Chloroplast Signaling and \textit{LESION SIMULATING DISEASE1} Regulate Crosstalk between Light Acclimation and Immunity in \textit{Arabidopsis} 

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Plants are simultaneously exposed to abiotic and biotic hazards. Here, we show that local and systemic acclimation in \textit{Arabidopsis thaliana} leaves in response to excess excitation energy (EEE) is associated with cell death and is regulated by specific redox changes of the plastoquinone (PQ) pool. These redox changes cause a rapid decrease of stomatal conductance, global induction of \textit{ASCORBATE PEROXIDASE2} and \textit{PATHOGEN RESISTANCE1}, and increased production of reactive oxygen species (ROS) and ethylene that signals through \textit{ETHYLENE INSENSITIVE2} (\textit{EIN2}). We provide evidence that multiple hormonal/ROS signaling pathways regulate the plant’s response to EEE and that EEE stimulates systemic acquired resistance and basal defenses to virulent biotrophic bacteria. In the \textit{Arabidopsis LESION SIMULATING DISEASE1 (lsd1)} null mutant that is deregulated for EEE acclimation responses, propagation of EEE-induced programmed cell death depends on the plant defense regulators \textit{ENHANCED DISEASE SUSCEPTIBILITY1} (\textit{EDS1}) and \textit{PHYTOALEXIN DEFICIENT4} (\textit{PAD4}). We find that \textit{EDS1} and \textit{PAD4} operate upstream of ethylene and ROS production in the EEE response. The data suggest that the balanced activities of \textit{LSD1}, \textit{EDS1}, \textit{PAD4}, and \textit{EIN2} regulate signaling of programmed cell death, light acclimation, and holistic defense responses that are initiated, at least in part, by redox changes of the PQ pool.

\section*{INTRODUCTION}

When low light adapted plants are exposed to a sudden increase in light intensity, they experience a large increase in absorbed light energy. Only a proportion of this energy can be used by photosynthetic metabolism (Asada, 1999; Niyogi, 2000). Excess excitation energy (EEE), the amount of energy exceeding that required for photosynthetic CO\textsubscript{2} assimilation, is dissipated in the form of heat via the xanthophyll cycle or by the provision of alternative electron acceptors, such as oxygen, in the water–water cycle and photorespiration (Willekens et al., 1997; Asada, 1999; Niyogi, 2000; Ort and Baker, 2002; Muller-Moule et al., 2003). Reactive oxygen species (ROS), such as singlet oxygen (\textit{O}_2\textsuperscript{\textbullet}), superoxide anion (\textit{O}_2\textsuperscript{\textbullet}\textsuperscript{−}), and hydrogen peroxide (\textit{H}_2\textit{O}_2), are produced during an increase in excitation energy (Asada, 1999; Karpińska et al., 1999; Niyogi, 1999; Karpińska et al., 2000; Fufezan et al., 2002). The response to EEE stress does not only involve changes in photosynthetic flux but is also accompanied by alterations in leaf water status and temperature and is as a consequence also associated with increased activity of heat shock transcription factors, raised abscisic acid (ABA) levels, changes of the redox state of glutathione, and decreased stomatal conductance (Karpińska et al., 1997, 1999; Panchuk et al., 2002; Fryer et al., 2003; Ball et al., 2004; Chang et al., 2004).

Light energy and photosynthetic flux also play important roles in the plant immune response to pathogens, and certain characteristics of plant defense are shared with its reaction to increased light intensity (Karpińska et al., 2003; Ball et al., 2004; Mateo et al., 2004). These include changes in chlorophyll \textit{a} fluorescence parameters and in foliar water status as well as triggering of cell death and bursts of ROS associated with local and systemic signal transmission (Karpińska et al., 2003; Ball et al., 2004; Mateo et al., 2004; Bechtold et al., 2005). Acclimation of plant leaves to EEE is controlled, at least in part, by specific changes in redox status of the photosynthetic electron carrier chain, namely the plastoquinone (PQ) pool (Karpińska et al., 1997, 1999; Pfannschmidt et al., 1999; Karpińska et al., 2000; Pfannschmidt, 2003; Kruk and Karpiński, 2006).

During the photoperiod, fine control of redox homeostasis is needed to prevent ROS overload due to excess light or pathogen attack since the largest producers of ROS are the chloroplasts and the peroxisomes (Kozaki and Takeba, 1996; Asada, 1999; Foyer and Noctor, 2003; Mateo et al., 2004). Thus, in conditions where plants are exposed to excess light, they are also at risk of overflowing ROS-dependent signaling systems and metabolic...
processes. Failure to maintain redox balance results in growth defects or initiation of cell death (Karpinski et al., 1999; Dat et al., 2000; Karpinska et al., 2000; Mateo et al., 2004). H$_2$O$_2$ and O$_2^-$ have emerged as the two key ROS that together with the hormones salicylic acid (SA) and ethylene contribute to the propagation of programmed cell death (Mazel and Levine, 2001; Overmyer et al., 2003).

In Arabidopsis thaliana, numerous mutants have been isolated that are defective in regulating programmed cell death. These initiate programmed cell death spontaneously or in response to a stress stimulus, such as pathogen infection or the application of ROS. In wild-type plants and in several of these mutants, programmed cell death is potentiated by light (Seo et al., 2000; Mach et al., 2001; Samulov et al., 2003; Mateo et al., 2004; Muhlenbock et al., 2007). One of the best-characterized deregulated programmed cell death phenotypes is caused by recessive mutations in the lesion simulating disease resistance1 (LSD1) gene (Jabs et al., 1996; Dietrich et al., 1997; Rusterucci et al., 2001; Epplle et al., 2003; Torres et al., 2005). The lsd1 mutant fails to limit the spread of programmed cell death under long (>16 h) photoperiods or after infection with an avirulent pathogen. LSD1 is also necessary for acclimation to conditions that promote photooxidative stress. The lsd1 plants have reduced stomatal conductance and lower peroxisomal catalase activity under permissive (low light) conditions that would make them vulnerable to ROS overload during EEE stress. Consistent with this idea, photooxidative stress will cause further limitations of stomatal gas exchange in lsd1 that in turn will cause an increase in EEE-induced photosynthetic H$_2$O$_2$ and exacerbated programmed cell death (Mateo et al., 2004). Notably, all defects of Arabidopsis lsd1 plants in restricting programmed cell death depended on the immune regulators ENHANCED DISEASE SENSITIVITY1 (EDS1) and PHYTOALEXIN DEFICIENT4 (PAD4) (Rusterucci et al., 2001; Mateo et al., 2004).

The SA pathway leading to systemic immunity is negatively controlled by MITOGEN PROTEIN KINASE4 (MPK4) (Petersen et al., 2000; Rusterucci et al., 2001; Brodersen et al., 2002, 2006). The SA signaling pathway also regulates light acclimatization processes (Karpinska et al., 2000; Mateo et al., 2003; Mateo et al., 2006). It has also been suggested that EDS1 and PAD4 amplify ethylene and SA signals by processing ROS that are essential for regulating the expression of cellular immunity against biotrophic pathogens (Rusterucci et al., 2001; Brodersen et al., 2006, Muhlenbock et al., 2007). EDS1 signaling complexes are nucleocytoplasmic, and evidence suggests that dynamic interactions between cell compartments are important for effective stress signal relay (Mateo et al., 2004; Feys et al., 2005).

Recently, two components of Arabidopsis immunity were identified 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 fungi, is in redox stress responses.

LSD1, PAD4, EDS1, and MPK4 affect many aspects of plant defense and acclimation by regulating SA, jasmonate/ethylene, and other as yet unidentified signal intermediates that lead to programmed cell death (Wiermer et al., 2005), systemic immunity, systemic acquired acclimation (SAA) to EEE (Mateo et al., 2004, 2006) as well as to hypoxia-induced, tissue-specific lysigenous aerenchyma formation in Arabidopsis (Muhlenbock et al., 2007). All of these features point to an intrinsic activity of LSD1, EDS1, and PAD4 in processing and transduction of redox-derived signals from different subcellular compartments and different plant organs and tissues during a variety of environmental stresses.

While the importance of ROS/hormonal cellular homeostasis is clear, research in both plants and animals raises more questions than answers. For example, how are different cellular responses elicited from ROS produced from different subcellular compartments? What are the key proteins that perceive changes in ROS/hormone levels and what are their functions under differing cellular redox states? How many genes, proteins, and metabolites are adjusted in response to a specific ROS/hormonal signaling? Which are the DNA cis-regulatory elements in ROS/hormonal-responsive genes that interact with trans-regulatory proteins? These questions apply equally to fungus, animal, and plant cells.

We aimed to unravel processes controlling plant acclimation to EEE and retrograde chloroplast to nucleus signaling. Here, we show that ethylene plays a role during acclimation to EEE of wild-type Arabidopsis plants and that its formation is dependent, in part, on the redox status of photosynthetic electron transport carriers (e.g., the PQ pool). We further establish that ethylene and ROS signaling are necessary for EEE-induced programmed cell death and acclimation in wild-type leaves and runaway cell death in lsd1 leaves (see also Muhlenbock et al., 2007). We also demonstrate that EEE regulates programmed cell death not only in exposed leaves but also in leaves undergoing SAA. Using photosystem I and photosystem II (PSII)–specific wavelengths of light, photosynthetic electron transport inhibitors, and lsd1 mutants as a test system for deregulated EEE acclimation (Mateo et al., 2004), we find that programmed cell death propagation signals and production of ethylene require EIN2, EDS1, and PAD4 activity. Activation of the SA signaling pathway in response to EEE was observed after an initial induction of ethylene/ROS signaling. We conclude that ethylene and ROS contribute to plant acclimation to EEE downstream of EDS1 and PAD4 in a pathway modulated by LSD1 and induced by redox changes in the photosynthetic electron transport carriers (e.g., PQ pool).

RESULTS

EEE-Induced Local and Systemic Programmed Cell Death Depends on Redox-Regulated Foliar Ethylene and ROS

In Arabidopsis wild-type (Columbia [Col-0]) leaves, excess light (for 1 h) caused programmed cell death that was detected by lActophenol–trypan blue (TB) staining (Figures 1A and 1B). The induction of programmed cell death in adjacent but untreated leaves (leaves undergoing SAA exposed to the ambient growth
light) reflects an active process that we refer to as EEE-induced programmed cell death. Acclimation to EEE is signaled by chloroplastic, peroxisomal, cytosolic, and plasma membrane–specific signal transduction pathways (e.g., Karpinski et al., 2003, Mateo et al., 2004; Kleine et al., 2007). The redox state of the PQ pool was demonstrated to be involved in signaling of EEE acclimation and can be selectively manipulated by treatment with light enriched in 680-nm (light-2 [L2]) or 700-nm (light-1 [L1]) wavelength or by use of specific photosynthetic electron transport inhibitors (Escoubas et al., 1995; Karpinski et al., 1999; Pfannschmidt et al., 1999; Kruk and Karpinski, 2006). Treatment of low light–grown plants with DCMU or 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) elicits similar effects on the redox status of the PQ pool as light enriched with the

**Figure 1.** Plant Programmed Cell Death in Response to Excess Light Is Modulated by Redox Status of the PQ Pool.

(A) to (J) TB staining of Col-0 leaves after low light (LL; 100 ± 20 μmol photons m⁻² s⁻¹) (A), 1 h of excess light (EL; 2200 ± 200 μmol m⁻² s⁻¹) (B), fumigated with 7.5 ppb ethylene for 24 h in LL (ET+LL) (C), fumigated with ethylene for 24 h and exposed to 1 h of EL (ET+EL) (D), or exposed to 4 h of light-1 (L1; enriched in 700-nm wavelength of energy of 10.24 J s⁻¹ m⁻²) (E); Col-0 leaves fumigated with ethylene for 24 h and then exposed to L1 (ET+L1) (F); leaves exposed to light-2 (L2; enriched in 680-nm wavelength of energy of 10.24 J s⁻¹ m⁻²) (G) or fumigated for 24 h with ethylene and exposed to 4 h of L2 (ET+L2) (H); leaves of the ethylene insensitive 2-1 null mutant exposed to 1 h of EL (EL on ein2) (I); and leaves of the ein2-1 null mutant exposed to 24 h of ethylene and then to 1 h of EL (ET+EL on ein2-1) (J). Images are representative of at least 27 leaves per treatment from three independent experiments (n = 27). Magnification is as indicated on each image (×15 or ×30).

(K) Areas of TB-stained foliar tissues in Col-0 and the ein2-1 null mutant cultivated in LL and then exposed to light and ethylene conditions as described in (A) to (J). Images are representative of at least nine leaves per treatment from three independent experiments (n = 3, n = 27 ± SD). Confidence levels were tested by a Student’s t test (*, P < 0.05, **, P < 0.01, ***, P < 0.001). Ethylene fumigation (for 24 h) was performed directly before exposure to different light conditions, and fumigated samples are indicated by a plus sign. Samples receiving different light treatments only are indicated by a minus sign. Symbols for light treatment are the same as in (A) to (J). Only sporadic programmed cell death appears in ein1-2 and in L1 and ET+L1 conditions.
Figure 2. Plant Cellular ROS/Ethylene Homeostasis, Programmed Cell Death, and Stomatal Conductance Are Modulated by Redox Status of the PQ Pool.
wavelengths 700 nm (L1) or 680 nm (L2), respectively (Kruk and Karpinski, 2006). DCMU and L1 keep the PQ pool more oxidized, while DBMIB and L2 keep the PQ pool more reduced (Escoubas et al., 1995; Karpinski et al., 1999; Pfannschmidt et al., 1999; Mateo et al., 2004, Kruk and Karpinski 2006). Arabidopsis wild-type leaves that were either exposed to DCMU and held under ambient light for 3 h followed by exposure to excess light (for 1 h) or that received the L1 treatment were associated with strongly reduced programmed cell death (Figure 1).

Fumigation of plants grown under low light conditions with low concentrations of ethylene (7.5 parts per billion) caused a significant increase in programmed cell death (Figures 1A and 1B) to ~30% of the level observed in plants treated with excess light. However, ethylene fumigation in combination with excess light (for 1 h) or light-2 (for 4 h) or DBMIB treatments increased the incidence of programmed cell death synergistically (Figures 1A and 1B).

These data suggest that a more reduced PQ pool (programmed cell death induced by EL and L2 and synergistically increased due to additional ethylene and lack or weak programmed cell death in L1 and L1 +ethylene of the wild type and in ein2; Figure 1) causes EEE-induced programmed cell death and light acclimatory responses that are regulated by EEE-induced ROS and ethylene homeostasis. Additionally, we quantified the areas of TB staining in leaves exposed to the different light regimens and observed that increases in programmed cell death occurred in wild-type leaves only under excess light stress or 4 h of light-2 (Figure 1B) and that it was strongly reduced in the ethylene insensitive2 (ein2) null mutant (Guzman and Ecker, 1990).

Previously, it was reported that ROS and ethylene regulate photosynthesis (Kays and Pallas, 1980; Asada, 1999; Karpinski et al., 1999) and that ethylene potentiates the oxidative burst (Ge et al., 2000; de Jong et al., 2002; Tuominen et al., 2004). Therefore, we measured levels of hydrogen peroxide (H2O2) and 1-aminocyclopentane-1-carboxylate (ACC), which is the immediate biosynthetic precursor of ethylene and a reliable marker of ethylene levels in the plant (Adams and Yang, 1979). We also measured relative (to the ambient growth condition) stomatal conductance (RSC), since it is known that EEE induces stomata closure and limitation of CO2 uptake and in consequence a photosynthetic burst of H2O2 (Mateo et al., 2004). Exposure of plants to 1 h of excess light caused a significant increase in foliar H2O2 and ACC concentrations in local and systemic leaves (Figures 2A to 2C) and a significant decrease in RSC (Figure 2D). Also, plants exposed to DBMIB treatment under low light conditions or plants exposed to L2 conditions for 4 h had significantly increased foliar levels of H2O2 and ACC and decreased levels of RSC (Figure 2). By contrast, low light–adapted plants that were treated with DCMU and then exposed to excess light or plants exposed to L1 had lower levels of H2O2 and ACC but not significantly reduced RSC compared with control plants grown under ambient (low light) conditions and exhibited only sporadic incidences of programmed cell death (Figures 1 and 2). Thus, we determined that there is a positive correlation between ethylene/ROS production and the incidence of programmed cell death and a negative correlation between RSC and the incidence of programmed cell death in the treatments that caused a stronger reduction of the PQ pool. Therefore, we concluded that the redox status of the PQ pool regulates cellular ROS/ethylene homeostasis, and consequently programmed cell death, in wild-type leaves exposed to EEE.

Recently we demonstrated that SA and glutathione signaling pathways are functionally and genetically integrated in the regulation of acclimation to EEE (Mateo et al., 2006). We therefore proceeded to measure the levels of free and bound foliar SA in response to EEE (Figure 3). We observed significant changes in free foliar SA levels after 2 h of excess light exposure, but only in leaves undergoing SAA (Figure 3A). In the case of conjugated foliar SA, we observed a significant increase in levels several hours after EEE stress (Figure 3B). These results indicate that SA signaling is activated in recovery and post-stress acclimation responses, both in leaves directly exposed to excess light and in leaves undergoing SAA. The results also suggest that the initial response to EEE (up to 1 h) is predominately governed by ethylene and ROS signaling (Figures 1 and 2).

Taking into consideration the above results, we also analyzed expression of two robust molecular markers, ASCORBATE PEROXIDASE2 (APX2), a marker of SAA, and PATHOGEN RESISTANCE1 (PR1), a marker of systemic acquired resistance (SAR), in ein2 and salicylic insensitive2 (sid2) null mutants.
We found that EIN2 is not required for appropriate induction of APX2 in leaves directly exposed to excess light (LO) and in leaves undergoing SAA (SY). In the case of PR1 regulation, we found that EIN2 is required for delay of PR1 induction in response to excess light (EEE) in both directly exposed leaves (LO) and in leaves undergoing SAA (SY) (see Supplemental Figure 1 online). SID2 delays the induction of APX2 in directly exposed leaves but not in leaves undergoing SAA (SY leaves). Consistent with previous observations that PR1 is regulated by SA (Dangl and Jones, 2001), SID2 is required for appropriate induction of PR1 in response to excess light in both LO and SY leaves (see Supplemental Figure 1 online). These results confirm that SAA and SAR are functionally and genetically integrated in acclimatory responses to EEE.

EEE Causes a Concerted Induction of Acclimation and of Defense-Related Genes

To further investigate the genetic mechanisms controlling responses to EEE and SAA, two separate suppression subtractive hybridization cDNA libraries were constructed and analyzed. In the first library (LO), we identified 730 ESTs representing 278 different genes induced in response to 40 min of excess light exposure (Figure 4; see Supplemental Data Set 1 online). In the second subtractive library constructed from leaves undergoing SAA (SY), we obtained 462 EST clones, representing 148 different genes (Figure 4; see Supplemental Data Set 1 online). We also found groups of genes that were only detected in excess light or SAA libraries (see Supplemental Data Set 1 online). The EST clones identified in both libraries were assigned to 15 different functional groups, including categories and subcategories (Figure 4). Taking the imperfection of the experimental system we used into consideration (Ji et al., 2002), we did not perform a comparative analysis between local and systemic libraries. However, we can conclude that plant global response to EEE involves gene expression reprogramming of different categories of genes that were previously classified either as biotic or abiotic stress-responsive genes.

Since redox status of the photosynthetic electron transport carriers plays an important regulatory role in EEE responses (Karpinski et al., 1997, 1999; Karpinska et al., 2000; Kruk and Karpinski, 2006), we tested the EEE-induced ESTs (see Supplemental Data Set 1 online) for opposite regulation by DCMU and DBMIB treatments. We found that 25 ESTs from the libraries (Table 1) that were induced by excess light or SAA were suppressed by DCMU and induced by DBMIB. We will refer to these 25 ESTs as the PQ regulon. In this novel regulon, we identified the presence of several robust markers for light acclimation, pathogen defense, ozone, drought, low-temperature responses, and genes regulated by ethylene, ROS, glutathione, SA, ABA, sugar, and auxin (indole-3-acetic acid [IAA] signaling (Figure 4, Table 1; Geisler et al., 2006). In the PQ regulon, we also detected a putative regulator of ribulose-1,5-bis-phosphate carboxylase/oxygenase and other nuclear-encoded chloroplast genes that are controlled by sugars and light signaling (Table 1). It is important to note that the set of genes presented in Table 1 does not represent the complete PQ regulon.

The above results indicate strong similarities between EEE acclimation and pathogen defense responses (SAR). Therefore, we tested whether leaves undergoing local and systemic acclimation to EEE were altered in pathogen defense and found that they limited growth of virulent biotrophic Pseudomonas syringae DC3000 after infection compared with nonacclimated control plants (Figure 5A). Leaves treated with DCMU for 3 h and then exposed to EEE for 1 h or leaves exposed to 4 h of L1 before infection permitted similar growth of P. syringae DC3000 as control plants (Figure 5A). By contrast, treatment of leaves with
DBMIB in ambient light conditions or with 4 h of exposure to L2 caused significantly reduced growth of *P. syringae* DC3000 compared with wild-type plants (Figure 5A). Together, these data show that the redox status of the PQ pool regulates, at least in part, acclimation to EEE, programmed cell death, and basal defense responses to virulent biotrophic bacteria.

**Redox Status of the PQ Pool Affects Runaway Cell Death and ROS/Ethylene Signaling in the *lsd1* Mutant**

Previously, we showed that excitation energy from PSII induces runaway cell death in *lsd1* mutant plants and that this is regulated by the same light conditions that control the redox status of the PQ pool (Mateo et al., 2004). We reasoned here and before (Karpinski et al., 1999; Karpinska et al., 2000; Slesak et al., 2003; Mateo et al., 2004) that EEE passing through PSII likely contributes to excess oxidation of components of the photosynthetic electron transport chain, causing increased production of ROS and changes in PQ redox status. Here, we observed that *lsd1* mutants and wild-type plants treated with DBMIB or DCMU in ambient light conditions developed localized chlorosis (DCMU) and runaway cell death (DBMIB) within 48 h (Figure 5B). Additionally, in Figure 1A, we demonstrated that DCMU inhibited EEE-induced programmed cell death in Col-0. However, only DBMIB caused spreading runaway cell death in *lsd1* (Figure 5B). This was associated with induction of gene markers for SAR (*PR1*) and a marker for programmed cell death (*PRXcb*) (Jabs et al., 1996) (Figure 5C). *PR1* and *PRXcb* induction was detected several hours after DBMIB treatment and preceded the development of runaway cell death. To determine the extent of oxidation or reduction of the PQ pool during DCMU or DBMIB treatments, respectively, we monitored chlorophyll a fluorescence parameters (Karpinski et al., 1999).

We concluded that reduction of the PQ pool contributes to the induction of pathogen defense genes and to propagation of runaway cell death in *lsd1*.

In a further analysis, we demonstrated that the induction of foliar ethylene and H$_2$O$_2$ in response to redox changes of the PQ pool is under negative control of LSD1 and under positive control of EDS1 and PAD4 (Figure 6). Single and double mutant analysis confirmed that LSD1 suppressed EDS1- and PAD4-dependent ROS and ethylene signaling (Figure 6) and that EIN2 acts downstream of EDS1 and PAD4 (Figures 6 and 7). Propagation of programmed cell death was dependent, at least in part, on the ethylene signal transduction encoded by *EIN2*, since the Col *ein2-1 lsd1* double mutant had significantly reduced runaway cell death compared with Col *lsd1* after restricting stomatal conductance (restricting gas exchange) (Figure 7). These results confirm that EEE-induced programmed cell death through *EIN2* signaling is regulated by LSD1.
Table 1. Pool of Genes Regulated by Changes in Redox Status of the PQ Pool (PQ Regulon) and Induced by Excess Light and by the SAA Mechanism

<table>
<thead>
<tr>
<th>Genes Detected in EL or SAA EST Libraries</th>
<th>DB Number</th>
<th>EL$^b$</th>
<th>SAA$^a$</th>
<th>DC$^a$</th>
<th>DB$^a$</th>
</tr>
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<tbody>
<tr>
<td>Heat shock protein 3 (HSC70-3)</td>
<td>AT3G09440</td>
<td>6.3</td>
<td>4.10</td>
<td>0.20</td>
<td>5.10</td>
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<tr>
<td>Beta Carotene Hydroxylase</td>
<td>AT5G52570</td>
<td>8.7</td>
<td>4.50</td>
<td>0.20</td>
<td>6.20</td>
</tr>
<tr>
<td>Plastid-lipid associated protein (PAP)</td>
<td>AT4G04020</td>
<td>11.3</td>
<td>6.50</td>
<td>0.10</td>
<td>5.30</td>
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<tr>
<td>Pathogenesis receptor kinase (PRSK)</td>
<td>AT4G36010</td>
<td>4.2</td>
<td>2.70</td>
<td>0.45</td>
<td>3.60</td>
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<tr>
<td>Plant defense-fusion protein (PDF1.2b)</td>
<td>AT2G26020</td>
<td>12.1</td>
<td>6.50</td>
<td>0.30</td>
<td>4.70</td>
</tr>
<tr>
<td>DNAj heat shock protein, putative (J3)</td>
<td>AT3G44110</td>
<td>4.4</td>
<td>2.60</td>
<td>0.40</td>
<td>2.50</td>
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<td>Encodes an auxin-inducible GST</td>
<td>AT2G29450</td>
<td>4.4</td>
<td>2.55</td>
<td>0.60</td>
<td>4.70</td>
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<td>Glycosyl hydrolase family 17 protein</td>
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<td>6.4</td>
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<td>Dehydrin Xero2</td>
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<td>8.3</td>
<td>6.10</td>
<td>0.10</td>
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<td>Disease resistance (TIR-NBS-LRR)</td>
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<td>4.30</td>
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<tr>
<td>Cytochrome P450 81D1 (CYP81D1)</td>
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<td>Phenylalanine ammonia-lyase (PAL1)</td>
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<td>Superoxide dismutase (Cu-Zn) (CSD1)</td>
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<td>Monodehydroascorbate reductase (MDR)</td>
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<td>9.4</td>
<td>5.80</td>
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<td>Phospholipase D α 1 (PLD1)</td>
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<td>Phospholipase C (PC2)</td>
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<td>Rubisco subunit binding protein β subunit</td>
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<td>0.40</td>
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<td>Aminotransferase, putative</td>
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<td>14.8</td>
<td>7.60</td>
<td>0.10</td>
<td>6.20</td>
</tr>
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<td>0.30</td>
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<td>Pfam domain, PFO0069: protein kinase</td>
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<td>6.40</td>
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<td>Vegetative storage protein (VSP2)</td>
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<td>S-Linalool synthase</td>
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<td>2.30</td>
<td>0.30</td>
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</table>

Custom gene arrays (see Supplemental Data Set 1 online) were hybridized to P$^{33}$-labeled cDNAs from Arabidopsis leaves exposed to EL (2200 ± 200 μmol m$^{-2}$ s$^{-1}$, 40 min), leaves undergoing SAA, leaves treated with 8 μM DCMU (DC) for 3 h, and leaves treated with 14 μM DBMIB (DB) for 3 h in ambient low light (LL) as described by Karpinski et al. (1999).

$^a$Relative transcript level values represent ratios of mean mRNA abundance to mean mRNA abundance in LL after normalization to mean actin mRNA expression. $^b$Relative transcript level values represent ratios of mean mRNA abundance to mean mRNA abundance for pooled samples of 10 leaves from four independent experiments (n = 4). χ$^2$ analysis based on the values observed in leaves induced and suppressed by DBMIB and DCMU, respectively, and values compared with that of neutral control. All presented differences in relative transcript abundance are significant for 2 degrees of freedom (P < 0.005).

The EEE-Induced Ethylene Response Is Modulated by LSD1

Previously, we showed that EEE acclimatory responses, stomatal conductance, and the photorespiratory burst of ROS require LSD1 activity (Mateo et al., 2004). Therefore, we decided to determine whether LSD1 had a similar regulatory impact on ethylene during the photorespiratory condition, due to EEE. Such a condition can be induced naturally, for example, during heat shock and/or drought stress or artificially, by restricting foliar gas exchange with a smear of lanoline wax applied on the adaxial side of a leaf or with semitransparent scotch tape (Figure 8). It is important to note that the lsd1 mutant plant can be cultivated for several days. Finally, we presented evidence that runaway cell death of the oldest rosette leaves 72 h after R.G. didn’t die under these conditions. In Ws-0 plants exposed to permissive conditions (permisssive low light conditions) led to runaway cell death of the oldest rosette leaves 72 h after R.G. (Figure 8, in lsd1 leaves 72 h after R.G., indicated by red arrows). Interestingly, younger lsd1 leaves (near the center of the rosette) didn’t die under these conditions. In Ws-0 plants exposed to permissive conditions (R.G.), only sporadic programmed cell death was detected 72 h after R.G. (Figure 8, in lsd1 leaves 72 h after R.G., indicated by red arrows). Similar results of runaway cell death induction were obtained with the smear of lanoline used for R.G. and induction of photorespiratory conditions (Mateo et al., 2004) and in Col-lsd1 (see Supplemental Figure 2 online).

Under low light conditions, artificially restricted gas exchange had no significant effect on foliar ACC levels during the first 24 h of R.G. treatment in Ws-0 plants (Figure 8B). By contrast, lsd1 mutants produced high levels of ACC and lsd1 leaves emitted ethylene (~350-fold higher than control plants within 24 h after R.G.) before exhibiting runaway cell death on systemic leaves.
Figure 5. Redox Status of the PQ Pool Affects Defense against Virulent Biotrophic Bacteria Infection in Arabidopsis Leaves.

(A) Growth of *P. syringae* pathovar DC3000 in infected Col-0 leaves 1 h and 0 d and 72 h and 3 d after infection and estimated in fresh weight (FW). Infections were made on leaves with various light acclimatory conditions and consequently changed the PQ redox status. Leaves before infection were acclimated to low light (control) (100 ± 20 μmol photons m⁻² s⁻¹), exposed for 1 h to excess light (2200 ± 200 μmol photons m⁻² s⁻¹), were developing SAA (Karpinski et al., 1999), or were exposed to 4 h of light-1 (L1 enriched in 700-nm wavelength of energy of 10.24 J s⁻¹ m⁻²), exposed to 4 h of light-2 (L2 enriched in 680-nm wavelength of energy of 10.24 J s⁻¹ m⁻²), treated with 8 μM of DCMU under LL conditions for 3 h and then exposed to 1 h EL, or treated with 14 μM DBMIB under LL conditions for 4 h. DCMU and DBMIB treatments caused an ~20 and 40% reduction of photosynthetic electron transport, respectively, as indicated by chlorophyll a fluorescence parameters. Results represent three independent experiments and 15 infections per experiment and treatment (n = 45 ± SD). Confidence levels were tested in a Student’s t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001). cfu, colony-forming units.

(B) Treatment of leaves with 8 μM DCMU in low light conditions (100 ± 20 μmol photons m⁻² s⁻¹) caused formation of discrete spots of chlorosis (indicated by arrows) in both Ws-0 and Ws lsd1. By contrast, application of 14 μM DBMIB under low light conditions induced spreading lesions only in
Previously, we demonstrated that foliar H$_2$O$_2$ levels in lsd1 mutants during restricted gas exchange follow similar patterns to those observed for ACC (Mateo et al., 2004). Collectively, these results indicate that in daylight, the hypersensitive disease response, SAR, and SAA are strongly exacerbated by EEE that was induced, for example, by excess light or photorespiratory conditions. It is important to note that restricted foliar gas exchange limits not only CO$_2$ uptake but also aeration of leaf vapors, such as H$_2$O$_2$, methyl jasmonate, methyl salicylate, and ethylene, and that it mimics the effects of elicitors of the hypersensitive disease response (Figure 8A).

Analysis of the single eds1-1 and pad4-5 mutants as well as of the 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 (Figures 6 and 8). Similar results were observed for foliar H$_2$O$_2$ levels after EEE was induced by restricting gas exchange (Mateo et al., 2004). To investigate the role of ethylene further, we measured the effects of application of ethylene on the propagation of runaway cell death in the lsd1 mutant. When exposed to 100 μM ACC for 48 h, lsd1 plants exhibited significantly larger areas of runaway cell death than the control plants (Figure 8C). Collectively, the data presented in Figures 6 and 8 and data presented in Figure 6 in Mühlenbock et al. (2007) lead us to conclude that EDS1 and PAD4 operate upstream of ethylene/ROS production in EEE stress signaling and result in the propagation of runaway cell death in the lsd1 mutant.

**DISCUSSION**

**Role of Ethylene in EEE-Induced Programmed Cell Death**

Exposure of wild-type Col-0 plants to excess light caused rapid increases in foliar ACC, the direct precursor of ethylene, in both leaves directly exposed to excess light and in leaves undergoing SAA (Figure 1). We provide several pieces of evidence that the production of ACC, ROS, and expression of genes regulated by ethylene, ROS, SA, glutathione, ABA, IAA, and sugars under excess light stress are regulated, at least in part, by signaling originating from redox changes in the photosynthetic transport carriers, for example, the PQ pool (Table 1, Figures 1 to 8; see Supplemental Data Set 1 and Supplemental Figures 1 to 3 online). Increase of free and conjugated foliar SA in response to EEE was detected after a significant increase of ethylene and ROS levels. This suggests that ethylene and ROS (Liu and Zhang, 2004, Geisler et al., 2006; Mühlenbock et al., 2007) rather than SA signaling are important for the initial and acute response to EEE.

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**Figure 5.** (continued).

Ws lsd1 (indicated by arrows) ($n = 5$ from two independent experiments, $n = 2$). Chlorosis in Ws-0 and in lsd1 was observed 24 h after DCMU, and runaway cell death in lsd1 mutants was also observed 24 h after DBMIB treatment. Bars = 1 cm.

**Figure 6.** LSD1 and Redox Status of the PQ Pool Regulate Foliar Ethylene and H$_2$O$_2$ Levels.

Foliar ACC (A) and foliar H$_2$O$_2$ (B) levels relative to those observed in control Ws-0 plants. ACC and H$_2$O$_2$ were measured in Ws-0, Ws-lsd1, Ws-eds1-1, and Ws-pad4-5 mutants and in Ws-pad4-5 lsd1 and Ws-eds1-1 lsd1 double mutants 4 h after treatment of leaves with 8 μM DCMU and 14 μM DBMIB under low light conditions (100 ± 20 μmol photons m$^{-2}$ s$^{-1}$). ACC and H$_2$O$_2$ were estimated in fresh weight (FW) of leaves. Data are representative of pooled leaf samples from four independent experiments ($n = 4 ± SD$). Confidence levels were tested by a Student’s t test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
could be observed in the lanoline smear apply on adaxial side of a single leaf). Foliar chlorosis being exposed to photorespiratory conditions for 24 h (by R.G. with double mutant. glutathione (Karpinski et al., 1997) and free and conjugated SA in recovery and acclimatory responses do, however, induce foliar response. After several hours of exposure to EEE, post-stress yet another signaling system predominates in the early EEE response. Additionally, we previously reported that SA- and jasmonate-dependent signaling pathways are not required for rapid EEE induction of the robust genetic marker for EEE acclimation, APX2 (Karpinski et al., 1997; Chang et al., 2004), indicating that yet another signaling system predominates in the early EEE response. After several hours of exposure to EEE, post-stress recovery and acclimatory responses do, however, induce foliar glutathione (Karpinski et al., 1997) and free and conjugated SA in leaves directly exposed and in leaves undergoing SAA (Figure 3). The recent observation that injecting SA or glutathione under the leaf epidermis induces glutathione or SA synthesis, respectively, demonstrates that foliar glutathione and SA levels are interconnected (Mateo et al., 2006). In addition, we have demonstrated that plants with deregulated SA synthesis or deregulated SA signaling are also deregulated in glutathione synthesis or signaling, respectively, and that SID2 function is required for optimal photosynthetic performance of a plant (Mateo et al., 2006). Moreover, higher foliar SA levels than those detected in low light acclimated plants were observed in plants acclimated to EEE (cultivated in high light conditions; Figure 1 in Karpinski et al., 2003). Collectively, these data suggest that ethylene and ROS production and signal relay precede changes in the glutathione pool and accumulation of SA in the early response to EEE in Arabidopsis leaves.

Our results reveal that changes in the redox status of the PQ pool regulate EEE-dependent ethylene/ROS signaling of both acclimatory and defense responses. Significantly, only 1 h of exposure to EEE was sufficient to restrict growth of virulent biotrophic bacteria (Figure 5). The effects of pharmacological (DCMU and DBMIB) or specific wavelength light treatments and restricted gas exchange argue against phytochromes playing a major role in regulation of EEE responses. Also, light-1/DCMU and light-2/DBMIB antagonistically regulate the redox status of the PQ pool (Kruk and Karpinski, 2006). Thus, ethylene and ROS production in response to the redox status of PQ appears to be a necessary component of an acute programmed cell death response to EEE and of the subsequent acclimatory and defense responses (Figures 1, 2, 5, 6, and 8, Table 1). The synergistic effect of ethylene fumigation (at low concentrations) combined with either excess light or light-2 (Figure 1) on programmed cell death supports our reasoning that ethylene and ROS combined (Figure 1, 5, and 6) are the initial signals promoting programmed cell death in plants under EEE stress. The observation that EEE-induced programmed cell death occurs in systemic tissues and that local and systemic tissues exposed to excess light or undergoing SAA are resistance toward biotrophic virulent bacteria infection suggests that the same processes may regulate SAA and SAR (Karpinski et al., 1999). This is supported by several observations. First, leaves directly exposed to EEE, leaves undergoing SAA, or leaves treated with DBMIB or light-2 (but not with DCMU or light-1) displayed significantly higher resistance toward biotrophic virulent pathogen infection than that observed in control plants. Second, EEE induced numerous genes involved in light acclimatory and pathogen defense responses (Figure 4, Table 1; see Supplemental Data Set 1 and Supplemental Figure 1 online). EEE and subsequent changes in redox status of the PQ pool also induced programmed cell death and higher resistance toward biotrophic virulent pathogen infection in unexposed parts of a plant undergoing SAA. Third, EEE induced by photosynthetic conditions (achieved by artificially restricting foliar gas exchange) led to runaway cell death in other leaves not directly challenged with such stress (Figure 8). Together, our results strongly suggest that light acclimatory and biotrophic pathogen defense responses are modulated by crosstalk at the level of ROS and hormone signaling that is regulated by the PQ pool redox status and in general redox changes in the chloroplast. Interestingly, it was demonstrated that chloroplasts indeed participate in regulation of programmed cell death in response to the infection of leaves by some biotrophic virulent pathogens (Krzymowska et al., 2007).

However, it was recently demonstrated that SAA is distinct from pathogen-stimulated SAR and apparently involves a novel...
Figure 8. Photorespiratory Conditions in Single Rosette Leaves Induce an Ethylene Burst and Systemic Runaway Cell Death in the Ws-lsd1 Rosette.
signal or combination of signals that specifically preacclimate photosynthetic tissues to upcoming EEE (Rosell et al., 2007). The authors mainly concluded this on the basis of mRNA profiling of wild-type plants and of mutant plants with several deregulated genes, including the Cys2/His2-type zinc finger transcription factor ZAT10. This conclusion was not supported by subsequent tests of virulent biotrophic bacterial growth after infection of leaves that are undergoing or developing SAA in wild-type and mutant plants (Rosell et al., 2007). Our results demonstrate that SAA is rapidly able to induce SAR (within the first hour of EEE treatment) in leaves directly exposed to excess light and in leaves undergoing SAA.

**LSD1, PAD4, and EDS1 Regulate EEE Stress-Induced Programmed Cell Death by Modulating Foliar ROS/ H第一部分

Previously, LSD1 was identified as a negative regulator of SA-dependent programmed cell death and plant defense responses to pathogens (Dietrich et al., 1997; Torres et al., 2005) and as a regulator of light acclimation processes (Mateo et al., 2004). Deregulated programmed cell death in both of these stresses depends on the plant defense components EDS1 and PAD4 (Rusterucci et al., 2001). Could the antagonistic activities of LSD1 and EDS1/PAD4 be important in processing the SA, ethylene, and ROS pathways? Here, we show that runaway cell death in response to EEE induced by photorespiratory conditions or by exposure to excess light in lsd1 mutants causes significantly increased production of the ethylene precursor ACC within hours of initiation and is promoted by ethylene and ROS signaling (Figures 1, 2, 6, 8B, and 8C). We further demonstrate that propagation of programmed cell death in lsd1 depends, at least in part, on the redox status of the PQ pool. Significantly, the enhanced production of ethylene and ROS in lsd1 under EEE conditions requires EDS1 and PAD4 (Figures 6 and 8B). This finding was unexpected since EDS1 and PAD4 are known to positively regulate the SA pathway in plant defense against pathogens (Wiermer et al., 2005). Also, EDS1 and PAD4 are required to repress jasmonate/ethylene signaling in the Arabidopsis mpk4 mutant that has constitutively active SA signaling through EDS1 and PAD4 (Brodersen et al., 2002, 2006). This apparent alternation of EDS1/PAD4-dependent signal relay may be governed by a particular input stimulus and points to a promiscuity in EDS1/PAD4 activities that was not previously appreciated, although signals other than SA were recently shown to be regulated by EDS1/PAD4 in pathogen-triggered defense and programmed cell death (Bartsch et al., 2006). We conclude that EDS1 and PAD4 act upstream of ethylene in the EEE response (Figures 6, 8B, and 9). In light stress, EDS1 and

**Figure 8. (continued).**

(A) R.G., obtained by applying semitransparent Scotch tape to the adaxial surface of a single leaf, induced local runaway cell death followed by systemic runaway cell death in older (red arrows), but not in younger, rosette leaves. In Ws-0 plants exposed to R.G., sporadic programmed cell death was observed in both the treated and in the oldest systemic leaves. The images are representative of 17 independent experiments. Similar effects were observed in Col-0 and in Col-lsd1 plants 24 h after R.G. was achieved by applying a smear of lanoline wax on the adaxial surface of a leaf significantly induced foliar ACC production in Ws-lsd1 but not in wild-type (Ws-0) plants after 24 h of treatment. The eds1 lsd1 and pad4 lsd1 double mutants similarly to Ws-0 did not significantly induce ACC in the same treatments (one-way analysis of variance with Tukey-Kramer procedure; ***, P < 0.01; ***, P < 0.001; n = 4 ± 50 for each treatment). Similar R.G. treatment induced foliar ethylene emission (see Supplemental Figure 3 online).

(B) Injection of 100 µM ACC solution into leaves induced runaway cell death after 48 h in Ws-lsd1 leaves. Compare with leaves injected with water (Student’s t test; ***, P < 0.001; n = 24 ± 50).
PAD4 positively control redox signal relay (Mateo et al., 2004). We propose now that this control is through promotion of ethylene/ROS signaling. However, partial reversion of runaway cell death in the double ein2-1 lsd1 mutant (Figure 7) indicates that ethylene is not the only programmed cell death signal that is suppressed by LSD1. Alternatively, another analysis reveals that a bZIP transcription factor in Arabidopsis, bZIP10, that is held in the cytoplasm by LSD1 contributes to programmed cell death in the oxidative stress response (Kaminaka et al., 2006), Kaminaka et al. (2006) suggested an alternative pathway to cell death that is controlled by bZIP, and this may be supported by our results presented above. Our recent results suggest complicated inter- and intracellular homeostasis between auxins, ABA, ethylene, glutathione, SA, ROS, and plasma membrane electrical action potential. It is also important to note here that the youngest leaves in lsd1 rosettes did not die after induction of runaway cell death (Figure 8). This effect may be due to hormonal sink-source regulation by auxins or cytokinins, and perhaps an increased understanding of bZIP function in Arabidopsis could provide an explanation for this exciting and largely unexplored phenomenon.

Additionally, many ethylene mutants have disrupted ABA signaling, and the ein2-1 mutant was shown to be ABA sensitive, which may have led to additional EEE stress and accounted for a faster induction of APX2. Importantly, however, ethylene signaling appears to act upstream of these effects, and the complicated and unresolved crosstalk between ABA and ET in light stress may have contributed to downstream effects that are outside the scope of this study.

The large increase in ACC observed here in response to EEE has been described in other studies and referred to as stress ethylene, an early response that amplifies programmed cell death signals (Ge et al., 2000; Overmyer et al., 2000; Tuominen et al., 2004). Our results show that LSD1 suppresses an ethylene/ROS-dependent programmed cell death pathway that requires the functions of EDS1 and PAD4 (Figure 9). Our evidence further points to an important role of the chloroplast in modulating defense responses through the redox status of its PQ pool (Figure 9, Table 1). It may be that plants have evolved unique defense mechanisms that depend on EEE and redox signaling originating from the chloroplast. Such response systems are likely to be crucial to plants growing in the natural environment where acclimation to prevailing abiotic stress factors (EEE) and defense responses have to be integrated. Rapid changes in light intensity and quality, in humidity, and temperature make acclimation to the natural environment an ever-present problem. Therefore, any biotic stress response in plants will be specifically adjusted in a concerted manner by a prevailing abiotic stress condition. LSD1, together with EDS1 and PAD4, may be key homeostatic regulators of a plant global acclimatory and defense mechanism. This was recently supported by the observation that LSD1, EDS1, and PAD4 are essential for regulation of lysigenous aerenchema formation in Arabidopsis during root hypoxia (Mühlenbock et al., 2007). Together, these results indicate that plants’ acclimatory and basal defense strategies are orchestrated through crosstalk by a genetic system that acts as a hub between redox signals from the chloroplast and ROS/hormonal signals arising in response to specific stress factors.

METHODS

Plant Materials, Growth, Light Conditions, and Pharmacological Treatments

The following mutants were in Arabidopsis thaliana accession Col-0: lsd1 (Jabs et al., 1996), ein2-1 (Guzman and Ecker, 1990), and the double mutant ein2 lsd1. All other mutants (lsd1, eds1-1, pad4-5, eds1-1 lsd1, and pad4-5 lsd1) were in accession Ws-0. The double mutant lsd1 ein2-1 was produced by crossing Col ein2-1 and Col lsd1. Plants were selected from a segregating F2 population by observing a triple response phenotype (for ein2-1) (Guzman and Ecker, 1990) and sensitivity to restricted gas exchange (lsd1). PCR was used to confirm homozygosity of lsd1 with the primer set forward 5'-GTCTGGTTGGTTTGGATGAATAGCAG-3' and reverse 5'-GCTTAATGCAACAGCTAGACGC-3' and ein2-1 with the primer set forward 5'-CTAGCTCGTCTATGTGTTGCG-3' and reverse 5'-CGGAATGAGGGGACCACG-3' followed by restriction enzyme cleavage with AflII (BspTI) to ensure correct fragment amplification.

Plants were grown in low light chambers at 100 ± 25 μmol photons m⁻² s⁻¹ (NIALOX NAV high-pressure sodium vapor lamps for experiments in Figure 1 and HQI ME-lamps 400 and 250 W [Osram] for experiments in Figures 2 and 3), with a relative air humidity of 50 to 60%, a temperature of 20 ± 1°C, and a short 9-h photoperiod. For excess light, Arabidopsis plants were treated with 2200 ± 250 μmol photons m⁻² s⁻¹, HMI (halogen metal lamps) 1200 W photo-optic lamp (Osram) for up to 2 h. Photoperiod, temperature (±3°C), and humidity (±5%) for all experiments were similar to those in the LL chamber. Treatment with light-1 and light-2 was performed as described by Mateo et al. (2004) with the following modifications. Four-week-old plants cultivated in low light were exposed to light enriched with either the 680- or 700-nm wavelength, and in both cases, the light provided at the leaf surface (~100 μmol photons m⁻² s⁻¹) was adjusted to the same energy levels for both wavelengths (10.24 J s⁻¹ m⁻²). During 4 h of exposure to such light conditions, plants were also exposed to ethylene gas (7.5 ppm). Plants were also sprayed with or exposed to a 30 to 40 μL drop of DCMU (8 μM) or DBMIB (14 μM) applied to the leaf 2 to 3 h before EL treatment as described (Karpinski et al., 1999). Approximately 20 μL of 100 μM ACC was injected into leaves, and water injection was used as a control (we conducted two replicates of 12 plants/treatment). The area covered by lesions was calculated by automated pixel analysis of grayscale digital photos of the same resolution, using the software Image J (National Institutes of Health; adapted from Chaerle et al., 2004).

TB Staining

Cell necrosis was detected as described by Koch and Slusarenko (1990). Areas of TB-stained leaves were calculated with the help of a raster grid (grid cell area of 1 mm²) and a light microscope (Olympus) at 15-fold magnification. TB staining was estimated per 0.5 cm² of randomly chosen leaf areas within the grid. In each area we scored TB staining at 25, 50, 75, and 100% (areas/treatment). The area covered by lesions was calculated by automatic pixel analysis of grayscale digital photos of the same resolution, using the software Image J (National Institutes of Health; adapted from Chaerle et al., 2004).

Bacterial Pathogen Infections

In the pathogen proliferation tests, 5-week-old Col-0 plants were exposed to excess light (2000 ± 250 μmol photons m⁻² s⁻¹) for up to 1 h or light-1 and light-2 for up to 4 h and then treated with DBMIB for up to 4 h in ambient (low) light or with DCMU for up to 3 h in ambient light and then exposed to excess light as above for 1 h. Two h after such treatments, leaves were infected with the virulent Pseudomonas syringae pathovar DC3000. Bacterial growth was inspected as described (Rusterucci et al., 2001) 1 h (0 time) and 72 h after infection (n = 5 for each treatment).
Stomatal Conductance and Restricting Gas Exchange

Stomatal conductance was measured as previously described (Mateo et al., 2004). Artificial restriction of gas exchange was achieved either by application of a lanolin wax smear or by adhering strips of semitransparent tape to the adaxial surfaces of leaves covering two-thirds of the leaf or the whole leaf blade (Mateo et al., 2004).

ACC, Ethylene, and SA Quantifications

ACC production was determined as previously described (Concepcion et al., 1979; Langebartels et al., 1991). Gas samples were analyzed using gas chromatography (GC-8A equipped with a 2 m Porapak N80/100 mesh, 3-mm i.d. column; Shimadzu). Determination of SA was performed as described by Meuwly and Métraux (1993) from leaves snap-frozen in liquid nitrogen. Ethylene was measured according to Du and Yamamoto (2003). The samples were injected into a gas chromatograph (Hitachi 2863-50) with a flame ionization detector and a spiral glass column (0.35 × 200 cm) packed with 60/80 mesh activated alumina. Chromatographic conditions were as follows: column, injector, and detector temperatures were 80, 120, and 120°C, respectively; the carrier gas was He at 35 mL·min⁻¹. Ethylene concentration was calculated on the basis of leaf surface area compared with the chromatographic data of a 10.6 mg L⁻¹ nitrogen balanced ethylene standard (Sanso). Experiments were performed at least in triplicate at different time points.

Hydrogen Peroxide Quantification

Hydrogen peroxide was quantified as described previously (Guilbault et al., 1988; Jimenez et al., 2002) with the following modification: 100 mg of fresh Arabidopsis leaf tissue of 5-week-old plants was used per 1 mL of extraction medium. We also visualized hydrogen peroxide by staining Arabidopsis leaves in the dark for 15 min with 10 µM 2′,7′-dichlorofluorescin diacetate and visualizing the stain by fluorescence microscopy (Axioskop; Zeiss).

Subtractive Suppression cDNA Libraries

A standard protocol was used for large-scale total RNA extraction (Karpinska et al., 1997; CTAB extraction buffer, lithium chloride precipitation followed by phenol/chloroform 1:1 [v/v] extraction). Subtractive suppression hybridization was performed using the PCR-Select cDNA subtraction kit (Clontech) according to the manufacturer’s instructions. Double-stranded cDNA was prepared from 2 µg of poly(A)⁺ mRNA of tester and driver populations. cDNA prepared from leaves directly exposed to EEE or from leaves undergoing SAA were used as the testers and that from the control samples (low light–treated plants) as the driver for the forward subtraction to isolate fragments corresponding to genes whose expression level was increased in response to EEE or during SAA. The cDNA was then digested with RsaI. In two separate ligations, tester cDNA was ligated to kit adapters 1 and 2. In the first hybridization, an excess of driver cDNA was hybridized at 68°C for 8 h with tester cDNA ligated to adapter 2 in reaction 2. In the second hybridization, reactions 1 and 2 were hybridized together in the presence of fresh driver cDNA at 68°C overnight. Hybrid sequences were then removed. The subtractive products were amplified by PCR using kit primers that were complementary to adapters 1 and 2. PCR was performed using the following conditions: 75°C for 5 min and 27 cycles at 94°C for 30s, 66°C for 30s, and 72°C for 1.5 min. Then, a nested PCR was performed as follows: 12 cycles at 94°C for 30s, 66°C for 30s, and 72°C for 1.5 min. The final PCR products were identified as upregulated in local and systemic leaves in relation to low light–treated leaves. Consequently, PCR fragments were cloned into the T/A cloning vector pGEM-T Easy (Promega) according to the manufacturer’s instructions and introduced into TOP10-competent E. coli (Invitrogen). Positive clones were then selected on ampicillin plates with IPTG and X-gal selection of recombinants and were later sequenced. Several hundred colonies were obtained from each subtrac tion experiment. Randomly picked single colonies were grown in 1 mL of liquid Luria-Bertani medium with 100 µg·mL⁻¹ of ampicillin. Plasmid DNA isolated from these cultures was subjected to sequencing. Sequencing and the analysis of NCBI blast function was performed by the Laboratory of DNA Sequencing at Umeå Plant Science Center.

Different cDNA ESTs identified from Arabidopsis custom EL- and SAA-induced subtractive suppression cDNA libraries (see Supplemental Data Set 1 online) selected for their high homology to known and reported Arabidopsis and other plant genes were assembled on nylon membranes. PCR amplification of the selected cDNA clones was performed using the universal M13 pair of primers. The amplification of a unique and specific cDNA fragment was confirmed by electrophoresis of an aliquot of the purified PCR product on agarose gel. Purified PCR product (100 ng) from each cDNA clone was loaded on the gene array. After hybridization with labeled cDNA, made from mRNA isolated from EEE-exposed leaves, from leaves undergoing SAA, and leaves treated with DCMU and DMBM, membranes were exposed to P₃₃-sensitive screens and scanned using a Typhoon 8600 (Molecular Dynamics, Amersham-Pharmacia Biotech). Quantitative analysis of the gene array images was made using Image-Quant 5.0 (Molecular Dynamics, Amersham-Pharmacia Biotech). Data represent pooled leaf samples from four independent experiments (n = 4). χ² P values < 0.05 were considered weakly significant and < 0.001 strongly significant.

RT-PCR Analysis

Transcript levels for PR1 and PRXcb (Jabs et al., 1996) and APX2 (Karpinska et al., 1997) were determined by RT-PCR using the following primers: forward 5′-GTCCTGCTGCTAACCCCTACC-3′ and reverse 5′-AGGTAGGGTGCGGAAACCTGTTGCG-3′; APX2, forward 5′-AAGAAAAGTCTGACTGAGATGC-3′ and reverse 5′-CGGTTGAGTT-GAAATGC-3′. They were analyzed as described by Karpinska et al. (2001) using the RETROscript first strand synthesis kit (Ambion) and the Ambion Quantum RNA kit using 18sRNA as an internal standard.

Microarray Data Meta-Analyses

Experimental statistics and normalization were performed by the Nottingham Arabidopsis Stock Centre (NASC) using MAS 5.0 scaling or by The Arabidopsis Information Resource using the print-tip-group Loess method. Normalized signal values were used to generate ratios of treated or mutant plants to untreated or wild-type plants in the same experiment. NASC-generated detection call and P values were used to evaluate whether a gene was induced, suppressed, neutral, or not expressed (in both the control and the treatment). χ² P values < 0.05 were considered weakly significant and < 0.001 strongly significant.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases and are listed in Table 1 (DB column).

Supplemental Data

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

Supplemental Figure 1. Robust Molecular Markers of SAA and SAR (APX2 and PR1, Respectively) Are Differentially Regulated by SIZ2 and EIN2 in Response to Excess Light.
Supplemental Figure 2. Photorespiratory Conditions in Single Rosette Leaves Induce Runaway Cell Death in Col-ldsd1 and Ws-ldsd1 Rosettes.

Supplemental Figure 3. Photorespiratory Conditions Induced Emission of Gaseous Ethylene in Ws-ldsd1 Plants.

Supplemental Data Set 1. List of Identified and Sequenced ESTs from Subtractive Suppression cDNA Libraries Generated from Leaves Undergoing Molecular Reprogramming in Arabidopsis Rosettes Partially Exposed to Excess Light.

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REFERENCES


Correction


The list of authors has been corrected to include Alfonso Mateo.