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Lysigenous Aerenchyma Formation in *Arabidopsis* Is Controlled by *LESION SIMULATING DISEASE1* [W] [OA]

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Aerenchyma tissues form gas-conducting tubes that provide roots with oxygen under hypoxic conditions. Although aerenchyma have received considerable attention in *Zea mays*, the signaling events and genes controlling aerenchyma induction remain elusive. Here, we show that *Arabidopsis thaliana* hypocotyls form lysigenous aerenchyma in response to hypoxia and that this process involves H_2O_2 and ethylene signaling. By studying *Arabidopsis* mutants that are deregulated for excess light acclimation, cell death, and defense responses, we find that the formation of lysigenous aerenchyma depends on the plant defense regulators *LESION SIMULATING DISEASE1* (*LSD1*), *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), and *PHYTOALEXIN DEFICIENT4* (*PAD4*) that operate upstream of ethylene and reactive oxygen species production. The obtained results indicate that programmed cell death of lysigenous aerenchyma in hypocotyls occurs in a similar but independent manner from the foliar programmed cell death. Thus, the induction of aerenchyma is subject to a genetic and tissue-specific program. The data lead us to conclude that the balanced activities of *LSD1*, *EDS1*, and *PAD4* regulate lysigenous aerenchyma formation in response to hypoxia.

INTRODUCTION

Each year, flooding greatly reduces global land crop harvests (Dennis et al., 2000; Setter and Waters, 2003). Since flooding leads to the depletion of soil oxygen, the main strategy for improving flooding and/or waterlogging tolerance in land crops has been directed toward improving the tolerance to hypoxia (<4% [O_2]) and anoxia (close to 0% [O_2]). The pores in aerated soils contain the same level of oxygen as the atmosphere (21% [O_2]). However, during flooding, the air in these pores is replaced by water, and because of the slow diffusion rates of oxygen in water and the aerobic activities of plant roots and microorganisms, the oxygen levels quickly fall (Pezeshki, 1994).

Adaptation to low oxygen levels in plants occurs in three stages. Initially, the plant rapidly induces a set of signal transduction components (Dennis et al., 2000). This is followed by metabolic adaptation involving fermentation pathways and, finally, depending on plant species, by morphological changes such as aerenchyma and adventitious root formation (Dennis et al., 2000). In species such as *Arabidopsis thaliana*, *Zea mays*, and *Iris pseudacorus*, hypoxia responses involve signaling pathways controlled by ethylene, reactive oxygen species (ROS), and abscisic acid (Monk et al., 1987; He et al., 1996; de Bruxelles et al., 1996; Peng et al., 2001; Baxter-Burrell et al., 2002; Klok et al., 2002; Fukao and Bailey-Serres, 2004). Physiological responses to hypoxia include wilting and stomata closure (Pezeshki et al., 1996a), Ca^{2+} signaling (Subbaiah and Sachs, 2003), and increased levels of hemoglobins (Dordas et al., 2003). The levels of hemoglobins may be affected by auxins (Watts et al., 2001) and may directly affect ethylene signaling (Manac'h-Little et al., 2005).

Hypoxia-induced aerenchyma occurs in some non-wetland plants to improve the aeration of the rhizosphere (Jackson and Armstrong, 1999). Plants form aerenchyma using two different processes, either schizogeny or lysigeny, or by their combination (Drew et al., 2000). Schizogenous aerenchyma involves cell wall reorganization and cell separation, whereas lysigenous aerenchyma is formed as a consequence of programmed cell death (PCD) and cell wall autolysis (Campbell and Drew, 1983; Gunawardena et al., 2001b; Evans, 2003). In *Z. mays*, ethylene has been shown to be associated with lysigenous aerenchyma formation (Jackson et al., 1985; He et al., 1996).

To date, studies have shown that *Arabidopsis* seedlings do not form aerenchyma in response to hypoxia. However, since waterlogging causes stratified oxygenation in soils, and the top layer maintains high oxygen levels, it would not be advantageous for small *Arabidopsis* seedlings to form aerenchyma. (Evans, 2003; Yu and Patrick, 2003; Colmer et al., 2004; Fukao and Bailey-Serres, 2004). However aerenchyma formation may be of importance in adult *Arabidopsis* plants, which have deeper root systems and larger rhizospheres.

Genetic and mechanistic links were found between light acclimation processes and defense against pathogen infection in studies of the *LESION SIMULATING DISEASE1* (*LSD1*),

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PHYTOALEXIN DEFICIENT4 (*PAD4*), and *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) functions (Mateo et al., 2004) that control ROS and regulate PCD in *Arabidopsis* leaves (Jabs et al., 1996; Rusterucci et al., 2001). Consistent with its role in both initiating and propagating foliar PCD, loss-of-function lsd1 mutants exhibit runaway cell death (RCD) in response to light stress or pathogen infection in leaves. RCD is a laterally spreading, unchecked cell death that affects a major part of the leafy rosette. (Rusterucci et al., 2001; Mateo et al., 2004). *LSD1* is an important regulator of plant PCD through several interactions (Jabs et al., 1996; Rusterucci et al., 2001). *LSD1* sequence predicts that the encoded protein functions as a transcription factor of the C2C2 zinc finger family. These types of proteins were shown to link different environmental stimuli to caspase activity in many organisms (Uren et al., 2000). We were able to link RCD in *lsd1* to the activity of photosystem II (RCD was induced by 680-nm light but not by 700-nm light), stomatal conductance, and ultimately to photorespiratory H_2O_2 (Mateo et al., 2004). Null mutations in *PAD4* and *EDS1* block *lsd1*-conditioned RCD triggered by long photoperiods, high light, photorespiratory conditions, pathogen inoculation, ROS provision, or supply of the phenolic signaling molecule, salicylic acid (SA) (Jabs et al., 1996; Rusterucci et al., 2001; Mateo et al., 2004). The results point to multiple roles of LSD1 in reducing cellular ROS content (1) by controlling PAD4- and EDS1-dependent stomatal closure and consequently foliar (photoresiratory) H_2O_2 production during excess excitation energy (EEE) and (2) by regulating the H_2O_2 scavenging capacity (Mateo et al., 2004).

The SA pathway leading to systemic immunity is under negative control by mitogen-activated protein kinase 4 (Petersen et al., 2000; Rusterucci et al., 2001; Brodersen et al., 2006). Current evidence suggests that EDS1 and PAD4 amplify ethylene and SA signals by processing ROS-derived molecules that are essential for expression of cellular immunity against biotrophic pathogens (Rusterucci et al., 2001; Brodersen et al., 2006). EDS1 signaling complexes are nucleocytoplasmic, and evidence suggests that dynamic interactions between cell compartments are important for effective stress signal relay (Feys et al., 2005). Two new components of *Arabidopsis* immunity were discovered using gene expression microarrays combined with reverse genetics (Bartsch et al., 2006). A flavin-dependent monooxygenase (FMO1) positively regulates the EDS1 pathway, and one member (NUDT7) of a family of cytosolic Nudix hydrolases exerts negative control of EDS1 signaling. A common theme underlying the functions of counterparts of these proteins in animals and yeast is in redox stress responses.

However, the role of *LSD1*, *PAD4*, and *EDS1* in acclimation to EEE, in regulation of PCD, and in defense responses was assessed only in ambient oxygen concentration and only in foliar tissues. We observed that LSD1 is a negative regulator of ethylene signaling. Therefore, we wondered if the above genetic system controls other PCD responses, for example, lysigenous aerenchyma formation, in different tissues than that observed in foliar tissues during defense and EEE acclimatory responses. Here, we report that *LSD1* and its associated genetic system also specifically control the induction of lysigenous aerenchyma in *Arabidopsis* hypocotyls under root hypoxia.

RESULTS

Arabidopsis Hypocotyls Form Aerenchyma in Response to Hypoxia

To investigate whether hypoxia response in *Arabidopsis* includes lysigenous aerenchyma induction, we used 4- and 12-week-old

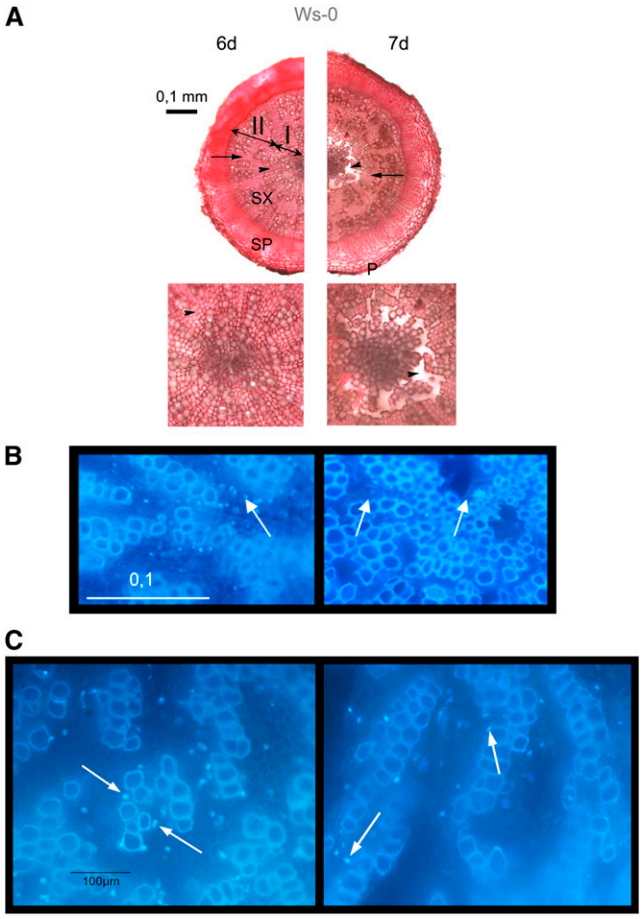

Figure 1. *Arabidopsis* Hypocotyls Form Aerenchyma in Response to Root Hypoxia.

(A) Anatomy of the root-hypocotyl axis in 12-week-old plants waterlogged for 6 and 7 d as seen in ruthenium red–stained cross sections. Representative samples from 6 and 7 d of waterlogging ($n = 8$). Mostly secondary tissues are seen, including secondary phloem (SP), secondary xylem (SX), and periderm (P) in the top panel. In secondary xylem, there is an inner zone with vessel elements and axial parenchyma cells (arrowhead) called xylem I (I). This zone is magnified in the bottom panel. Note a disappearance of axial parenchyma cells between days 6 and 7. The outer zone of secondary xylem is composed of vessel elements and fibers (arrow) called xylem II (II). **(B)** DAPI-stained xylem I in control and waterlogged plants forming aerenchyma. Representative samples from 6 and 7 d of waterlogging ($n = 3$). Note the presence of whole nuclei on the sixth day of waterlogging and DNA condensation and cell walls lysis (arrows) visible in plants waterlogged for 7 d. **(C)** Difference in nuclei from representative samples on the sixth and seventh days of waterlogging. Normal nuclei can be observed in plant waterlogged for 6 d, while condensated and moon-shaped nuclei were found in plants waterlogged for 7 d (arrows).

flowering plants grown in soil. We submerged the soil entirely in water for a period of 8 d. After 7 d, the solution surrounding the pots had reached hypoxic levels (~4% O_2) (see Supplemental Figure 1 online). The hypocotyl-root axis was examined under the microscope each day during waterlogging for signs of aerenchyma formation. The hypocotyl-root axis of 4- and 12-week-old plants was mostly composed of secondary tissues. All primary extrastellar tissues had been shed, and new tissues had been initiated from the cork cambium (cork and secondary cortex) and the vascular cambium (secondary phloem and secondary xylem). In the secondary xylem of 12-week-old plants, two distinct zones were observed, termed xylem I and xylem II (Chaffey et al., 2002) (Figure 1A). Xylem I contained vessel elements surrounded by axial parenchyma, whereas xylem II was composed of vessel elements and lignified fibers. However, only xylem I was produced in 4-week-old plants.

No aerenchyma was observed in 4-week-old plants during the period of waterlogging and reduction of oxygen concentration to 4% (see Supplemental Figure 2 online). The 12-week-old plants did not show any anatomical changes during the first 6 d of waterlogging. After 7 d of waterlogging, the axial parenchyma cells of the secondary xylem I in the central part of the hypocotyl disappeared, forming air spaces (Figure 1A). Parenchyma cells of periderm or secondary phloem appeared normal. In the air spaces that formed in the central part, no sign of cell separation was detected. By contrast, the cell walls apparently vanished, indicating that the formation of aerenchyma was due to cell lysis. The air spaces formed all along the shoot-root axis (Figure 2). Lysigeny was confirmed by the disappearance of nuclei of these cells as evidenced by 4′-6-diamidino-2-phenylindole (DAPI) staining (Figure 1B). Analysis of the DAPI-stained nuclei revealed

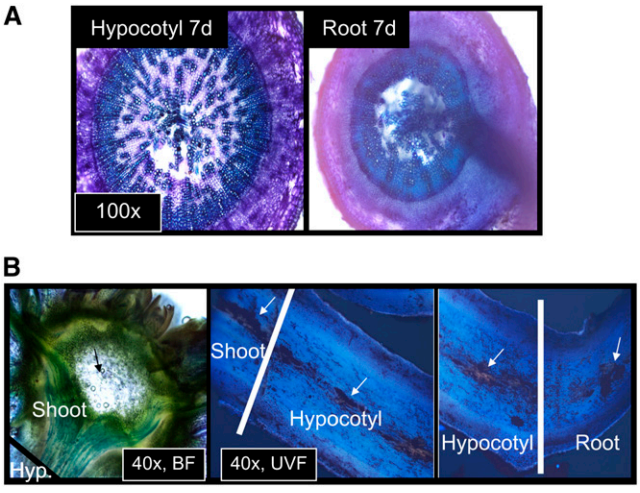

Figure 2. Aerenchyma Stretches from Shoot to Root.

(A) Aerenchyma was detected at several different distances from the shoot in both hypocotyl and in root tissues.

(B) Aerenchchyma (white arrows) stretches continuously from the naturally occurring cavities of the *Arabidopsis* shoot (black arrow) through the unexposed parts of the hypocotyl to hypoxia-exposed hypocotyl and root tissues. BF, bright-field microscopy; UVF, UV field microscopy; x indicates objective magnification.

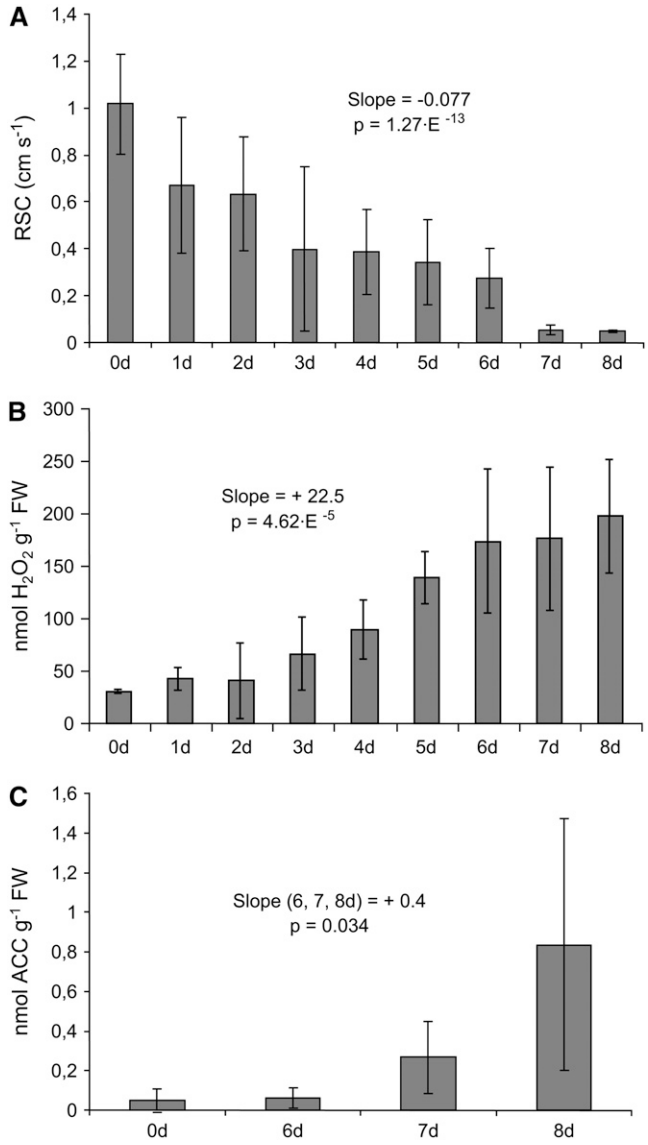

Figure 3. Stomatal Conductance Decreases and Hydrogen Peroxide and Ethylene Are Increased during the Course of Aerenchyma Formation in *Arabidopsis*.

(A) A gradual decrease in relative stomatal conductance (RSC) was detected during 8 d of waterlogging in *Arabidopsis* leaves (slope value and P value of simple linear regression is presented in the chart, $n > 6$ for each time point, error bars are ± SD).

(B) During the same period and treatments, there was a gradual increase in hydrogen peroxide (slope value and P value of simple linear regression is presented in the chart, $n = 4$ sets of at least four grouped samples for each time point, error bars are ± SD). FW, fresh weight.

(C) The ethylene precursor ACC increased in the *Arabidopsis* hypocotyls after 6 d of waterlogging (slope value and P value of simple linear regression is presented in the chart, $n = 3$ sets of at least four grouped samples for each time point, error bars are ± SD). Observe that decrease in stomatal conductance and increase in hydrogen peroxide level precede the increase of ACC by several days.

characteristics of PCD, such as moon-shaped nuclei and DNA condensation (Figure 1C) (Pennell and Lamb, 1997).

Since it has been shown that responses to hypoxia involve closure of leaf stomata and reduction of photosynthetic capacity in several angiosperm species (Pezeshki et al., 1996a, 1996b), we monitored stomatal conductance during the 8 d of waterlogging. Stomata closure decreased continuously during the 8 d of waterlogging, indicating that the plants were responding to the rhizospheric hypoxia (Figure 3A). Reduced stomatal conductance is known to play an important role in increased formation of H_2O_2 that functions as EEE acclimatory systemic signal (Karpinski et al., 1999; Mateo et al., 2004). Thus, we hypothesized that the same elements of systemic signaling might be used to induce PCD signals in axial parenchyma cells. Consistent with this hypothesis, we have observed that aerenchyma could be induced faster when plants were exposed to EEE (high light 500 μE or long photoperiod 18 h) (see Supplemental Table 1 online). These data suggest that light conditions affect formation of lysigenous aerenchyma during waterlogging. Thus, we propose that the plant induces an interactive systemic signaling network during waterlogging and light acclimation.

To investigate if aerenchyma induction in *Arabidopsis* involves ethylene and ROS signaling, we monitored the levels of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of de novo ethylene synthesis (Adams and Yang, 1979), and hydrogen peroxide (H_2O_2) during the 8-d waterlogging period leading to aerenchyma formation in hypocotyls. The levels of H_2O_2 increased in a gradual manner from day 1 to day 8 (i.e., before the formation of aerenchyma was detected) (Figure 3B). ACC is the immediate biosynthetic precursor of ethylene and is a reliable marker of ethylene levels in the plant (Adams and Yang, 1979). ACC level in the waterlogged plants remained unchanged for 6 d, but the level strongly increased after 6 d (Figure 3C), notably coinciding with the formation of the lysigenous aerenchyma (Figure 1A).

ASCORBATE PEROXIDASE1 (*APX1*) is a ubiquitous H_2O_2 scavenging enzyme that has been shown to be regulated by H_2O_2 and ABA (Karpinski et al., 1997, 1999; Storozhenko et al., 1998). To analyze induction of APX1 expression, we have used transgenic *Arabidopsis* plants that harbor the *APX1*-promoter fused to the firefly luciferase gene (*APX1:LUC*). Luciferase activity in this transgenic line mirrors induction of the native *APX1* gene (Karpinski et al., 1997, 1999). We observed that the *APX1:LUC* transgene was induced by 7 d of waterlogging (Figure 4A). We also found that the *APX1:LUC* transgene was induced more in roots than in shoots (Figure 4B), indicating root-specific ROS (H_2O_2) signaling. Altogether, these data show that *Arabidopsis* plants induce lysigenous aerenchyma in secondary xylem I of the hypocotyl-root axis in response to root hypoxic conditions and that this response may involve H_2O_2, ethylene, systemic root-to-shoot signaling, and PCD (Figures 1 to 4).

LSD1, *EDS1*, and *PAD4* Control Lysigenous Aerenchyma Formation

Previously, we have shown that *LSD1* inhibits stomatal closure and ROS signaling during acclimation to EEE (Mateo et al., 2004). We were interested if the same genetic system was involved in regulation of the aerenchyma formation and ethylene signaling. Therefore, we compared the aerenchyma formation in the wild type (Wassilewskija [*Ws-0*]) and in the *lsd1* null mutant. Image analysis was used to quantify the area of induced aerenchyma in cross sections of *lsd1* plants and the corresponding Ws-0 ecotype plants that had been waterlogged for 7 d. While Ws-0 ecotype produced on average 4.7% of aerenchyma per unit area of the secondary xylem core, the *lsd1* mutant had an on average 9.6% of aerenchyma (Figures 5A and 5C) even though the amount of parenchyma was similar in the *lsd1* mutant as in the wild-type plants (see Supplemental Figure 3 online). This indicates that LSD1 acts as a negative regulator of lysigenous aerenchyma formation, and this is in agreement with the previously described role of LSD1 role in PCD (Jabs et al., 1996; Rusterucci et al., 2001; Mateo et al., 2004).

RCD in *lsd1* leaves is dependent on the functions of *EDS1* and *PAD4* (Rusterucci et al., 2001; Mateo et al., 2004). Therefore,

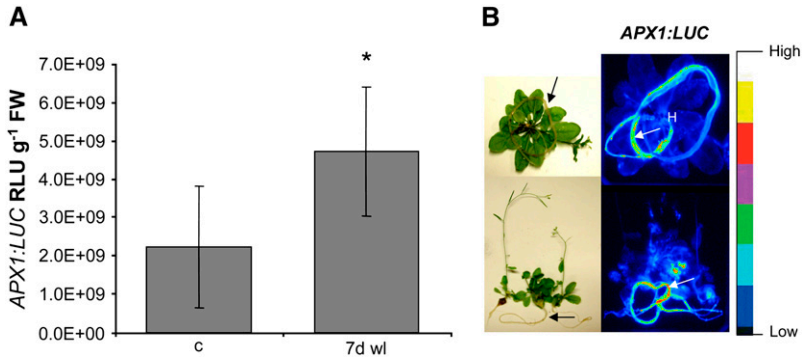

Figure 4. Analyses of *APX1* Expression Reveals Root-Specific Signals.

(A) Quantification of luciferase level controlled by the *APX1* promoter in transgenic *APX1promoter:LUC Arabidopsis* Col-0 rosettes, shoots, hypocotyls, and roots (Karpinski et al., 1999; Karpinska et al., 2000) showed that 7 d of waterlogging [7 d wl]) induced *APX1:LUC* expression compared with untreated plants (c) (*$P < 0.05$, Students *t* test, $n = 5$, error bars are ± SD).

(B) Imaging of luciferase level in the same transgenic lines revealed a significantly higher *luciferase* activity in roots than in shoots during waterlogging. Arrows indicate root systems, and H indicates hypocotyls of the investigated plants.

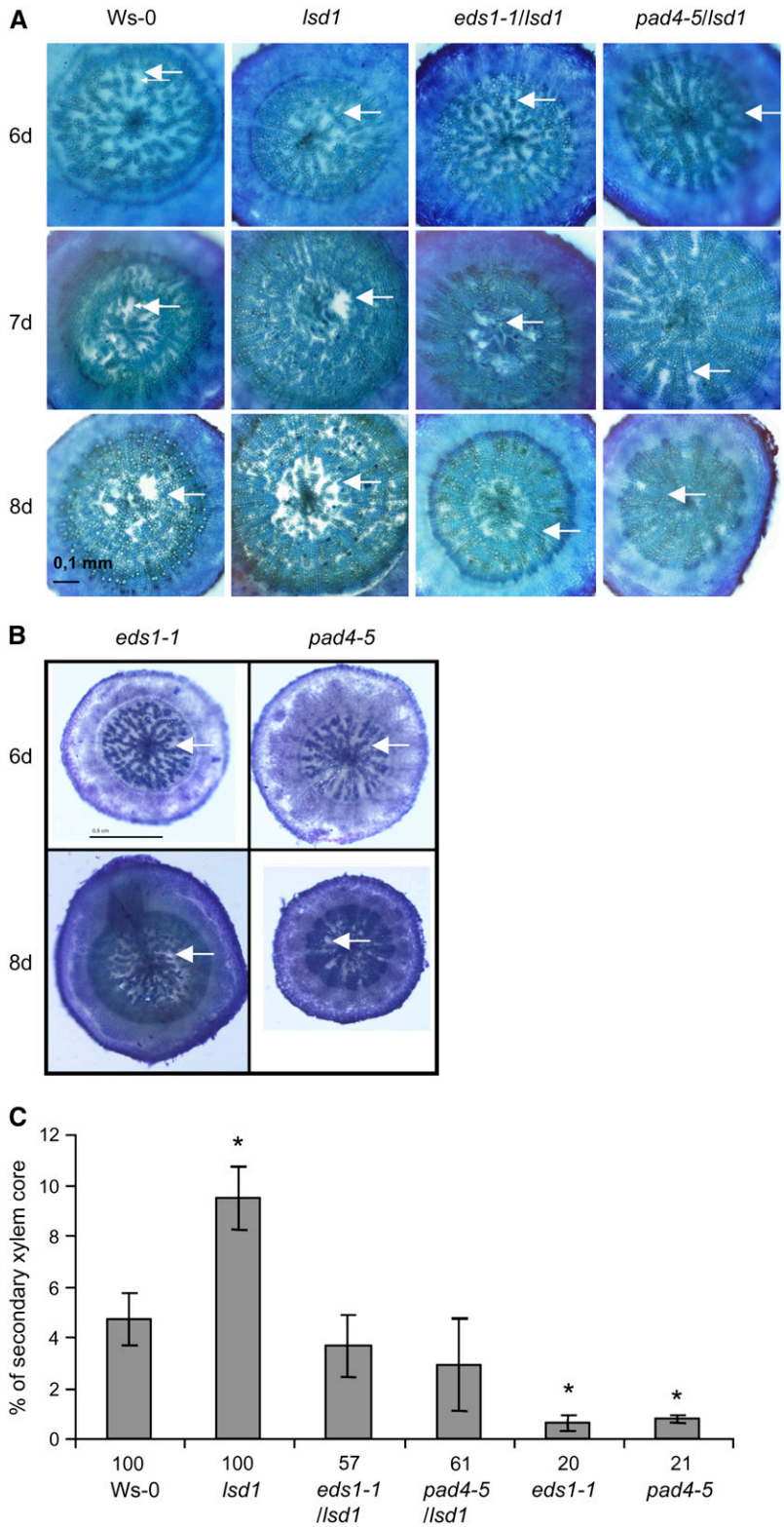

Figure 5. *LSD1*, *EDS1*, and *PAD4* Control Aerenchyma Formation in *Arabidopsis*.

(A) Toluidine blue–stained cross sections of *Arabidopsis* Ws-0, *lsd1*, *lsd1 eds1-1*, and *lsd1 pad4-5* hypocotyls of plants subjected to waterlogging for 6, 7, and 8 d. Representative samples are shown ($n = 8$). Arrows indicate parenchyma (6 d) and aerenchymatous lacunae (7 and 8 d).

(B) Toluidine blue–stained cross sections of *Arabidopsis eds1-1* and *pad4-5* hypocotyls waterlogged for 7 d. Representative samples are shown ($n = 8$). White arrows indicate parenchyma (6 d waterlogged) and aerenchymatous lacunae (7 d).

(C) Quantification of cross-section areas of aerenchymatous lacunae in *Arabidopsis* Ws-0, *lsd1*, *eds1-1 lsd1*, *pad4-5 lsd1*, *eds1-1*, and *pad4-5* hypocotyls waterlogged for 7 d (*$P < 0.05$, Students *t* test, $n = 8$, error bars are ± SE). The numbers beneath each column denote the percentage of plants that formed aerenchyma.

we quantified formed aerenchyma per secondary xylem area formation in *eds1-1 lsd1* and *pad4-5 lsd1* double mutants and in *eds1-1* and *pad4-5* single null mutants (all in the Ws-0 ecotype), thinking that they may revert the *lsd1* phenotype. Interestingly, only 60% of the *eds1-1 lsd1* and *pad4-5 lsd1* double mutant plants induced aerenchyma at days 7 and 8 compared with 100% plants in the wild-type and *lsd1* plants (Figures 5A and 5C). The plants that formed aerenchyma had the area of aerenchymatous lacunae comparable to that of the wild type (i.e., two times less than the *lsd1* mutant) (Figure 5C). Consistently, only 20% of *eds1-1* and *pad4-5* mutant plants induced aerenchyma at days 7 and 8 (Figures 5B and 5C). In these lines (*eds1-1* and *pad4-5*), the plants that did induce aerenchyma had only 0.6 and 0.75% of aerenchyma per unit area of the secondary xylem core, respectively, which was significantly less than the wild type or the *eds1-1 lsd1* and *pad4-5 lsd1* double mutants (Figure 5C). These data indicate that *EDS1* and *PAD4* positively regulate both induction and the amount of this tissue counteracting the inhibitory action of *LSD1*.

To further investigate the signaling network that regulates aerenchyma formation, we measured ACC and H_2O_2 levels during EEE exposure of wild-type and mutant plants (Figure 6A). EEE exposure caused higher increases of ACC levels in *lsd1* leaves compared with the wild type (twofold versus sixfold, respectively; Figure 6A). Analysis of the single *eds1-1* and *pad4-5* mutants as well as *eds1-1 lsd1* and *pad4-5 lsd1* double mutants revealed that the increase in ACC under EEE conditions in *lsd1* requires the defense regulators *EDS1* and *PAD4* (Figure 6B). Similar results were obtained for H_2O_2 levels (Figure 6B). Thus, *EDS1* and *PAD4* operate upstream of ethylene and ROS production in EEE stress signaling, leading to propagation of cell death in *lsd1*.

We wondered if the development of foliar RCD had an impact on aerenchyma formation and vice versa. Development of RCD in older leaves does not induce aerenchyma in hypocotyls under normal oxygen concentration (Figure 7). In addition, plants that developed foliar RCD and then were exposed to waterlogging for 7 d could also not develope lysigenous aerenchyma (Figure 7). This observation indicated that the control exerted by LSD1 in lysigenous aerenchyma formation was separated from that of foliar PCD. Additionally, these results indicated that the systemic signals produced during RCD blocked the competence for lysigenous aerenchyma formation (Figure 7).

We also analyzed *LSD1*, *EDS1*, and *PAD4* expression and biologically active *cis*-regulatory elements (*CRE*s) in the promoter region for these genes. *LSD1* was upregulated by high CO_2 levels, and *EDS1* and *PAD4* were both upregulated by short exposures to drought (Table 1). Further analysis revealed that all three genes were regulated by phosphate deficiency and heat (Table 1). This analysis revealed a similar regulation of *EDS1* and *PAD4* and partially overlapping regulation of *LSD1*. We have also used the PLACE algorithm (Prestridge, 1991; Higo et al., 1999) and our new algorithm (Geisler et al., 2006) for CRE analysis. All three genes contain possible *CRE*s involved in drought, heat, light, ROS/sugar, abscissic acid, ethylene, gibberellin, and auxin responses. These data suggest that factors that directly influence plant metabolism are integrated into *LSD1*-, *EDS1*-, and *PAD4*-mediated redox and cell death signaling.

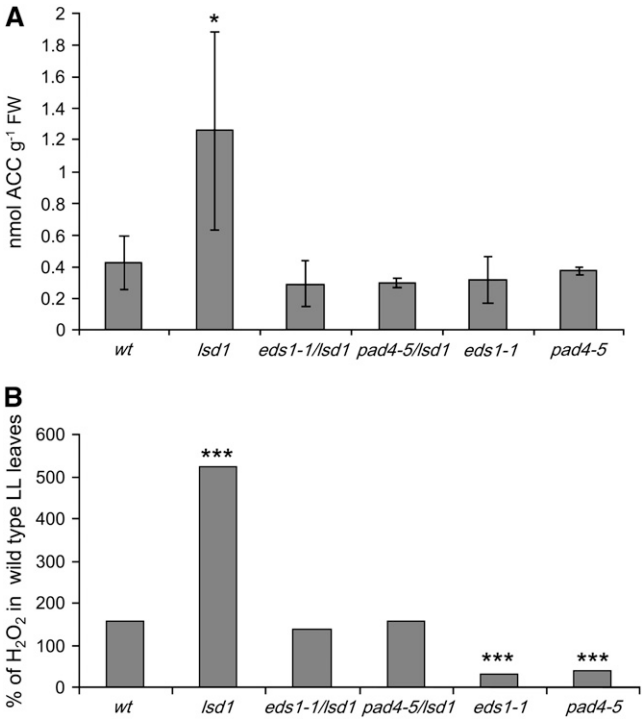

Figure 6. *LSD1*, *EDS1*, and *PAD4* Control Stress Induced Ethylene and Hydrogen Peroxide Levels in *Arabidopsis*.

(A) EEE treatment (2000 ± 250 μmol m^2 s^{-1}) induces significantly higher production of ACC in Ws-0 *lsd1* mutants than in the Ws-0 wild type (Student's *t* test P < 0.05*, $n = 4 \pm$ SD for each treatment). *lsd1* mutants produce threefold higher amounts of the ethylene precursor ACC in response to EEE compared with wild-type plants. Therefore, LSD1 is a negative regulator of stress ethylene. Ethylene signaling in the absence of functional LSD1 was positively dependent on EDS1 and PAD4 since *eds1-1 lsd1* and *pad4-5 lsd1* double mutants reverted to wild-type levels of ACC in EEE and single *pad4-4* and *eds1-1* mutants could not increase ACC levels. Control levels of ACC in Ws-0 and all mutants vary between 0.15 and 0.24 nmol ± 0.12 SD of ACC per g FW.

(B) EEE treatment induces significantly higher production of H_2O_2 in Ws *lsd1* mutants than in the Ws-0 wild type (Student's *t* test; ***P < 0.001, $n = 4 \pm$ SD for each treatment). The *lsd1* mutant produce twofold higher amounts of H_2O_2 in response to EEE compared with wild-type plants. Therefore, LSD1 is a negative regulator of stress H_2O_2. H_2O_2 signaling in absence of functional LSD1 was positively dependent on *EDS1* and *PAD4* since *eds1-1 lsd1* and *pad4-5 lsd1* double mutants reverted to wild-type levels of H_2O_2 levels in EEE, and single *pad4-5* and *eds1-1* actually had decreased H_2O_2 levels (Student's *t* test; ***P < 0.001, $n = 4$ ±S.D. for each treatment). Control levels of H_2O_2 in Ws-0 and all mutants were set to 100%

DISCUSSION

Arabidopsis Forms Lysigenous Aerenchyma in Secondary Xylem I in Response to Flooding

We found that flooding of 12-week-old *Arabidopsis* plants induces autolysis of the axial parenchyma cells in secondary xylem I. This tissue is formed during the secondary growth of the root-hypocotyl axis, and it is composed of axial parenchyma cells and

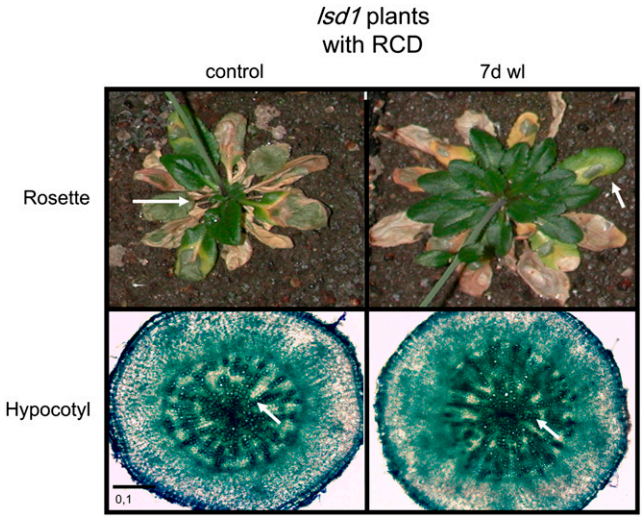

Figure 7. Development of Aerenchyma and Foliar RCD in *lsd1* Plants Follows Tissue-Specific Regulation.

In *lsd1* plants cultivated in normal oxygen concentration and with strongly induced foliar RCD, we could not detect aerenchyma in hypocotyls of 12-week-old control plants. Waterlogging for 7 d of such plants also did not induce aerenchyma after 7 d of waterlogging (7d wl). Arrows indicate leaves in *lsd1* in the top panels with RCD and parenchyma in hypocotyl cross sections of the same plants in the bottom panels. It is concluded that development of foliar RCD before root hypoxia restricts aerenchyma formation.

vessel elements (Chaffey et al., 2002; A. Banasiak and E. Mellerowicz, unpublished data). The autolysis creates air spaces continuously throughout the stem-root axis where vessel elements with remaining parenchyma cells are apparently suspended. By definition, the aerenchyma is a tissue frequently induced by flooding where enlarged air spaces between cells create gas exchange channels (Evans, 2003; Evert, 2006); therefore, in *Arabidopsis*, we called this tissue aerenchyma.

Aerenchyma is commonly induced in young roots of hydrophytes and mezophytes but is has not been previously observed in *Arabidopsis* (Justin and Armstrong, 1987; Evans, 2003; Seago et al., 2005). Most commonly it has been found in the cortical parenchyma close to the root apical meristem (Justin and Armstrong, 1987; Seago et al., 2005). Relatively little is known about the occurrence of aerenchyma in older roots with secondary growth where the primary cortex has been destroyed. In soybean (*Glycine max*), the secondary aerenchyma developed in the secondary cortex of roots and hypocotyls (Laan et al., 1989; Shimamura et al., 2003). *Lythrum salicaria* and some other species form the aerenchyma in the phellem (Stevens et al., 2002). By contrast, pea (*Pisum sativum*; Gladish et al., 2006) and lodgepole pine (*Pinus contorta*; Topa and McLeod, 1986) induced aerenchyma in root stele when exposed to waterlogging. *Arabidopsis* plants also formed aerenchyma within the secondary tissues of the vascular cylinder, but in contrast with the species mentioned above, the aerenchymatous lacunae were mostly restricted to xylem I (Figure 1A). The younger plants that did not develop lignified secondary xylem II failed to develop aerenchyma under hypoxia conditions. The failure of younger plants to develop aerenchyma (see Supplemental Figure 2 online) might be related to the lack of a lignified xylem core that could isolate the tissues by creating compartments that entrap ethylene (Evans, 2003). In young roots of wetland plants, the compact cell layers of endodermis and exodermis always surround the developing aerenchyma tissues (Seago et al., 2005), whereas suberized or lignified tissues surround aerenchyma in secondary growth (Stevens et al., 2002; Shimamura et al., 2003). Thus, the developmental stage of the plants appears to play a role in their ability to form aerenchyma.

Classically, two basic mechanisms of aerenchyma formation have been described: cell lysis (lysigeny) and cell separation (shizogeny) (Justin and Armstrong, 1987; Evans, 2003; Evert, 2006). In addition, cell division and/or differential cell expansion (expansigeny) is involved in many but not in all species studied to date (Seago et al., 2005). In hypocotyls of 12-week-old *Arabidopsis* plants, no signs of cell expansion, division, necrosis, or cell separation were observed. Instead, the cells disappeared completely by lysigeny (Figure 1).

The cell wall autolysis in *Arabidopsis* plants was rapid. During the first 6 d of flooding, no signs of any differentiation were observed at the light microscopy level, and at day 7, condensed and moon-shaped nuclei were detected in not fully digested

Table 1. Expression of *LSD1*, *EDS1*, and *PAD4* after Different Treatments of Wild-Type and *PHO1* and *PHO3* Null Mutant Plants

	Gene		
Treatment	*LSD1*	*EDS1*	*PAD4*
Phosphate starvation 24 h	Induced, P = 0.006520	Not changed	Not changed
pho1 mutant	Not changed	Induced, P = 0.000419	Induced, P = 0.000419
pho3 mutant	Not changed	Induced, P = 0.000697	Induced, P = 0.000697
Elevated CO_2, 0.08%	Induced, Pearson correlation >0.8	Not changed	Not changed
Heat shock shoot 15 min	Induced, P = 0.0011	Inhibited, P = 0.009515	Inhibited, P = 0.009515
Drought <1 h	Not changed	Induced, P = 0.000259	Induced, P = 0.000184

Significant changes in transcript levels (indicated as induced or inhibited) 24 h after phosphorous starvation in *pho1* or *pho3* null mutants, 48 h after treatment with elevated CO_2 levels (0.08%) in wild-type plants, 15 min after heat shock treatment in wild-type plants, and 1 h after drought stress in wild-type plants. Plants were fumigated with 0.08% CO_2 in ambient oxygen and nitrogen concentration for 2 d. Experiments were performed in triplicate on pooled samples ($n = 3$, ±SD).

cells, while other parenchyma cells were completely digested, including their walls and protoplasts (Figures 1A and 1B). Similar timing of lysigenous aerenchyma formation has been reported in maize and rice (*Oryza sativa*; Evans, 2003). The rapidity of the autolysis process during aerenchyma development resembled that during the PCD of tracheary elements, except that in the latter case, the lignified walls and primary cell walls that are in contact with them remained intact (Ohdaira et al., 2002). However, when the deposition of secondary walls is prevented as in the *gapped xylem* mutant, the entire cell with its primary walls is autolysed (Kozela and Regan, 2003) in an apparently similar manner to the lysigenous aerenchyma.

A Genetic System That Controls Aerenchyma Formation in *Arabidopsis*

From the analysis of stomatal conductance and H_2O_2 and ACC levels, the following sequence of events leading to aerenchyma formation in *Arabidopsis* is proposed (Figures 1 to 8). First, the stomatal conductance in leaves decreases, which we propose results from a systemic signaling of hypoxia, for example, due to reduced root pressure, followed by the initiation of a gradual increase in H_2O_2 in hypocotyls for 6 d and an increase in ACC in hypocotyls preceding the aerenchyma formation after 7 d (Figures 1 and 5). In previous work, we have found that photoproduced H_2O_2 is strongly correlated with a decrease in leaf stomatal conductance and foliar RCD (Mateo et al., 2004). Additionally, we showed that light sensitivity, stomatal conductance, and ROS accumulation are controlled by *LSD1*, *EDS1*, and *PAD4* in foliar tissues (Figure 6; Mateo et al., 2004). Therefore, we tested if this genetic system regulated the aerenchyma formation. Here, we show that *LSD1*, *PAD4*, and *EDS1* also control lysigenous aerenchyma formation via ethylene and ROS (Figures 4 to 7). It is important to note, however, that the PCD of lysigenous aerenchyma in hypocotyls occurs in a similar but independent manner to the foliar PCD when roots are exposed to hypoxia. This is indicated by the observations that simultaneous exposure of rosettes to mild EEE and hypoxia stress induce RCD (Mateo et al., 2004) and accelerate aerenchyma formation (see Supplemental Table 1 online). However, it is important to note that under root normoxia and induced foliar RCD or under root hypoxia of plants that already developed RCD in majority of leaves, aerenchyma was not formed (Figure 7). In addition, the observation that *APX1* was induced more in roots than shoots (Figure 5) confirms that root-specific signaling is a prerequisite for aerenchyma formation. Thus, the induction of aerenchyma is subject to a genetic and tissue-specific program. Moreover, roots and shoots respond differently to waterlogging (Liao and Lin, 2001) in several plant species as well as in *Arabidopsis* (Ellis et al., 1999; Liao and Lin, 2001), and our results confirm this observation.

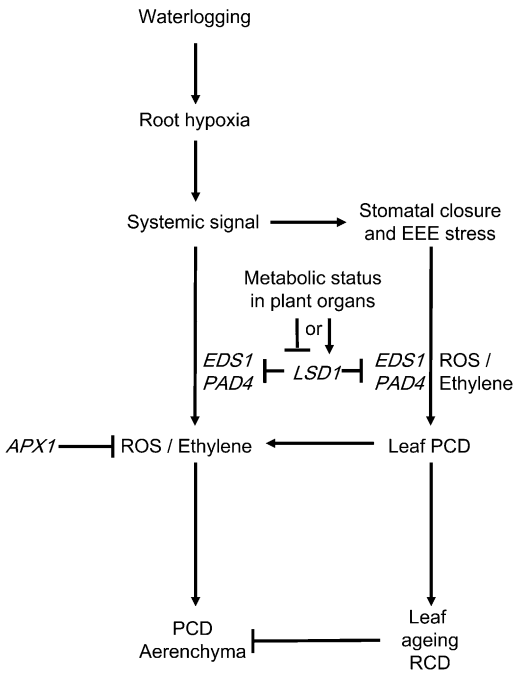

Figure 8. Proposed Working Model for Signaling Pathways That Regulate Aerenchyma Formation in *Arabidopsis*.

Root hypoxia produces a systemic signal that promotes ROS/ethylene formation, leading to the induction of aerenchyma and stomatal closure. Stomatal closure leads to EEE stress, which enhance ROS/ethylene systemic signaling and PCD in leaves (Mateo et al., 2004) and aerenchyma formation (Figures 1 to 7; see Suplemental Table 1 online). ROS/ethylene signaling operates in a positive feed-forward loop, which is negatively controlled by LSD1 and positively controlled by PAD4 and EDS1 (Figures 5 to 7). PCD during aerenchyma formation is independent from that in leaves and needs a tissue-specific signal. Severe PCD development (RCD) in leaves somehow restricts the development of aerenchyma.

We suggest that several environmental factors are integrated for the induction of lysigenous aerenchyma. First, light quantity has been shown to affect stellar development (Wahl et al., 2001), and we observed that stellar development determines the ability of *Arabidopsis* hypocotyls to produce aerenchyma. We also observed that different light conditions affect the timing of aerenchyma induction (see Supplemental Table 1 online). Second, CO_2 has been shown to accumulate in waterlogged soils to levels (Drew et al., 1979; Jackson, 1985) that are ~5 times higher than what is needed to promote expression of *LSD1* (Table 1). High CO_2 levels are also known to affect both ethylene signaling and aerenchyma formation (Drew et al., 1979; Jackson, 1985; Constable and Longstreth, 1994; Bragina et al., 2001; Liao and Lin, 2001). Third, root hypoxia reduces phosphorus accumulation in roots and reduces total phosphorus uptake in the plant (Drew et al., 1979; Topa and Cheeseman, 1994). Phosphorous deficiency also induces aerenchyma formation and enhances production and sensitivity to ethylene and other hormones (such as auxin) involved in hypoxia (He et al., 1992; Fan et al., 2003). Our transcript level analysis and meta-analysis of DNA microarray experiments show that phosphorous deficiency strongly influences *LSD1*, *EDS1*, and *PAD4* gene expression. Phosphorous deficiency caused either by limitation of Pi in the growth medium or through mutation in the *PHOSPHOLIPIDS DEFICIENT1* (*PHO1*) or *PHO3* genes that cause phosphate-deficient phenotypes induce *LSD1*, *EDS1*, and *PAD4* (Abel et al., 2002;

Table 1). Additionally, reduced respiration is known to be a common denominator for phosphorous deficiency, hypoxia, and high CO_2 (Qi et al., 1994; Fan et al., 2003), indicating that the metabolic status in the plant is an important determinant for aerenchyma formation. Therefore, we propose that metabolic signals derived from hypoxia upstream of *LSD1*, *EDS1*, and *PAD4* contribute to the regulation of the cellular redox status during aerenchyma formation as suggested in the proposed model (Figure 8).

It is possible that these genes in their functions as redox regulators have effects on the whole-plant metabolism that may have affected aerenchyma formation. Nevertheless, *LSD1*, *EDS1*, and *PAD4* control the extent and the timing of aerenchyma formation (Figure 3). These data suggested that *LSD1* is a negative regulator, while *PAD4* and *EDS1* are positive regulators of the amount of aerenchyma. Additionally, *PAD4* and *EDS1* also regulate the timing of the event (Figure 3B). Since both *PAD4* and *EDS1* functions are needed to initiate aerenchyma, these genes likely act in a common pathway, as previously described (Rusterucci et al., 2001; Mateo et al., 2004). These findings also indicate that the *LSD1*, *EDS1*, and *PAD4* genes may have important functions in other responses to hypoxia (Mateo et al., 2004; Table 1).

We have concluded that hypocotyls and roots of the model plant species, *Arabidopsis*, can form lysigenous aerenchyma associated with PCD. We have also confirmed that some of the known inducers and regulators of lysigenous aerenchyma in other species, such as hypoxia, H_2O_2, and ethylene are involved in aerenchyma development in *Arabidopsis*. We have demonstrated that timing of ROS signaling is ahead of ethylene signaling in aerenchyma development. Moreover, we have identified genes essential for this process (Figures 5 to 8). Therefore, we report here that this genetic system does not only control leaf PCD during normoxia but also controls a tissue-specific PCD during hypoxia.

Future Perspectives

Our findings open new possibilities of using the genetic system of *Arabidopsis* to elucidate the underlying mechanism of this important process of stress adaptation in plants. An interesting aspect of the flooding response in *Arabidopsis* is the predictable and complete autolysis of cell walls. This gives another opportunity, namely, to use this system to study cell wall autolysis. This process is very important, for example, in the vessel differentiation of angiosperm plants, but because no good experimental system has been developed to study this process so far, the enzymes involved and their dynamics remain elusive.

Moreover, we have shown that the lysigenous aerenchyma of *Arabidopsis* is formed at a late stage of development when the secondary growth is advanced. It is possible that most species with profuse secondary growth, especially trees, form a similar type of aerenchyma, and this is an area that desperately needs to be investigated. Since *Arabidopsis* is already a well-explored model system for wood formation (Chaffey et al., 2002; Nieminen et al., 2004), our findings open up new possibilities of using the *Arabidopsis* model to specifically address issues relating to tree responses to flooding and hypoxia.

METHODS

Growth and Light Conditions

Arabidopsis thaliana Ws-0 and five different mutants of the same accession were used: *lsd1*, *eds1-1*, and *pad4-5* mutants and the *lsd1 eds1-1* and *lsd pad4-5* double mutants (Mateo et al., 2004). Plants were grown in low-light chambers at PFD 100 $\pm$ 25 μmol m^2 s^{-1}, relative air humidity 60%, and temperature 20 $\pm$ 2°C in a 9-h photoperiod. Oxygen levels were analyzed using a Schott minilab 12 (Schott-Geräte). For excess light, *Arabidopsis* plants were treated at PFD 2000 $\pm$ 250 μmol m^2 s^{-1} (HMI 1200 W/GS photo-optic lamp; Osram) for up to 2 h. Photoperiod, temperature ($\pm$4°C), and humidity ($\pm$5%) for all experiments were similar to those in the low-light chamber.

Stomatal Conductance

Stomatal conductance was measured in growth conditions by measuring the speed of rehydration (cm/s) of a cyclically desiccated chamber by 1-cm^2 leaf areas. We used a portable AP4 Porometer (Delta-T Devices) according to the manufacturer's instructions.

Aerenchyma Detection and Quantification

Hypocotyls of waterlogged and control plants (12 weeks old) were fixed in FAA (70% ethanol, 5% acetic acid, and 1.75% formaldehyde) for 2 d. The fixed material was then transferred to 80% ethanol and gradually rehydrated before hand-sectioning with a razor blade. Cross sections were stained with 0.05% Toluidine Blue containing 1% boric acid for general anatomy staining or with 0.1% ruthenium red to visualize pectin-rich cell walls of parenchyma cells. Area analysis of the aerenchymatous lacunae and parenchymatous tissues was determined from grayscale digital images as previously described (Gunawardena et al., 2001a; Chaerle et al., 2004).

Root-shoot aerenchyma distribution was visualized by first dipping sections in red ink and then in 99.5% ethanol. The hardened ink was carefully wiped off the surface of the sections, leaving only the cavities ink filled. Sections were then analyzed under UV fluorescence for increased visibility. For DAPI staining, hypocotyls were collected and hand-sectioned with a razor blade. Sections were stained with DAPI solution (1 μg/mL) and observed under UV fluorescence.

Hydrogen Peroxide Quantification

Hydrogen peroxide was quantified as described (Guilbault et al., 1968; Jimenez et al., 2002), with the following modification: 100 mg of fresh *Arabidopsis* hypocotyl or leaf tissue of 12-week-old plants was used per 1 mL of extraction medium.

ACC Quantification

ACC production was determined as described by Lizada and Yang (1979) and Langebartels et al. (1991). Fresh hypocotyl or leaf tissues of 12-week-old plants were used.

Molecular Analyses

For in vitro analysis of *APX1 promoter:LUC* induction, hypocotyls of 12-week-old *APX1promoter:LUC* transgenic plants (Karpinska et al., 2000; Chang et al., 2004) were used. A luciferase assay kit (Promega) and a Berthold LB950 luminometer was used to measure the luciferase expression as previously described (Karpinski et al., 1997, 1999; Chang et al.,

2004). For in vivo visualization, *APX1promoter:LUC* transgenic plants were grown hydroponically as described (Norén et al., 2004) in Hoagland solution modified for *Arabidopsis* (Gibeaut et al., 1997). Detection of luciferase was done by spraying entire plants with a 2 mM D(−)-luciferin (Promega) solution containing 0.01% Tween and imaging of luciferase activity with a Typhoon 8600 variable mode imager (Molecular Dynamics).

Quantitative PCR reactions for *LSD1*, *PAD4*, and *EDS1* were performed with the following gene-specific primers: *LSD1* forward, 5′-GAGGAGCATCTAATGTGCGTTGT-3′, and reverse, 5′-GAGGGTGGTGTTGAAGTTGATGTA-3′; *EDS1* forward, 5′-TCAAGCTTCTGTGGAAATGG-3′, and reverse, 5′-CGCTTCCAGTCAATTCACAA-3′; *PAD4* forward, 5′-AGTTAAAGATCAAGGAAGGATTGG-3′ and reverse, 5′-CCTCTGATGTTCCTCGGTG-3′ Reactions were performed as described before (Chang et al., 2004). Putative *CRE*s of *LSD1*, *EDS1*, and *PAD4* were identified by analyzing 1000-bp 5′ upstream (promoter) regions using the publicly available PLACE (Higo et al., 1999) database algorithms and verified by our algorithm (Geisler et al., 2006), which generates a list of element identities, sequence, and position on the promoter region. Sequences of *LSD1*, *EDS1*, and *PAD4* were obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

In silico analysis of gene expression was performed on publicly available microarray data as described by Geisler et al., (2006). Data for the effect of elevated CO_2 (800 ppm) on *LSD1* expression are publicly available at http://www.arabidopsis.org (expression of clone ID 268g12XP, locus At4g20380), and we performed our elevated CO_2 experiments as described before (Mateo et al., 2004).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: At4g20380 (*LSD1*), At3g52430 (*PAD4*), and At3g48090 (*EDS1*).

Supplemental Data

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

Supplemental Figure 1. Oxygen Levels Decrease during Waterlogging.

Supplemental Figure 2. Four-Week-Old Plants Do Not Make Aerenchyma.

Supplemental Figure 3. Wild-Type (Ws-0) and lsd1 Xylem Cores Contain Similar Amounts of Parenchyma.

Supplemental Table 1. Simultaneous Exposure to EEE and Root Hypoxia Accelerates Aerenchyma Formation.

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