root tips. Ethylene, which accumulates rapidly under flooding stress, prior to the decline in O_2 levels causes significant changes to the transcriptome. These include modulation of genes associated with various other hormonal synthesis and signaling pathways, facilitating energy prioritization and redox balance maintenance. These changes, together with potential epigenetic modulation, eventually result in hypoxia and re-oxygenation acclimation.

Given that ERFVIIs are well-established regulators of hypoxia responses and subject to the N-end rule –driven turnover, the question remains whether ERFVIIs are involved in the

ethylene-mediated hypoxia tolerance observed in this study. Among the transcription factors that were identified in chapter 3, two of the ERFVIIs, *HYPOXIA RESPONSE ERF 1* (*HRE1*) and *RELATED TO AP2 3* (*RAP2.3*), were identified as ethylene specific pre-treatment and hypoxia DEGs, respectively. However, this does not rule out an ethylene effect on the constitutively expressed master hypoxia regulators, RAP2.2 and 2.12. Ethylene could still influence RAP activity at the post translational level. The expression of many core hypoxia genes was higher in the ethylene pre-treated samples pointing to elevated ERFVII activity in these samples.

The potential involvement of ERFVIIs could also be linked to the light-driven antagonism of the ethylene effects. The regulation of ERFVIIs via the N-end rule pathway requires the availability of NO (Gibbs *et al.*, 2015). Under high light intensity, NO levels in the roots were found to increase dramatically compared to samples in the dark (Figure 6.2). However, whether this difference in NO levels is linked to hypoxia survival via the stability of ERFVIIs still needs to be further investigated.

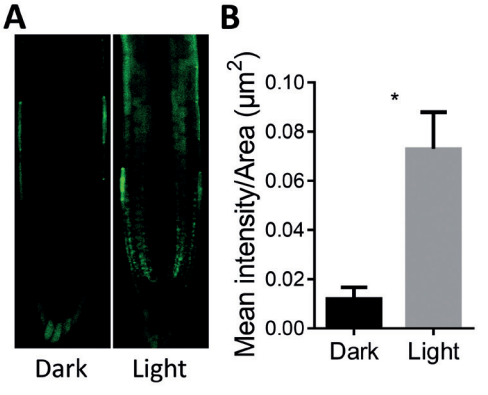

Figure 6.2 Light boosts nitric oxide (NO) levels in root tips of *Arabidopsis* Col-0 seedlings. Four-day-old seedlings were placed under darkness or light (120 µmol·m^{-2}·S^{-2}) conditions for 4 h from 9:00 h to 13:00 h before being incubated in Diaminofluorescein-FM diacetate (DAF-FM DA) staining solution. The DAF-FM DA dye reacts with NO to give a fluorescent signal. (**A**) Representative images of an *Arabidopsis* root tip incubated in the light (120 µmol·m^{-2}·S^{-2}) or complete darkness and then stained for NO with DAF-FM DA. (**B**) Quantification of the fluorescent signal shown in (A). Data shown is mean ± SE, n=10 (Biological replicates). Each replicate represents quantitative fluorescent signal of one root. Asterisk indicates statistically significant differences. Statistical significance was calculated using a student's t-test, $p<0.05$.

Ethylene was also found to exert its effect in the re-aeration period. Several ethylene-specific genes were associated with oxidative stress and excessive ROS accumulation during re-aeration compromises survival. The higher oxidative stress tolerance of *prt6-1* implies that N-end rule targets are required to cope with oxidative stress during re-aeration. Since N-end rule targets are expected to be destabilized with available O_2 during re-oxygenation, it seems more likely that the beneficial effects of these genes are triggered during the hypoxia period. Interestingly, ERFVIIs were reported to regulate hypoxia and oxidative stress responses in an age-dependent manner. Apart from the basal developmental differences, the lower oxidative stress tolerance of older seedlings could in part contribute to the age-specific hypoxia responses.

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Layman summary

Global warming has led to considerable changes in weather patterns over the past decades. Extreme climate events such as flooding, are expected to become even more frequent and severe in the future. Flooding being detrimental to major crop species, will have significant negative effects on global food production. Gas exchange rate is 10^4 times slower underwater than in the air. Consequently, plant growth underwater suffers from a lack of oxygen (O_2) and carbon dioxide, which are essential for respiration and photosynthesis, respectively. The resulting reduction in photosynthesis and respiration lead to a carbohydrate and energy crisis ultimately affecting plant performance. Restricted gas diffusion underwater also results in rapid accumulation of the plant hormone ethylene in flooded plant tissues. Ethylene has been shown to be one of the key regulators of major flood adaptive responses. Unlike other flooding signals such as O_2 and light availability that can be variable depending on other factors, ethylene accumulation under flooding conditions will always occur due to physical entrapment. Therefore, it is considered a consistent and reliable early flooding signal for plants. Previous studies have suggested that, when exposed to flooding stress, this early accumulation of ethylene prepares plants for subsequent O_2 crisis. However, little is known about the molecular mechanisms underlying this improved hypoxia tolerance. This thesis aims to further investigate this ethylene-mediated hypoxia tolerance using the model plant *Arabidopsis thaliana*.

Chapter 2 describes the development of a root tip survival assay as a tool to study the beneficial effect of ethylene on hypoxia tolerance. It was shown that an early ethylene pre-treatment improved root tip survival under subsequent hypoxia stress in *Arabidopsis* seedlings. Hypoxia survival was affected by several factors including light conditions, developmental stage of seedlings and the ability to adapt to post-hypoxic conditions, which is the re-oxygenation period following hypoxia. Above all, the limited cell death in ethylene pre-treated seedlings during both hypoxia and post-hypoxia demonstrated the importance of ethylene in facilitating adaptations to not only hypoxia, but also re-oxygenation.

In **chapter 3**, a transcriptomics approach was used to study the ethylene-mediated hypoxia and post-hypoxia responses described in the previous chapter. This approach allowed us to identify global changes in gene expression that prolong hypoxia survival in ethylene pre-treated seedlings. The transcriptome study implied that the beneficial influence of ethylene pre-treatment includes the enhanced regulation of multiple hormones, down-regulation of growth related processes, various epigenetic modification processes and reactive oxygen

species (ROS) homeostasis. Based on the transcriptome study, we suggest that ethylene-mediated hypoxia and re-oxygenation survival could be attributed to energy conservation from down-regulated growth and the ability to maintain redox balance post-hypoxia.

As stated, re-aeration following hypoxia affects recovery capacity. Re-oxygenation post-hypoxia is typically associated with excessive production of ROS. ROS over-accumulation leads to oxidative stress and results in extensive damage in plant cells. In **chapter 4**, we confirmed high ROS accumulation in hypoxia treated *Arabidopsis* root tips, which correlated with decreased root tip survival and the induction of oxidative stress related genes identified in chapter 3. Besides, limiting ROS accumulation through genetic and chemical approaches led to improved hypoxia root tip survival. More importantly, the ethylene pre-treatment correlated with a much stronger induction of oxidative stress related genes and less ROS accumulation post-hypoxia. These results suggest that ethylene-mediated hypoxia tolerance could be linked to the enhanced oxidative stress tolerance upon re-oxygenation. In addition, the increased hypoxia survival was also related to N-end-rule targets since the N-end-rule mutant *prt6-1* displayed higher survival as well as limited ROS accumulation post-hypoxia.

An age-dependent hypoxia sensitivity was identified in chapter 2 of the thesis. In **chapter 5**, the same transcriptomics approach used in chapter 3 was applied to study this age-dependent response in 4- and 7-day-old *Arabidopsis* seedlings. The expression of many genes involved in various hormone signaling and responses were found to be different between the young and old seedlings regardless of pre-treatment conditions. Besides, the ability to maintain redox homeostasis, coordinate energy metabolism and stress sensitivity could also be attributed to the different hypoxia sensitivity in 4- and 7-day-old seedlings. Overall, these age-specific responses correlated with the observed development-dependent hypoxia sensitivity.

In summary, the findings in this thesis demonstrated the potential ability of plants to benefit from the early flooding signal ethylene in adaptation to hypoxia stress. We identified several ethylene specific responses, including hormones interaction, energy metabolism, redox balance maintenance and epigenetic modifications, which could be facilitating the increased hypoxia survival in *Arabidopsis*.

Samenvatting

De opwarming van onze aarde heeft de laatste tientallen jaren forse veranderingen in het weer veroorzaakt. Extreme weersomstandigheden zoals overstromingen zullen in de toekomst vaker gaan voorkomen. Omdat de meeste gewassen niet tolerant zijn tegen overstroming zal dit leiden tot een daling van de voedselproductie op onze aarde. Gasdiffusie in water voltrekt zich ongeveer 10.000 keer langzamer dan in lucht. Een van de gevolgen hiervan is dat overstroomde planten te maken krijgen met een tekort aan zuurstof (hypoxia) en koolstofdioxide. Deze gassen zijn belangrijk voor respectievelijk ademhaling en fotosynthese. Limitaties in deze sleutelprocessen zal leiden tot forse tekorten in beschikbare koolhydraten en energie van de plant. Een ander gevolg van de langzamere gasdiffusie onder water is dat het gasvormige plantenhormoon ethyleen ophoopt in overstroomde plantenweefsels. Dit hormoon is een belangrijke regulator van aanpassingsprocessen tegen overstroming. In tegenstelling tot zuurstof en kooldioxide is ethyleen een zeer betrouwbare en snelle indicator voor overstroming. Eerder onderzoek heeft er op gewezen dat vroege ophoping van ethyleen planten beschermt tegen zuurstof tekort dat pas later tijdens overstroming optreedt. Het moleculaire mechanisme dat dit reguleert is echter nog grotendeels onbekend. Dit proefschrift bestudeert deze ethyleen gecontroleerde hypoxia tolerantie in de modelplant *Arabidopsis thaliana* (de Zandraket).

Hoofdstuk 2 beschrijft de ontwikkeling van een onderzoeksmethode om op een eenduidige manier het voordeel van ethyleen op hypoxia tolerantie te kwantificeren. Een voorbehandeling met ethyleen verbeterde de overleving van wortelpunten van *Arabidopsis* zaailingen tijdens hypoxia. Ook bleek dat de overleving tijdens hypoxia beïnvloed wordt door de licht condities, het ontwikkelingsstadium van de zaailingen en het vermogen van de zaailingen om zich aan te passen aan de condities na de hypoxia (post-hypoxia condities). De beperkte celsterfte in wortelpunten tijdens hypoxia en post-hypoxia na voorbehandeling met ethyleen illustreert de belangrijke faciliterende rol van ethyleen bij overstromingsaanpassingen aan.

In **Hoofdstuk 3** is een transcriptoom methodologie toegepast om ethyleen-gemedieerde hypoxia en post-hypoxia tolerantie te bestuderen. Deze methode maakte het mogelijk om generieke genexpressie veranderingen te bestuderen tijdens ethyleen-gemedieerde hypoxia tolerantie. Hieruit bleek dat een ethyleen voorbehandeling leidt tot een sterke regulatie van diverse plantenhormonen, een reductie van de expressie van groei-gerelateerde genen, diverse epigenetische modificaties en de homeostase van reactieve zuurstof moleculen (ROS). Op basis van deze transcriptoom analyse suggereren we dat

ethyleen-gemedieerde hypoxia tolerantie gereguleerd wordt door genen die het gebruik van energie reduceren gedurende hypoxia en die het vermogen hebben om een juiste redox balans in cellen te handhaven tijdens post-hypoxia.

De blootstelling van zaailingen aan zuurstof na een hypoxia behandeling heeft een negatieve invloed op het herstelvermogen. Een dergelijke behandeling wordt geassocieerd met de productie van vrije zuurstof radicalen (ROS). Te veel ROS leiden tot de inductie van oxidatieve stress en resulteert in schade aan plantencellen. In **Hoofdstuk 4** bevestigen we de hoge ROS productie na hypoxia, hetgeen correleert met een afname van de overleving van de wortelpunt en de inductie van genen gerelateerd aan oxidatieve stress zoals geïdentificeerd in hoofdstuk 3. Het reduceren van ROS accumulatie via genetische en chemische benaderingen leidde tot verbeterde overleving van wortelpunten na hypoxia. De voorbehandeling van ethyleen correleerde met een sterke inductie van genen gerelateerd aan oxidatieve stress en met minder ROS ophoping tijdens de post-hypoxia periode. Deze resultaten suggereren dat ethyleen-gemedieerde hypoxia overleving gerelateerd is aan toegenomen oxidatieve stress tolerantie tijdens de post-hypoxia periode. Verder bleek de toegenomen hypoxia tolerantie na een ethyleen voorbehandeling ook te correleren met de aanwezigheid van N-end rule target genen. De N-end rule mutant *prt6-1* bleek namelijk een hogere overleving te hebben alsook een beperkte ROS accumulatie tijdens post-hypoxia.

In hoofdstuk 2 is geconstateerd dat hypoxia tolerantie afhankelijk van de leeftijd van de zaailing. In **Hoofdstuk 5** is dezelfde transcriptoom techniek als in hoofdstuk 3 gebruikt om verschillen in genregulatie tussen zaailingen van 4 en 7 dagen oud te ontdekken. Er werden duidelijke verschillen in genregulatie betreffende diverse hormoon signaleringen en reacties op deze hormonen gevonden tussen de twee stadia met een verschillende leeftijd. Deze verschillen waren onafhankelijk van de voorbehandelingen. Ook bleken er verschillen te bestaan tussen deze twee leeftijden ten aanzien van het vermogen om de redox homeostase te handhaven en het vermogen om energie metabolisme en stress gevoeligheid te coördineren. Deze leeftijdsspecifieke reacties correleerden sterk met de verschillen in hypoxia tolerantie van de twee leeftijden.

Samenvattend laat dit proefschrift zien dat het vroege overstromingssignaal ethyleen helpt bij het ontwikkelen van aanpassingen aan hypoxia stress. We hebben diverse ethyleen-specifieke reacties geïdentificeerd zoals hormoon interacties, energie metabolisme, handhaving van de redox balans en epigenetische veranderingen. Deze veranderingen verklaren de hogere hypoxia tolerantie na een ethyleen voorbehandeling.

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Zeguang (泽光)
Feb 2019, Utrecht

Curriculum vitae

Zeguang Liu was born on 11 November 1988 in Hebei, China. He started his bachelor study in September 2007 in the Academy of Agricultural Sciences, Northwest A&F University, Yangling, Shaanxi, China. His research experience started with an internship in the lab of Prof. dr. Xiang Gao, where he worked on drought tolerance evaluation and gene cloning in wheat cultivars. After graduation in June 2011, he was enrolled in the master program in the College of Plant Protection at the same university. Under the supervision of Prof. dr. Baotong Wang, he focused on the identification of wheat stripe rust resistance genes from wild relatives of wheat. Before Zeguang graduated in June 2014, he obtained a fellowship from China Scholarship Council in May, which allowed him to work as a PhD candidate in the Plant Ecophysiology group, Institute of Environmental Biology, Utrecht University, the Netherlands. From September 2014 to February 2019, he worked on ethylene-mediated hypoxia tolerance in *Arabidopsis thaliana* under the supervision of Prof. dr. L.A.C.J. Voesenek and Dr. Rashmi Sasidharan. This thesis is the result of his PhD research.

List of publications

Sasidharan, R., Hartman, S., **Liu, Z.**, Martopawiro, S., Sajeev, N., van Veen, H., Yeung, E. and Voesenek, L.A.C.J. (2018). Signal dynamics and interactions during flooding stress. *Plant Physiology* Feb 2018, 176 (2) 1106-1117; DOI: 10.1104/pp.17.01232

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