EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*

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Shortly after the release of singlet oxygen ($^1O_2$), drastic changes in nuclear gene expression occur in the conditional *flu* mutant of *Arabidopsis* that reveal a rapid transfer of signals from the plastid to the nucleus. In contrast to retrograde control of nuclear gene expression by plastid signals described earlier, the primary effect of $^1O_2$ generation in the *flu* mutant is not the control of chloroplast biogenesis but the activation of a broad range of signaling pathways known to be involved in biotic and abiotic stress responses. This activity of a plastid-derived signal suggests a new function of the chloroplast, namely that of a sensor of environmental changes that activates a broad range of stress responses. Inactivation of the plastid protein EXECUTER1 attenuates the extent of $^1O_2$-induced up-regulation of nuclear gene expression, but it does not fully eliminate these changes. A second related nuclear-encoded protein, dubbed EXECUTER2, has been identified that is also implicated with the signaling of $^1O_2$-dependent nuclear gene expression changes. Like EXECUTER1, EXECUTER2 is confined to the plastid. Inactivation of both EXECUTER proteins in the *ex1*/*ex2*/*flu* triple mutant is sufficient to suppress the up-regulation of almost all $^1O_2$-responsive genes. Retrograde control of $^1O_2$-responsive genes requires the concerted action of both EXECUTER proteins within the plastid compartment.

oxidative stress | retrograde signaling | singlet oxygen | chloroplast

In plants, continuous generation of reactive oxygen species (ROS) is an unavoidable consequence of aerobic metabolic processes such as photosynthesis and respiration that has necessitated the evolution of various scavengers to minimize the cytotoxic impact of ROS on cells. Sensing changes of ROS concentrations that result from metabolic disturbances is being used by plants to evoke stress responses that support plants to cope with environmental variation (1–3). Plants may also produce ROS in a genetically controlled way (e.g., by NADPH oxidases) and use these molecules as signals to control a broad range of processes that comprise defense reactions against pathogens (4), the closure of stomata (5), the regulation of cell expansion and plant development (6), and the control of plant–fungus interactions (7). Chloroplasts and peroxisomes have been shown to be major sites of ROS production (3, 8). The enhanced generation of ROS in these cellular compartments has been attributed to the disturbance of photosynthetic electron transport by a variety of environmental factors (such as high light, high or low temperatures, salt, and drought) that trigger various stress responses (3, 8). One of the difficulties in elucidating the biological activities of ROS during these processes stems from the fact that, in plants under stress, several chemically distinct ROS are generated simultaneously within different intracellular compartments, thus making it very difficult to link a particular stress response to a specific ROS (3, 9). This problem has been alleviated by using the conditional *flu* mutant of *Arabidopsis* to study the biological activity of only one of these ROS at a given time (9).

In the dark, the *flu* mutant accumulates protochlorophyllide (Pchlide), a potent photosensitizer that upon illumination generates singlet oxygen ($^1O_2$) (9–11). Immediately after a dark-to-light shift, mature *flu* plants stop growing, whereas *flu* seedlings bleach and die. By varying the length of the dark period, one can modulate noninvasively the level of the photosensitizer Pchlide and define conditions that minimize the cytotoxicity of $^1O_2$ and reveal the genetic basis of $^1O_2$-mediated signaling as indicated by the inactivation of the *EXECUTER1* gene that is sufficient to abrogate $^1O_2$–dependent stress responses (12). The enhanced generation of $^1O_2$ within plastids that triggers drastic phenotypic changes would be expected to modulate nuclear gene expression as well. Indeed, 2 h after the release of $^1O_2$, rapid changes in the expression of nuclear genes have been shown to affect ≈5% of the total genome of *Arabidopsis* (9). However, as reported in the present work, inactivation of the *EXECUTER1* gene of the *flu* mutant is not sufficient to fully suppress $^1O_2$-induced changes in nuclear gene expression, suggesting that a residual $^1O_2$-induced transduction of signals from the plastid to the nucleus still operates in the absence of EXECUTER1. We have identified a second signaling component closely related to EXECUTER1 that is also present inside the plastid compartment and, together with EXECUTER1, is required for $^1O_2$-dependent signaling of nuclear gene activities. This protein has been dubbed EXECUTER2. The *EXECUTER1* and *EXECUTER2* genes are highly conserved among higher plants and thus seem to play an important but hitherto unknown role during the transfer of stress-related signals from the plastid to the nucleus.

Results

Identification and Localization of EXECUTER2. During an extensive second-site-mutant screen of the *flu* mutant, 15 different allelic lines of *executer1* (*ex1*) were identified. In three of these mutant lines, the mutations led to an amino acid exchange (12). These amino acid residues are conserved among all EXECUTER1 proteins of higher plants for which sequence data are available (12). A second *EXECUTER1*-like gene was found in *Arabidopsis* that was dubbed *EXECUTER2* and that was considered to be a candidate gene for a second putative signal component involved in $^1O_2$-dependent signaling. The predicted overall amino acid sequence identity between EXECUTER1 and EXECUTER2 is 38%, but the sequence identity increases to 42%, if only sequences of mature proteins without the signal sequences are compared (Fig. 1). EXECUTER1 and EXECUTER2 of *Arabi-*

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Abbreviations: ROS, reactive oxygen species; Pchlide, protochlorophyllide; T-DNA, portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells.

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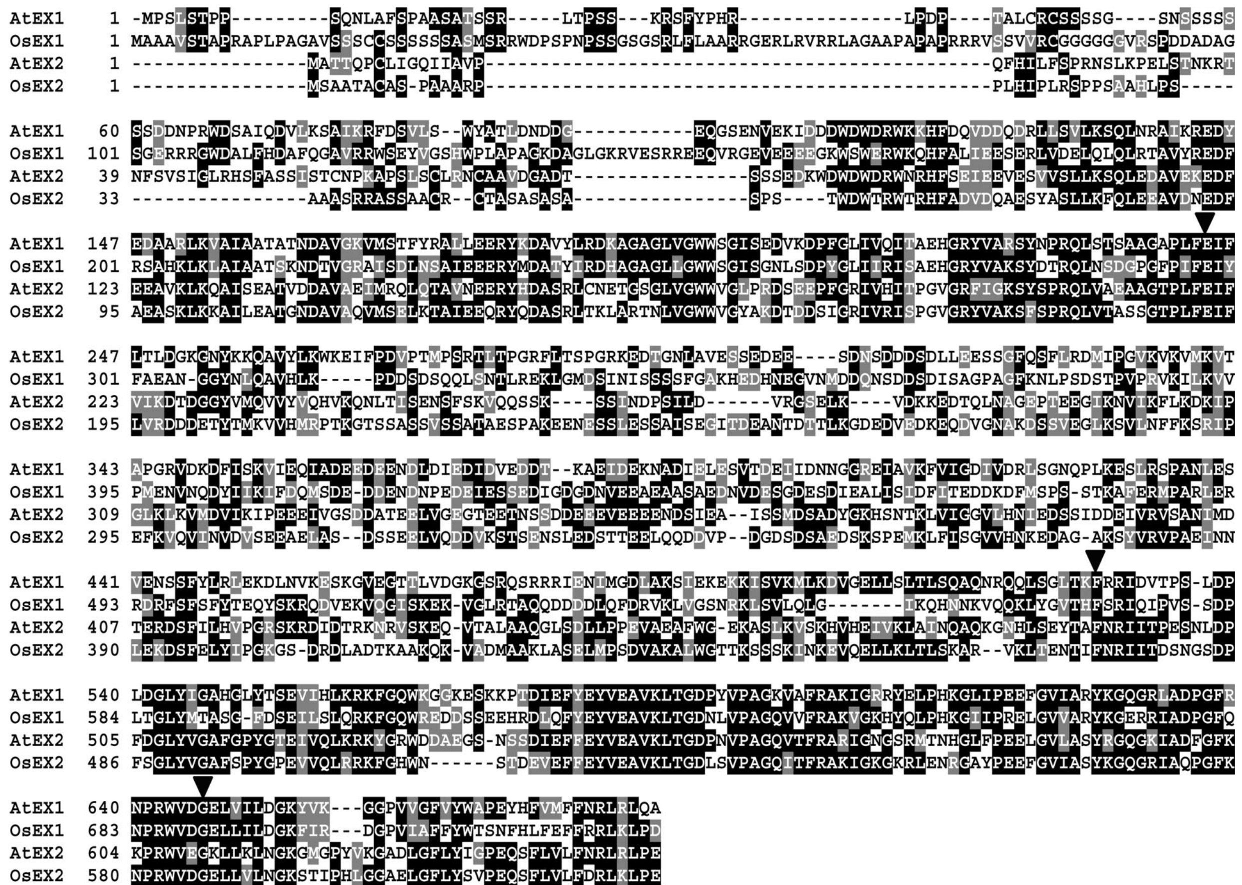

Fig. 1. Multiple alignments of deduced amino acid sequences of full-length cDNAs of *EXECUTER1* and *EXECUTER2* from *Arabidopsis* and rice. The three highly conserved amino acid residues of EXECUTER1 that were identified in a previous suppressor mutant screen of *flu* (12) are indicated by arrow heads. The amino acid sequences were aligned by using the ClustalW program. Gaps, which were introduced to maximize the alignment, are indicated by dashes. AtEX1(NP_567929) and AtEX2(NP_564287) from *A. thaliana*, OsEX1(AAL59023) and OsEX2 (BAD44852) from rice.

dopsis are closely related to the corresponding proteins of the monocotyledonean plant rice (Fig. 1). In particular the C termini of the EXECUTER proteins are highly conserved (Fig. 1). The three highly conserved amino acid residues of EXECUTER1 that seem to be essential for its activity are also conserved in all EXECUTER2 proteins of higher plants for which sequence data are available (Fig. 1; unpublished data). The ORF of EXECUTER2 predicts a protein of 652 aa with a molecular mass of 72 kDa. Like EXECUTER1, it is unrelated to known proteins, except that its N-terminal part resembles import signal sequences of nuclear-encoded plastid proteins. This prediction was confirmed experimentally by expressing EXECUTER1- and EXECUTER2-GFP fusion proteins in stably transformed *Arabidopsis* plants and determining their intracellular localization under the confocal microscope (Fig. 2). As controls also plants expressing the small subunit (SSU) of the ribulose-1,5-bisphosphate carboxylase-GFP fusion protein and the NADPH-protochlorophyllide oxidoreductase(POR)B-GFP fusion protein were analyzed (Fig. 2). The former accumulates within the stroma of plastids, whereas PORB is part of the chloroplast membranes (13, 14). Both EXECUTER1 and EXECUTER2 accumulate within chloroplasts and seem to be associated with thylakoid membranes (Fig. 2).

Functional Characterization of EXECUTER2. During the second-site mutant screen of *flu* a large number of allelic *ex1* mutant lines, but no *executer2* (*ex2*) mutants have been found (12). These results suggest that the EXECUTER2 protein is not essential for mediating the visible stress responses that have been used for the selection of second-site mutants (12). However, this conclusion does not preclude the possibility that EXECUTER2 is involved in mediating other $^1O_2$-dependent stress responses. These predictions were tested experimentally by first identifying an *EXECUTER2* mutant line and crossing it with *flu* and then studying the effect of *EXECUTER2* inactivation on $^1O_2$-mediated stress responses in the *flu* background.

We have identified an *Arabidopsis* T-DNA [portion of the Ti (tumor-inducing) plasmid that is transferred to plant cells] insertion line from the SALK collection with a predicted insertion of the T-DNA in the *EXECUTER2* gene. The genetic background of this line was Columbia (Col-0). Because the *ex1* mutation had been found originally in Ler we searched for and identified a Col-0 line with the insertion of the T-DNA also predicted to be in the *EXECUTER1* gene. This prediction could be confirmed by PCR (data not shown). Both T-DNA-insertion lines were crossed with each other and a *flu* Col-0 line. Mature plants of the resulting *ex1*/*flu*, *ex2*/*flu* and *ex1*/*ex2*/*flu* mutant lines, *flu*, and wild type, all in Col-0, were subjected to the same dark/light shift experiment used previously to characterize the *flu* and *ex1* mutations in the Ler lines (12).

Mutant and wild-type plants were grown under continuous light until they reached the rosette leaf stage and were ready to bolt. Plants were then shifted from continuous light to a 16 h light/8 h dark program for the next 30 days. Once they were transferred to the long day conditions, *flu* and *ex2*/*flu* plants stopped growing, whereas *ex1*/*flu* and *ex1*/*ex2*/*flu* plants contin-

Fig. 2. Intracellular accumulation of GFP fusions with the small subunit of ribulose-1,5-bisphosphate carboxylase (SSU), the NADPH-Pchlide oxidoreductase B (PORB), EXECUTER1 (EX1), and EXECUTER2 (EX2) in cotyledons of transgenic seedlings grown for 5 days under continuous light. The green fluorescence of GFP fusion proteins (*A*) and the red fluorescence of chlorophyll (*B*) were monitored separately by using a confocal laser scanning microscope, and the the two fluorescence images were merged (*C*).

ued to grow similar to wild type except that their growth was slightly reduced and their final height was ≈80% of that of wild type. Under continuous light all five lines grew equally well and finally reached the same height (Fig. 3). When mature plants grown under continuous light were shifted to the dark for 8 h free Pchlide accumulated in all four mutant lines to similar levels in rosette leaves and were 3- to 4-fold higher than in wild-type controls.

Collectively, these results demonstrate that in the Col-0 background the effect of the *flu* mutation is similar to that in Ler (9, 12). Furthermore, also in Col-0 the *ex1* mutation in the *flu* background suppresses singlet oxygen-mediated growth inhibition of mature plants, whereas inactivation of the *EXECUTER2* gene of the *flu* mutant has only a minor effect on this $^1O_2$-mediated stress response, as one would expect based on our previous failure to isolate *ex2/flu* double mutants during an extensive second-site mutant screen of *flu* (12). Mature *ex1/ex2* mutant plants without the *flu* mutation are phenotypically similar to wild type (data not shown).

The Effects of EXECUTER1 and EXECUTER2 Inactivation on $^1O_2$-Dependent Signaling of Nuclear Gene Expression. The impact of the *ex1* and *ex2* mutations on rapid $^1O_2$-mediated changes in nuclear gene expression was analyzed by growing plants for 3 weeks under continuous light, until they were ready to bolt. Plants at the rosette leaf stage were transferred to the dark for 8 h and reexposed to light for 30 min. Total RNA was extracted from the leaves and was first transcribed into cDNAs and then into biotinylated complementary RNAs that were hybridized to Affymetrix gene chips. Genes with a 2-fold or greater transcript level than the control were considered to be significantly up-regulated. After 30 min of reillumination, a total of 245 genes had been up-regulated in *flu* relative to wild type (Figs. 4 and 5). This number in ecotype Col-0 was lower than that in *flu* Ler reported earlier (9). Inactivation of EXECUTER1 led to a dramatic drop in the number of up-regulated genes from 245 in *flu* down to 54 in *ex1/flu* (Fig. 5). This suppressive effect of *EXECUTER1* inactivation in *flu* can also be seen by comparing scatter plots of nuclear transcripts of genes significantly up-regulated in *flu* and suppressed in *ex1/flu* (Fig. 4). EXECUTER1 seems to play a major role during the up-regulation of nuclear

Fig. 3. $^1O_2$-mediated growth inhibition of mature *ex1/flu*, *ex2/flu*, *ex1/ex2/flu*, *flu*, and wild-type (wt) plants. Plants were grown for 21 days under continuous light until they were ready to bolt. Plants were then either shifted to a 16 h light/8 h dark program (*A*) or kept under continuous light (*B*), and the elongation of the inflorescence was followed over the next 30 days. Long-day conditions were used instead of short-day conditions to avoid the overaccumulation of excess amounts of Pchlide during an extended dark period. Under these light conditions, toxic effects of $^1O_2$ could be minimized. In contrast to Ler plants used previously (9, 12), the onset of bolting of Col-0 varied greatly between different plants. Growth curves of individual plants were corrected for these differences. Each value represents the average growth measurements of 10 different plants. (*C*) The accumulation of Pchlide in 21-day-old plants grown under continuous light and transferred to the dark for 8 h. Total Pchlide was extracted from aerial parts of single plants and analyzed by HPLC. For each genotype, seven independent Pchlide measurements are shown.

genes in the *flu* mutant, but its absence in *ex1/flu* double mutants does not completely eliminate activation of $^1O_2$-responsive genes. Therefore, additional components must be implicated with the $^1O_2$-induced transduction of signals from the plastid to the nucleus.

EXECUTER2 may play a supplementary role during $^1O_2$-mediated signaling that may account for the residual activation of $^1O_2$-responsive genes in *ex1/flu* after a dark-to-light shift. This proposition was tested experimentally first by analyzing in *ex2/flu* the transcript profiles of those genes that in *flu* were at least 2-fold up-regulated relative to wild type. The scatter plot analysis of these transcripts in *ex2/flu* revealed that inactivation of

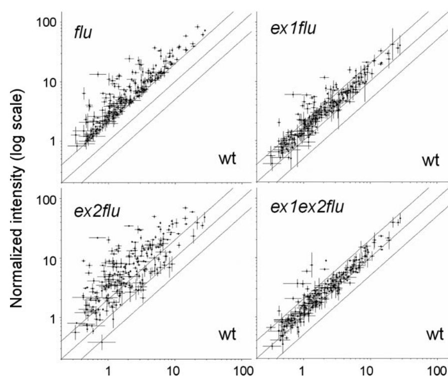

Fig. 4. The impact of *EXECUTER1* and *EXECUTER2* mutations on the up-regulation of $^1O_2$-responsive nuclear genes in the *flu* mutant. Plants (Col-0) were grown for 21 days under continuous light, shifted to the dark for 8 h, and reexposed to light for 30 min. Global changes in transcript levels were determined by using Affymetrix gene chips. Among 13,600 genes that were selected as present in all replicas, 245 were up-regulated at least 2-fold in *flu* relative to wild type. Transcript levels of these selected genes are shown in scatter plots of *flu* versus wild type, *ex1/flu* versus wild type, *ex2/flu* versus wild type, and *ex1/ex2/flu* versus wild type. The individual dots shown on the scatter plots were derived as average expression values from both replicate experiments.

EXECUTER2 modified drastically the up-regulation of $^1O_2$-responsive genes in the *flu* mutant by further enhancing or reducing the transcript levels of these genes (Fig. 4). In a subsequent step, additional genes were included in this analysis that were up-regulated in the *ex1/flu*, *ex2/flu* and *ex1/ex2/flu* mutant lines relative to wild type [Fig. 5 and supporting information (SI) Data Set 1]. The majority of $^1O_2$-up-regulated genes in the *flu* mutant are found in a cluster of 178 genes that are up-regulated both in *flu* as well as in *ex2/flu* (Fig. 5, groups A, I, and M). Unexpectedly, in *ex2/flu* the up-regulation of a larger part of these genes is significantly higher than in *flu* (Fig. 4). Half of the genes with an assigned function have been associated with signaling, gene transcription and stress responses. Among the genes predicted to encode transcription factors and DNA-binding proteins, nine belong to the large gene family of WRKY transcription factors that have been associated with various disorders such as stress, aging, senescence and diseases (15). The enhanced expression of $^1O_2$-responsive genes caused by the inactivation of EXECUTER2 is also reflected in the appearance of additional $^1O_2$-responsive genes that are significantly up-regulated in *ex2/flu* but not in *flu* (Fig. 5, groups L and G). Approximately half of these genes are of unknown function. Similar to the gene groups A, I, and M, also in groups L and G >50% of the remaining genes encode proteins predicted to be involved in transcription, signaling or stress-related responses (SI Data Set 1).

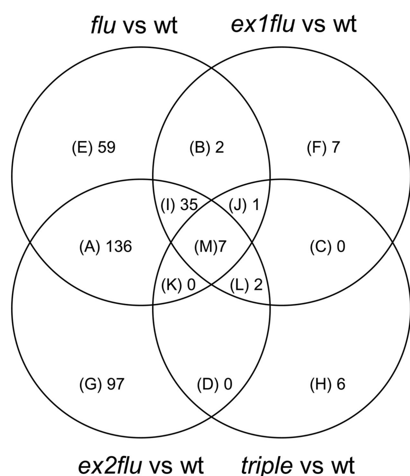

Fig. 5. The impact of *EXECUTER1* and *EXECUTER2* mutations on $^1O_2$-mediated changes in nuclear gene expression. The relationships of four selected groups of genes up-regulated at least 2-fold in *flu* versus wild type, *ex1/flu* versus wild type, *ex2/flu* versus wild type, and *ex1/ex2/flu* versus wild type were analyzed by using a Venn diagram. A subset of five genes up-regulated only in *flu* and *ex1/ex2/flu* relative to wild type is not shown in the Venn diagram, but has been included in SI Data Set 1. Plants were grown and treated as described under Fig. 4.

Inactivation of EXECUTER2 does not only accelerate the expression of a large number of $^1O_2$-responsive genes, but at the same time also evokes the down-regulation of a subset of 62 $^1O_2$-responsive genes (Fig. 5, groups E, B, and J). Up-regulation of 59 of these genes in response to $^1O_2$ generated in chloroplasts of the *flu* mutant depends on the combined activities of EXECUTER1 and EXECUTER2 (Fig. 5, group E). In *ex1/flu* and *ex2/flu*, but also in the *ex1/ex2/flu* triple mutant the $^1O_2$-induced enhanced expression of these genes is suppressed. Several of these genes have been associated with various stress-related responses such as two trehalose 6-phosphate synthetase genes which have been implicated in conferring desiccation tolerance to plants (16, 17). Collectively, these results reemphasize a key role of EXECUTER1 in stimulating the up-regulation of a larger number of nuclear genes that comprise the majority of $^1O_2$-responsive genes in the *flu* mutant. At the same time, they reveal a striking regulatory role of EXECUTER2 that seems to attenuate and antagonize the activity of EXECUTER1. However, EXECUTER2 alone in the absence of active EXECUTER1 has only a limited effect on the expression of $^1O_2$-responsive genes. Because of the reciprocal activities of the two EXECUTER proteins in the *flu* mutant that impact each other during the $^1O_2$-induced transfer from the plastid to the nucleus, it was of interest to see whether inactivation of both these proteins in the *ex1/ex2/flu* triple mutant would completely abrogate the singlet oxygen-mediated up-regulation of nuclear genes. Almost all of the transcripts that in *flu* had been up-regulated at least twofold remained in the triple mutant below the 2-fold threshold value, but were still slightly higher than in the wild-type control. Six of the $^1O_2$-responsive genes were significantly up-regulated only in the triple mutant relative to wild type (Fig. 5, group H). Two of these genes encode proteins of unknown function. One of the four genes with an assigned function is predicted to encode an auxin-responsive transcription factor. At the same time two of seven genes that are significantly up-regulated in *ex1/flu* are also involved in auxin-dependent responses (Fig. 5, group F; SI Data Set 1).

Among the genes that had been shown by the Affymetrix chip analysis to be induced stronger in *ex2/flu* than in *flu*, four were selected and changes in their transcript levels were quantified independently by using real-time PCR to test the reliability of the Affymetrix chip analysis (Fig. 6). The expression of genes that encode the WRKY33 (At2g38470) and WRKY46 (At2g46400) transcription factors (15), a disease resistance protein (At1g66090) and the 1-amino-cyclopropane-1 carboxylic acid (ACC) synthase 6 (At1g11280) (18) were up-regulated in *flu* during the first 30 min of reillumination. For each gene the transcript level was 2- to 3-fold higher in *ex2/flu* than in *flu*, whereas in *ex1/flu* and the triple mutant these levels were down-regulated and similar to those of wild type.

Discussion

In our present work, we have used the conditional *flu* mutant to characterize the physiological role of $^1O_2$ that is generated within the plastid compartment after a dark-to-light shift. Shortly after the release of $^1O_2$ drastic changes in nuclear gene expression occur that reveal a rapid transfer of signals from the plastid to the nucleus. Because $^1O_2$ is very unstable and unlikely to leave the plastid

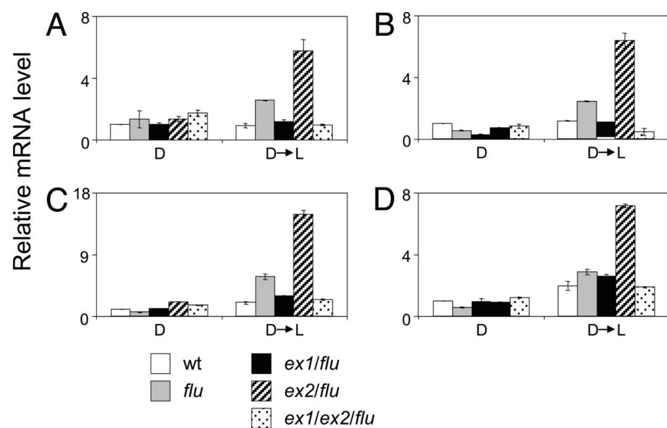

Fig. 6. Activation of four $^1O_2$-responsive genes in *flu* and *ex2/flu* and their suppression in *ex1/flu* and *ex1/ex2/flu* mutant plants. Plants were grown for 21 days under continuous light, transferred to the dark for 8 h, and in some cases reexposed to light for 30 min. Transcript levels of *WRKY33* (At2g38470) (*A*), *WRKY46* (At2g46400) (*B*), a putative disease resistance gene (At1g66090) (*C*), and the gene encoding the 1-amino-cyclopropane-1 carboxylic acid (ACC) synthase 6 (At1g11280) (*D*) were determined by Real-Time PCR. The results represent average values of measurements from three independent experiments ± SE. RNA was extracted at the end of the dark period (D) or after 30 min of reillumination (D → L).

compartment (19, 20), its physiological impact has been attributed to the generation of more stable second messengers within the plastid that are assumed to activate a signaling pathway and control the expression of a large number of nuclear genes (9). EXECUTER1 seems to play a key role during the transfer of signals from the plastid to the nucleus. Its biological activity, however, depends on its interaction with a second closely related protein, EXECUTER2. Even though it is not known yet whether EXECUTER1 and EXECUTER2 physically interact with each other, such a direct contact would be in line with some of the results of our present work. The two proteins localize in chloroplasts and seem to be both associated with thylakoid membranes. Upon inactivation of EXECUTER2 in the *flu* mutant, additional $^1O_2$-responsive genes emerge and genes that were already up-regulated in *flu* are either further stimulated or down-regulated. In the absence of EXECUTER1, EXECUTER2 has only a relatively minor effect on the expression of $^1O_2$-responsive genes (see e.g., Fig. 4). Thus, the primary function of EXECUTER2 seems to be that of a modulator attenuating and controlling the activity of EXECUTER1. Inactivation of EXECUTER1 greatly reduces but does not completely eliminate the up-regulation of nuclear $^1O_2$-responsive genes. Only when both EXECUTER proteins are inactive is the up-regulation of the vast majority of $^1O_2$-responsive genes abolished.

The EXECUTER1- and EXECUTER2-dependent signaling in the *flu* mutant bears a striking resemblance to retrograde signaling that has been shown to play a central role in controlling gene expression in the nucleus and the plastid (21, 22). Chloroplast proteins are encoded by both nuclear and plastid genomes (23). Because of this separation of the genetic information, the expression of these two genomes needs to be coordinated. It is well established that the development and activity of chloroplasts depend on the synthesis and import of a large number of nuclear-encoded plastid proteins (24). On the other hand, the expression of at least some of the nuclear genes depends on the functional state of the plastid by means of a process known as retrograde signaling (25–27).

Initially the biological impact of plastid-derived signals had been considered to be confined to the fine-tuning and coordination of nuclear and chloroplast gene activities that are required for the optimization and protection of chloroplast-specific functions such as e.g., photosynthesis (21, 22, 25). The results of our work demonstrate that the primary function of singlet oxygen in the *flu* mutant does not seem to be the control of chloroplast performance but the activation of a stress-related signaling cascade that encompasses numerous signaling pathways known to be activated by pathogen attack, wounding, light and drought stress (28–30).

Less than 15% of the $^1O_2$-responsive genes of the *flu* mutant are predicted to encode plastid proteins and none of these genes can be linked to photosynthesis or the control of chloroplast development, whereas a large fraction of $^1O_2$-responsive genes are known to be involved in different stress responses. The $^1O_2$-activated cell death program and growth inhibition resemble stress-related resistance strategies of higher plants (31, 32). These $^1O_2$-dependent stress responses of the *flu* mutant were suppressed after the inactivation of EXECUTER1 and EXECUTER2. Both the generation of $^1O_2$ within plastids and the plastid-specific localization of the EXECUTER1 and 2 proteins reiterate the importance of chloroplasts as a major source of stress-related signals.

The activation of a suicidal program in seedlings and the block of growth in mature plants of *flu* has not been reported to occur in wild-type plants even under conditions that would be expected to stimulate the release of $^1O_2$. This apparent difference between *flu* and wild type may question the physiological relevance of $^1O_2$-mediated stress responses of the *flu* mutant. EXECUTER1 and EXECUTER2 are highly conserved among all higher plants for which sequence data are available. This conservation is consistent with EXECUTER1 and EXECUTER2 being involved in processes that are both beneficial and common to higher plants. The overaccumulation of the photosensitizer Pchlide and the sudden shift from the dark to the light that in the *flu* mutant evokes the instantaneous release of $^1O_2$ does normally not occur in wild-type plants. Conditions to which wild-type plants are genetically adapted and that endorse the enhanced production of $^1O_2$ would thus be expected to induce the release of modulating factors that control and subdue the extreme $^1O_2$-mediated stress responses as seen in *flu*. Two such modulating activities have recently been identified. Various stress conditions may lead to the hyperreduction of the photosynthetic electron transfer chain that blocks electron transfer by PSII and enhances the production of $^1O_2$ (33, 34). Plants may use additional electron sinks to maintain the acceptor site of PSII in a partially oxidized state (8). One of these additional electron acceptors is molecular oxygen. It can be reduced by PSI to superoxide that is rapidly converted to hydrogen peroxide (35). Hydrogen peroxide has been shown recently to antagonize the biological activity of $^1O_2$ and to suppress $^1O_2$-mediated cell death and growth inhibition (36). Another modulation of $^1O_2$-dependent stress responses has been attributed to acclimation activated by minor stress conditions that precede the release of $^1O_2$ (M. Würsch and K.A., unpublished results). Therefore, EXECUTER1- and EXECUTER2-dependent signaling of stress responses in wild-type plants seems to form an integral part of a complex signaling network and is subject to the control by various modulators that weaken the extreme consequences of this signaling as seen in the *flu* mutant. As shown in the present work, the *flu* mutant offers a way of how to penetrate and dissect this complexity and identify individual signaling pathways.

Methods

Plant Material. The *EX1* (At4g33630) T-DNA insertion line SALK_002088 and *EX2* (At1g27510) T-DNA insertion line SALK_012127 were obtained from the European *Arabidopsis* Stock Centre (NASC). Homozygous mutant lines were identified by PCR analysis by using T-DNA-, *EX1*- and *EX2*-specific primers. Both T-DNA-lines were crossed with a *flu* Col-0 line that had been obtained by 5 backcrosses of *flu1-1* in Landsberg erecta with wild-type Columbia. The *ex1/flu* and *ex2/flu* mutant lines were crossed, and within the segregating F2 population triple mutants were identified by PCR-based genotyping. For the cultivation of

mature plants, seeds of wild type, *flu*, *ex1*/*flu*, *ex2*/*flu*, and *ex1*/*ex2*/*flu*, all in Col-0 ecotype, were sown on soil and plants were grown under continuous light (100 μmol·m^{-2}·s^{-1}).

Extraction and Measurement of Protochlorophyllide. Pchlide was extracted separately from seven biological samples of each of the 5 genotypes (wild type, *flu*, *ex1*/*flu*, *ex2*/*flu*, and *ex1*/*ex2*/*flu*) growing under continuous light (100 μmol·m^{-2}·s^{-1}) for 21 days and then transferred to the dark for 8 h. After the end of dark periods, samples were harvested and homogenized with liquid nitrogen under green safety light. About 0.1g of the powdered samples were suspended in 1 ml of cold 90% acetone, and centrifuged for 5 min at 9,300 $\times$ *g*. The supernatants were used to determine the level of Pchlide by HPLC according to Kim and Apel (13).

RNA Extraction and Real-Time PCR. Total RNA was extracted by using an RNeasy plant mini kit (Qiagen, Hilden, Germany) and quantified spectrophotometrically at 260 nm. For the real-time PCR, RNAs were treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and reverse-transcribed by using oligo(dT)15 primer (Promega) and and Improm II reverse transcriptase (Promega) according to the manufacturer's recommendations. Real-time PCR was performed with equal amounts of cDNAs by using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), a SYBR Green PCR kit from Applied Biosystems, and gene-specific primers. Relative mRNA abundance was calculated by using the comparative delta-Ct method and normalized to the *ACT2* (At3g18780) gene levels. The sequences of the primers for the selected genes are: At2g38470, GAAACAAATGGTGGGAATGG and TGTCGTGTGATGCTCTCTCC; At2g46400, GATCCTTAAGCGAAGCCTTG and TCGATGCGTGCATCTGTAAT; At4g11280, GACGAGTTTATCCGCGAGAG and ACACGCCATAGTTCGGTTTC; At1g66090, AACCGGAGTACACGTCCAAG and CGGAGATCCCAACGATCTTA.

Microarray Hybridization and Analysis. Two individual biological replicates, each containing material of five mature plants of wild type, *flu*, *ex1*/*flu*, *ex2*/*flu*, and *ex1*/*ex2*/*flu*, respectively, were used for the microarray analysis. Plants were germinated on soil and kept under continuous light until the beginning of bolting and then transferred to the dark for 8 h. Dark-incubated mature plants were reilluminated for 30 min and subsequently harvested for RNA extraction. Total RNA was prepared as described in *SI Materials and Methods*.

Growth Measurements. Growth of the primary stem was determined by measuring its length daily for 30 days with wild type, *flu*, *ex1*/*flu*, *ex2*/*flu*, and *ex1*/*ex2*/*flu* growing under continuous light (100 μmol·m^{-2}·s^{-1}) or under long day conditions (16 h light/8 h dark).

Construction and Detection of the GFP Fusion Proteins *in Vivo*. A modified pCAMBIA 3300 binary vector containing the CaMV 35S promoter, a NcoI cloning site, the *EGFP*-sequence and the terminal polyadenylation site was used as a basis for all subsequent constructions (13). For the *in vivo* localization of the fusion protein, full-length *EXECUTER1* and *EXECUTER2* without their stop codons were amplified from the cDNA of *Arabidopsis thaliana* (Col-0) and subcloned between the promoter and *EGFP* of the modified pCAMBIA 3300 vector. To amplify this plasmid, competent *Escherichia coli* cells (DH5α) were used. Competent cells of *Agrobacterium tumefaciens* C58 were transformed with the plasmid and then used for stable *in planta* transformation of *Arabidopsis* Col-0. The primary transgenic plants were selected on MS agar plates containing phosphinothricin (25 mg/l) and transferred to soil to harvest seeds. The green fluorescence of GFP and the red fluorescence of chlorophyll were monitored by using a Confocal Laser Scanning Microscope (TCS-NT; Leica Microsystems, Heidelberg, Germany) according to Kim and Apel (13).

Other Methods. For homology searches and protein structure predictions, National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/) and ExPASy Molecular Biology Server (www.expasy.ch) were used.

For multiple sequence alignment, ClustalW (www.ebi.ac.uk/clustalw/) and Boxshade 3.21 (www.ch.embnet.org/software/BOX_form.html) were used.

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