The High Light Response in Arabidopsis Involves ABA Signaling between Vascular and Bundle Sheath Cells

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Previously, it has been shown that Arabidopsis thaliana leaves exposed to high light accumulate hydrogen peroxide (H2O2) in bundle sheath cell (BSC) chloroplasts as part of a retrograde signaling network that induces ASCORBATE PEROXIDASE2 (APX2). Abscisic acid (ABA) signaling has been postulated to be involved in this network. To investigate the proposed role of ABA, a combination of physiological, pharmacological, bioinformatic, and molecular genetic approaches was used. ABA biosynthesis is initiated in vascular parenchyma and activates a signaling network in neighboring BSCs. This signaling network includes the Gα subunit of the heterotrimeric G protein complex, the OPEN STOMATA1 protein kinase, and extracellular H2O2, which together coordinate with a redox-retrograde signal from BSC chloroplasts to activate APX2 expression. High light-responsive genes expressed in other leaf tissues are subject to a coordination of chloroplast retrograde signaling and transcellular signaling activated by ABA synthesized in vascular cells. ABA is necessary for the successful adjustment of the leaf to repeated episodes of high light. This process involves maintenance of photochemical quenching, which is required for dissipation of excess excitation energy.

INTRODUCTION

In the natural environment, plants are frequently exposed to fluctuating light intensities and often absorb more light energy than can be consumed by photosynthetic metabolism and thus require that excess excitation energy be dissipated. Many abiotic and biotic stresses limit photosynthesis, which causes further increases in excess excitation energy needing to be dissipated (Long et al., 1994; Asada, 1999; Baker 2008). Failure to dissipate excitation energy results in overreduction of the photosynthetic chain components that direct linear electron flux (LEF) from water to NADPH (Baker et al., 2007). Part of the absorbed light energy is dissipated as heat in the light-harvesting complexes of photosystem II (PSII) through non photochemical quenching (NPQ; Horton et al., 1996; Muller et al., 2001). Additional dissipation of excitation energy is also achieved by photochemical quenching (Baker et al., 2007; Baker, 2008) and reflects that action of processes such as the reduction of molecular oxygen at photosystem I by the Mehler reaction (Asada, 1999; Ort and Baker, 2002; Baker et al., 2007) and through photorespiration (Asada, 1999; Douce and Neuberger, 1999). These two processes produce reactive oxygen species (ROS) that are scavenged by lipid- and water-soluble low molecular weight antioxidants and antioxidant enzymes (Mittler et al., 2004; Apel and Hirt, 2004; Van Breusegem et al., 2008). Sustained exposure to very high light intensities, well in excess of light intensities optimal for growth (hereafter called excess light), will exceed the antioxidant and excitation energy dissipation capacity of the leaf and cause oxidative damage to the photosynthetic apparatus (Aro et al., 1993; Asada, 1999; Krieger-Liszkay, 2005; Triantaphylides et al., 2008), photobleaching, and cell death (Karpinski et al., 1999; Mühlénbock et al., 2008).

In excess light–stressed plants, damaged chloroplasts initiate retrograde signaling to the nucleus (Nott et al., 2004; Pogson et al., 2008) to downregulate the expression of photosynthetic genes and upregulate stress defense genes to mitigate oxidative stress (Rossel et al., 2002, 2007; Kimura et al., 2003; Koussevitzky et al., 2007; Mühlénbock et al., 2008). In contrast with excess light treatments, Arabidopsis thaliana leaves that are exposed to a moderate increase (typically <10-fold) over growth light intensity (hereafter called high light [HL]) do not suffer oxidative stress (Fryer et al., 2003; Davletova et al., 2005) or irreversible photoinhibition (Russell et al., 1995; Karpinski et al., 1997; Fryer et al., 2003). However, these plants do accumulate H2O2 in the chloroplasts of bundle sheath cells (BSCs) and neighboring mesophyll cells (Fryer et al., 2003; Mullineaux et al., 2006). BSCs in Arabidopsis form a single layer of elongated cells around the vasculature (Kinsman and Pyke, 1998; Leegood,
2008). Limitations in the supply of CO2 to such cells (Morison et al., 2005) may cause them to more readily produce ROS via the photoreduction of O2 (Hibberd and Quick, 2002; Fryer et al., 2003; Leegood, 2008). The accumulation of H2O2 in BSC chloroplasts is associated with the rapid induction of the antioxidant gene ASORBATE PEROXIDASE2 (APX2; Fryer et al., 2003; Ball et al., 2004; Karpinski et al., 1997, 1999). HL-mediated induction of APX2 requires LEF, redox signals from reduced glutathione, and abscisic acid (ABA; Karpinski et al., 1999; Fryer et al., 2003; Ball et al., 2004; Chang et al., 2004). These latter features are common to a majority of other HL-responsive genes examined to date (Rossel et al., 2002; Ball et al., 2004; Bechtold et al., 2008). However, in contrast with most HL-responsive genes so far examined, the induction of APX2 expression also requires extracellular H2O2 (Karpinski et al., 1999; Bechtold et al., 2008). The BSC-specific expression of APX2 in response to HL allows this gene to be used as a BSC-specific reporter (Mullineaux et al., 2006), in contrast with the expression of many HL-responsive genes that may not be confined to a single leaf tissue (Bechtold et al., 2008).

The induction of APX2 expression and increased capacity to dissipate excitation energy in BSCs can be prevented if the leaf is exposed to high humidity (Fryer et al., 2003). This observation suggests that at low humidity, HL-exposed BSCs experience a loss of water (Fryer et al., 2003). Water loss leads to the accumulation of ABA, which plays a central role in the regulation of plants’ water status (Davies et al., 2002; Christmann et al., 2006). HL-mediated APX2 induction is attenuated in the ABA signaling mutants abi1-1 and abi2-1 (Fryer et al., 2003). In the mutant altered in APX2 expression&1 that constitutively expresses APX2, ABA content is threefold elevated under non-stress conditions, providing a correlation between ABA accumulation and APX2 expression (Rossel et al., 2006). Supply of ABA to plants under low light conditions has shown that many HL-expressed genes are responsive to ABA (Fryer et al., 2003; Rossel et al., 2006; Bechtold et al., 2008).

Based on the requirement of APX2 induction for an extracellular source of H2O2 in BSCs (Karpinski et al., 1999; Bechtold et al., 2008), we speculate that an ABA-regulated plasma membrane NADPH oxidase as shown in guard cells (Pei et al., 2000; Murata et al., 2001; Mustilli et al., 2002; Kwak et al., 2003; Li et al., 2006) may be a source of ROS in BSCs (Mullineaux et al., 2006). BSCs may contain additional ABA signaling components that regulate extracellular ROS, such as the heterotrimERIC G protein complex (Suharsono et al., 2002; Booker et al., 2004; Joo et al., 2005; Li et al., 2006), the OPEN STOMATA1 (OST1) protein kinase (Mustilli et al., 2002; Xie et al., 2006), and ABI1 (Murata et al., 2001).

ABA is synthesized in response to a reduction in water potential (Davies et al., 2002; Nambara and Marion-Poll, 2005; Christmann et al., 2006, 2007). ABA biosynthesis is partitioned between plastids and the cytosol (Qin and Zeevaart, 1999; Nambara and Marion-Poll, 2005). The oxidative cleavage of the precursor carotenoid 9-cis-neoxanthin to xanthoxin, catalyzed by the plastidial enzyme 9-cis-epoxycarotenoid dioxygenase (NCED), is the committed step for ABA biosynthesis (Nambara and Marion-Poll, 2005). Xanthoxin is then exported from the plastid, and the two remaining biosynthetic steps to ABA, catalyzed by enzymes coded by ABA DEFICIENT2 (ABA2) and ABA ALDEHYDE OXIDASE3 (AAO3), occur in the cytosol (Nambara and Marion-Poll, 2005).

In this study, we set out to establish how ABA, secreted from vascular parenchyma cells (Endo et al., 2008), regulates HL-responsive gene expression and integrates into H2O2- and redox-mediated retrograde signaling from chloroplasts in BSCs. We conclude that in HL-exposed leaves at ambient or lower humidity (1) paracrine (i.e., cell-to-nearby-cell) signaling occurs between vascular parenchyma cells and BSCs, (2) ABA signaling integrates into H2O2- and redox-mediated retrograde signaling from BSC chloroplasts, and (3) ABA is required for an effective physiological response of leaves to a fluctuating light environment.

**RESULTS**

**Exposure to Moderate HL Treatment Does Not Induce Signaling Pathways Associated with Exposure to Excess Light**

To determine the effects of exposure to HL in the BSCs, we measured the expression of the antioxidant gene APX2 and chlorophyll fluorescence as an indicator of photosynthetic performance and photo inhibition. The response of leaves was determined to a fivefold increase in PPFD over their growth PPFD (150 μmol m−2 s−1), hereafter referred to as HL. Exposure to HL caused detectable accumulation of APX2 transcript after 20 min, which continued to rise throughout the duration of the experiment (Figure 1A). The exposure of leaves to HL resulted in a decline in the chlorophyll fluorescence parameter Fv/Fm, which defines the maximum quantum efficiency of PSII photochemistry (see Methods; Baker, 2008; see Supplemental Figure 1 online). Fv/Fm values reverted to pre-HL exposure values when plants were returned to their growth conditions (see Supplemental Figure 1 online). Under these conditions, the expression of genes controlled by retrograde signaling pathways responsive to excess light (Danon et al., 2005; Koussevitzky et al., 2007; Pryzbyla et al., 2008) showed no significant change (see Supplemental Figure 2 online). Taken together, these data indicate that the plant’s responses to HL exposure did not induce permanent damage to leaves and did not activate retrograde signaling associated with such photooxidative stress.

**ABA Accumulation in HL-Exposed Leaves Is Due to Changes in Leaf Water Status**

To examine the possible role of ABA in the response of leaves to HL, the ABA content of leaves was determined. ABA content increased in attached and detached leaves by 65 and 60%, respectively, when exposed to HL at 25% RH (hereafter called low humidity; see Methods). This indicates that the ABA accumulation is leaf autonomous; no other source of ABA, such as from roots (Nambara and Marion-Poll, 2005), is involved. The increase in ABA was observed after 15 min in HL and was followed by an increase in APX2 expression (Figure 1A). At 80%
Figure 1. ABA Levels and APX2 Expression in HL-Exposed Leaves and Osmotically Stressed Petioles.

(A) Increase in ABA content (closed symbols, dashed line) and APX2 transcript levels (open diamonds, solid line) in leaves attached to the rosette and exposed to HL (750 μmol m⁻² s⁻¹ PPFD) and 25% RH. Each leaf (one per plant) was clamped into a CIRAS leaf chamber to control humidity and temperature (see Methods). NCED3 transcript levels are shown (closed squares, dotted line). ABA levels were determined using a radioimmunoassay (see Methods) from fully expanded outer leaves attached to rosettes during HL exposure. The data presented, expressed as μg per gram dry weight (gm DW⁻¹), are the means (±SE; n = 6) from one expanded leaf from each of three plants for each time point in two experiments. Foliar transcript levels were determined by quantitative real-time PCR on single-stranded cDNA prepared from total leaf RNA (see Methods) harvested at each time point. Each data point is the mean cDNA level (±SE) relative to the zero time point, low-light cDNA level. Transcript levels were normalized with respect to CYCLOPHILIN transcript levels, which do not respond to HL (Rossel et al., 2006).

(B) Foliar ABA content in detached leaves exposed to HL (PPFD of 750 μmol m⁻² s⁻¹) for 45 min at either low humidity (25% RH) or high humidity (80% RH) or kept at the growth PPFD of 150 μmol m⁻² s⁻¹ (LL). The difference in ABA levels between the mean (±SE) HL and LL samples at low humidity was significant (P = 0.024 from t test; n = 8 from two experiments). One fully expanded leaf from each rosette was used and clamped into a CIRAS leaf chamber to control humidity and temperature (see Methods).

(C) APX2LUC expression in osmotically stressed petioles is light dependent. Fully expanded leaves of APX2LUC/Col-0 plants (Karpinski et al., 1999) were detached and infiltrated with PEG-400 between 0 and 0.5 M for 2 h at a PPFD of 20 μmol m⁻² s⁻¹ and then petioles were detached and bathed in the same concentrations of PEG-400 plus 1 mM D (−) luciferin for a further 1.5 h either at growth PPFD (low light) or in the dark. At the end of this period,
RH (hereafter termed high humidity; see Methods), HL did not increase the ABA level (Figure 1B).

In the first 10 min of exposure to HL under low humidity, the transpiration rate increases (Fryer et al., 2003), which transiently lowers leaf water status and provides the necessary cue to initiate ABA accumulation. However, the sudden increase in light intensity may also directly contribute (a) signal(s) that triggers ABA accumulation in leaves. To distinguish between these two possibilities, ABA accumulation was measured in petioles of Columbia-0 (Col-0) plants harboring an APX2 promoter LUCIFERASE gene fusion (APX2LUC; Karpinski et al., 1999) subjected to a range of osmotic stress treatments under low-light conditions and in the dark. Petioles contain BSCs, which express APX2 (Fryer et al., 2003; Ball et al., 2004), and vascular parenchyma that carry out ABA biosynthesis in the Arabidopsis leaf (Endo et al., 2008). Petioles of Col-0/APX2LUC were chosen as an experimental system because they could be bathed in solutions of different osmotic potential, and on exposure to light, APX2 expression could be rapidly monitored by imaging the luminescence produced by luciferase (Figure 1C). Incubation of isolated APX2LUC petioles in a range of polyethylene glycol (PEG-400) solutions of increasing osmotic potential under low-light conditions activated APX2 expression (Figure 1C), with osmotic potential thresholds of −0.5 to −0.8 MPa (calculations based on Money, 1989). Similar observations were made when petioles were treated with sorbitol or mannitol solutions of equivalent osmotic potential (data not shown). The 0.4 M PEG-400 that induced APX2 expression also caused a decrease in the chlorophyll fluorescence parameters $F_o/F_m$ and $F_v/F_m$ and an increase in $q_L$ and $NPQ$ (Table 1). $F_o/F_m$ provides an estimate of the quantum efficiency at which PSII operates under a given PPFD (Baker, 2008). $q_L$ and $NPQ$ provide information of the redox state of the primary quinone electron acceptor of PSII ($Q_A$) and $NPQ$, respectively (Baker, 2008). Similar changes in these characteristics accompany the induction of APX2 expression (Karpinski et al., 1997, 1999; Fryer et al., 2003; Chang et al., 2004).

ABA levels in petioles increased in low light and in the dark by 30 and 27%, respectively, when 0.4 M PEG-400, a concentration above the threshold for APX2 expression, was applied (Figure 1D). These data indicate that ABA accumulation in petioles is not dependent on a light-associated signal in addition to a change in water status. Similarly, treatment of HL-exposed leaves with the LEF inhibitor DCMU (Duy sens, 1972) did not inhibit ABA accumulation (Figure 1E), confirming the observations in dark-incubated osmotically stressed petioles.

### Table 1. Chlorophyll Fluorescence Parameters for Water- and 0.4 M PEG-400–Treated Petioles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Water-Treated Petioles</th>
<th>0.4 M PEG-400–Treated Petioles</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_v/F_m$</td>
<td>0.71 ± 0.003</td>
<td>0.65 ± 0.005</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>$F_o/F_m$</td>
<td>0.34 ± 0.012</td>
<td>0.29 ± 0.01</td>
<td>0.013</td>
</tr>
<tr>
<td>$NPQ$</td>
<td>1.38 ± 0.024</td>
<td>1.51 ± 0.05</td>
<td>0.030</td>
</tr>
<tr>
<td>$q_L$</td>
<td>0.91 ± 0.01</td>
<td>1.09 ± 0.013</td>
<td>&lt;0.001</td>
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One petiole per plant was used from an outer rosette leaf ($n = 4$).

#### A Capacity for Foliar ABA Biosynthesis Is Required for Induction of HL-Responsive Genes

ABA has been implicated in the induction of BSC-specific APX2 expression and some additional HL-responsive genes whose expression occurs in several leaf tissues (Fryer et al., 2003; Rossel et al., 2006; Bechtold et al., 2008). To examine the role of the foliar ABA biosynthetic pathway, the compound ABAMineSG, a specific inhibitor of NCED activity that is involved in the synthesis of ABA (Kitahata et al., 2006), was applied to detached HL-illuminated leaves of Col-0/APX2LUC. ABAMineSG inhibited the rise in foliar ABA levels and the increase in APX2 expression in leaves exposed to HL at low humidity (Figure 2A).

The predominant NCED gene expressed in Arabidopsis leaves is NCED3 (Luchini et al., 2001; Tan et al., 2003; Ruggiero et al., 2004; Endo et al., 2008). When rosettes of a null mutant of NCED3 (nced3-2; see Supplemental Figure 3 online) were exposed to HL, induction of APX2 expression was attenuated (Figure 2B) consistent with the data obtained with ABAMineSG (Figure 2A). Leaves from nced3-2 plants did not show an increase in ABA content under HL (see Supplemental Figure 3 online), although prestress levels of ABA were no different from those of wild-type leaves (see Supplemental Figure 3 online). A second T-DNA insertion mutant allele of NCED3, sto1-1, which has reduced expression of the gene (Ruggiero et al., 2004; see Supplemental Figure 3 online), also showed attenuated induction of APX2 expression in HL-exposed leaves (Figure 2B), although not to the same degree as nced3-2 (Figure 1D). Null mutant alleles of ABA2, aba2-11, and aba2-14, which have low levels of foliar ABA under a range of conditions (Gonzalez-Guzman et al., 2002; Barrero et al., 2006), failed to induce APX2 expression in response to HL (Figure 2B). NCED3 expression did not increase upon HL and low humidity treatment (Figure 1A), suggesting that the mechanism of action is posttranscriptional.

**Figure 1.** (continued).

Luciferase activity (top panels) and reflected light (bottom panels) were imaged using a CCD camera and false color images for luciferase activity generated (see Methods). The experiment shown was typical of replicates of these treatments. (D) ABA levels in osmotically stressed petioles in the low light and the dark. Petioles of APX2LUC leaves were treated with 0.4 M PEG-400 or water as described in the legend for (C) and then their ABA content determined as in the legend for (A). Some additional petioles of low light-incubated petioles were imaged for luciferase activity, as shown in (C), to confirm their response to the PEG-400 treatment. The values are the means ($\pm$SE) for three separate experiments from eight petioles pooled in each experiment from four plants. (E) DCMU has no effect on HL-induced foliar ABA accumulation. Detached leaves were infiltrated with 30 $\mu$M DCMU for 2 h (see Methods) prior to exposure to HL (750 $\mu$mol m$^{-2}$ s$^{-1}$) at 25% RH. Leaf ABA content was then determined as in the legend for (A). These data are the means ($\pm$SE) of two experiments with four leaves each from a separate plant ($n = 8$).
Figure 2. The Expression of HL-Responsive Genes Require a Foliar Capacity for ABA Biosynthesis.

(A) APX2LUC expression in leaves treated with the NCED inhibitor ABAmineSG (Kitahata et al., 2006). Image of luciferase activity from Col-0/APX2LUC infiltrated with ABAmineSG and D (-) luciferin (1 mM) or luciferin only for 2 h prior (see Methods) prior to exposure to HL (750 μmol m⁻² s⁻¹) at 25% RH for 45 min. Luciferase activity was imaged using a CCD camera system and image processing to color code luciferase activity (see Methods). Red indicates regions of highest luciferase activity. The effect of ABAmine on foliar ABA levels (Kitahata et al., 2006) was confirmed in a single experiment using four leaves from separate APX2LUC/Col-0 plants for each treatment as described in the legend of Figure 1 and Methods.

(B) APX2 transcript levels in HL-exposed leaves of ABA biosynthesis mutants. Whole rosettes of the wild type (Col-0), nced3-2, aba2-11, and aba2-14 were exposed to HL (750 μmol m⁻² s⁻¹) for 45 min at growth humidity (see Methods). APX2 expression was determined as transcript levels in the mutants relative to the wild type using real-time quantitative RT-PCR (see Methods and legend of Figure 1). Data were normalized to cDNA levels of CYCLOPHILIN, which shows no significant variation in expression under HL or where ABA levels differ between genotypes (Rossel et al., 2006). All data shown are the means (±SE) combined from two biological replications of six plants. All differences between mutants and the wild type were significant (P < 0.001 from t-test).

(C) Transcript levels of five HL-responsive genes in leaves of ABA biosynthesis mutants. The genes used in this study are to compare with the BSC-specific expression of APX2. The conditions and methods are as in legend for (B). Asterisks show where differences between mutants and the wild type were significant (P < 0.05 from t-test).

(D) Hierarchical clustering of shared gene expression responses to HL exposure and exogenous ABA application. A total of 816 genes were identified as representing a significant overlap between HL and ABA responses and were clustered with data from the Gene Expression Omnibus (GEO) and NASCARRAYS databases. The 30 min, 1 h, and 3 h ABA data come from NASCARRAYS-176, 4 h ABA from GSE7112, 3 h ABA #2 from GSE6171, and 3 h HL from GSE7743. In this TREEVIEW representation, red indicates upregulation relative to mock or control treatment, while green indicates downregulation. The scale bar indicates log (base 2) ratios of treatment to control for the heat map.
Additional HL-responsive genes (Bechtold et al., 2008) were tested for their expression in HL-exposed ABA biosynthesis-deficient mutants. All mutants caused a significant reduction in the expression of the test genes compared with the wild-type controls (Figure 2C). A meta-analysis of publicly available microarray data for treatment of seedlings with ABA (Goda et al., 2008) compared with data from HL-exposed seedlings (Kleine et al. 2007; see Methods) revealed that 916 genes were coresponsive to ABA and HL (P value < 0.00001). Expression of 496 genes was induced under both conditions, while 320 were suppressed in response to both treatments (see Supplemental Data Set 1 online). When expression data for these genes were clustered with other publicly available ABA treatment data (Figure 2D), a strong correlation was observed between 3 h of HL exposure and plants 3 or 4 h after ABA application at a variety of concentrations (uncentered correlation = 0.780). Thus, a significant number of HL-responsive genes are also responsive to ABA levels and may require foliar ABA biosynthesis, implying an important role for this hormone in the response of the leaf to its prevailing light environment.

A Capacity for Foliar ABA Biosynthesis Is Required for Response to a Change in Light Intensity

Based on the above observations on the correlation between HL- and ABA-responsive gene expression, we hypothesized that a capacity to synthesize foliar ABA would be important for the leaf’s ability to adjust to changing light conditions. To test this hypothesis, nced3-2 was subjected to HL and lowered humidity for 60 min at the same time each day for 5 d, and chlorophyll fluorescence parameters were imaged before, immediately after, and 2 h after each HL episode. The treatment protocol and times at which fluorescence parameters were imaged are shown in Figure 3A (see Methods).

After a single HL treatment, only minor differences in dark-adapted Fv/Fm between mutant and wild-type plants were observed (Figures 3B, top panel and 3C). However, after 5 d, the outer, fully expanded leaves of nced3-2 rosettes had lower Fv/Fm values than the equivalent wild-type leaves (Figures 3B, bottom panel, and 3C), indicating that the mutant plants, unlike the wild type, experienced photoinhibition of PSII that was not recoverable during a 20-min dark period or after 22 min back in growth conditions. Measurements of the PSII operating efficiency at the growth and HL treatment PPFs (Figure 3D) confirmed that nced3-2, but not wild-type leaves, experienced an increase in photoinhibition with increasing days of treatment. There were no significant differences in NPQ between the mutant and the wild type during the course of the HL treatments, but there were decreases in qL values for the mutant (Figure 3D). Thus, the photoinhibition observed in nced3-2 was attributable to a reduced ability for photochemical quenching since Qo was more reduced in the mutant than in the wild type. This would imply that the mutant is less able than the wild type to use the products of LEF, ATP, and reductants. Consequently, nced3-2 leaves experienced increasing photoinhibition and, presumably, photodamage to PSII, with increasing periodic exposure to HL.

The same experiments were also conducted on aba2-11 and aba2-14, but the leaves wilted severely during the HL exposures, which made the accurate determination of chlorophyll fluorescence parameters impossible.

Taken together, these data indicate that induction of ABA biosynthesis is a key factor in the response of fully expanded leaves to repeated HL episodes at low RH.

HL Response Is Associated with Biphasic Accumulation of Extracellular H2O2 in Vein-Associated Cells and the Leaf Lamina

While previous studies reported a role for the production of extracellular ROS in the regulation of HL-responsive genes, direct observation of ROS accumulation during HL exposure was not reported. Real-time measurement of H2O2 accumulation in leaves can be achieved using a sensitive derivative of the H2O2-specific fluorogenic probe 10-acetyl-3,7-dihydroxyphenoxazine, called Amplex Red Ultra (ARU; see Methods). ARU is oxidized by H2O2 in a peroxidase-catalyzed reaction to resorufin (Zhou et al., 1997). ARU-resorufin fluorescence reports accumulation of extracellular H2O2 in leaf lamina tissues (Śnyrychová et al., 2008). At high magnification, ARU fluorescence was observed as a diffuse overlay of tissues of the veins and immediately adjacent leaf lamina (see Supplemental Figure 4 online), confirming the observations of Śnyrychová et al. (2008). Laser scanning confocal microscopy could not be used to establish more precise location of extracellular H2O2 in leaf lamina and periveinal tissue, since the laser light source used induced H2O2 production, rapidly caused bleaching of tissues and quenching of resorufin fluorescence.

To simultaneously image resorufin accumulation in periveinal and adjacent lamina, initial experiments relied on the colocalization of APX2LUC expression with ARU-resorufin fluorescence (Figure 4A). These experiments showed that 30 min after HL exposure at low humidity, elongated cells displayed readily detectable resorufin fluorescence compared with adjacent lamina tissue (Figure 4A). Since APX2LUC expression is confined to BSCs of the periveinal region in HL-exposed leaves (Fryer et al., 2003; Ball et al., 2004), we conclude that extracellular H2O2 accumulation is associated with these cells, although we do not rule out that other vein-associated tissues would also produce extracellular H2O2.

In a series of experiments with leaves exposed to HL at low humidity (Figures 4B and 4C), ARU-resorufin fluorescence in periveinal tissue was detected at 15 min after exposure to HL, although the peak of fluorescence varied between 15 and 30 min (cf. Figures 4B and 4C). A weaker secondary burst of resorufin fluorescence was detected at 90 min after HL exposure. In adjacent lamina tissue, a similar biphasic burst of fluorescence was detected; the second burst was of the same or greater magnitude than the first (Figure 4C). The biphasic changes in fluorescence were inhibited if leaves were placed in HL at high humidity (Figure 4C) or if the leaves were pretreated with ABAmineSG prior to HL exposure at low humidity (Figure 4B).

To verify accumulation of extracellular H2O2 in HL-exposed leaves, we used transmission electron microscopy to visualize cerium perhydroxide deposits, formed by reaction of infiltrated cerium trichloride (CeCl3) with H2O2 (Bestwick et al., 1997; Hu et al., 2005). Dark cerium perhydroxide deposits were detected in the appoplast adjoining cells of the bundle sheath and the...
Figure 3. A Foliar Capacity for ABA Biosynthesis Is Required for Fully Expanded Leaves to Adjust to Repeated Episodes of HL at Growth Humidity.
vascular parenchyma (Figure 4D, right panel; see Supplemental Figure 5 online). At 150 μmol m⁻² s⁻¹ PPFD, weaker and less extensive deposits were observed, showing that the dense cerium perhydroxide accumulation was due to the HL treatments (Figure 4D, left panel; see Supplemental Figure 5 online). However, no staining was detected in the apoplast on the mesophyll side of BSCs nor in the apoplast surrounding mesophyll cells of the leaf lamina (Figure 4D; see Supplemental Figures 5 and 6 online). Previous work has shown that the electron-dense cerium perhydroxide deposits in Arabidopsis leaf cell walls are almost entirely caused by H₂O₂ (Solyu et al., 2005).

**HL-Induced Accumulation of H₂O₂ in BSCs in Response to HL Is Rapid, Humidity Insensitive, and Directly Implicated in Signaling to the Nucleus**

Previous studies showed that the chloroplasts principally of periveinal cells, but not exclusively of BSCs, accumulate H₂O₂ in response to HL (Fryer et al., 2003; Mullineaux et al., 2006). Infiltration of leaves using 3’3’-diaminobenzidine (DAB) penetrates cells and stains for H₂O₂ accumulation in chloroplasts (Fryer et al., 2003; Liu et al., 2007; Driever et al., 2008; Šnyrychová et al., 2008), appearing as a brown precipitate (Thordal-Christiansen et al., 1997). Using DAB, H₂O₂ accumulation in chloroplasts of periveinal cells was visualized after 10 min exposure (Figure 5A), and the intensity of DAB staining in the veins of HL-exposed leaves was not susceptible to prevailing humidity (Figure 5B). Therefore, it was concluded that there was no direct role for leaf water status in the HL-induced H₂O₂ accumulation in the chloroplasts of periveinal cells, such as BSCs and flanking mesophyll cells.

BSC-specific APX2 expression was elevated in the sulfiredoxin 1 mutant srx1 and the vitamin C-1 deficient mutant vtc1, which are both partly defective in ROS scavenging capacity in the chloroplasts (Conklin et al., 1997; Rey et al., 2007; see Discussion). Figure 5C. There was no greater degree of photoinhibition nor was it any less reversible than in wild-type plants (see Supplemental Figure 1 online). Other HL-expressed genes used in this study were unaffected in their expression in these mutants (see Supplemental Table 1 online). These observations show that while the accumulation of H₂O₂ in HL-exposed BSC chloroplasts plays no role in the activation of ABA accumulation, H₂O₂ has a BSC-specific function in the induction of APX2 expression under HL. The specificity of the effect of these mutants on APX2 expression may reflect the propensity for BSC chloroplasts to accumulate H₂O₂ more than other leaf tissues under HL conditions (Figure 5A; Fryer et al., 2003) and therefore affect BSC-specific gene expression more than genes expressed elsewhere in the leaf.

**ABA Signaling Is Associated with Extracellular ROS Production and HL-Responsive Gene Expression**

ABA signaling in guard cells involves the production of ROS at the plasma membrane (Murata et al., 2001; Kwak et al., 2003). At least two components of the guard cell ABA signaling network were proposed to be involved: OST1 (Mustilli et al., 2002; Li et al., 2006) and heterotrimeric G proteins (Joo et al., 2005; Li et al., 2006). Null mutant alleles of ost1 conferred a more than twofold reduction in APX2 expression under HL (Figure 6A), but no consistent effect of the loss of OST1 was observed upon the expression of the five ABA- and HL-responsive genes studied in Figure 2C (see Supplemental Table 1 online). Throughout the HL treatment, stomatal conductance values for ost1-1 leaves were fourfold higher than for wild-type plants (see Supplemental Figure 7 online), which enhanced transpiration and a more rapid change in leaf water status than in the wild type.

Total foliar H₂O₂ content in ost1-1 did not increase upon HL exposure, in contrast with an increase of 175% in wild-type plants (Figure 6G). Staining of HL-exposed leaves with DAB...
Figure 4. Extracellular $\text{H}_2\text{O}_2$ Production in Veins and Adjacent Lamina Tissue in HL-Exposed Leaves Is Sensitive to Prevailing Humidity and the ABA Biosynthesis Inhibitor ABAmine.
showed brown precipitate predominantly in periveinal tissue to equal levels in both ost1-1 and the wild type (see Supplemental Figure 8 online). We conclude that OST1 positively regulates both HL-induced APX2 expression and an increase in nonchloroplastic H₂O₂ levels, although this regulation may be confined to BSCs.

Null mutants (gpa1-3 and gpa1-4; Ullah et al., 2001; Jones et al., 2003) in genes encoding the Arabidopsis Gα subunit (G PROTEIN ALPHA1 [GPA1]) and the mutants agb1-2 (Jones et al., 2003), and agb1-9 in the gene ARABIDOPSIS G PROTEIN BETA1 (AGB1), encoding the Gβ subunit of the heterotrimeric G protein complex, all showed three- to fivefold stimulation of APX2 expression under HL conditions (Figure 6B). Furthermore, a gpa1-4 agb1-2 double mutant (Jones et al., 2003) showed the same increase in APX2 expression as gpa1-3 and gpa1-4 plants (Figure 6B). The HL-responsive genes coding for lipocalin, consistent with expression of this gene in BSCs (Fryer et al., 2003; Ball et al., 2004). The images are from a single typical experiment. The yellow bar in used. Note that denotes areas of high resorufin fluorescence, which is mainly in the vein but also may be immediately adjacent lamina; hence, the term periveinal is captured (see Methods). In Transverse sections of the mesophyll (M), bundle sheath (BS), and vascular parenchyma (VP; Kinsman and Pyke, 1998; Evert, 2006) of HL-exposed Arabidopsis; Ullah et al., 2001; Jones et al., 2003) in genes encoding the Arabidopsis Gα subunit (G PROTEIN ALPHA1 [GPA1]) and the mutants agb1-2 (Jones et al., 2003), and agb1-9 in the gene ARABIDOPSIS G PROTEIN BETA1 (AGB1), encoding the Gβ subunit of the heterotrimeric G protein complex, all showed three- to fivefold stimulation of APX2 expression under HL conditions (Figure 6B). Furthermore, a gpa1-4 agb1-2 double mutant (Jones et al., 2003) showed the same increase in APX2 expression as gpa1-3 and gpa1-4 plants (Figure 6B). The HL-responsive genes coding for lipocalin, consistent with expression of this gene in BSCs (Fryer et al., 2003; Ball et al., 2004). The images are from a single typical experiment. The yellow bar in used. Note that denotes areas of high resorufin fluorescence, which is mainly in the vein but also may be immediately adjacent lamina; hence, the term periveinal is captured (see Methods). In (A), the focal plane highlights the vein (V) and the surrounding darker area is the leaf lamina (L). In (B), the false red color denotes areas of high resorufin fluorescence, which is mainly in the vein but also may be immediately adjacent lamina; hence, the term periveinal is used. Note that APX2LUC expression in (C) is confined more tightly to the veins and includes the elongated cells seen in the reflected light image (a), consistent with expression of this gene in BSCs (Fryer et al., 2003; Ball et al., 2004). The images are from a single typical experiment. The yellow bar in the right panel denotes 100 μm.

(B) Resorufin fluorescence in HL leaves inhibited for foliar ABA biosynthesis. Col-0 detached leaves were infiltrated with 50 μM ABAminesSG and 40 μM ARU or ARU alone (closed and open circles, respectively) as in Methods. All leaves were then exposed to HL (750 μmol m⁻² s⁻¹ PPFD) for 60 min at low humidity in a leaf chamber as described for (A) and in Methods. Resorufin fluorescence from the venal focal plane was quantified as described in Methods. The data are combined from three independent sets of measurements (±SE; n = 3). The relative fluorescence values were normalized to the starting low light values for resorufin fluorescence in each leaf.

(C) Images of resorufin fluorescence in HL (750 μmol m⁻² s⁻¹) exposed leaves at low humidity (25% RH) and high humidity (80% RH) sealed in a leaf chamber as described for (A) and in Methods. The images are from a typical single experiment and leaves were infiltrated with ARU only as described for (A) and in Methods. The numbers under each panel refer to the time in minutes from the beginning of exposure to HL. In this focal plane, the periveinal region (V) and the adjacent leaf lamina (L) are as described for (A). False color yellow indicates resorufin fluorescence, and red color denotes higher resorufin fluorescence. The graphs show the mean values at each time point (±SE; n = 3) for resorufin fluorescence, normalized to starting low light values, in the periveinal and lamina focal planes of HL-exposed leaves at low humidity (closed circles) and high humidity (open circles).

(D) Transverse sections of the mesophyll (M), bundle sheath (BS), and vascular parenchyma (VP; Kinsman and Pyke, 1998; Evert, 2006) of HL-exposed leaves at 4000 magnification (the bar on each panel denotes 5 μm) infiltrated with CeCl₃ prior to fixation, sectioning, and examination by transmission electron microscopy (see Methods). Cerium perhydroxide deposition (yellow arrows highlight prominent examples in the apoplast) denotes accumulation of H₂O₂. The leaves were exposed to HL (750 μmol m⁻² s⁻¹ PPFD) for 45 min at low humidity (right panel) or kept at growth PPFD at low humidity (left panel) as in the legend of (A), except that the leaves (one per plant) remained attached to the rest of the plant when clamped into the leaf chamber. The two panels show typical images. More examples from two separate batches of plants can be seen in Supplemental Figure 5 online.
tissue is the site of foliar ABA synthesis (Endo et al., 2008). Petioles contain both vascular parenchyma cells and BSCs (Kinsman and Pyke, 1998; Evert, 2006; Endo et al., 2008) and have the capacity to synthesize ABA and express APX2 in these cell types, respectively (Fryer et al., 2003; Endo et al., 2008). ABA levels in osmotically stressed petioles in both low light and in the dark increased by a similar amount (Figure 1D; see Results), and DCMU had no effect on ABA accumulation in HL-exposed leaves at low humidity (Figure 1E). From these experiments, we conclude that in HL at low humidity, increased ABA accumulation requires a decline in leaf water content, but not active LEF or other light-associated signals.
Figure 6. HL-Responsive Gene Expression in ABA Signaling Mutants.

(A) Whole rosettes of ost1-1 and ost1-2 and wild-type plants (Landsberg erecta [Ler]) were exposed to HL (750 μmol m\(^{-2}\) s\(^{-1}\) for 45 min) at growth humidity, and APX2 cDNA levels were normalized with respect to those of CYCLOPHILIN (see legend of Figure 1A) and expressed as mutant relative to the wild type. All data shown (means ± SE) are of two biological replications with a total of six to eight plants. The differences between the mutants and the wild type were significant; P = 0.03 and 0.02 from t tests for ost1-1 and ost1-2, respectively.

(B) APX2 transcript levels in HL exposed heterotrimeric G protein null mutants compared with wild-type plants. Rosettes of null mutants of GPA1 (gpa1-3 and gpa1-4), AGB1 (agb1-2 and agb1-9), and a double mutant (gpa1-4 agb1-2) were exposed to HL at growth humidity along with Col-0 and assayed for relative APX2 cDNA levels as described in (A). All data shown (means ± SE) are of two biological replications with a total of six to eight plants. The differences between the mutants and the wild type are significant (P < 0.05 from t test).

(C) to (F) Transcript levels of HL-responsive genes encoding lipocalin (C), RD20 (D), HSP17.6B-C1 (E), and HSP17.6C-C1 (F) assayed in HL-exposed rosettes (see legend of [A]) of gpa1-3, gpa1-4, agb1-2, and gpa1-4 agb1-2 relative to Col-0. All cDNA levels were normalized with respect to those of CYCLOPHILIN and expressed as mutant relative to the wild type. All data shown (means ± SE) are of two biological replications with a total of six to eight plants. The histograms marked with asterisks are significant (P < 0.05 from t test) between mutant and the wild type.

(G) Foliar H\(_2\)O\(_2\) levels in low light and HL-exposed ost1-1 and wild-type (Ler) leaves. H\(_2\)O\(_2\) levels were determined from cell-free acid extracts of fully expanded leaves of plants exposed to HL at growth humidity as described in (A) or from plants kept at growth PPFD and humidity (LL). The H\(_2\)O\(_2\) amount in each sample was determined using an amplex red-based in vitro assay (see Methods). Data are the means (± SE) of two separate experiments with four plants per experiment (n = 8). Three replicate determinations were carried per sample. The differences between the mutants and the wild type are significant (P < 0.05 from t test).
The rise in foliar ABA levels in intact leaves exposed to HL and low humidity occurred at a rate ~10-fold lower than in dehydration-stressed detached leaves (calculated from Endo et al., 2008 and data in Figure 1A). This difference is reflected in the strong induction of NCED3 expression during dehydration stress (Iuchi et al., 2001; Tan et al., 2003; Endo et al., 2008). By contrast, NCED3 transcript levels did not rise in HL-exposed leaves at growth or lower humidity (Figure 1A). However, in our experiments, NCED3 transcripts could be readily detected in well-watered plants at low light (see Supplemental Figure 3 online) using a similar RT-PCR technique (see Methods) to that of Endo et al. (2008), who could not detect this transcript under fully turgid conditions. This may reflect a difference in watering regimes, although it should be noted that in nced3-2, the levels of ABA under well-watered, low-light conditions were higher than in wild-type plants (see Supplemental Figure 3 online), suggesting that ABA levels can be maintained under nonstress conditions by NCED3-independent means. By contrast, under HL, foliar ABA levels did not rise in the mutant (see Supplemental Figure 3 online), underscoring the requirement for an extant capacity for foliar ABA biosynthesis for many aspects of the HL response (Figures 2 to 4).

The lack of NCED3 induction (Figure 1A), but the observed rise in foliar ABA levels (Figures 1A and 1B) and the requirement for an extant foliar ABA biosynthetic capability once HL is applied (Figures 2 to 4), suggest that NCED3 could also be subject to posttranslational regulation. This notion is supported by the observation that two different sized NCED3 isoforms of 64 and 58 kD were detected in dehydration-stressed Arabidopsis leaves (Endo et al., 2008), showing that posttranslational modification of NCED3 can occur. Posttranscriptional activation of foliar NCED by ethylene in cleavers (Gallium aparine) is proposed to occur concomitant with a slower induction of NCED transcription by auxin (Kraft et al., 2007).

Under high humidity conditions, ABA accumulation did not occur (Figure 1B). This could be due to a failure to activate ABA biosynthesis, since a transient change in leaf water content would be required. However, we cannot rule out that ABA catabolism is activated, since recently it has been shown that the vascular-located ABA 8′hydroxylase gene CYP707A3 is induced in 10 min of exposure to high humidity conditions (Okamoto et al., 2009).

A Capacity for Foliar ABA Biosynthesis Is Required for Maintenance of Photochemical Quenching and Acclimation to HL

The maximum expression of HL-responsive genes requires ABA (Figures 2A to 2C; Bechtold et al., 2008). This requirement may implicate >800 HL- and ABA-coreponsive genes (Figure 2D; see Supplemental Data Set 1 online), suggesting that ABA biosynthesis and signaling in different leaf tissues should be important for the response of the whole leaf to HL. This was the case since nced3-2 was unable to adjust to a daily exposure to 60 min of 10-fold HL (Figure 3A) and suffered a degree of irreversible photo-inhibition (Figures 3B and 3C). NPQ was unaffected in the mutant (Figure 3D), but it displayed a lower capacity for photochemical quenching throughout these treatments (Figure 3D; see Results). Therefore, in wild-type plants, an extant capability for foliar ABA biosynthesis is required to maintain or induce additional metabolic capacity to dissipate excitation energy. Failure to do so results in damage to the photosynthetic apparatus, increased photooxidative stress, and accelerated foliar senescence. These symptoms were clearly observed in nced3-2 (Figure 3B) and are consistent with changes in chlorophyll fluorescence in senescing leaves (Jenkins et al., 1981). Increases in photochemical quenching observed in BSC chloroplasts after 30 min exposure to HL are humidity sensitive (Fryer et al., 2003), supporting the suggestion that the ABA-mediated induction of additional electron sink capacity in this tissue is occurring.

These observations provide a new role for foliar ABA biosynthesis in addition to those described for dehydration stress (Christmann et al., 2007; Endo et al., 2008), stomatal responses to low humidity (Xie et al., 2006), and exploitation by the bacterial pathogen Pseudomonas syringae to subvert host defenses (de Torres-Zabala et al., 2007).

ABA Signaling in Veins and Lamina in Response to HL at Low Humidity

For ABA to participate in the regulation of HL-expressed genes in a range of leaf tissues, it must be secreted from the vascular parenchyma and induce signaling responses in other cell types. The interaction of ABA with BSCs is demonstrated by the ABA biosynthesis- and humidity-dependent biphasic production of extracellular H₂O₂, associated with these cells (Figure 4). This begins at 15 min for the first phase and a much weaker but evident second burst at 90 min after onset of HL (Figures 4B and 4C). This extracellular H₂O₂ production may be more precisely located in the intercellular spaces between BSCs and vascular parenchyma cells (Figure 4D; see Supplemental Figure 5 online). These observations are consistent in different experimental systems with descriptions of ABA-, excess light-, and ozone-stimulated signaling in guard cells, epidermal cells, and other leaf tissues requiring plasma membrane NADPH oxidase isoforms (Kwak et al., 2003; Davletova et al., 2005; Joo et al., 2005; Li et al., 2006). Moreover, APX2, which is inducible by exogenous ABA (Fryer et al., 2003; Rossel et al., 2006; Bechtold et al., 2008), is also inducible by provision of exogenous H₂O₂ to leaves (Karpinski et al., 1999; Bechtold et al., 2008), and is completely inhibited in excess light stressed leaves prefiltrated with catalase (Karpinski et al., 1999), shows lowered expression in a...
double null mutant defective for NADPH oxidases D and F (Bechtold et al., 2008) and where NADPH oxidase activity is inhibited (Volkov et al., 2006). In other plant species, these observations are consistent with ABA treatment of maize (Zea mays) leaves, which causes an increase in apoplastic H$_2$O$_2$ levels (Hu et al., 2005; Jiang and Zhang, 2003) and similar distribution patterns of extracellular vascular H$_2$O$_2$ levels preceded by foliar ABA accumulation in the Mediterranean shrub Cistus albidus subject to summer drought (Jubany-Mari et al., 2009).

Mutants defective in OST1 protein kinase function (Mustilli et al., 2002) showed marked attenuation of APX2 induction in response to HL (Figure 6A), suggesting that OST1 positively regulates induction of APX2 expression under HL conditions. These observations are consistent with the ABI1 protein phosphatase 2C (PP2C)-mediated positive regulation of APX2 induction (Fryer et al., 2003) and its interaction with and activation of OST1 protein kinase (Yoshida et al., 2006). OST1 also may have a positive role in the production of extracellular H$_2$O$_2$ in HL-exposed leaves, similar to its proposed role in the production of ROS at the plasma membrane of guard cells (Mustilli et al., 2002). This is because total foliar H$_2$O$_2$ levels did not rise in response to HL treatment, in contrast with a near doubling in Col-0 (Figure 6G), but accumulation of H$_2$O$_2$ in BSC chloroplasts, estimated by DAB staining, appears to be no different between mutant and wild-type plants (see Supplemental Figure 8 online). Therefore, we suggest that OST1 positively regulates extracellular H$_2$O$_2$ production upon activation by ABA synthesized under HL conditions. In addition, in HL-exposed osst1-1, the normal induction of the HL-responsive genes (see Supplemental Table 1 online), other than APX2, and accumulation of H$_2$O$_2$ in BSC chloroplasts (see Supplemental Figure 8 online) rules out any pleiotropic affect in this mutant caused by enhanced stomatal conductance (see Supplemental Figure 7 online; Mustilli et al., 2002).

The induction of APX2 expression was enhanced by four- to fivefold over wild-type plants in HL-exposed GPA1 and AG1 null mutants (Figure 6B). In the double mutant gpa1-4 agb1-2, there was no additive effect of these combined mutations (Figure 6B). These data suggest that in BSCs, the heterotrimeric G protein complex is a negative regulator of APX2 expression. Similar results were reported for Rab18 gene expression (Pandey et al., 2006). Under low-light conditions, the GPA1 null mutants had elevated levels of total foliar H$_2$O$_2$ (Figure 6H) in the absence of any increase in vein chloroplast H$_2$O$_2$ content (see Supplemental Figure 8 online). Thus, GPA1, and potentially the G protein complex, also negatively regulates extracellular H$_2$O$_2$ levels in veins under ambient light conditions.

In contrast with the situation in animal cells, activation of the Arabidopsis Gx subunit does not require a G-protein coupled receptor (i.e., specifically a receptor having guanine nucleotide exchange factor function since GPA1 is a rapid nucleotide exchanger) (Johnston et al., 2007; Temple and Jones, 2007). Indeed, the hydrolysis of GTP is probably the rate-limiting step in the plant G protein cycle. Therefore, induction of APX2 by ABA would include triggering a deactivation of some or all of the BSC GPA1 complement, which would release downstream signaling from negative regulation. This could include a release of extracellular H$_2$O$_2$ production from negative control, which could induce APX2 expression. RGS1, a seven-transmembrane-domain GTPase activating protein, controls the activation state of GPA1 (Chen et al., 2003). Based on the current body of evidence, control of the GTPase activating protein activity of RGS1 occurs upon glucose binding or by binding of some other, but related, photosynthetic product.

H$_2$O$_2$- and Redox-Mediated Retrograde Signaling from Chloroplasts Is Independent of Humidity

The above considerations show that ABA and its biosynthesis in leaves are necessary, but not sufficient, for the induction of HL-responsive genes. BSC chloroplasts and those of flanking mesophyll cells accumulate H$_2$O$_2$ within 10 min of exposure of the leaf to HL (Figures 5A and 5B), and this accumulation is independent of prevailing humidity (Figure 5C). This suggests that any ABA signal from vascular parenchyma cells does not affect the accumulation of H$_2$O$_2$ in BSC chloroplasts, which is likely to result from the photoreduction of O$_2$ at photosystem I (Fryer et al., 2003).

The induction of APX2 expression by mild osmotic stress of isolated petioles requires light (Figure 1C) and is associated with a lowering of the oxidation state of Q$_A$ and the operating efficiency of PSII photochemistry (Table 1). The change in these parameters and the repeated observation that the LEF inhibitor DCMU suppresses the induction of a large majority of nuclear-encoded HL-responsive genes (Karpinski et al., 1997; Rossel et al., 2002; Yabuta et al., 2004; Bechtold et al., 2008), including APX2 expression in petioles (Chang et al., 2004), is consistent with a requirement for LEF to provide additional redox and ROS signals sourced from BSC chloroplasts.

Null mutants of SRX1, which codes for sulfredoxin, a key enzyme component of the plastidial peroxiredoxin-based H$_2$O$_2$ scavenging system in Arabidopsis leaves (Rey et al., 2007), showed a significant elevation of APX2 expression (Figure 5D). Similarly, vtc1-1, which has depleted levels of ascorbic acid (Conklin et al., 1997), also showed a marked stimulation of only APX2 expression upon exposure to HL (Figure 5C). Neither set of mutants showed any significantly increased sensitivity to the HL exposure as determined by chlorophyll fluorescence measurements (see Supplemental Figure 1 online). This discrepancy is explained by the observation that partial loss of ROS scavenging capacity occurs in every tissue, but under these moderate HL treatments, an effect is only noted for redox- and ROS-responsive APX2 in BSCs which readily accumulate chloroplastic H$_2$O$_2$.

The other HL-responsive genes used in this study did not give a consistent response across the mutants (see Supplemental Table 1 online), but this is consistent with the lack of accumulation of H$_2$O$_2$ in any other leaf tissue under these mild HL conditions. No doubt more extreme photooxidative stress would provoke alterations in the expression of a much wider group of ROS- and redox-responsive genes. Nevertheless, many of these HL-responsive genes still require a chloroplast-sourced redox- or LEF-associated signal (Karpinski et al., 1997; Rossel et al., 2002; Ball et al., 2004; Bechtold et al., 2008).

In summary, along with previous observations (Karpinski et al., 1997, 1999; Fryer et al., 2003; Ball et al., 2004; Chang et al., 2004), the data presented here confirm that in BSCs, APX2...
expression requires an increased chloroplast oxidative state and active LEF to activate its expression, along with a requirement for an extracellular ABA signal.

Interdependence of a Transcellular ABA Signal and Chloroplast Retrograde Signals in Leaves Responding to HL

In the BSCs of HL exposed leaves, all the key signaling events involving H$_2$O$_2$ sourced from chloroplasts and ABA secreted from neighboring vascular parenchyma cells are complete within 30 min of exposure. This series of signaling events in HL-exposed BSCs supports the generic hypothesis proposed by Pfannschmidt et al. (2009), which suggested that retrograde signals from chloroplasts may typically act by merging with an external tissue-sourced signal.

In other leaf tissues, the topology of combined retrograde signaling and ABA signaling networks may be different. This is evidenced by less extensive and slower extracellular H$_2$O$_2$ production across leaf tissues (Figure 4B), which may reflect a later or less responsive interaction with external ABA, and no evidence for an involvement of OST1 in signaling and differing responses to G protein mutants (Figure 6). In addition, most leaf cell types do not accumulate chloroplastic H$_2$O$_2$, but most HL-responsive genes require an active LEF, supporting the argument that retrograde redox signals may vary from tissue to tissue (Ball et al., 2004).

METHODS

Growth of Plants

*Arabidopsis thaliana* genotypes (Col-0, Ler, or C24) and mutants used were grown in a peat-based compost in a controlled environment room under an 8/16-h light/dark cycle at a PPFD of 150 μmol m$^{-2}$ s$^{-1}$, 22°C ± 1°C, and a RH of 50%. All plants studied were at 5 to 6 weeks after germination.

Genotypes

The mutants used and their ecotype background are as follows: APX2LUC (Col-0; Karpinski et al., 1999); nced3-2 (Col-0; see below); sto1-1 (C24; Ruggiero et al., 2004); aba2-11 and aba2-14 (Col-0; Gonzalez-Guzman et al., 2002; Barrero et al., 2006); srx1-1 and srx1-2 (Col-0; Rey et al., 2007); vct1-1 (Col-0; Conklin et al., 1997); ost1-1 and ost1-2 (Ler; Mustilli et al., 2002); gpa1-3 and gpa1-4 (Col-0; Jones et al., 2003); agb1-2 and agb1-9 (Col-0; Jones et al., 2003; see below); and gpa1-4 agb1-2 (Jones et al., 2003). All genotypes were confirmed.

Isolation of Mutants nced3-2 and agb1-9

The nced3-2 mutant line (N331021) for NCED3 was obtained from the Nottingham Arabidopsis Stock Centre. The T-DNA insertion site is in the coding region of the unique exon of NCED3 (see Supplemental Figure 3 online) and is identical to the mutant recently published by Urano et al. (2009).

The mutant agb1-9 was isolated from a screen of *Arabidopsis* displaying agb1-2 null mutant phenotypes. agb1-9 contains a W$_{115}$S-to-stop codon mutation that causes premature termination of AGB1 synthesis, rendering the truncated protein nonfunctional.

Exposure of Leaves to HL

Fully expanded outer leaves detached from the plant were exposed to a PPFD of 750 (±50) μmol m$^{-2}$ s$^{-1}$ at a RH of 25% (low humidity) or 80% (high humidity) using a fiber optic white light source of 1 cm diameter as described previously (Fryer et al., 2003). The temperature (27°C ± 1°C) and humidity during HL exposure was kept constant using a CIRAS leaf chamber (PP Systems) as described previously (Fryer et al., 2003). Prior to clamping in the chamber, detached leaves were infiltrated with luciferin (see below), DCMU (30 μM; Sigma-Aldrich), DAB, or ARU for staining H$_2$O$_2$ (see below) via their transpiration stream for 2.5 h in low-light conditions as described previously (Fryer et al., 2002).

Whole rosettes were exposed to a PPFD of 750 (±100) μmol m$^{-2}$ s$^{-1}$ at ambient (growth) RH. This was done using lamps and water filters as described previously (Karpinski et al., 1999). Under the lamps, leaf temperatures increased by ±5°C over 90 min, the maximum time of exposure. Temperature increases of <5°C do not induce expression of any of the genes in this study (Bechtold et al., 2008).

Osmotic Stress Treatment of Petioles

Solutions of (0.1 to 0.5 M) PEG-400, sorbitol, mannitol, or water were infiltrated into detached fully expanded leaves of APX2LUC/Col-0 (Karpinski et al., 1999) for 2 h as described below for the infiltration of ARU. Then, an ~15 to 20 mm length of petiole was cut from each leaf and immersed for a further 1.5 h in the same solution also containing 1 mM D (-) luciferin under low light or in the dark. At the end of this period, luciferase activity was imaged and the petioles harvested for determination of their ABA content.

Chlorophyll Fluorescence Imaging

Chlorophyll fluorescence parameters were measured using a Flourimag chlorophyll fluorescence imaging system (Technologica). The application of preprogrammed regimes of actinic growth light exposure times, saturating light pulses, and dark periods and the calculation and imaging of the parameters F$\text{v}$/$F$\text{m}$, F$\text{a}$/$F$\text{m}'$, F$\text{v}$/$F$\text{m}'$, and NPQ (Baker, 2008) were performed automatically by the Flourimag’s software. qL was calculated postmeasurement from images of F$\text{a}$/$F$\text{m}'$, F$\text{a}$, and F$\text{v}$.

Visualization of H$_2$O$_2$ Accumulation and Its Measurement in Total Foliar Extracts

Use of ARU

ARU (a proprietary derivative of 10-acetyl-3,7-dihydroxyphenoxazine; Invitrogen; Zhou et al., 1997) was used to detect accumulation of extracellular H$_2$O$_2$ in the veins and periveinal regions of *Arabidopsis* leaves. It should be noted that this dye is different in its penetration properties compared with Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) and depending on which tissues in the leaf are being examined (Šnyrychová et al., 2008). ARU was dissolved in dimethylsulfoxide and then diluted into 50 mM sodium phosphate buffer, pH 7.5, to give a final concentration of 40 μM for infiltration into leaves. A detailed description of how dyes can be infiltrated into leaves via the transpiration stream is given in two publications (Fryer et al., 2002; Driever et al., 2008). Infiltration was performed into fully expanded detached leaves for ARU to 2 h under low light (20 to 50 μmol m$^{-2}$ s$^{-1}$) conditions. The leaves were cut from the plant under liquid to ensure no airlock formed at the cut petiole surface. At the end of the infiltration period, the leaf was then clamped into the CIRAS leaf chamber and exposed to the HL conditions with the 500- to 570-nm region of the spectrum being removed using a Rose Pink filter (Lee Filters). This was to prevent absorption of these wavelengths by ARU during the course of the

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CeCl₃ reacts with hydrogen peroxide to form cerium perhydroxides, experiment and to prevent photodegradation of ARU. Resorufin exposed leaves at low humidity were rapidly sliced into ion electron microscopy (Bestwick et al., 1997). Control and HL-buffered osmium tetroxide for 1 h at 4°C. Hydrochloric acid (0.1 M) extracts from 100 mg of fresh charcoal as described by Creissen et al. (1999). H₂O₂ concentrations in band-pass filter (Edmund Optics) in front of the lens of the CCD camera. The leaves were controlled by FluorImager V1.01 software (Technological), which was purposely designed for image acquisition (576 × 384 pixels) and control of exposure time, which was 5 s for all experiments. Acquired images were processed using ImageJ software (Abramoff et al., 2004).

**Use of DAB**

DAB (5 mM at pH 3.8; Fryer et al., 2002; Driever et al., 2008) was infiltrated into detached leaves as described above for ARU. The leaves were placed in the CIRAS leaf chamber and subjected to HL at low or high humidity. At the end of the experiment, leaves were infiltrated with lacto- glycerol-ethanol to fix them and remove chlorophyll (Fryer et al., 2002; Driever et al., 2008) prior to imaging.

**Use of CeCl₃**

CeCl₃ reacts with hydrogen peroxide to form cerium perhydroxides, which forms electron-dense deposits that can be visualized by transmission electron microscopy (Bestwick et al., 1997). Control and HL-exposed leaves at low humidity were rapidly sliced into pieces. These strips (3 × 3 mm) were then prefixed in 2.5% (v/v) glutaraldehyde buffered with 0.1 M cacodylate buffer, pH 7.2, and incubated for 2 h. Leaf segments (0.1 M Na cacodylate buffer, pH 7.2. The leaves were then postfixed in 1% (v/v) buffered osmium tetroxide for 1 h at 4°C followed by three washes in distilled water. The leaves were then stained in 1% (w/v) uranyl acetate for 1 h followed by dehydration in an ethanol series (50, 70, 90, and 100% ethanol for 10 min each). They were then treated with propylene oxide for 1 h prior to transfer to TAAB LV resin (TAAB Laboratories Equipment). The leaves were then transferred to 100% TAAB LV for 2 h followed by fresh TAAB LV for 24 h. Finally, the leaf segments were transferred to molds with TAAB LV and cured for 16 h at 60°C. Sections (90 mm) were cut with a diamond knife, stained with lead citrate, and viewed with a Jeol JEM-1400 electron microscope at an accelerating voltage of 80 kV.

**Total Foliar H₂O₂ Measurements**

Hydrochloric acid (0.1 M) extracts from 100 mg of fresh Arabidopsis tissue ground in liquid nitrogen were prepared and purified over activated charcoal as described by Creissen et al. (1999). H₂O₂ concentrations in purified extracts were determined using an Amplex Red assay kit (Invitrogen) according to the manufacturer’s instructions.

**Imaging of Luciferase Activity in Leaf Veins**

High-magnification imaging of HL-induced luciferase activity in Col-0/ APX2LUC was as previously described (Fryer et al., 2003). Plants were sprayed with 1 mM D (-) luciferin (Biosynth), or it was infiltrated into detached leaves as described above for ARU and for HL experiments. Images were taken using the same CCD camera system and processed as described for resorufin (see above) using an image acquisition time of 2 min.

**ABA Measurements**

All measurements of foliar ABA content were performed on leaves clamped in the CIRAS chamber, and only one leaf per plant was used. After treatments, leaves were frozen in liquid nitrogen, freeze-dried, and ABA content measured using a radio-immunooassay procedure (Quarrie et al., 1988), as described previously (Xie et al., 2006). Following exposure to osmotic stress, individual petioles were immediately frozen in liquid nitrogen and similarly assayed for ABA content.

**RNA Extraction and Quantitative Real-Time RT-PCR**

RNA was extracted from 100 to 200 mg of fully expanded leaves using TRIzol reagent (Sigma-Aldrich) according to the manufacturer’s instructions, except that an additional ethanol precipitation step was included at the end of the procedure to ensure the RNA was of appropriate quality (A₂₆₀/A₂₈₀ ≥ 2.0). RNA (3 µg) was treated with RNase-free DNase1 (Ambion) and the absence of contaminating genomic DNA confirmed using a PCR test as described previously (Bechtold et al., 2008). RNA (2 µg) was used to make random-primed cDNA as previously described (Ball et al., 2004), except that the MuMLV reverse transcriptase was purchased from Fermentas. Quantitative real-time PCR was performed as described previously (Ball et al., 2004; Bechtold et al., 2008) using a cyanogen-blue-luciferase-fluorescence based procedure with reagents purchased in kit form from Sigma-Aldrich. Relative cDNA levels between two sets of threshold cycle (Ct) values were calculated using the ∆∆CT method (Kubista et al., 2006) and normalized with respect to relative cDNA levels for CYCLOPHILIN. This reference gene was chosen because it shows unchanging transcript levels in excess light-exposed leaves and in conditions where foliar ABA content varies (Rossel et al., 2006). The primers used in this study for quantitative RT-PCR are given in Supplemental Table 2 online.

**Bioinformatics**

Data for Affymetrix ATH1 GeneChips were downloaded from the GEO repository (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gds) and the NASCAarrays database (http://affymetrix.Arabidopsis.info/narrrays/experimentbrowse.pl). The HL exposure data (GEO accession number GSE7743) and ABA treatment data (NASCARRAYS-176) were normalized using GCRMA procedures in the Bioconductor package within the R statistical environment (Gentleman et al., 2004; Wu et al., 2004). Values for replicate arrays were averaged and ratios calculated between treatment and control; these ratios were used to rank genes for the responses to 3 h HL exposure and 3 h following ABA treatment. Significant similarities between these two ranked lists were then calculated using the Ordered List Bioconductor package (Lottaz et al., 2006). The genes commonly upregulated and commonly downregulated that contributed to the significant weighted similarity score were clustered together with other ABA treatment time points from the NASCAARRAYS-176 data set and additional ABA treatment data sets (GSE7112 and GSE6171). Hierarchical clustering was performed using CLUSTER (Eisen et al., 1998) and visualized with the program TREEVIEW (Eisen et al., 1998). Complete linkage clustering using an uncentered Pearson correlation was applied after the genes were first ordered by self-organizing maps to produce better-arranged clusters.

**Accession Numbers**

The Arabidopsis Genome Initiative locus identifiers of genes used or mentioned in this study are as follows: AAO3, At2g27150; ABA2, At1g52340; ABA4, At1g67080; ABA1, At4g26080; ABA2, At3g67505; AGB1, At1g34460; APX2, At3g09840; BAP1, At3g61190; CYCLOPHILIN, At2g29860; ELIP1, At3g22840; HSP17.6B-C1, At1g229900; HSP17.6C-C1, At1g53540; GFA1, At2g55200; LIPOCALIN, At5g58070; LHCB1.2,
Supplemental Data

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

Supplemental Figure 1. Dark-Adapted \( F_{v}/F_{m} \) Measurements on Col-0, srx1-1, srx1-2, and vtc1-1 before, immediately after HL Exposure, and 24 h Later.

Supplemental Figure 2. Expression under HL Conditions of Genes Controlled by the GUN1/ABI4 and \( \cdot O_{2} \)-Related Chloroplast-to-Nucleus Retrograde Signaling Pathways.

Supplemental Figure 3. Characterization of nced3-2.

Supplemental Figure 4. Resorufin Fluorescence from the Periveinal Region of Ampelx Red Ultrainfiltrated Leaves Exposed to Fivefold HL at Low Humidity.

Supplemental Figure 5. Further Electron Micrographs of CeCl3-Stained High Light and Control Leaf Sections through Vein Tissue.

Supplemental Figure 6. Further Electron Micrographs of CeCl3-Stained High Light and Control Leaf Sections through Vein Tissue.

Supplemental Figure 7. Stomatal Conductance of ABA Signaling Mutants.

Supplemental Figure 8. DAB Staining Showing \( H_{2}O_{2} \) Accumulation in the Voiis of ostl-1 and gpa1-4 Compared with Wild-Type Controls.

Supplemental Table 1. HL-Responsive Gene Expression in Mutants Used in This Study Where No Significant Effect Was Discerned.

Supplemental Table 2. A List of the Primers and Their Target Genes Used for qRT-PCR in This Study.

Supplemental Data Set 1A. A List of Genes Whose Expression Is Upregulated by Both High Light and ABA Treatment.

Supplemental Data Set 1B. A List of Genes Whose Expression Is Downregulated by Both High Light and ABA Treatment.

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The High Light Response in *Arabidopsis* Involves ABA Signaling between Vascular and Bundle Sheath Cells

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