Hypoxia and the group VII ethylene response transcription factor HRE2 promote adventitious root elongation in Arabidopsis

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ABSTRACT

- Soil water-logging and flooding are common environmental stress conditions that can impair plant fitness. Roots are the first organs to be confronted with reduced oxygen tension as a result of flooding. While anatomical and morphological adaptations of roots are extensively studied, the root system architecture is only now becoming a focus of flooding research. Adventitious root (AR) formation shifts the root system higher up the plant, thereby facilitating supply with oxygen, and thus improving root and plant survival.
- We used Arabidopsis knockout mutants and overexpressors of ERFVII transcription factors to study their role in AR formation under hypoxic conditions and in response to ethylene.
- Results show that ethylene inhibits AR formation. Hypoxia mainly promotes AR elongation rather than formation mediated by ERFVII transcription factors, as indicated by reduced AR elongation in erfVII seedlings. Overexpression of HRE2 induces AR elongation to the same degree as hypoxia, while ethylene overrides HRE2-induced AR elongation.
- The ERFVII transcription factors promote establishment of an AR system that is under negative control by ethylene. Inhibition of growth of the main root system and promotion of AR elongation under hypoxia strengthens the root system in upper soil layers where oxygen shortage may last for shorter time periods.

INTRODUCTION

Flooding as a consequence of weather extremes is a major cause of crop loss (Voosenek & Bailey-Serres 2015). During flooding, plants are exposed to reduced O$_2$ as a result of oxygen consumption and limited gas exchange. In addition to O$_2$ shortage, the gaseous hormone ethylene accumulates due to reduced diffusion rates in water (Grable 1966). Both, reduced O$_2$ and elevated ethylene levels serve as signals that induce flooding stress responses. In flooding-tolerant species several traits have evolved that improve O$_2$ supply and metabolic adjustments to compensate for limited oxidative ATP synthesis through substrate-level ATP synthesis and NAD$^+$ recovery in fermentation (Mustroph et al. 2014). To improve gas exchange, flooding-tolerant plants form aerenchyma (Steffens et al. 2011; Yamauchi et al. 2013), adventitious roots (Lorbiecke & Sauter 1999) and gas films (Herzog et al. 2018), or induce hyponastic growth (Millenaar et al. 2005; Pierik et al. 2005) and stem or petiole elongation (Kende et al. 1998; Millenaar et al. 2005) to raise leaves above the water surface.

Adventitious root (AR) growth is a common adaptive response to flooding, e.g. in deepwater rice (Oryza sativa L.), tamarack (Larix laricina), Rumex palustris and tomato (Solanum lycopersicum; Visser et al. 1996; Lorbiecke & Sauter 1999; Vidoz et al. 2010; Calvo-Polanco et al. 2012). ARs facilitate gas exchange, uptake of minerals, water and O$_2$ and anchor the plant during the post-submergence phase. In rice, nodal AR initiation is part of the regular developmental programme. However, the emergence of ARs depends on an environmental stimulus such as flooding, which traps ethylene and triggers the outgrowth of AR primordia.

Arabidopsis thaliana is an intermediate flooding-tolerant species in which ethylene and low O$_2$ trigger metabolic acclimation (Voosenek & Sasidharan 2013). Petiole elongation and hyponastic growth improve the O$_2$ supply of submerged tissues (Millenaar et al. 2005). The APETALA2/ETHYLENE-RESPONSE FACTOR (AP2/ERF) transcription factors of group VII (ERFVIIIs) are key mediators of low-oxygen stress responses in rice and Arabidopsis. ERFVIIIs regulate metabolic changes and developmental reprogramming under low-oxygen conditions (Licausi et al. 2013; Abbas et al. 2015; Paul et al. 2016). Arabidopsis has five ERFVIIIs, RAP2.2, RAP2.3, RAP2.12, HRE1 and HRE2. Expression of ERFVIIIs is differentially controlled by ethylene and low O$_2$ and differs spatially, suggesting that ERFVIIIs play specific roles in hypoxia adaptation and act in a tissue-specific manner. While ethylene induces the expression of HRE1 and RAP2.2, low O$_2$ induces the expression of HRE1 and HRE2 (Hinz et al. 2010; Hess et al. 2011; Bailey-Serres et al. 2012). ERFVII activity is further controlled at the level of protein stability. Under normoxic conditions ERFVII proteins are degraded via the N-end rule pathway, whereas in hypoxic conditions they escape degradation (Bailey-Serres et al. 2012).
Since roots are the first to encounter hypoxic stress during flooding, it is not surprising that the root system adjusts to these conditions, as recently shown for the primary root growth direction in Arabidopsis (Eysholdt-Derzsó & Sauter 2017). The root system includes a primary root, lateral roots and possibly ARs (Smith & De Smet 2012). In this study, we analysed AR formation in Arabidopsis in response to hypoxia and ethylene, two main signals of flooding stress, with a particular focus on the involvement of ERFVIIs in AR development.

**MATERIAL AND METHODS**

**Plant material and growth conditions**

All experiments were carried out with A. thaliana Col-0. The ein3 eil1 knockout line (An et al. 2010) was kindly provided by Hongwei Guo (Peking-Tsinghua Center of Life Sciences, Beijing, China). The hre1-1 and hre2-1 single knockout, the hre1-1, hre2-1 double knockout, the HRE1ox1, HRE2ox1 and HRE2ox5 overexpression lines (Hess et al. 2011; Eysholdt-Derzsó & Sauter 2017) as well as rap2.3-2 (Marín-de la Rosa et al. 2014), rap2.12-1 (Gibbs et al. 2014) and the rap2.12 rap2.2 rap2.3 hre1 hre2 pentuple mutant erfVII (Abbas et al. 2015) were described previously. HRE1ox1 and HRE2ox1 were crossed to generate the double overexpression line HRE1ox1 x HRE2ox1.

Seeds were surface-sterilised and plated as described (Eysholdt-Derzsó & Sauter 2017). To induce synchronous germination, seeds were exposed to light for 6 h before they were transferred to the dark for the times indicated. For all treatments, we used 5-day-old dark-grown seedlings that were kept either at 21% O2 or 2% O2 as previously described (Eysholdt-Derzsó & Sauter, 2017), or exposed to 5 µl−1 (5 ppm) ethylene (Air Liquide, Paris, France) or 2 µl−1 (2 ppm) 1-methylcyclopropene (1-MCP) for 6 days in the dark.

**Cloning and plant transformation**

Gateway technology based on site-specific recombination was used to generate overexpressing lines of RAP2.3 and RAP2.12 by introducing the open reading frames into the pB7WG2 destination vector through ligation into pENTR 1A DS (Thermo-Fisher Scientific, Waltham, MA, USA; Karimi et al. 2002). The primer pairs ForRAP2.3-ox1 and RevRAP2.3-ox1, and ForRAP2.12-ox1 and RevRAP2.12-ox1 (Table S1) were used to amplify the open reading frames of RAP2.3 and RAP2.12 from cDNA generated from leaf RNA. The pB7WG2 expression clones were transformed into Agrobacterium tumefaciens strain GV3101, followed by plant transformation of Col-0 with the floral dip method (Clough & Bent 1998). Plant selection was carried out based on gluconsamine resistance from the pB7WG2 vector. Homozygous plants were selected and expression of RAP2.3 and RAP2.12 was analysed in selected plants with RT-PCR using leaf material for RNA isolation. The gene-specific primers ForRAP2.3-ox1 and RevRAP2.3-ox1 for RAP2.3, RAPForRAP2.12-ox1 and RevRAP2.12-ox1 for RAP2.12, were used to verify overexpression (Table S1). Actin2 was used as a reference gene with primers Actin2F1 and Actin2R1 (Table S1).

**RESULTS**

HRE2 overexpression promotes AR formation and elongation growth of AR primordia

To study development of AR in Arabidopsis in response to hypoxic conditions, 5-day-old seedlings were transferred to a controlled gas atmosphere with 2% O2, 5 ppm ethylene at 21% or 2% O2 or kept in air for 6 days in the dark. Darkness induced formation of ARs from the hypocotyl of Arabidopsis seedlings (Fig. 1). For a detailed analysis of AR development three categories were distinguished: (i) young AR primordia of stages I–IV according to the nomenclature described previously for lateral root development (Casimiro et al. 2003), (ii) mature primordia prior to emergence at stages V–VII, and (iii) emerged ARs of stage VII as well as elongated ARs (Fig. 1A).

The gene HRE2, but not its related transcription factor gene HRE1, was shown to be up-regulated by hypoxia in the shoot (Licausi et al. 2010), and HRE2 overexpression improved anoxia survival (Figure S1). To establish a functional link between hypoxia signalling of AR development and ERFFVII transcription factors as hypoxia signal mediators we analysed the roles of the ERFFVIIIs and of ethylene in AR development using gene knockout and overexpression lines (Fig. 1B and C). HRE2 protein was previously shown to accumulate under non-stressed conditions when overexpressed (Gibbs et al. 2011). The ein3 eil1 mutant that is deficient in ethylene signalling displayed a significantly higher AR density, indicating that basal ethylene signalling takes place in normoxic seedlings that partially inhibits AR formation (Fig. 1B). Among the knockout mutants and overexpressors of ERFFVII transcription factors analysed, HRE2ox1 showed a significantly higher AR density compared to the wild type suggesting that HRE2 and HRE1 have non-redundant developmental activities. The hypocotyl lengths were not changed in ein3 eil1 or hre1-1 and hre2-1 knockout lines compared to the wild type (Figure S2), suggesting that AR organogenesis was a highly specific response to HRE2 overexpression. Knockout and overexpressing mutants of RAP2.3 and RAP2.12 (Fig. 1B; Figure S3) showed a wild-type phenotype. Based on the finding that HRE2 but not other ERFFVIIIs, control AR formation, we looked in more detail at AR development in hre2-1 and HRE2ox1 seedlings in comparison to ein3 eil1 and the wild type (Fig. 1C). Compared to the wild type, ein3 eil1 seedlings had more AR in all three categories, indicating that ethylene inhibits AR initiation but does not alter further AR development. HRE2ox1 seedlings had more ARs than the wild type. Hence, ethylene- and hypoxia-induced HRE2 inversely regulate AR formation. Furthermore, HRE2, unlike ethylene, controlled the late stages of AR development, resulting in more elongated ARs (Fig. 1C).

The AR density did not differ much between seedlings kept in air and seedlings exposed to hypoxia (Figs 1D and 2A). In normoxia, most ARs were at the young primordia stage, whereas hypoxia promoted AR emergence and growth.

**Statistics**

Minitab 14 software (www.minitab.com) was used for one-way ANOVA with Tukey’s post-hoc test. For non-parametric samples, a Kruskal–Wallis test with Dunn’s post-hoc test was used. Comparison of two means was performed with Student’s t-test.
Fig. 1. Hypoxia promotes AR elongation while ethylene acts as an inhibitor of AR initiation and growth. A: ARs were grouped into three categories based on the developmental stages described by Casimiro et al. (2003) for lateral root development. Representative ARs are shown from each category. B: AR density of 11-day-old wild type and mutant seedlings at normoxia. Error bars represent ± SE from three biological replicates and letters show statistically different values (one-way ANOVA with Tukey’s test, n = 22–26, P < 0.05). C: AR of wild type, eir3 eir1, hre2-1 and HRE2ox1 seedlings were counted and categorised as described in (A) in three biological replicates. Values are means (±SE) with single lowercase, capital or double capital letters indicating significant differences within each category (Kruskal–Wallis test, n = 22–26, P < 0.05). D: Average AR densities (±SE) were obtained in three biological replicates. Different letters indicate significantly different values (one-way ANOVA with Tukey’s test, n = 26–29, P < 0.05). E: 5-day-old seedlings were transferred to 21% or 2% O2 with or without 5 μl l⁻¹ ethylene for 6 days. ARs were categorised as shown in (B). Values are means (±SE) with single lowercase, capital and double capital letters indicating significant changes within the respective developmental category (Kruskal–Wallis test, n = 26–29, P < 0.05).
resulting in a significant increase in category three ARs (Fig. 1E). As ethylene is known to control root growth and as it mediates flooding responses (Sasidharan & Voesenek 2015), we next looked into AR development in the presence of 5 μl/l ethylene under normoxic and hypoxic conditions (Fig. 1E). Ethylene inhibited formation of ARs at both O2 levels and inhibited AR development at all developmental stages, overriding hypoxia-induced AR emergence and growth (Fig. 1E).

Ethylene overrides HRE2 activity

We next analysed the role of HRE2 in AR formation and elongation under hypoxic conditions (Fig. 2A and B). The AR density was not significantly altered in hre2-1 compared to the wild type, whereas knockout of all ERFVII in the erfVII pentuple knockout line resulted in significantly fewer ARs under both normoxic and hypoxic conditions (Fig. 1E). Ethylene inhibited formation of ARs at both O2 levels and inhibited AR development at all developmental stages, overriding hypoxia-induced AR emergence and growth (Fig. 1E).

DISCUSSION

Adventitious root growth in rice is a well-known adaptive trait for survival under prolonged flooding that is primarily
regulated by ethylene (Lorbiecke & Sauter 1999; Lin & Sauter 2018). However, apart from favouring ethylene accumulation, flooding causes O_2 deprivation that also acts as a submergence signal. In rice and *Rumex palustris*, AR primordia are formed as part of the developmental programme and emerge upon flooding (Visser et al. 1996; Lorbiecke & Sauter 1999), while in other plants, AR primordia are initiated only when an environmental change occurs. In tomato and sunflower, flooding, and in *Arabidopsis*, darkness or wound promoting AR formation (Wample & Reid 1978; Vidoz et al. 2010; da Rocha Correa et al. 2012; Dawood et al. 2014).

In this study, we analysed AR formation at the hypocotyl of *Arabidopsis* seedlings. Our results reveal a role for hypoxia signalling in AR growth and repression of hypoxia-induced AR growth by ethylene. While in *Arabidopsis* growth of ARs is enhanced by hypoxia, growth of the main root system is inhibited (Ellis et al. 1999). As a result, the root system architecture changes with a shift of root mass from lower ground toward higher ground, possibly to invest the limited energy reserves in roots that are closer to the soil surface and hence more likely to reach aerated zones. Unlike hypoxia, ethylene inhibits AR initiation (Velocci et al. 2016). High levels of ethylene inhibited AR initiation to the same degree in normoxic and hypoxic conditions, indicating that ethylene can override the hypoxia signal (Figure S4). In normoxia, AR growth is inhibited by ethylene in a dose-dependent manner (Velocci et al. 2016), suggesting that at lower ethylene levels AR are formed that can elongate faster when exposed to hypoxia. Future work may clarify if the sensitivity to ethylene changes under hypoxia. In contrast to *Arabidopsis*, ethylene has a promotive effect on AR emergence and growth in several flood-tolerant species including rice (Lorbiecke & Sauter 1999; Lin & Sauter 2018). The contrasting regulation of ARs by ethylene may reflect different adaptive strategies in flooding-tolerant rice compared to moderately tolerant *A. thaliana* (Vashishth et al. 2011).

Expression of HRE2 is induced by hypoxia but not ethylene in the shoot and root (Licausi et al. 2010; Hess et al. 2011) and was suggested to induce cell expansion (Lee et al. 2015). Interestingly, loss of HRE2 in hre2-1 did not reduce or abolish AR elongation under hypoxia, suggesting that a redundant regulatory factor exists. Since the response in erfVII seedlings is altered, redundancy likely comes from the ERFVII group, possibly from RAP2.2 that was not studied here due to the lack of a knockout line. It is further conceivable that knockout of HRE2 results in activation of other ERFVII that then compensate for its function. HRE1 expression is induced in roots by hypoxia and ethylene (Licausi et al. 2010; Hess et al. 2011; Eysholdt-Derzsó & Sauter 2017) and was shown to reduce ethylene inhibition of primary root growth. The molecular mechanism of HRE1 activity is not known, except that it does not alter ethylene production (Yang et al. 2011). While overexpression of HRE1 did not alter AR density, seedlings that over-expressed both HRE1 and HRE2 appeared to have an intermediate phenotype. It is conceivable that HRE1 and HRE2 can heterodimerize, which would contribute to fine-tuning of flooding responses. Taken together, ERFVII's promote elongation growth of emerged AR and this growth-promoting activity can be mimicked by overexpression of HRE2 but not HRE1, RAP2.3 or RAP2.12. Hence, a specific ERFVII contributes to the remodelling of the *Arabidopsis* root system in response to flooding.

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**AUTHOR CONTRIBUTIONS**

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

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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

**Figure S2.** Hypocotyl length in ein3 eil1, HRE1 and HRE2 mutants.

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

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

**Table S1.** Primers used for cloning and RT-PCR.

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Hypoxia promotes adventitious root elongation

Eyholdt-Derzsö & Sauter

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**Hypoxia promotes adventitious root elongation**

Eyholdt-Derzsö & Sauter

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Eyholdt-Derzsö & Sauter

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