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Chloroplasts of *Arabidopsis* Are the Source and a Primary Target of a Plant-Specific Programmed Cell Death Signaling Pathway[W]

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Enhanced levels of singlet oxygen ($^1O_2$) in chloroplasts trigger programmed cell death. The impact of $^1O_2$ production in chloroplasts was monitored first in the conditional *fluorescent* (*flu*) mutant of *Arabidopsis thaliana* that accumulates $^1O_2$ upon a dark/light shift. The onset of $^1O_2$ production is rapidly followed by a loss of chloroplast integrity that precedes the rupture of the central vacuole and the final collapse of the cell. Inactivation of the two plastid proteins EXECUTER (EX1) and EX2 in the *flu* mutant abrogates these responses, indicating that disintegration of chloroplasts is due to EX-dependent signaling rather than $^1O_2$ directly. In *flu* seedlings, $^1O_2$-mediated cell death signaling operates as a default pathway that results in seedlings committing suicide. By contrast, EX-dependent signaling in the wild type induces the formation of microlesions without decreasing the viability of seedlings. $^1O_2$-mediated and EX-dependent loss of plastid integrity and cell death in these plants occurs only in cells containing fully developed chloroplasts. Our findings support an as yet unreported signaling role of $^1O_2$ in the wild type exposed to mild light stress that invokes photoinhibition of photosystem II without causing photooxidative damage of the plant.

INTRODUCTION

Programmed cell death (PCD) is a genetically regulated physiological process that is of central importance for the development and homeostasis of multicellular organisms (Green and Reed, 1998). In several instances, the execution of PCD involves the participation of mitochondria that act as sensors of cellular stress and initiate the onset of the cell death response (Green and Reed, 1998; Green and Kroemer, 2004). Permeabilization of mitochondrial membranes and the release of mitochondrial proteins are hallmarks of these PCD processes (Adrain and Martin, 2001; Joza et al., 2001; Green and Kroemer, 2004). As shown in this work, another cell death response implicates chloroplasts as a source of a cell death signaling pathway. In analogy to PCD associated with impaired mitochondria, this cell death program leads to a rapid loss of chloroplast integrity and the subsequent collapse of the affected cell. In contrast with PCD associated with mitochondria, which has been linked to the release of hydrogen peroxide (H_2O_2)/superoxide (Vacca et al., 2006), the plastid-derived PCD is initiated by the release of singlet oxygen ($^1O_2$).

Plants under oxidative stress suffer from damages that previously have been interpreted as unavoidable consequences of injuries inflicted upon plants by toxic levels of reactive oxygen species (ROS) (Apel and Hirt, 2004.). For instance, plants under severe light stress generate enhanced levels of ROS and may bleach. Based on the analysis of lipid peroxidation products, this bleaching has been attributed to the cytotoxicity of $^1O_2$ that causes extensive photooxidative damage (Triantaphylidès et al., 2008). However, as shown in this study, under less severe stress conditions that reflect more closely environmental fluctuations often experienced by plants in their natural habitat, $^1O_2$ may also act as a signal, activating a PCD pathway that leads to the formation of microlesions, but does not seem to impair the viability of the affected plant.

The role of $^1O_2$ as a trigger of cell death was initially revealed in the *fluorescent* (*flu*) mutant of *Arabidopsis thaliana* (op den Camp et al., 2003). In the dark, plastids of the *flu* mutant accumulate excess amounts of protochlorophyllide (Pchlide) due to the absence of negative feedback control of tetrapyrrole biosynthesis (Meskauskiene et al., 2001). In the light, Pchlide acts as a photosensitizer and generates $^1O_2$, which leads to a rapid collapse of seedlings and growth inhibition of mature plants (op den Camp et al., 2003). The nucleus-encoded and chloroplast-localized EXECUTER1 (EX1) and EX2 proteins have been identified as essential components of $^1O_2$ signaling. Inactivation of EX proteins in an *ex1 ex2 flu* triple mutant is sufficient to suppress the upregulation of almost all $^1O_2$-responsive genes and to restore the wild-type phenotype (Wagner et al., 2004; Lee et al., 2007). In this work, this block of $^1O_2$-mediated responses in an *ex1 ex2* genetic background has been used to

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identify a signaling role of $^1O_2$ in wild-type plants and to define a genetically controlled PCD pathway unique to photosynthetic eukaryotes that operates under mild stress conditions that impede photosystem II (PSII) without causing photooxidative damage of the plant.

RESULTS

$^1O_2$-Mediated Cell Death

Continuous light–grown seedlings of *flu*, *flu ex1*, *flu ex2*, and *flu ex1 ex2* together with the wild type were transferred to the dark for various lengths of time ranging from 4 to 16 h (Figure 1A), which leads to the accumulation of increasing amounts of Pchlide (Figure 1B). During reillumination of *flu* and *flu ex2* plants, the number of visible lesions in true leaves steadily increased with increasing lengths of the preceding dark period (Figures 1A and 1B), indicating that during reillumination of seedlings different amounts of $^1O_2$ had been generated, causing cell death in a dose-dependent manner. In *flu ex1* seedlings, this cell death response was strongly attenuated, and in *flu ex1 ex2*, it was completely suppressed, suggesting that lesion formation in *flu* seedlings was due to the activation of $^1O_2$-mediated signaling that is blocked in *ex1* and *ex1 ex2*, but not in *ex2* mutant lines (Lee et al., 2007). The same differences between these mutant lines were seen when the spreading of cell death was visualized by trypan blue staining (Figure 1C). In wild-type seedlings, lesion formation did not occur.

Collectively, these results suggest that $^1O_2$-mediated cell death originates within chloroplasts and is initiated by the activation of an EX1/EX2-dependent signaling pathway. These conclusions are based on two assumptions: First, during dark incubation, seedlings of *flu*, *flu ex1*, *flu ex2*, and *flu ex1 ex2* accumulate similar excess amounts of Pchlide within their plastids. Second, during reillumination, all four mutant lines generate similar enhanced amounts of $^1O_2$ relative to the wild type. These predictions were tested experimentally.

At the end of an 8-h dark period *flu*, *flu ex1*, *flu ex2*, and *flu ex1 ex2* seedlings accumulate similar amounts of Pchlide that were seven- to eightfold higher than in wild-type controls (Figure 1D). $^1O_2$ production during reillumination was monitored in seedlings that had been infiltrated with dansyl-2,2,5,5-tetramethyl-2,5-

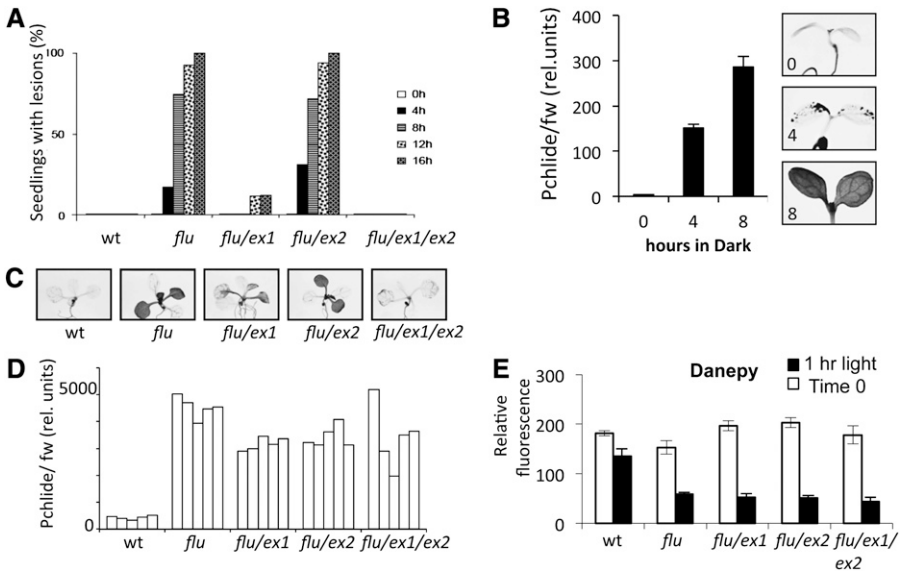

Figure 1. The Genetic Regulation of $^1O_2$-Mediated Cell Death.

(A) Dose-dependent $^1O_2$-mediated lesion formation in 10-d-old seedlings of the wild type (wt), *flu*, *flu ex1*, *flu ex2*, and *flu ex1 ex2*. Seedlings were grown under continuous light, transferred to the dark for 0, 4, 8, 12, and 16 h, and reexposed to light for 24 h. The number of seedlings with visible lesions relative to the total number of seedlings was used to determine the frequency of lesion formation. A minimum of 100 seedlings was counted per sample. The experiment was repeated three times and gave very similar results.

(B) Accumulation of different levels of Pchlide in *flu* seedlings during 4 h (4) and 8 h (8) of dark incubation and dose-dependent lesion formation in these seedlings after 24 h of reillumination. Cell death was measured by trypan blue staining. Control *flu* seedlings (0) were kept under continuous light. Results represent the mean and SD of three samples. For each sample, 10 seedlings were used. fw, fresh weight.

(C) $^1O_2$-mediated cell death as revealed by trypan blue staining of 10-d-old seedlings. Seedlings were transferred to the dark for 8 h and reexposed to light for 24 h.

(D) Pchlide levels in seedlings of the wild type, *flu*, *flu ex1*, *flu ex2*, and *flu ex1 ex2* at the end of a 16-h dark period. For each line, five pigment extracts from five plants each were analyzed. Note that in *flu ex1 ex2* triple mutants, no lesion formation was detectable, even though Pchlide reached similar excess levels as in *flu ex1* and *flu ex2* and only slightly lower levels than in *flu*.

(E) Release of $^1O_2$ in light-grown seedlings transferred for 8 h to the dark. $^1O_2$ trapping was measured as relative quenching of Danepy fluorescence before (0) or after 1 h (1) of reillumination. Values represent the mean and SD of three samples. For each sample, 10 seedlings were used.

dehydro-*1H*-pyrrole (Danepy) (Hideg et al., 1998; Kálai et al., 1998). Danepy is used as a double (spin and fluorescent) $^1O_2$ sensor, consisting of a fluorophore and a nitroxide precursor. In the absence of $^1O_2$, Danepy is highly fluorescent but shows no electron paramagnetic resonance signal. Upon reaction with $^1O_2$, conversion of the spin trap moiety into an electron paramagnetic resonance–active nitroxide results in partial fluorescence quenching (Kálai et al., 1998). Prior to reexposure to light, infiltrated seedlings of the wild type and the four mutant lines displayed similar Danepy fluorescence levels (Figure 1E). After 1 h of light exposure, this fluorescence was strongly quenched in *flu*, *flu ex1*, *flu ex2*, and *flu ex1 ex2*, whereas in wild-type seedlings, the fluorescence was only slightly reduced (Figure 1E). These results indicate that in all mutant lines, Pchlide acts as a photosensitizer and produces similar amounts of $^1O_2$. Since cell death responses were observed only in *flu* and *flu ex2* but not in *flu ex1* or *flu ex1 ex2*, photooxidative damage of chloroplasts by $^1O_2$ seems unlikely to be the cause of cell death.

Monitoring peroxidation of polyunsaturated fatty acids during reillumination of predarkened *flu* seedlings also supported this conclusion. Linolenic acid is the most prominent polyunsaturated fatty acid in chloroplast membranes (Murakami et al., 2000) and has been shown to be a preferred target of ROS. After 1 h of reillumination of predarkened *flu* seedlings, peroxidation of linolenic acid occurred almost exclusively enzymatically by lipoxygenases and not by direct attack of $^1O_2$, as illustrated by the rapid accumulation of the *S*-enantiomer of 13-hydroxy octadecatrieonic acid (13-HOT), a specific marker of enzymatic peroxidation of linolenic acid in *flu* (Berger et al., 2001; op den Camp et al., 2003). At this early time of reillumination, the nonenzymatic peroxidation products of linolenic acid 10-HOT and 15-HOT are hardly detectable (Figure 2A).

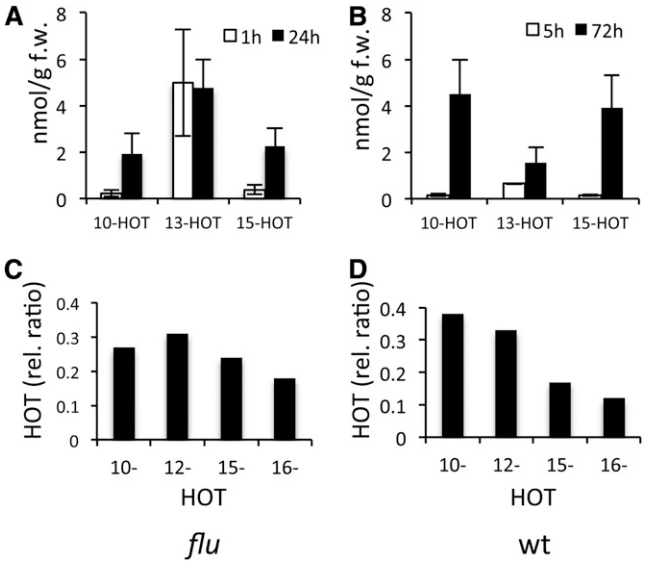

Figure 2. Enzymatic and Nonenzymatic Peroxidation of Linolenic Acid in 10-d-Old Seedlings of *flu* and the Wild Type.

(A) Continuous light–grown *flu* seedlings were shifted to the dark for 8 h and reexposed for 1 or 24 h to light. f.w., fresh weight.

(B) Wild-type (wt) seedlings shifted from low light (15 μmol m^{-2} s^{-1})/room temperature (22°C) to high light (270 μmol m^{-2} s^{-1})/low temperature (12°C) for 5 or 72 h. The $^1O_2$-specific nonenzymatic peroxidation products 10-HOT, 15-HOT, and 13-HOT generated both enzymatically and nonenzymatically were determined. Results in **(A)** and **(B)** represent the mean and SD of three biological samples.

(C) and **(D)** Lipid peroxidation signatures of *flu* (8 h dark/24 h light) **(C)** and the wild type (72 h of low-temperature/high-light stress) **(D)** indicate that nonenzymatic peroxidation occurs primarily through $^1O_2$.

$^1O_2$-Mediated Chloroplast Leakage in the *flu* Mutant

The first consequence of $^1O_2$ production in the *flu* mutant visible to the eye was a rapid loss of chloroplast integrity. Chloroplast integrity was assessed under the confocal microscope by monitoring the fluorescence distribution of the green fluorescent protein (GFP) in transgenic plants that express a chimeric reporter protein consisting of the nucleus-encoded and chloroplast-localized small subunit of the ribulose-1,5-bisphosphate carboxylase (SSU) and GFP. In nontransgenic *flu* seedlings kept in the dark or transferred from the dark to light, no GFP fluorescence signals were detectable (Figures 3A1 and 3A2). In transgenic wild-type plants, the fusion protein was confined to the plastid compartment (Figure 3A3). An identical distribution of the fusion protein was also seen in transgenic *flu* plants grown under continuous light (Figure 3A4). Under these growth conditions, *flu* seedlings do not overaccumulate Pchlide and do not exhibit enhanced $^1O_2$ production (op den Camp et al., 2003). However, after keeping transgenic *flu* plants in the dark for 4 h, chloroplast integrity was impaired during reillumination and the fusion protein was released from the chloroplast to the surrounding cytoplasm (Figure 3A5). The molecular weight of the SSU-GFP fusion protein as revealed by its electrophoretic mobility during SDS-PAGE and its relative amount did not change during the first hour of reillumination (Figure 3B). Thus, during initiation of $^1O_2$-mediated cell death, the presence of the reporter protein outside of chloroplasts does not seem to be due to an enhanced accumulation of nonprocessed higher molecular weight precursors of SSU-GFP prior to its translocation into chloroplasts. A similar change in the intracellular distribution of GFP fluorescence as in *flu* occurred also in *flu ex2* seedlings (Figure 3A7), whereas in transgenic *flu ex1* and *flu ex1 ex2* seedlings subjected to the same dark/light shift, the fusion protein was retained within chloroplasts (Figures 3A6 and 3A8). Since all four mutant lines generate similar amounts of $^1O_2$ during reillumination (Figure 1E), loss of chloroplast integrity in *flu* and *flu ex2* seedlings cannot be accounted for by a rupture of chloroplast envelopes by direct attack of $^1O_2$ but rather seems to be the result of $^1O_2$ signaling that is blocked in *ex1* and *ex1 ex2* lines.

Loss of chloroplast integrity could be one of the initial steps triggering $^1O_2$-mediated PCD, or it could merely be a secondary consequence of cellular disintegration during the spreading of $^1O_2$-mediated PCD. In the former case, loss of chloroplast integrity should precede the collapse of cells, whereas in the latter case, it should occur together with the disintegration of other intracellular compartments. To address this question, we exploited the unique properties of the *flu* mutant. Because of the precision with which generation of $^1O_2$ can be triggered in *flu*,

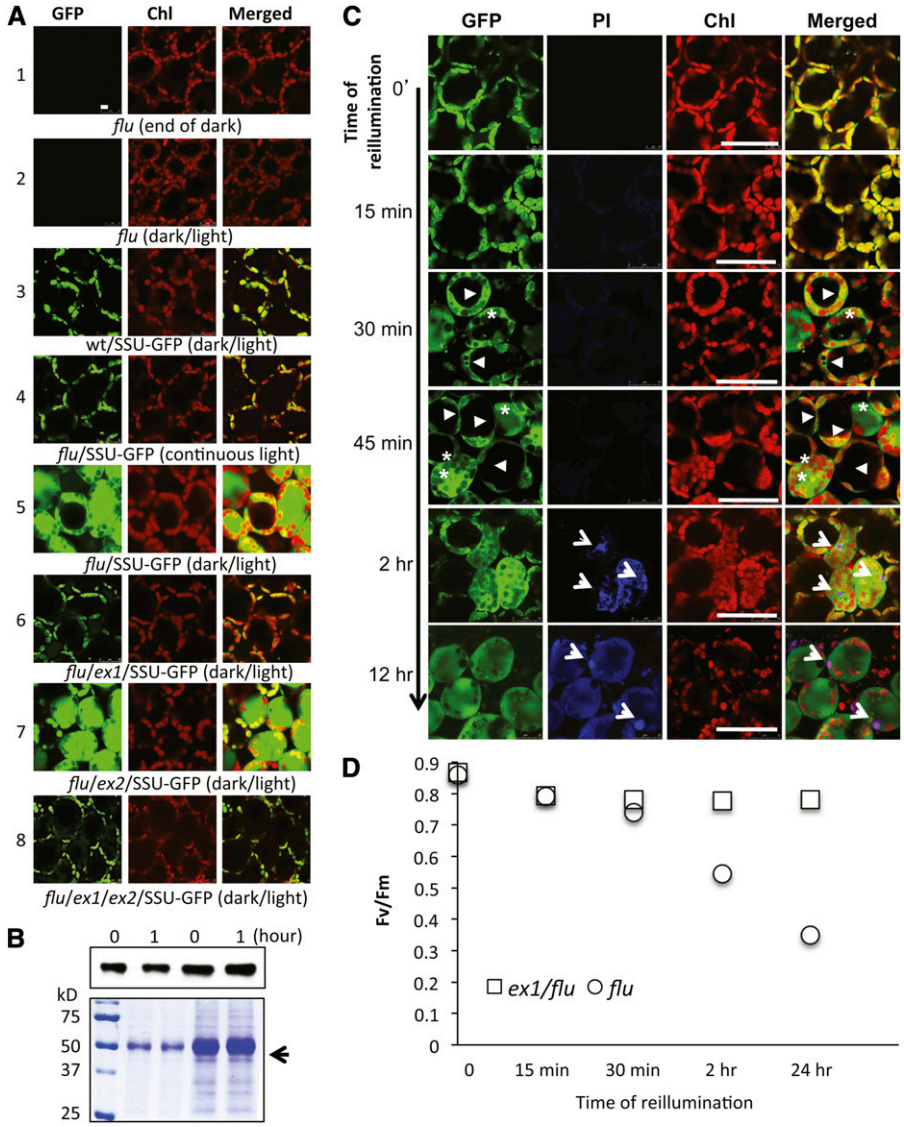

Figure 3. Genetic Control of $^1O_2$-Mediated Chloroplast Leakage.

(A) Ten-day-old wild-type (wt), *flu*, *flu ex1*, and *flu ex1 ex2* seedlings expressing the SSU-GFP fusion protein were grown under continuous light, shifted to the dark for 8 h, and reexposed to light for 1 h. The green fluorescence of GFP and red fluorescence of chlorophyll (Chl) were monitored separately by CLSM, and the two fluorescence images were merged. Bar = 10 μm.

(B) Size and relative concentration of the SSU-GFP fusion protein in total extracts of *flu* seedlings. Ten and 30 μg of total protein extracted from seedlings before (0) or 1 h (1) after reillumination were separated electrophoretically by SDS-PAGE and blotted, and the fusion protein was detected immunologically. Only the mature-sized SSU-GFP fusion protein was detected using the GFP antiserum. As a loading control, the stained protein gel prior to blotting is shown. Arrow marks the position of the fusion protein.

(C) Kinetics of $^1O_2$-mediated chloroplast leakage, vacuole rupture, and initiation of cell death in *flu*. Ten-day-old *flu* seedlings expressing the SSU-GFP fusion protein were initially grown under continuous light, shifted to the dark for 8 h, and reexposed to light for various lengths of time (0 to 12 h). Chloroplast leakage (asterisk) and intactness of vacuoles (triangle) were assessed by the intracellular distribution of the GFP fusion protein, and the onset of cell death (arrowhead) was visualized by staining with PI. The green fluorescence of GFP, the red fluorescence of chlorophyll, and the blue fluorescence of PI were monitored separately by CLSM, and the three fluorescence images were merged. Bars = 50 μm.

(D) $^1O_2$-mediated changes of the maximum quantum efficiency (F_v/F_m) of PSII in *flu* and *flu ex1* seedlings during reillumination. Results represent the mean of three samples. For each sample, at least 50 seedlings were analyzed.

the sequence of intracellular changes that occur during initiation of $^1O_2$-mediated cell death can be analyzed and early targets of $^1O_2$-mediated signaling can be identified and distinguished from secondary changes at a later time during disintegration of cells.

The kinetics of chloroplast leakage, rupture of the central vacuole, and onset of cell death were determined in *flu* seedlings. Continuous light-grown *flu* seedlings were transferred to the dark for 4 h and reexposed to light for various lengths of time. Generation of $^1O_2$ in *flu* seedlings has been shown previously to occur within <1 min following the dark/light shift (op den Camp et al., 2003). During the first 15 min of reillumination, the SSU-GFP fusion protein remained within the plastid compartment and chloroplast integrity was not visibly perturbed, but during the following 15 min, widespread chloroplast leakage started to occur (Figure 3C, asterisk). At this time the majority of affected cells still maintained their central vacuole intact (Figure 3C, triangle). Loss of chloroplast integrity clearly preceded vacuole rupture and cellular collapse. Over the next 15 min, the number of cells that not only lost chloroplast integrity but also contained ruptured vacuoles gradually increased. However, these cells still appeared intact. The onset of cell death in these cells was determined by propidium iodine (PI) staining. This reagent is excluded from intact cells but penetrates dying or dead cells and intercalates into double-stranded nucleic acids (Oparka and Reed, 1994; Kirik et al., 2001). PI staining was first detected in *flu* seedlings 2 h after the beginning of reillumination (Figure 3C, arrowhead). During the next few hours, the number of cells with positive PI staining rapidly increased throughout the seedling, and this was accompanied by visible lesion formation. At the same time, the intensities of GFP and chlorophyll fluorescence declined.

The kinetics of these $^1O_2$-mediated changes that lead to the collapse of the affected cells were in line with rapid changes of the functional state of PSII that was determined by measuring the maximum quantum efficiency of PSII expressed as the ratio of variable to maximum fluorescence of chlorophyll (F_v/F_m) (Figure 3D). During the first 15 to 20 min following the dark/light shift, the F_v/F_m ratios dropped slightly in *flu* and *flu ex1* seedlings, suggesting that in both lines PSII activity was similarly affected by the release of $^1O_2$. Afterwards, the F_v/F_m ratio continued to decline rapidly in *flu* seedlings, whereas in *flu ex1* seedlings, it remained constant over the next 24 h (Figure 3D). The time at which F_v/F_m ratio changes in *flu* and *flu ex1* seedlings started to diverge coincides with the beginning of chloroplast leakage in *flu*. Hence, the initial steps of $^1O_2$-mediated signaling in the *flu* mutant that induce the loss of chloroplast integrity and depend on the activity of EX1 and EX2 must be completed prior to this divergence of PSII activity changes.

With the progression of cell death, nonenzymatic peroxidation products of linolenic acid started to accumulate. Two nonenzymatic reaction types may lead to peroxidation of polyunsaturated fatty acids (Stratton and Liebler, 1997; Mueller et al., 2006). Type I reactions are initiated by free radicals, such as hydroxyl radicals, and type II reactions are affected by $^1O_2$. H_2O_2 and superoxide radical do not directly oxidize polyunsaturated fatty acids, but in the presence of transition, metal ions such as Fe^{2+} are converted to hydroxyl radical (Halliwell and Gutteridge, 2007). Nonenzymatic peroxidation of linolenic acid may result in the formation of six possible hydroxy fatty acid isomers. Two of them, 10-HOT and 15-HOT, are exclusively formed by $^1O_2$ during a type II reaction, whereas both reaction types generate the other four, 9-HOT, 12-HOT, 13-HOT, and 16-HOT. The signature of these various HOT isomers has been used to determine the relative impact of $^1O_2$ and H_2O_2 and superoxide radical on lipid peroxidation (Triantaphylidès et al., 2008). The relative abundance of the $^1O_2$-specific peroxidation products of type II reactions, 10-HOT and 15-HOT, to that of 12- and 16-HOT demonstrates that in *flu* seedlings during this late stage of cell death progression nonenzymatic peroxidation can be attributed predominantly to the cytotoxicity of $^1O_2$ (Figures 2A and 2C).

$^1O_2$-Mediated Release of Chloroplast Proteins

So far, $^1O_2$-mediated release of chloroplast proteins to the surrounding cytoplasm had been deduced from intracellular changes of GFP fluorescence distribution in transgenic *flu* plants. A second independent approach was used to verify the rapid $^1O_2$-mediated loss of chloroplast integrity by comparing the proportion of SSU-GFP recovered in the cytosol fractions of *flu* and wild-type seedlings subjected to a dark/light shift. For such an experiment, isolated protoplasts rather than intact seedlings were used to minimize chloroplast rupture by mechanical stress during the homogenization and fractionation steps. As shown previously (Danon et al., 2005), protoplasts of *flu* subjected to a dark/light shift also generate $^1O_2$ that induces cell death. At the end of the dark period, protoplasts contained intact chloroplasts as indicated by the localization of GFP fluorescence within the chloroplast compartment (Figures 4A and 4B). During reillumination, the number of *flu* protoplasts with intact chloroplasts rapidly declined, and after 3 h of reillumination, it had dropped to <20% (Figure 4B). By contrast, throughout reillumination of wild-type protoplasts, the majority of chloroplasts remained intact (Figures 4A and 4B). As in the case of seedlings, in *flu* protoplasts, chloroplast leakage and the release of SSU-GFP also seemed to be genetically determined and a result of photooxidative damage. In protoplasts of *flu ex1* seedlings subjected to the same dark/light shift, chloroplasts remained intact and did not release SSU-GFP to the surrounding cytoplasm (see Supplemental Figure 1 online). Death of protoplasts as revealed by staining with PI occurred only after chloroplast leakage and rupture of vacuoles had taken place (Figure 4A).

After various lengths of time of reillumination, aliquots of the protoplast suspension were collected and protoplasts were lysed. The intactness of chloroplasts within a given protoplast sample was determined by centrifuging lysed protoplasts and comparing the amounts of the SSU-GFP fusion protein in the supernatant by immunoblot analysis using an antiserum against GFP. Control experiments with protoplasts isolated from wild-type and *flu* plants grown under continuous light demonstrated that during protoplast lysis most chloroplasts remained intact. Only small fractions of the reporter protein were recovered in the supernatant of both protoplast samples (see Supplemental Figure 2 online).

During reillumination of predarkened wild-type protoplasts, minor amounts of SSU-GFP in the supernatant increased over

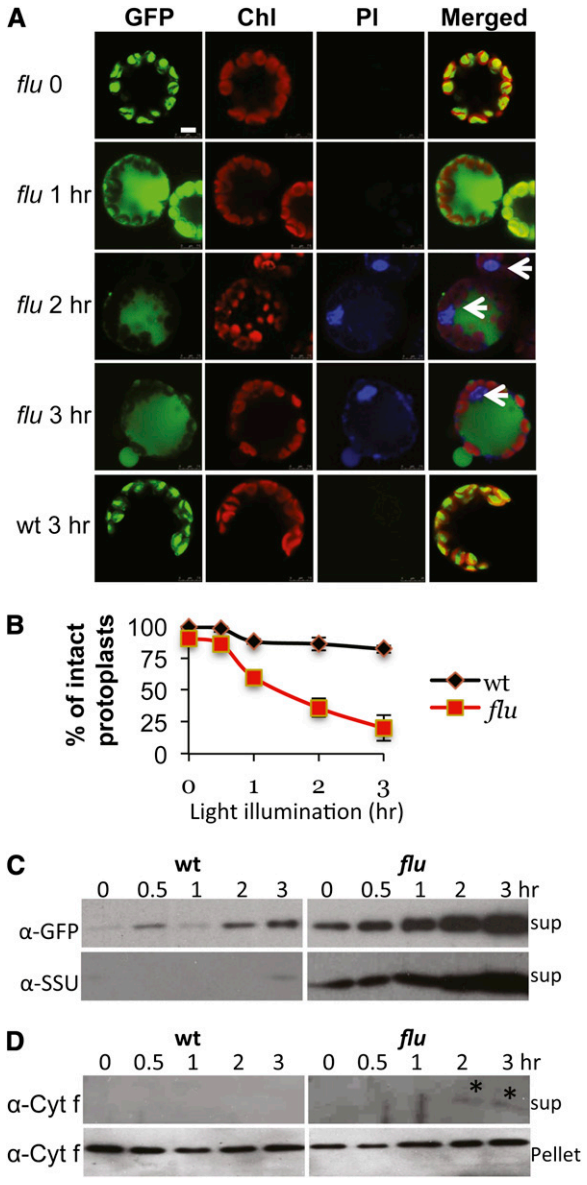

Figure 4. The Release of Chloroplast Proteins during $^1O_2$-Mediated Chloroplast Leakage.

(A) $^1O_2$-mediated chloroplast leakage and cell death in protoplasts isolated from *flu* seedlings expressing the SSU-GFP fusion protein. Chloroplast leakage and cell death were monitored by CLSM as described in Figure 3. Arrows indicate PI staining of nuclei of dead cells. Wild-type (wt) protoplasts after 3 h of reillumination are shown as controls. Bar = 10 μm.

(B) Changes of the percentage of protoplasts with intact chloroplasts of *flu* and the wild type during reillumination. Average and SD of three replicates are shown.

(C) The release of SSU-GFP and SSU from chloroplasts to the cytosol during reillumination of wild-type and *flu* protoplasts. After various lengths of time, protoplasts were lysed and the concentrations of SSU-GFP and SSU in the supernatant (sup) were assessed by immunoblot analysis using antisera against GFP and SSU.

(D) The release of thylakoid membrane-bound cyt *f* from chloroplasts to the cytosol during reillumination of wild-type and *flu* protoplasts. After various lengths of time, lysed protoplasts were centrifuged and the concentration of cyt *f* in the supernatant and pellet fractions was determined by immunoblot analysis, using an antiserum against cyt *f*. Trace amounts of cyt *f* were seen only in the supernatant fractions of *flu* protoplasts 2 h after the beginning of reillumination (asterisk).

time (Figure 4C). Much higher levels of SSU-GFP were found in the supernatant fraction of lysed *flu* protoplasts, regardless of whether proteins were probed with antisera against GFP or SSU (Figure 4C). Differences in the integrity of chloroplasts between the wild type and *flu* were also evident when chloroplasts were isolated from protoplasts before and after the beginning of reillumination and examined under the confocal microscope. Prior to reillumination, chloroplasts isolated from both protoplast samples retained the bright fluorescence of GFP. The majority of chloroplasts isolated from wild-type protoplasts 3 h after the beginning of reillumination still retained GFP fluorescence, whereas chloroplasts isolated from *flu* protoplasts had lost the GFP fluorescence and upon excitation showed only the bright red fluorescence of chlorophyll. At the same time, their size had increased due to swelling (see Supplemental Figure 3 online).

Previously, the release of cytochrome *f* (cyt *f*) from chloroplast membranes of stressed plants had been proposed to trigger a cell death response, similar to cytochrome *c* (cyt *c*) that acts as a trigger of PCD associated with impaired mitochondria (Peters and Chin, 2005; Zuppini et al., 2009). During the first hour of reillumination of predarkened *flu* protoplasts, when chloroplast leakage was first detected, no cyt *f* was found in the supernatant fraction of lysed protoplasts. The release of trace amounts of cyt *f* from thylakoid membranes was seen only after the onset of cell death as revealed by PI staining of protoplasts (Figures 4A and 4D, asterisk). The concentrations of cyt *f* in pellet fractions of wild-type and *flu* protoplasts were the same and did not change significantly throughout reillumination (Figure 4D). As the release of minute amounts of cyt *f* from thylakoid membranes of the *flu* mutant occurs only after cells start to disintegrate, cyt *f* is not a likely candidate for a molecular trigger that initiates $^1O_2$-mediated cell death but rather seems to be a marker for the final stage of cellular collapse.

$^1O_2$-Mediated Cell Death in Wild-Type Plants

Previously, cell death and bleaching of plants exposed to high light stress had been attributed to photooxidative damage caused by the cytotoxicity of $^1O_2$ (Triantaphylidès et al., 2008). These results raise the question of whether in the wild type an enhanced generation of $^1O_2$ in chloroplasts may also activate a genetically controlled PCD signaling pathway as seen in the *flu* mutant. Seedlings that express the SSU-GFP reporter protein were initially grown for 5 d at room temperature (22°C) under low light (15 μmol m^{-2} s^{-1}) and were then transferred to a combination of higher light (270 μmol m^{-2} s^{-1}) and lower temperature (12°C) (Figure 5A). As shown previously, at reduced temperatures, moderate light intensities are sufficient to cause severe light stress for photosynthetic membranes and to induce bleaching of seedlings that resembles closely the bleaching of *flu*

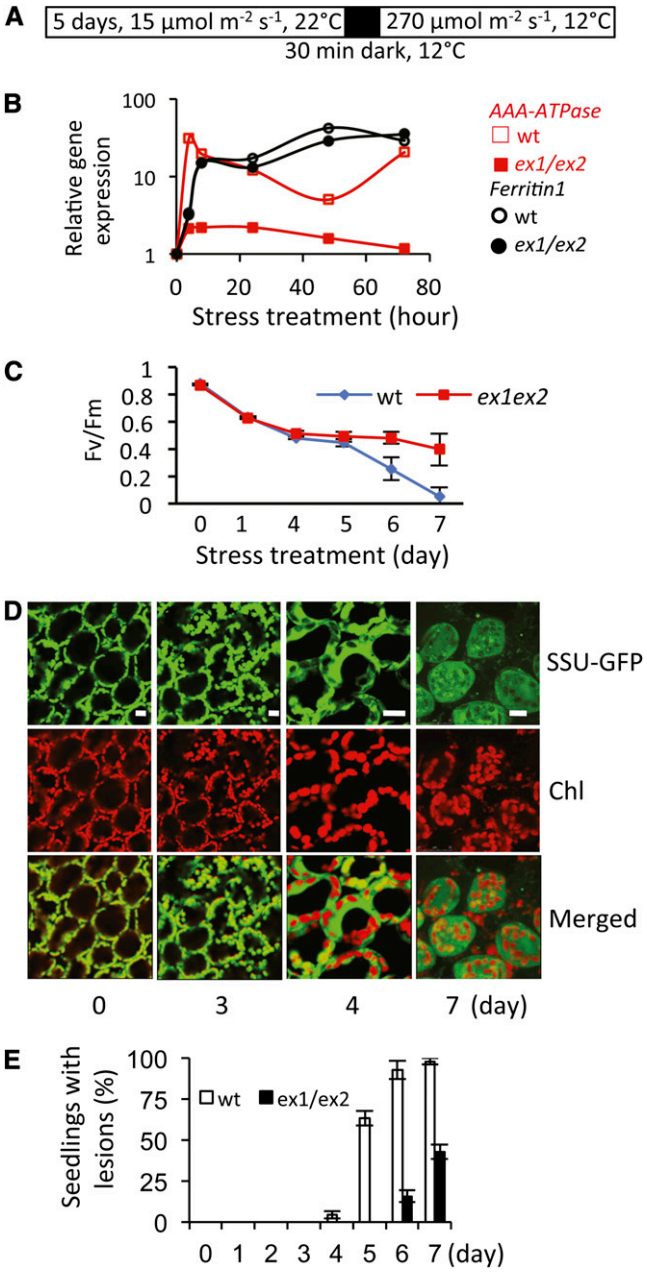

Figure 5. Stress-Induced Cell Death of Wild-Type and *ex1 ex2* Seedlings.

(A) Seedlings were initially grown for 5 d at 22°C and 15 µmol m^{-2} s^{-1} and were then exposed to a combined low-temperature (12°C)/high-light (270 µmol m^{-2} s^{-1}) stress program. Prior to the beginning of the low-temperature/high-light treatment, seedlings were placed for 30 min in the dark at 12°C to avoid high light–induced stress preacclimation during the lowering of the temperature.

(B) Stress-induced changes in the expression of the $^1O_2$-responsive *AAA-ATPase* and the H_2O_2-responsive *FER1* during low-temperature/high-light treatment of wild-type (wt) and *ex1 ex2* seedlings as revealed by quantitative PCR analysis. Results represent means of three independent biological replicates. *Actin2* was used as a control for normalization.

seedlings exposed to nonpermissive light/dark cycles (op den Camp et al., 2003; Meskauskiene et al., 2009). Shortly after the beginning of stress treatment, seedlings released enhanced levels of $^1O_2$ and H_2O_2 as indicated by the rapid upregulation of the $^1O_2$-responsive *AAA-ATPase* followed by the enhanced expression of the H_2O_2-responsive *FERRITIN1* (*FER1*) marker gene (Figure 5B). The upregulation of *AAA-ATPase* was strongly suppressed in *ex1 ex2* seedlings, whereas the enhanced expression of *FER1* was not affected (Figure 5B), in line with our earlier finding that enhanced levels of $^1O_2$ and H_2O_2 in chloroplasts activate two separate and distinct signaling pathways (op den Camp et al., 2003).

During this initial phase of stress treatment, photoinhibition of PSII occurs (Figure 5C), but photooxidative damage was hardly detectable as indicated by the very low levels of the nonenzymatic peroxidation products of linolenic acid 10- and 15-HOT after 5 h of stress treatment (Figure 2B). The presence of 13-HOT at this time was primarily due to the accumulation of its *S*-enantiomer that has previously been identified as a product of enzymatic peroxidation of linolenic acid by lipoxygenases in chloroplasts in response to enhanced levels of $^1O_2$ (op den Camp et al., 2003) (Figure 2B). Similar to the *flu* mutant, also in the wild type loss of chloroplast integrity occurred prior to the stress-induced collapse of seedlings. However, contrary to *flu*, loss of chloroplast integrity in the wild type did not immediately follow stress-induced $^1O_2$ production but was delayed and occurred only after an extensive light stress treatment (Figure 5D). Initiation of cell death started 4 d after the beginning of low-temperature/high-light treatment with chloroplast leakage preceding the rupture of the vacuole and the collapse of the cell (Figures 5D and 5E).

ex1 ex2 seedlings were less susceptible to the combined low-temperature/high-light stress than the wild type as shown by differences in lesion formation and the decline of the maximum quantum efficiency of PSII (F_v/F_m) (Figures 5C and 5E). Prior to stress, seedlings of both lines showed F_v/F_m values of 0.85 to 0.87. After 24 h of stress treatment, this ratio had dropped to 0.6 (Figure 5C). In wild-type seedlings, the quantum efficiency of PSII continued to decline further. After 4 d of stress, the first wild-type seedlings started to collapse and the number of bleached seedlings increased rapidly during the next 3 d (Figure 5E). In contrast with the wild type, *ex1 ex2* seedlings retained their chlorophyll for up to 5 d of stress treatment and were still viable with F_v/F_m values > 0.4 (Figure 5C).

(C) Stress-induced decline of the maximum quantum efficiency (F_v/F_m) of PSII in wild-type and *ex1 ex2* seedlings kept for various lengths of time under the combined low-temperature/high-light stress. Results represent the mean and SD of three samples. For each sample, 25 seedlings were analyzed.

(D) GFP and chlorophyll (Chl) autofluorescence images of leaf cells of wild-type seedlings expressing SSU-GFP were examined by CLSM before (0) and 3, 4, and 7 d after the beginning of low-temperature/high-light stress. Bars = 30 µm.

(E) Stress-induced bleaching of wild-type (white bars) and *ex1 ex2* seedlings (black bars). Values represent the mean and SD of four samples. For each sample, 50 seedlings were analyzed.

However, during the following days, *ex1 ex2* seedlings kept under low-temperature/high-light stress also started to bleach (Figure 5E). Hence, the block of $^1O_2$ signaling in *ex1 ex2* seedlings exposed to low temperature/high light did not abrogate but only delayed the final collapse of seedlings by 1 to 2 d. $^1O_2$-dependent PCD seems to be involved in triggering a cell death response in low-temperature/high-light-treated wild-type seedlings, but progression of cell death in these plants is not exclusively driven by $^1O_2$-mediated and EX1/EX2-dependent signaling.

Prior to the bleaching of seedlings, the concentration of nonenzymatic peroxidation products increased drastically. The relative abundance of $^1O_2$-specific markers of type II reactions, 10- and 15-HOT, and that of 12- and 16-HOT that are also formed by type I reactions indicates that this nonenzymatic lipid peroxidation is primarily due to $^1O_2$ (Figures 2B and 2D). Hence, under severe light stress conditions, the genetically determined PCD response of the wild type seems to be masked and superimposed by photooxidative damage caused by the cytotoxicity of $^1O_2$.

If correct, this notion would suggest that under less severe stress conditions photooxidative damage should be attenuated and activation of the genetically determined $^1O_2$-mediated cell death response should prevail. This prediction was tested by initially growing wild-type and *ex1 ex2* seedlings at room temperature for 5 d under moderate (90 μmol m^{-2} s^{-1}) rather than low light intensities, before transferring them to the same low-temperature/high-light stress program used in the previous experiment (Figure 6A). Similar to seedlings grown under low light, moderate light–grown seedlings also started to activate the $^1O_2$-dependent signaling pathway right after the beginning of the stress treatment, as indicated by the activation of the $^1O_2$-responsive marker gene *AAA-ATPase* and its suppression in *ex1 ex2* seedlings (Figure 6B). However, moderate light–grown wild-type and *ex1 ex2* seedlings did not bleach but retained their chlorophyll and remained viable throughout the stress treatment. There was a minor initial reduction of the F_v/F_m values from 0.86 to 0.74 (the wild type) and 0.79 (*ex1 ex2*) after 24 h of low-temperature/high-light treatment, but during the following stress treatment, the maximum quantum efficiency of PSII in both lines remained stable and did not further decline (Figure 6C). Chloroplast leakage in wild-type seedlings was first seen between 48 and 72 h of stress treatment (Figure 6D) and preceded formation of microlesions that were detected by staining with trypan blue (Figures 6D and 6E). In *ex1 ex2* seedlings, these cell death responses were completely blocked. Even when kept for an extended period of time under stress, *ex1 ex2* seedlings did not develop microlesions and their chloroplasts remained intact (Figures 6D and 6E).

$^1O_2$-Mediated PCD Is Activated in Green Leaf Sectors of the *variegated2* Mutant with Fully Developed Chloroplasts but Not in Its White Sectors with Undifferentiated Plastids

To confirm the proposed role of chloroplasts as a source of a plant-specific PCD pathway, seedlings of the leaf-variegated mutant *variegated2* (*var2*) were first grown for 10 d at room temperature and 90 μmol m^{-2} s^{-1}. Cells in the green leaf sectors of this mutant contained morphologically normal chloroplasts, whereas in the white sectors, they contained undifferentiated plastids that were reduced in size and lacked pigments and developed lamellar structures (Sakamoto et al., 2009). In white sectors, undifferentiated plastids were still able to accumulate the SSU-GFP fusion protein. Chloroplasts in the green leaf sectors of *var2* like those in the wild type accumulated high levels of the fusion protein (Figure 7A).

Seedlings of *var2* and *var2 ex1 ex2* grown at room temperature under moderate light (90 μmol m^{-2} s^{-1}) for 10 d were transferred to the low-temperature/high-light stress program described in Figure 6A. Soon after the beginning of the stress treatment, $^1O_2$-dependent signaling was activated in green cotyledons of *var2* seedlings, as indicated by the rapid upregulation of the $^1O_2$-responsive marker gene *AAA-ATPase* and its suppression in *var2 ex1 ex2* (Figure 7B). *AAA-ATPase* transcripts accumulated faster in *var2* and reached higher levels during the first 12 h of stress treatment than in the wild type (Figure 7B), in line with the reported enhanced susceptibility of *var2* to light stress (Rosso et al., 2009; Liu et al., 2010). In green sectors of variegated true leaves, chloroplast leakage occurred like in the wild type (Figure 7C) and preceded the final collapse of the cell, whereas in white leaf sectors, plastids remained intact (Figure 7C).

Previously, enhanced levels of superoxide radical and H_2O_2 had been shown to accumulate in green leaf sectors of variegated mutants (Kato et al., 2009). These ROS do not seem to be involved in triggering the cell death response of *var2* seedlings exposed to the low-temperature/high-light stress; cell death in green leaf sectors of *var2 ex1 ex2* seedlings was completely suppressed (Figure 7D). Hence, PCD in *var2* seedlings takes its origin in chloroplasts and seems to be under strict control of $^1O_2$-mediated and EX-dependent signaling.

DISCUSSION

The main finding of our study implicates chloroplasts as being the source and a primary target of a genetically determined, $^1O_2$-mediated cell death response unique to plants. In the *flu* mutant, activation of $^1O_2$-mediated signaling ends with seedlings committing suicide. The analysis of lipid peroxidation in these seedlings revealed two distinct, sequentially occurring biological activities of $^1O_2$. During initiation of the cell death response, $^1O_2$ acts as a signal without causing photooxidative damage. Lipid peroxidation at this stage occurs almost exclusively enzymatically. However, during the final collapse and bleaching of *flu* seedlings, the cytotoxicity of $^1O_2$ prevails, causing massive nonenzymatic lipid peroxidation and photooxidative damage. The initiation of $^1O_2$-mediated cell death signaling in *flu* following a dark-to-light shift was due to the photosensitizing activity of Pchlide that had accumulated in the dark. As overaccumulation of this photosensitizer stops in illuminated *flu* seedlings (Meskauskiene et al., 2001; Goslings et al., 2004), generation of $^1O_2$ during the final stage of the cell death response is likely to occur via another photosensitizer, chlorophyll, that is released during the $^1O_2$-induced and EX-dependent disintegration of thylakoid membranes (Matile et al., 1999). The $^1O_2$-mediated bleaching of *flu* seedlings is completely blocked in *flu ex1 ex2* seedlings that in the dark reach similar excess levels of free Pchlide and during reillumination generate similar amounts of $^1O_2$ as the parental *flu* line (Lee et al., 2007).

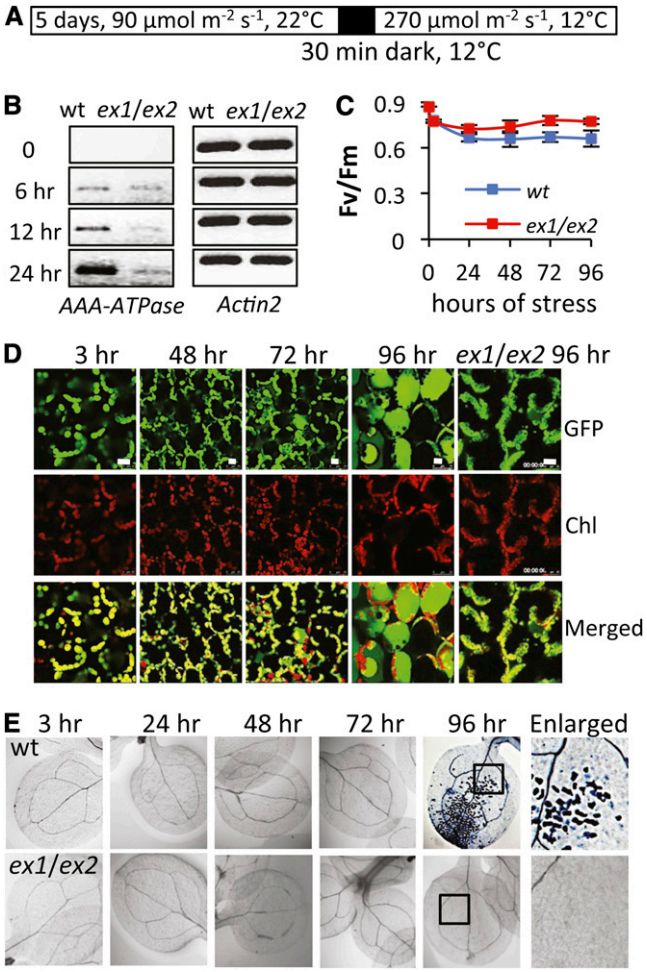

Figure 6. Activation of the $^1O_2$-Dependent PCD Pathway in Wild-Type Seedlings Exposed to Moderate Light Stress.

(A) Seedlings were initially grown for 5 d at 22°C and 90 µmol m^{-2} s^{-1} and were then exposed to a combined low-temperature (12°C)/high-light (270 µmol m^{-2} s^{-1}) stress program as described in Figure 5.

(B) A qualitative assessment of stress-induced changes in the expression of the $^1O_2$-responsive *AAA-ATPase* (At3g28580) during moderate low-temperature/high-light stress treatment of wild-type (wt) and *ex1 ex2* seedlings by RT-PCR analysis. *Actin2* expression was taken as a control. This experiment was repeated three times with independent biological samples and gave similar results.

(C) Stress-induced decline of the maximum quantum efficiency (F_v/F_m) of PSII in wild-type and *ex1 ex2* seedlings kept for various lengths of time under the combined low-temperature/high-light stress. Results represent the mean and SD of three samples. For each sample, 25 seedlings were analyzed.

(D) GFP and chlorophyll (Chl) autofluorescence images of leaf cells of wild-type seedlings expressing SSU-GFP were examined by CLSM 3, 48, 72, and 96 h after the beginning of the moderate low-temperature/high-light stress treatment. As a control, images of *ex1 ex2* seedlings after 96 h of stress treatment are included. Bars = 30 µm.

(E) Stress-induced activation of the $^1O_2$-dependent PCD pathway in wild-type seedlings during the moderate low-temperature/high-light stress treatment. Cell death was visualized by trypan blue staining. It affected only single cells or cell clusters without impairing the viability of seedlings. This cell death response was abrogated in *ex1 ex2* seedlings that are unable to activate $^1O_2$-mediated signaling.

Wild-type plants exposed to very severe light stress also bleach, very similar to *flu* seedlings (Meskauskiene et al., 2009). Under such stress conditions, similar sets of $^1O_2$-responsive genes are activated in the wild type as in the *flu* mutant following a dark/light shift (Bechtold et al., 2008; Triantaphylidès et al., 2008; González-Pérez et al., 2011). Furthermore, inactivation of EX1 and EX2 suppresses the expression of $^1O_2$-responsive genes, and lesion formation in the wild type and *flu* coincide with drastic nonenzymatic lipid peroxidation that could be ascribed to $^1O_2$. Collectively, these results seem to suggest that also in the wild type during severe light stress the $^1O_2$-dependent PCD pathway is active and responsible for the collapse of stressed seedlings. However, contrary to what has been found in *flu* seedlings, bleaching of the wild type does not mark the final consequence of activating a PCD pathway that depends strictly on the activities of EX1 and EX2. Nonenzymatic lipid peroxidation and lesion formation in the wild type occur under exceedingly high light stress that surpasses the plant's photochemical and nonphotochemical scavenging capacities to protect photosynthetic membranes from photooxidative damage. Under such severe light stress, activation of the $^1O_2$-dependent PCD pathway is masked and superimposed by an EX1- and EX2-independent cell death response that is mainly caused by the toxicity of $^1O_2$ and leads to the rapid bleaching and collapse of seedlings. Under less severe light stress conditions, photooxidative damage caused by the cytotoxicity of $^1O_2$ was suppressed, but genetically regulated $^1O_2$-mediated cell death signaling was still operating in wild-type seedlings and was fully dependent on EX1 and EX2. This latter finding is consistent with the stress-induced cell death response of the *var2* mutant exposed to the same mild stress program. PCD was induced only in cells containing fully developed chloroplasts. In neighboring cells of white leaf sectors that contain undifferentiated plastids and lack pigments, cell death did not occur. Hence, activation of this PCD pathway in green parts of the leaf does not affect adjacent areas that lack chloroplasts and seems to operate cell autonomously. Even though higher levels of H_2O_2 and superoxide radical accumulate constitutively in green leaf sectors of variegated mutants (Kato et al., 2009), these ROS do not seem to interfere with the stress-induced onset of PCD. Blocking $^1O_2$-mediated signaling in *var2 ex1 ex2* seedlings was sufficient to suppress the stress-induced cell death response.

Unlike in *flu*, in the wild type not Pchlide but chlorophyll acts as a photosensitizer that generates $^1O_2$ and initiates cell death. Generation of $^1O_2$ may happen either in light-harvesting antenna complexes (Rinalducci et al., 2004) or in the reaction centers of PSII (Krieger-Liszkay et al., 2008). Chlorophylls in light-harvesting complexes are in direct contact with carotenoids that efficiently quench excess light energy and suppress $^1O_2$ formation (Niyogi, 1999). Only when the absorbed light strongly exceeds the scavenging capacity of light-harvesting structures or in carotenoid-deficient plants does generation of $^1O_2$ cause photooxidative damage and nonenzymatic lipid peroxidation (Rebeiz et al., 1988;

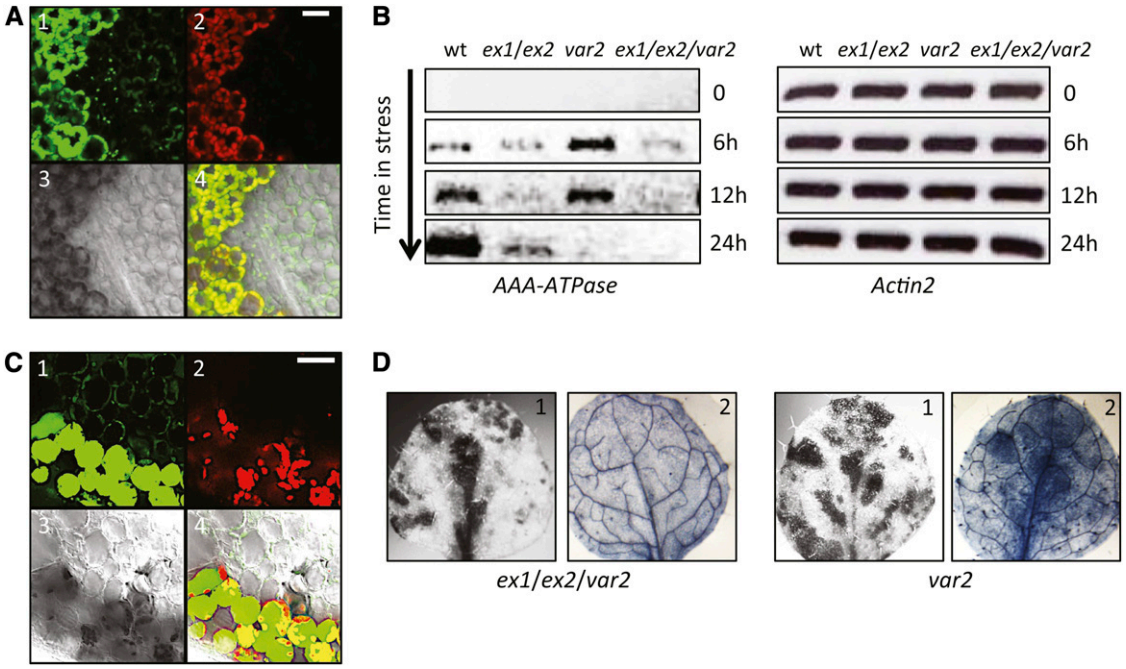

Figure 7. $^1O_2$-Mediated Cell Death and Loss of Plastid Integrity in Leaves of the *var2* Mutant Are Confined to Cells Containing Fully Developed Chloroplasts.

(A) The distribution of intact chloroplasts in green and undifferentiated plastids in white leaf sectors of 10-d-old *var2/SSU-GFP* seedlings grown at 22°C and 90 μmol m^{-2} s^{-1}. The green fluorescence of GFP (1) and red fluorescence of chlorophyll (2) were monitored separately by CLSM. (3) Light microscopy image of the leaf sector. (4) Merged images of chlorophyll and GFP fluorescence images. Bar = 60 μm.

(B) $^1O_2$-mediated gene expression changes in cotyledons of wild-type (wt), *ex1 ex2*, *var2*, and *ex1 ex2 var2* seedlings. Unlike true leaves, cotyledons of *var2* are uniformly green. A qualitative assessment of transcript levels of the $^1O_2$-responsive marker gene *AAA-ATPase* and the reference gene *Actin2* was done by RT-PCR. This experiment was repeated three times with independent biological samples and gave similar results.

(C) Stress-induced plastid leakage. Ten-day-old *var2/SSU-GFP* seedlings grown as in **(A)** were exposed for up to 4 d to the combined low-temperature/high-light regime described in Figure 6A. Loss of plastid integrity occurred in cells with fully developed chloroplasts in green leaf sectors but not in cells of the white leaf sector with undifferentiated plastids. The distribution of GFP fluorescence (1) and chlorophyll fluorescence (2) was determined by CLSM. (3) Light microscopy image of the leaf sector and (4) merged images of GFP and chlorophyll fluorescence. Bar = 60 μm.

(D) Stress-induced cell death was monitored by trypan blue staining in leaves of *ex1 ex2 var2* and *var2* seedlings kept under the same growth/stress conditions as described in **(C)**. Cell death occurred in green leaf sectors of *var2* but not of *ex1 ex2 var2* seedlings.

Chamovitz et al., 1991; Triantaphylidès et al., 2008). In animals, some of the nonenzymatic peroxidation products are biologically active and may act as signaling molecules (Lindshield et al., 2007; Kalariya et al., 2008). In plants, signaling under such severe stress conditions could be associated with responses to insults detrimental to the organism.

In the reaction center of PSII, carotenoids are far less involved in light scavenging. β-Carotenes bound to the PSII reaction center chlorophyll would compete for light energy, thereby reducing the efficiency of light-driven electron transport. Not surprisingly, they are localized away from the reaction center P680 chlorophyll (Ferreira et al., 2004; Loll et al., 2005). $^1O_2$ production by this special chlorophyll seems to be part of a trade-off of this optimization step and has become an inherent property of PSII (Vass and Cser, 2009). $^1O_2$ is produced via interaction of $^3O_2$ with the excited triplet reaction center chlorophyll that is formed whenever the electron acceptor site of PSII is reduced and unable to oxidize the excited P680 chlorophyll. This may frequently occur under fluctuating higher light conditions but also, at a reduced rate, under dim light (Tyystjärvi and Aro, 1996; Edelman and Mattoo, 2008).

One of the first visible consequences of $^1O_2$-mediated signaling in *flu* seedlings was the release of plastid proteins to the extraplastidic cytoplasm. This $^1O_2$-mediated loss of chloroplast integrity bears a striking resemblance to the release of mitochondrial proteins during the onset of PCD associated with an impairment of mitochondrial activity (Kroemer and Reed, 2000). Whereas in numerous organisms the release of mitochondrial proteins, such as cyt *c*, has been shown to activate a proteolytic cascade responsible for the breakdown of cells (Adrain and Martin, 2001; Tait and Green, 2010), in plants, the possible role of mitochondrial proteins as a trigger of cell death is still under debate (Balk and Leaver, 2001; Balk et al., 2003; Yao et al., 2004; Vacca et al., 2006; Reape et al., 2008). Similarly, it is not clear yet whether the $^1O_2$-mediated chloroplast leakage and the release of chloroplast proteins to the surrounding cytoplasm have any bearing on the $^1O_2$-mediated collapse of cells.

Current forms of PCD associated with an impaired mitochondrial activity have been traced back to an ancient hypothetical prokaryotic form acquired by primitive eukaryotic host cells following the integration of the prokaryotic ancestor of mitochondria as an endosymbiont (Blackstone and Green, 1999). It is tempting to speculate that the chloroplast-associated cell death program as defined in this work may originate from a second, independent endosymbiotic event, in which chloroplasts arose from a cyanobacterial ancestor acquired by a eukaryotic host (Koonin and Aravind, 2002). Under stress conditions that interfere with the photosynthetic electron transport, hyperreduction of the photosynthetic electron transport chain promotes the release of $^1O_2$ by the reaction center of PSII (Krieger-Liszkay et al., 2008). During the subjugation of the cyanobacterial endosymbiont, the host cell might have refined by selection the function of $^1O_2$ as a stress signal and subsequently used it to activate a cell death program that targets the chloroplast and impairs its integrity. Strikingly, the two plastid proteins EX1 and EX2, essential components of the $^1O_2$-dependent PCD pathway, are highly conserved among all plants for which genome sequence information is available, but they are not present in prokaryotic cyanobacteria (Lee et al., 2007).

As a genetically regulated cell death response must have evolved under selective pressure, it should be beneficial to the plant. However, at present, possible benefits of the $^1O_2$-dependent PCD pathway have not yet been identified. Previously, formation of microlesions was shown to be closely associated with an enhanced resistance against pathogens (Alvarez et al., 1998; Šimková et al., 2012), and many of the $^1O_2$-responsive genes are expressed in response to abiotic and biotic stress (op den Camp et al., 2003). Hence, it seems likely that activation of the $^1O_2$-dependent PCD pathway in wild-type plants may be part of an acclimation response that enhances stress resistance.

METHODS

Plant Material and Growth Conditions

Transgenic *Arabidopsis thaliana* Columbia-0 plants expressing the SSU-GFP transgene under the control of the cauliflower mosaic virus 35S promoter (Kim and Apel, 2004) were crossed with *flu* (Meskauskiene et al., 2001), *flu ex1* (Wagner et al., 2004), *flu ex2*, *flu ex1 ex2* (Lee et al., 2007), and *var2* (Sakamoto et al., 2009), and the resulting double, triple, and quadruple mutants were selected from segregating F2 generations. *Arabidopsis* (Columbia-0), *var2*, *var2 ex1 ex2*, and *ex1 ex2* seedlings were used for the low-temperature/high-light stress experiments as described previously (Meskauskiene et al., 2009).

Determination of the Subcellular GFP Distribution

The subcellular GFP distributions in protoplasts and seedlings were analyzed using a Leica TCS SP5 confocal laser scanning microscope (CLSM). GFP was excited with the 488-nm line of an argon laser, and the emission was monitored using a 510- to 540-nm band-pass filter. The chlorophyll autofluorescence and bright-field images were obtained as previously described (Šimková et al., 2012). To detect concurrent cell death, protoplasts and seedlings were incubated with 50 μM PI (Molecular Probes) diluted with water. After removing the excess dye by washing with water, samples were observed under the CLSM. The DNA and PI complexes in cells undergoing cell death were visualized with the 561 diode-pumped solid-state laser, and the emission was captured using a 613- to 630-nm band-pass filter.

Determination of $^1O_2$ Levels

Danepy solution was prepared as described earlier (Hideg et al., 2007). Danepy was excited with a 404 UV laser line and traced using a 510- to 625-nm band-pass filter. Relative fluorescence of each plant sample was analyzed and processed with CLSM.

Staining of Dead Cells

Trypan blue staining of seedlings was performed as described (op den Camp et al., 2003).

RNA Extraction and RT-PCR

Total RNA was extracted from seedlings using an RNeasy plant mini kit (Qiagen). cDNA was synthesized from 0.7 μg RNA, treated with DNase (Promega) using oligo(dT)15 primers (Promega) and Improm II reverse transcriptase (Promega) according to the manufacturer's instructions. RT-PCR was performed with equal amounts of cDNA using the GeneAmp PCR system 9700 (Applied Biosystems). *AAA-ATPase* was selected as an early $^1O_2$-responsive gene (Gene Expression Omnibus accession number for microarray data, GSE10509; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10509). *FER1* was selected as a H_2O_2-responsive gene. Primer sequences for these genes are shown in Supplemental Table 1 online.

Isolation of Protoplasts and Chloroplasts

Transgenic wild-type and *flu* seedlings expressing SSU-GFP under the control of the cauliflower mosaic virus 35S promoter were used to isolate intact protoplasts to monitor chloroplast integrity under the CLSM. Progression of cell death was determined using Evans Blue (Danon et al., 2005). Seedlings initially grown under continuous light for 4 d were harvested under green safe light and incubated in protoplast extraction buffer for 15 h in the dark. Afterwards, protoplasts were isolated under green safe light as previously described (Danon et al., 2005). Isolated intact protoplasts in culture medium were illuminated for 1, 2, or 3 h and used for protein analysis or were lysed and used for chloroplast isolation.

Identification of Intact versus Broken Protoplasts or Chloroplasts

At time 0, aliquots were taken from the protoplast suspension prior to illumination. During illumination (100 μmol m^{-2} s^{-1}), aliquots were taken at various time intervals (0, 0.5, 1, 2, and 3 h) and intact and nonintact protoplasts were counted under the CLSM. For chloroplast isolation, the procedure of Aronsson and Jarvis (2011) was slightly modified. For each time point, protoplasts were resuspended (400 mM sorbitol, 20 mM MES-KOH, pH 6, and 0.5mM $CaCl_2$) and centrifuged at 60*g*, and subsequently the protoplast pellet was resuspended in lysis buffer (300 mM sorbitol, 20 mM Tricine-KOH, pH 8.4, 10 mM EDTA, 10 mM $NaHCO_3$, and 0.1% BSA). The lysed protoplast suspension was centrifuged at 355*g*, and the pellet was resuspended either in HS buffer (50 mM HEPES-KOH, pH 8.0, and 0.33 M D-sorbitol) to monitor the intactness of chloroplasts under CLSM or in 90% acetone for pigment extraction.

Protein Analysis

During reillumination of predarkened protoplasts of the wild type and *flu* expressing SSU-GFP, aliquots were taken at various time points and lysed to harvest pellets and the corresponding supernatant fractions. Pellets were washed two times with lysis buffer, and supernatants were

directly mixed with 2× protein SDS loading buffer. Electrophoretic protein separation and immunoblot analysis were done as described previously (Šimková et al., 2012). SSU and cyt *f* were detected immunologically using rabbit polyclonal antibodies (Stephan Greiner, Max Planck Institute, Golm, Germany). GFP fusion proteins were detected using a GFP monoclonal antibody (Roche).

Extraction and Measurement of Pchlide

Pchlide measurement was done as described previously (Kim and Apel, 2004). Pchlide was extracted with 90% acetone and 10% 0.1 M NH_4OH in water and separated by HPLC on a C18 reverse-phase silica gel column (Nucleosil ODS 5 μm, 250 × 4.6 mm; Machery Nagel). The relative fluorescence unit of Pchlide was determined using 430-nm excitation and 630-nm emission (ex/em) wavelengths.

Measurement of the Maximum Quantum Efficiency of PSII Photochemistry

F_v/F_m was determined before and during stress treatment with a FluorCam 800MF system (Photon Systems Instruments). F_v/F_m was determined using the standard quenching analysis protocol provided by Photon Systems Instruments.

Determination of Peroxidation Products of Linolenic Acid

Enzymatic and nonenzymatic peroxidation products of linolenic acid were measured as described previously (Przybyla et al., 2008). Free oxylipins were analyzed by HPLC or by gas chromatography–mass spectrometry. For the analysis of esterified oxylipins, the samples were transmethylated (op den Camp et al., 2003). Esterified compounds were quantified using triricinoleate as an internal standard to determine the recovery of esterified hydroxyl fatty acids, whereas free compounds were quantified using 13γ-HOT as an internal standard.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At1g27510 (*EX2*), At2g30950 (*VAR2*) At3g14110 (*FLU*), At3g18780 (*Actin2*), At3g28580 (*AAA-ATPase*), At4g33630 (*EX1*), and At5g01600 (*FER1*).

Supplemental Data

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

Supplemental Figure 1. Genetic Control of $^1O_2$-Mediated Chloroplast Leakage in *flu* Protoplasts.

Supplemental Figure 2. Probing the Intactness of Chloroplasts during Incubation of Protoplasts.

Supplemental Figure 3. The Intactness of Chloroplasts Isolated from Wild-Type and *flu* Seedlings Expressing the SSU-GFP Fusion Protein.

Supplemental Table 1. List of Selected Genes and Primer Sequences.

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AUTHOR CONTRIBUTIONS

C.K. and K.A. designed the research. C.K., R.M., S.Z., K.P.L., M.L.A., K.B., C.H., and I.F. performed research. All the authors analyzed data. C.K. and K.A. wrote the article.

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