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A mutation in the Arabidopsis mTERF-related plastid protein SOLDAT10 activates retrograde signaling and suppresses $^1O_2$-induced cell death

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SUMMARY

The conditional *flu* mutant of *Arabidopsis thaliana* generates singlet oxygen ($^1O_2$) in plastids during a dark-to-light shift. Seedlings of *flu* bleach and die, whereas mature plants stop growing and develop macroscopic necrotic lesions. Several suppressor mutants, dubbed singlet oxygen-linked death activator (*soldat*), were identified that abrogate $^1O_2$-mediated cell death of *flu* seedlings. One of the *soldat* mutations, *soldat10*, affects a gene encoding a plastid-localized protein related to the human mitochondrial transcription termination factor mTERF. As a consequence of this mutation, plastid-specific rRNA levels decrease and protein synthesis in plastids of *soldat10* is attenuated. This disruption of chloroplast homeostasis in *soldat10* seedlings affects communication between chloroplasts and the nucleus and leads to changes in the steady-state concentration of nuclear gene transcripts. The *soldat10* seedlings suffer from mild photo-oxidative stress, as indicated by the constitutive up-regulation of stress-related genes. Even though *soldat10/flu* seedlings overaccumulate the photosensitizer protochlorophyllide in the dark and activate the expression of $^1O_2$-responsive genes after a dark-to-light shift they do not show a $^1O_2$-dependent cell death response. Disturbance of chloroplast homeostasis in emerging *soldat10/flu* seedlings seems to antagonize a subsequent $^1O_2$-mediated cell death response without suppressing $^1O_2$-dependent retrograde signaling. The results of this work reveal the unexpected complexity of what is commonly referred to as 'plastid signaling'.

Keywords: singlet oxygen, acclimation, light stress, mTERF, *flu* mutant.

INTRODUCTION

Reactive oxygen species (ROS) are generated continuously as unavoidable by-products of aerobic metabolism (Apel and Hirt, 2004). In plants chloroplasts and peroxisomes are the main sites of production of ROS (Foyer and Noctor, 2000). The enhanced release of ROS in these cellular compartments has been attributed to the disturbance of light-driven photosynthetic electron transfer by various environmental factors, such as high light, high or low temperatures or drought (Long *et al.*, 1994; Niyogi, 1999). Under these environmental conditions, plants are exposed to light intensities that exceed their capacity to assimilate CO_2 and lead to the hyper-reduction of the photosynthetic electron transport chain that ultimately inhibits photosynthesis.

One of the difficulties in studying the biological activities of ROS stems from the fact that several chemically distinct ROS are generated simultaneously in cells under stress, thus making it difficult to link a particular stress response to a specific ROS. To study the specific activity of singlet oxygen ($^1O_2$) this problem has been overcome by using the conditional *flu* mutant of Arabidopsis, which allows the induction of $^1O_2$ within plastids in a non-invasive and controlled way (Kim *et al.*, 2008). Immediately after the release of $^1O_2$ mature *flu* plants stop growing, whereas seedlings bleach and die (op den Camp *et al.*, 2003). FLU is a nucleus-encoded plastid protein that plays a key role during negative control of chlorophyll (Chl) biosynthesis (Meskauskiene *et al.*, 2001).

Inactivation of this protein in the *flu* mutant leads to the accumulation of free protochlorophyllide (Pchlide) in plastids whenever plants are transferred to the dark. Once these plants are shifted back to the light, Pchlide acts as a photosensitizer and generates $^1O_2$ (op den Camp *et al.*, 2003). By extending the length of the dark period the concentration of Pchlide grows steadily up to a certain level and upon exposure to light it generates increasing amounts of $^1O_2$. By modifying the length of the dark period conditions have been established that minimize the cytotoxic effect of $^1O_2$ and reveal its signaling role (Kim *et al.*, 2008; Przybyla *et al.*, 2008). Under these conditions a larger number of suppressor mutations have been identified that abrogate $^1O_2$-mediated cell death and/or growth inhibition of *flu*. One of the mutated genes, *EXECUTER1* (*EX1*), has been shown previously to be indispensable for the transfer of $^1O_2$-dependent signals that mediate growth inhibition and cell death from the plastid to the nucleus (Wagner *et al.*, 2004).

Here we have analyzed a member of another group of suppressor mutants, dubbed singlet oxygen-linked death activator (*soldat*). The *soldat10* mutation does not interfere directly with the transfer of $^1O_2$-derived signals but disturbs chloroplast homeostasis and suppresses a subsequent $^1O_2$-mediated cell death response in *flu* seedlings.

RESULTS

Isolation of *soldat/flu* suppressor mutants

We have isolated second-site mutants of *flu* in which the $^1O_2$-mediated cell death response is suppressed at the seedling stage. Approximately 2000 seeds from each of 60 different M_2 seed batches that had been obtained by mutagenizing *flu* with ethyl methane sulfonate (EMS) were germinated separately on MS agar plates and kept for 5 days under 16-h light/8-h dark conditions. The original *flu* seedlings bleached and died under these growth conditions, whereas some of the M_2 seedlings started to green and survived the light/dark cycles. From the suppressor mutants the only ones selected for further studies were those that retained the ability of the parental *flu* line to overaccumulate free Pchlide in the dark (Figure 1). Mutants that did not bleach as seedlings but which as mature plants were still retarded in their growth were dubbed *soldat*. A total of 19 independent *soldat* mutations were found, that comprise 17 different loci. One of these mutants, *soldat10*/*flu*, was chosen randomly for a more detailed analysis.

Characterization of the *soldat10/flu* double mutant

Etiolated seedlings of *solda10*/*flu* and *flu* exposed to blue light emitted the strong red fluorescence of excited free Pchlide. This red fluorescence was not seen in etiolated wild-type seedlings (Figure 1a; Meskauskiene *et al.*, 2001). When seedlings of *soldat10*/*flu* initially grown under continuous light for 5 days were shifted to the dark their free Pchlide content increased and upon re-illumination generated $^1O_2$ that up-regulated subsets of nuclear genes (op den Camp *et al.*, 2003; Danon *et al.*, 2005) (Figure 2). Within the first 30 min of re-illumination of 5-day-old *soldat10*/*flu* seedlings transcripts of *ERF5*, *ERF6* and *BAP1* reached levels that were between 40-fold and more than 300-fold higher than those in the wild type and similar to transcript levels in seedlings of the parental *flu* mutant treated in the same way (Figure 2).

Identification of the *soldat10* gene by map-based cloning

In contrast to *executer1*, that together with a mutation in the closely related *EXECUTER2* gene suppresses $^1O_2$-mediated changes of nuclear gene expression in *flu* (Lee *et al.*, 2007), *soldat10* did not interfere with the release and transfer of

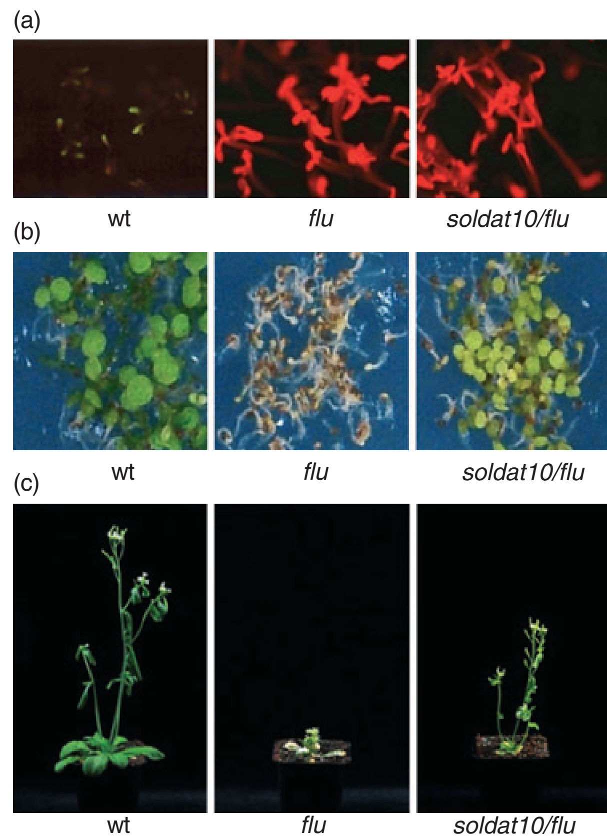

Figure 1. Singlet oxygen-mediated stress responses and phenotypic differences between wild-type (wt), *flu* and *soldat10*/*flu* mutant plants.
(a) Free protochlorophyllide (Pchlide) accumulates in etiolated seedlings of *flu* and *soldat10*/*flu* and upon excitation with blue light emits a strong red fluorescence, whereas in wild-type seedlings Pchlide forms part of the photoactive ternary NADPH–Pchlide oxidoreductase (POR) complex.
(b) Seedlings grown for 5 days under 16-h light/8-h dark conditions. In *soldat10*/*flu* seedlings the singlet oxygen-mediated cell death response of *flu* is suppressed and these seedlings resemble wild-type seedlings, except that their chlorophyll (Chl) content is lower and their size is reduced.
(c) Plants were initially grown for 20 days under continuous light and were subsequently transferred to 16-h light/8-h dark conditions. The *soldat10* mutation partially suppresses the singlet oxygen-mediated severe growth inhibition of the *flu* mutant.

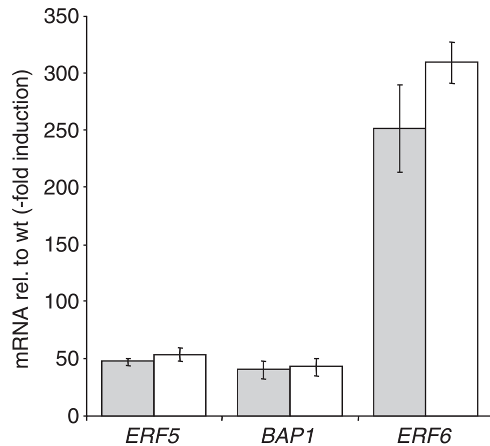

Figure 2. Singlet oxygen-mediated rapid up-regulation of three nuclear genes, *ERF5*, *ERF6* and *BAP1*, in *flu* (gray bars) and *soldat10*/*flu* (white bars) seedlings.
Transcript levels were determined relative to the wild type in seedlings first grown for 5 days under continuous light, then transferred to the dark for 8 h and re-exposed to light for 30 min. The relative mRNA abundance was normalized to the corresponding *ACTI2* (*At3g18780*) transcript level. The values represent averages and standard deviations of three biological samples. Each cDNA was analyzed twice by real-time PCR.

$^1O_2$-dependent signals from the plastid to the nucleus (Figure 2). Hence, *soldat10* is likely to suppress $^1O_2$-mediated cell death in *flu* seedlings more indirectly. One way to elucidate possible mechanisms responsible for the suppression of $^1O_2$-mediated stress responses in *soldat10*/*flu* seedlings is the identification of the mutated gene by map-based cloning. We genetically mapped *SOLDAT10* using the F_2 generation of a cross between the homozygous *soldat10*/*flu* mutant in ecotype Landsberg *erecta* (L*er*) and the *flu* mutant in ecotype Columbia (Col-0). The *flu* line of ecotype Col-0 had been obtained by five backcrosses of *flu* L*er* with wild-type Col-0. Homozygous *soldat10*/*flu* seedlings were selected by growing the F_2 population for 5 days on plates under non-permissive 16-h light/8-h dark conditions. Whereas *flu* seedlings died under these conditions, *soldat10*/*flu* seedlings survived (Figure 1b). Out of 200 F_2 seedlings, 56 surviving seedlings were found, suggesting that the *soldat10* phenotype is caused by a single recessive mutation (χ^2 = 0.96). In mature *soldat10*/*flu* double mutant plants kept under non-permissive dark/light conditions $^1O_2$-mediated growth inhibition was less severe than in the parental *flu* line (Figure 1c). Plates with seedlings exposed to five dark/light cycles were shifted to continuous light and surviving seedlings were transferred to soil. With the selected 56 *soldat10*/*flu* plants *SOLDAT10* was mapped on chromosome 2 (Figure 3a). For the subsequent fine mapping, the size of the mapping population was increased to a total of 982 homozygous *soldat10*/*flu* plants. *SOLDAT10* was located on a genomic fragment of approximately 45 kb that contained nine open reading frames (ORFs; http://www.arabidopsis.org/) (Figure 3a). In one of the genes (*At2g03050*) a single point mutation at position 161 changed cytosine to thymine leading to an exchange from proline to leucine in *soldat10*/*flu.*

Complementation of the *soldat10* mutation

The identification of the *SOLDAT10* gene was confirmed by complementing the *soldat10*/*flu* mutant. A genomic fragment spanning from 721 bp upstream to 705 bp downstream of the wild-type *SOLDAT10* ORF was stably integrated into the genome of the homozygous *soldat10*/*flu* double mutant. For complementation analysis T_3 seeds homozygous for the transgene were germinated under light/dark cycles. Under these conditions the wild type and *soldat10*/*flu* developed similarly, except that the chlorophyll content and the size of the mutant seedlings was reduced (Figure 3b). In contrast, seedlings of the *soldat10*/*flu* double mutant transformed with wild-type *SOLDAT10* were bleached and phenotypically indistinguishable from seedlings of the parental *flu* line (Figure 3b). Mature *soldat10*/*flu* plants complemented with the wild-type copy of *SOLDAT10* showed a similar growth inhibition to the parental *flu* line (Figure 3C). Thus, suppression of the $^1O_2$-mediated stress responses of *flu* was caused by the mutation of the *SOLDAT10* gene.

Subcellular localization of SOLDAT10

SOLDAT10 encodes a protein related to the human mitochondrial transcription termination factor mTERF (Fernandez-Silva *et al.*, 1997). The mTERF proteins have in common a modular architecture with a variable number of an approximately 30 amino acid signature termed the mTERF motif (Roberti *et al.*, 2009). The distinctive feature of this motif is the conservation of a proline at position 8 and a leucine or another hydrophobic amino acid at positions 11, 18 and 25 (Roberti *et al.*, 2009). The number and positions of mTERF motifs as well as the remaining sequences are extremely variable among the different members of the family. An alignment of mTERF motifs from SOLDAT10 and three other mTERF proteins is shown in Figure S1 in Supporting Information. Arabidopsis contains at least 35 different mTERF proteins (http://smart.embl-heidelberg.de/). One of them, PTAC15, has been shown previously to be associated with transcriptionally active DNA and to be localized in plastids (Pfalz *et al.*, 2006). MOC1, a putative transcription termination factor of *Chlamydomonas* was found in mitochondria (Schönfeld *et al.*, 2004). In SOLDAT10, five mTERF motifs are predicted by the SMART tool (http://smart.embl-heidelberg.de/). A sixth closely related sequence signature that in *soldat10* has its proline mutated into a leucine is found upstream of the five mTERF domains (Figure S1). The N-terminal part of SOLDAT10 resembles import signals that are predicted to direct proteins either to mitochondria or

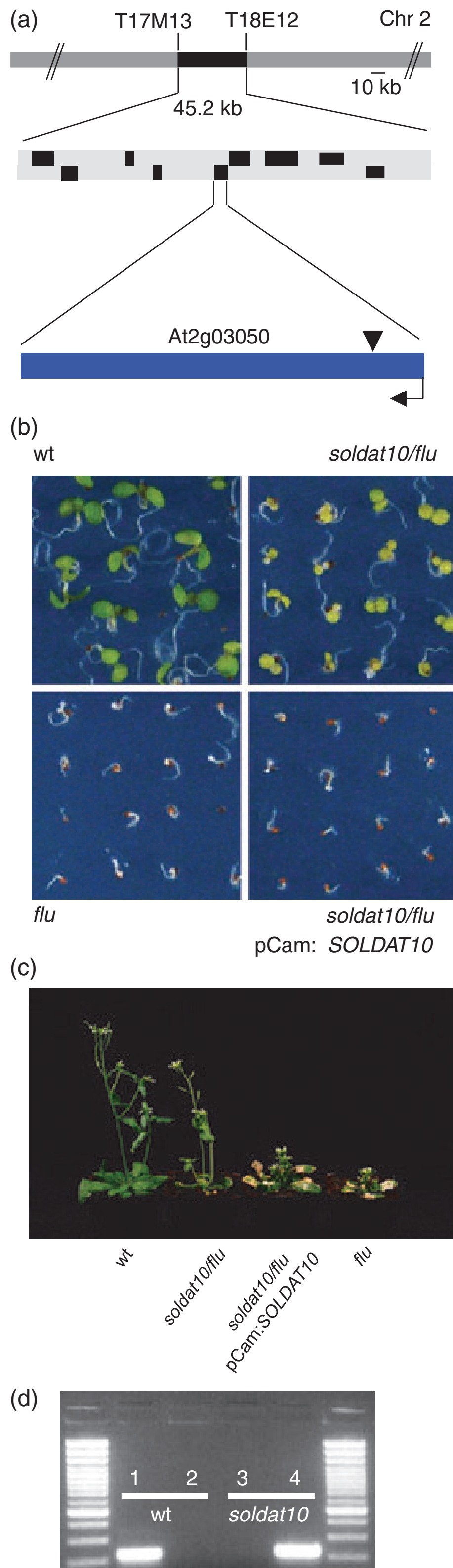

Figure 3. Identification of the *SOLDAT10* gene by map-based cloning, sequencing and complementation.
(a) Map-based cloning of the *SOLDAT10* gene. The gene was localized on a 45.2-kb genomic fragment of chromosome 2. This fragment contained nine open reading frames (black boxes). After sequencing the gene *At2g03050* was shown to carry a mutation in *soldat10/flu* when compared with *flu*. The mutation leads to an amino acid exchange from proline to leucine at position 54 of the predicted polypeptide (arrowhead).
(b, c) Complementation of the mutation by the *SOLDAT10* gene. (b) Seedlings of the *soldat10/flu* double mutant complemented with the *SOLDAT10* gene behaved like the parental *flu* line, when grown under non-permissive dark/light conditions. (c) In mature double mutant plants, the wild-type *SOLDAT10* copy restored the severe growth inhibition of the *flu* parental line after these plants had been shifted from continuous light (20 days) to non-permissive light/dark cycles.
(d) Identification of *soldat10* mutants devoid of the *flu* mutation by PCR analysis. Wild-type sequence-specific forward primer, in combination with reverse *SOLDAT10* primer, amplified DNA fragments only from wild-type but not from *soldat10* genomic DNA (1, 3). When using the *soldat10* mutation-specific forward primer, DNA fragments were amplified only from *soldat10* but not from wild-type genomic DNA (4, 2).

chloroplasts. The WoLF PSORT program (http://wolfpsort.org/) predicts SOLDAT10 to be chloroplastic with a score of 10 and mitochondrial with a score of 3. The TargetP program (http://www.cbs.dtu.dk/services/TargetP/) predicts SOLDAT10 to be chloroplastic with a score of 0.204 and to be mitochondrial with a score of 0.206. The subcellular localization of SOLDAT10 was determined experimentally by expressing a SOLDAT10–GFP fusion protein in protoplasts of Arabidopsis. Two types of transient protoplast transformation experiment were performed. First, the intracellular distribution of SOLDAT10–GFP signals was determined by confocal microscopy in protoplasts isolated and transformed according to Jin *et al.* (2001) (Figure 4a). As a control, the mitochondrial formate dehydrogenase (FDH) pre-sequence fused to GFP was expressed in a separate protoplast sample (Figure 4b). SOLDAT10–GFP signals were detected only in chloroplasts (Figure 4a). In the second experiment, the SOLDAT10–GFP construct was transiently expressed in protoplasts isolated as described by Yoo *et al.* (2007). MitoTracker Red CMX-ROS was added to protoplasts immediately before monitoring the fluorescence. Different excitation and emission wavelengths were selected for the detection of Chl, GFP and MitoTracker in the same protoplast preparation. The overlay of different fluorescence images confirms that SOLDAT10 only accumulates inside the chloroplasts (Figure 4c).

Characterization of *soldat10* in the wild-type background

The physiological consequences of the *soldat10* mutation and the assessment of its possible impact on $^1O_2$-mediated stress responses were determined in *soldat10* mutants lacking the *flu* mutation. Two strategies were used to isolate such mutants. Firstly, two T-DNA insertion lines, SALK_141368 and SALK_020778 (http://signal.salk.edu/cgi-bin/tdnaexpress), carrying insertions within exons of the *At2g03050* (*SOLDAT10*) gene were analyzed. The sites of insertions could be confirmed by PCR analysis. However, no homozygous mutant could be rescued from these lines, suggesting that inactivation of the *SOLDAT10* gene may be

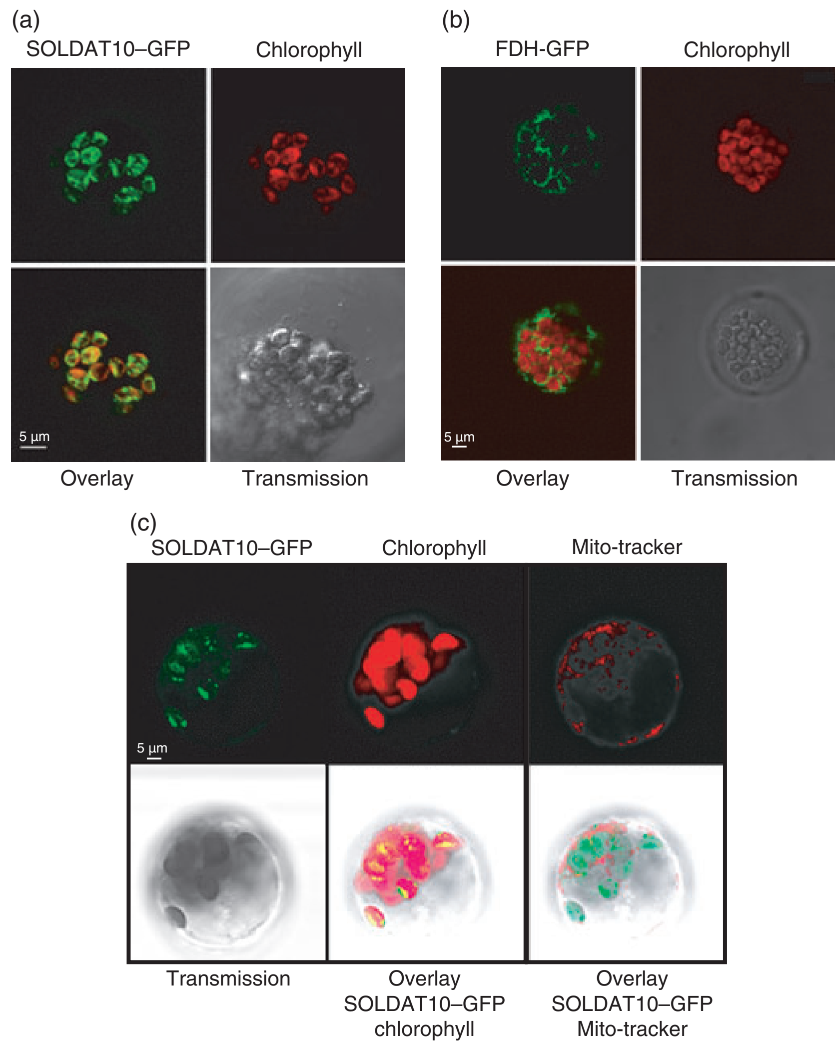

Figure 4. Intracellular localization of SOLDAT10–GFP as revealed by confocal microscopy.
(a) Arabidopsis protoplasts were transiently transformed with *SOLDAT10::GFP*. The distribution of GFP and chlorophyll fluorescence signals indicate a chloroplast-specific localization of SOLDAT10.
(b) A construct encoding the mitochondrial formate dehydrogenase pre-sequence fused to GFP (FDH::GFP) was used as control.
(c) Overlay of fluorescence images of chlorophyll, SOLDAT10–GFP and the mitochondrial marker MitoTracker from the same protoplast preparation confirm the localization of SOLDAT10 in chloroplasts. For details see Experimental Procedures.

lethal. Segregation of white embryos in an approximate 1:3 ratio in siliques of plants heterozygous for T-DNA insertion in *SOLDAT10* supports this notion (Figure S2). Secondly, the *soldat10*/*flu* double mutant was crossed with the wild type (L*er*) and homozygous *soldat10* mutants devoid of the *flu* mutation were selected from the segregating F_2 generation of this cross. First, seedlings with lighter green cotyledons were identified as putative homozygous *soldat10* mutants. A PCR analysis was then used to confirm the presence of two mutated alleles of the *SOLDAT10* locus and to identify among the selected mutants those that carried two wild-type copies of the *FLU* gene (Figure 3d; Goslings *et al*., 2004).

Based on the previously reported biological activities of human mitochondrial mTERF, MOC1 and PTAC15, all of which seem to be involved in transcriptional control, the expected primary consequence of the modification of SOLDAT10 could be a change in transcript levels within chloroplasts of *soldat10*. The expression of 15 plastid genes was analyzed by quantitative real-time PCR (Figure 5). These included *psbA* and *psbD* and *psaA* and *psaB* that encode polypeptides of the reaction centers of photosystem II (PSII) and PSI, respectively. One of the genes, encoding a subunit of the chloroplast-specific protease ClpP was significantly up-regulated in *soldat10* relative to the wild type. Previously, an up-regulation of this gene had been found in Arabidopsis plants exposed to high-light or low-temperature stress (Zheng *et al*., 2002). Among the selected chloroplast genes only those encoding the 16S and 23S RNAs were clearly down-regulated in *soldat10*, suggesting that the *soldat10* mutation does not lead to a general impairment of chloroplast RNA accumulation but seems to affect more selectively levels of rRNAs. The reduction of these RNAs is expected to affect the synthesis of proteins in chloroplasts, particularly of those with a high turnover rate. Among the proteins synthesized within chloroplasts, the D1 protein of PSII has probably the highest turn-over rate and hence its synthesis and steady-state concentration are likely to be reduced in *soldat10* seedlings. This suggestion was tested experimen-

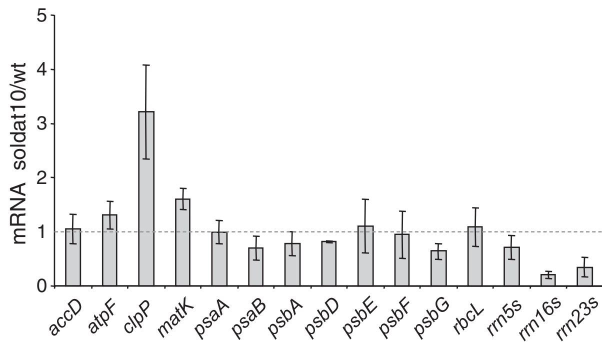

Figure 5. Transcript levels of a selected subset of 15 plastid genes were analyzed by real-time PCR in 5-day-old seedlings grown under 100 μmol m^{-2} sec^{-1} continuous light.

The vertical line indicates ratio of '1'. The values represent the mean and standard deviations of three independent experiments.

tally by a short-term *in vivo* labeling of proteins in seedlings pre-treated with cycloheximide. The *de novo* synthesis of the D1 protein was strongly reduced in *soldat10* when compared with the wild type (Figure 6a). The steady-state level of the D1 protein in *soldat10* mutant seedlings was also much lower than in the wild type. The levels of two other proteins associated with photosynthesis, LHCP and RbcL, were also reduced, whereas amounts of the FLU protein were even slightly increased (Figure 6b). The D1 protein forms part of the PSII core complex and plays a crucial role in photosynthetic electron transport and photoprotection of PSII (Andersson and Aro, 2001). The functional state of PSII in wild-type and *soldat10* seedlings was assessed in 3- to 14-day-old seedlings grown on MS agar plates under continuous light at 100 μmol m^{-2} sec^{-1}. The ratio of variable to maximum fluorescence (F_v/F_m) was 0.85 in 3-day-old wild-type and only 0.57 in *soldat10* seedlings (Figure 7). This value gradually increased in the mutant seedlings during the following days, but even in 14-day-old mutant seedlings remained significantly lower than in wild-type controls. A similar reduction of F_v/F_m occurs in wild-type seedlings suffering from severe light stress (Krause and Weis, 1991). As the possible enhanced light sensitivity of *soldat10* seedlings may lead to the activation of stress-related genes, global gene expression profiles of *soldat10* and wild-type seedlings were compared using Affymetrix ATH1 gene chips. For gene expression analysis wild-type and *soldat10* seedlings were grown under low light (12 μmol m^{-2} sec^{-1}) to minimize possible damage of mutant seedlings by photo-oxidative stress. Genes with two-fold or greater differential expression were identified. A total of 199 genes were up-regulated in *soldat10* relative to the wild type. Among these genes, 31 had previously been shown to be up-regulated in response to enhanced levels of $^1O_2$ and/or hydrogen peroxide (op den Camp *et al.*, 2003). More than 20 additional genes up-regulated in *soldat10* have been associated with

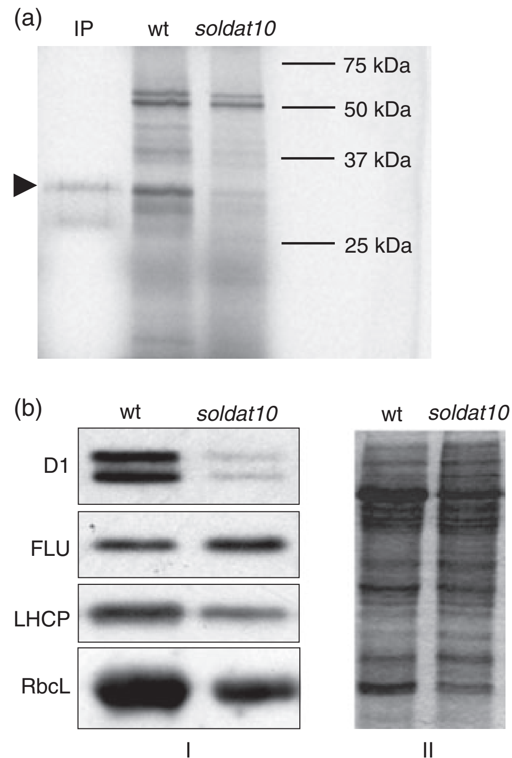

Figure 6. The effect of the *soldat10* mutation on the biosynthesis and steady-state levels of plastid proteins in 5-day-old seedlings grown under 100 μmol m^{-2} sec^{-1} continuous light.

(a) The labeling of the D1 protein of photosystem II (PSII) in wild-type (wt) and *soldat10* seedlings with ^{35}S-methionine in the presence of cycloheximide. Seedlings were pre-incubated with cycloheximide for 1 h before adding the radioactively labeled amino acid for 2 h. The arrowhead marks the position of the D1 protein. As a control, immunoprecipitation with a D1-specific antibody was performed from wild-type samples and immunoprecipitated proteins were separated on the same gel (IP).

(b) Western blot analysis of four plastid proteins. Proteins were isolated from 5-day-old seedlings grown under 100 μmol m^{-2} sec^{-1} continuous light, subjected to SDS–PAGE, blotted on polyvinylidene fluoride membranes and analyzed with specific antibodies (I). Coomassie Blue staining of the gel served as a loading control (II).

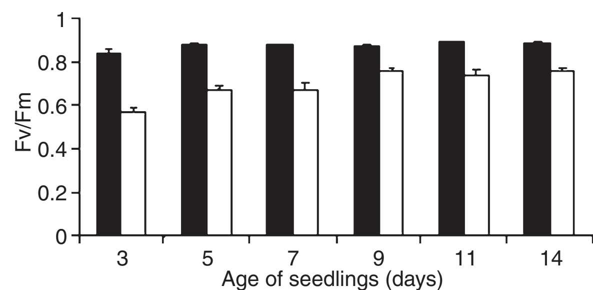

Figure 7. Measurements of photosystem II efficiency (F_v/F_m, ratio of variable to maximum fluorescence) in *soldat10* (white bars) and wild-type (black bars) seedlings grown under 100 μmol m^{-2} sec^{-1} continuous light.

For each time point F_v/F_m values of 20–30 seedlings were determined. The values represent average and standard deviations of these measurements.

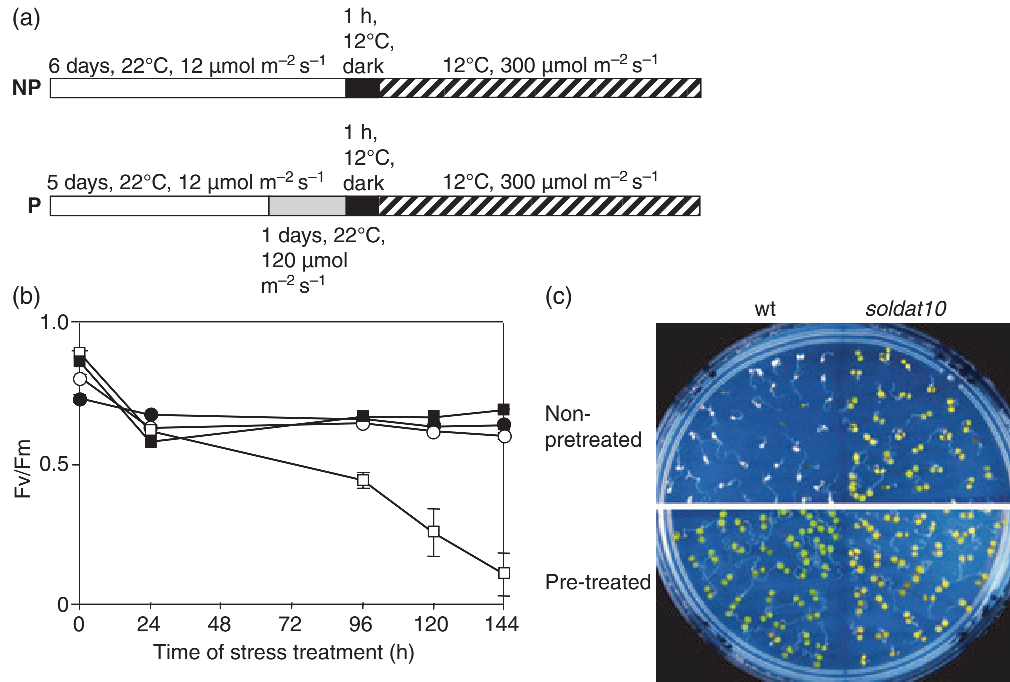

Figure 8. The effect of the *soldat10* mutation on the stress susceptibility of seedlings.
Wild-type and *soldat10* seedlings were first grown under low light at 22°C for 6 days and then exposed to the combined light and cold stress (a, NP). Changes of F_v/F_m (ratio of variable to maximum fluorescence) were monitored throughout the stress treatment of wild-type (b, □) and *soldat10* (b, ○) seedlings. The pre-treatment with 120 μmol m^{-2} sec^{-1} light before the light/cold stress (a, P) improved the stress tolerance of wild type (b, ■) but did not further improve the stress tolerance of *soldat10* (b, ●). The picture of non-pretreated and pre-treated mutant and wild-type seedlings was taken at the end of the light/cold stress (c). At the beginning of the stress treatment (time point zero), the F_v/F_m ratio of non-pretreated *soldat10* seedlings was significantly lower than that of wild-type controls and was even more reduced in *soldat10* seedlings that had been pre-treated (b). These results are consistent with an enhanced susceptibility of *soldat10* seedlings to light stress that seems to evoke an acclimatory response both in non-pretreated and pre-treated mutant. NP, non-pretreated; P, pre-treated.
Each of the F_v/F_m values represent average and standard deviations of measurements of 50 seedlings. The experiment was repeated three times and gave similar results.

other stress-related stimuli (Table S1). These data suggest that *soldat10* seedlings are stressed even when grown under low light intensities.

A possible consequence of this mild stress in *soldat10* could be stress acclimation, which might confer an enhanced resistance against a more severe light stress to *soldat10* seedlings initially grown under low light. This premise was tested experimentally. First *soldat10* and wild-type seedlings were grown for 6 days at 22°C under continuous low light (12 μmol m^{-2} sec^{-1}) (Figure 8a). Afterwards, the light was switched off and over the next hour the temperature of the growth chamber was lowered to 12°C before seedlings were re-exposed to continuous light of higher intensity (300 μmol m^{-2} sec^{-1}) and kept at 12°C for the next 144 h (Figure 8a). Responses of seedlings were registered by measuring changes in F_v/F_m. At the beginning of the combined high-light/low-temperature stress the F_v/F_m value of *soldat10* seedlings grown in low light (0.80) was lower than that of the wild-type (0.88) (Figure 8b) but higher than in *soldat10* seedlings grown at 100 μmol m^{-2} sec^{-1} (Figure 7). During the first 24 h of high-light/low-temperature stress both wild-type and *soldat10* seedlings suffered similarly, as can be seen from the reduction of the F_v/F_m values to 0.61 and 0.60 for wild-type and *soldat10* seedlings, respectively. During the following days the F_v/F_m values of wild-type seedlings were further reduced, and finally the stress treatment resulted in the bleaching and collapse of the seedlings (Figure 8b,c). In contrast, the F_v/F_m values of *soldat10* seedlings were not reduced further; seedlings remained viable and were not visibly damaged even after 144 h of stress (Figure 8b,c). Thus, contrary to the wild type, *soldat10* seedlings grown initially under low light were clearly able to withstand the following more severe combined high-light/low-temperature stress.

If young *soldat10* mutant seedlings already perceive the initial continuous low light as a minor stress leading to acclimation, only wild-type but not mutant seedlings would be expected to activate acclimation in response to an additional light stress treatment preceding the following more severe high-light/low-temperature stress. This prediction was tested with seedlings subjected to the same light/temperature program as used before, except that on the fifth day seedlings were exposed for 24 h to an enhanced light intensity of 120 μmol m^{-2} sec^{-1} without changing the

temperature (Figure 8a). In the wild type this pre-treatment caused only a slight reduction of the F_v/F_m values when compared with non-pretreated seedlings (Figure 8b). The F_v/F_m values of pre-treated *soldat10* mutants were clearly lower than those of non-pretreated ones (Figure 8b), being in line with an enhanced light sensitivity of the mutant. After 144 h of combined high-light/low-temperature stress treatment none of the pre-treated wild-type seedlings was bleached and their F_v/F_m values were retained at a similar level as in non-pre-treated *soldat10* (Figure 8b). Thus, the light pre-treatment of wild-type seedlings was clearly effective in improving the seedlings' ability to withstand the following severe combined high-light/low-temperature stress (Figure 8b,c). Responses of pre-treated *soldat10* seedlings to the low-temperature/high-light stress were very similar to those of the non-pretreated *soldat10* seedlings (Figure 8b,b). Hence, *soldat10* seedlings even without receiving the higher light pre-treatment seem to already be acclimated to the following more severe stress. This constitutive acclimation to light stress appears to be the likely cause of suppression of $^1O_2$-mediated collapse of *soldat10/flu* seedlings.

DISCUSSION

As shown in the present work, the *SOLDAT10* gene encodes a plastid-localized protein related to the human mitochondrial transcription factor mTERF (Fernandez-Silva *et al.*, 1997) and the mitochondrial protein MOC1 of *Chlamydomonas* (Schönfeld *et al.*, 2004). Inactivation of MOC1 in *Chlamydomonas* drastically enhances the light sensitivity of the mutant (Schönfeld *et al.*, 2004). Light sensitivity is also enhanced in *soldat10* seedlings, as indicated by their reduced F_v/F_m ratio and the up-regulation of stress-related nuclear genes. The mutant showed the same maximum fluorescence after a saturating light pulse as the wild type, but the basal level of fluorescence (F_0) was higher, resulting in lower F_v/F_m values (data not shown). An increase in F_0 has been interpreted as the result of a reduced rate constant of energy trapping of PSII centers (Havaux, 1993). In young *soldat10* seedlings the contents of both total chlorophyll and LHCP were reduced (Figures 6b and S3), but the chlorophyll *a*/*b* ratios of wild type and mutant were similar, reaching values of 3.36 and 3.22, respectively. By contrast, the D1 level in mutant seedlings relative to wild type was drastically reduced. This change in stoichiometry of light harvesting complexes and active PSII core particles is expected to contribute to an enhanced release of ROS that may cause the stress symptoms seen in *soldat10* seedlings grown under low light.

Right after *soldat10* seedlings were transferred from low light to the combined low-temperature/high-light stress, they showed a drop in their F_v/F_m ratio that in wild-type seedlings had been attributed to photoinhibition of PSII (Krause and Weis, 1991). In contrast to wild-type seedlings that during the following days of stress bleached and died, *soldat10* seedlings surprisingly survived this harsh stress treatment. Acclimation to stress caused by a low dose of ROS, high light, lower or higher temperature and wounding enhances a plant's ability to cope with a following more severe stress (Vierling, 1991; Prasad *et al.*, 1994; Karpinski *et al.*, 1999; Iida *et al.*, 2000; Orozco-Cardenas *et al.*, 2001; Chang *et al.*, 2004; Ledford *et al.*, 2007). Such acclimation may not only provide protection against the same type of stress that was initially perceived by the plant but may also include cross-tolerance to other stresses (Wu *et al.*, 1995; Bowler and Fluhr, 2000; Funatsuki *et al.*, 2003; Mateo *et al.*, 2004; Mühlenbock *et al.*, 2008).

As shown in our present work, exposure to a minor light stress markedly enhanced the stress tolerance of wild-type seedlings during the following severe low-temperature/high-light stress, whereas it did not appear to further enhance the stress tolerance of *soldat10* seedlings. These results implicate activation of an acclimatory response in *soldat10* seedlings grown under low light.

The primary cause for phenotypic changes in *soldat10* resides within chloroplasts, whereas the enhanced expression of genes associated with acclimation takes place within the nucleus. Hence, signaling factors that are exported from the chloroplast and act in the nucleus must be affected in their activity by the mutation. Plastid-to-nucleus signaling plays a central role in controlling gene expression in the nucleus (Taylor, 1989; Mullineaux and Karpinski, 2002; Beck, 2005; Nott *et al.*, 2006; Pesaresi *et al.*, 2007). The biological impact of plastid-derived retrograde control of nuclear gene activity had been thought to be confined to the fine-tuning and coordination of nuclear and chloroplast genes that are required for the optimization and protection of chloroplast-specific functions primarily associated with photosynthesis (Surpin *et al.*, 2002; Pfannschmidt, 2003; Ball *et al.*, 2004; Heiber *et al.*, 2007). However, more recently plastid-derived retrograde signals have also been shown to control other, stress-related reactions (Ochsenbein *et al.*, 2006; Lee *et al.*, 2007; Mühlenbock *et al.*, 2008; Belhaj *et al.*, 2009).

Plastid signals have been thought to derive either from the tetrapyrrole pathway (Strand *et al.*, 2003), or from plastid gene expression in young seedlings (Sullivan and Gray, 1999), ROS (Karpinski *et al.*, 1999; op den Camp *et al.*, 2003), or the redox state of the organelle (Dietz, 2003; Pfannschmidt, 2003). So far the existence of different retrograde signaling pathways has been inferred mostly from correlations between a particular disturbance of a plastid-specific activity and a corresponding change in the steady-state concentration of nuclear gene transcripts. The *soldat10* mutation impairs plastid gene expression, but may also affect more indirectly the production of ROS or the redox state of the plastid. Hence, we consider 'the disturbance of plastid homeostasis' to be a likely cause for the suppression

of $^{1}O_2$-mediated cell death in *soldat10/flu* double mutants, emphasizing the fact that at present the identity of retrograde signals responsible for phenotypic changes in *soldat10* are unknown and that their activity cannot be linked exclusively to any of the putative retrograde signals discussed previously.

The disturbance of plastid homeostasis in *soldat10/flu* seedlings did not block the transfer of $^{1}O_2$-dependent retrograde signals from the plastid to the nucleus and the subsequent activation of $^{1}O_2$-responsive genes, but it did suppress $^{1}O_2$-mediated cell death. This seemingly paradoxical outcome of an interaction between different plastid-derived signaling events reveals an unexpected complication one is faced with when trying to dissect and describe the consequences of the activation of retrograde signaling. Previously, $^{1}O_2$-mediated stress responses have been shown to be antagonized by at least two plastid signals derived from H_2O_2 and $^{1}O_2$, respectively (Laloi *et al.*, 2007; Ledford *et al.*, 2007). The intensity of $^{1}O_2$-mediated stress responses such as cell death, growth inhibition and expression of $^{1}O_2$-responsive nuclear genes was significantly enhanced when the concentration of H_2O_2 in plastids was reduced due to the overexpression of the plastid-specific thylakoid-bound ascorbate peroxidase. This result suggests that in addition to acting on its own as a plastid-derived signal, H_2O_2 may also modify either directly or indirectly the response to another retrograde signal activated by the release of $^{1}O_2$ (Laloi *et al.*, 2007).

At a low dose $^{1}O_2$ activates an acclimatory response that enhances resistance against a subsequent more severe stress caused by a higher dose of $^{1}O_2$ (Ledford *et al.*, 2007). Some of the genes up-regulated constitutively in *soldat10* turned out to be responsive to H_2O_2 (e.g. *FERRITIN1*) and/or to be up-regulated in the *flu* mutant after the release of $^{1}O_2$ (op den Camp *et al.*, 2003) (Table S1). Other $^{1}O_2$-responsive genes such as *BAP1* and *ERF5*, however, were not affected in *soldat10* prior to the release of $^{1}O_2$. These seemingly conflicting data could be explained if cell death and acclimation represent two qualitatively different stress responses that are triggered differentially by $^{1}O_2$ in a dose-dependent manner (Kim *et al.*, 2008). This suggestion is supported by a comparison of global changes in gene expression induced by a very low and a higher dose of $^{1}O_2$. Whereas some of the $^{1}O_2$-responsive genes were activated under both conditions, the majority of them were unique to either acclimation- or cell-death-inducing conditions (CK and KA, in preparation).

In addition to H_2O_2 and $^{1}O_2$, the disturbance of plastid homeostasis in *soldat10* is also likely to affect other putative plastid-derived signaling factors such as the redox state and the ATP/AMP ratio that are known to activate distinct signaling pathways (Dietz, 2003; Pfannschmidt, 2003; Baena-Gonzalez *et al.*, 2007) and, in the case of ATP/AMP, may enhance stress acclimation of plants (Baena-Gonzalez *et al.*, 2007). In conclusion, changes induced by the disturbance of plastid homeostasis in *soldat10* cannot be attributed exclusively to just one of the previously discussed retrograde signals, but rather result from the activation of several signaling events whose relative contributions overlap and may change depending on the primary defect in plastids that causes a disturbance of plastid homeostasis. As some of these signaling pathways, for example starvation signaling, H_2O_2- and $^{1}O_2$-signaling, regulate the expression of distinct sets of nuclear genes that are largely different from each other (op den Camp *et al.*, 2003; Gadjev *et al.*, 2006; Baena-Gonzalez *et al.*, 2007), integration of these various signaling activities is likely to occur within the nuclear compartment. This notion is in clear conflict with a recent model put forward by Koussevitzky *et al.* (2007) that predicts that different retrograde signaling pathways already converge within the plastid compartment and form part of a common signal transduction pathway that conveys information from the plastid to the nucleus. The dissection of the complexity of signaling events triggered by a disturbance of plastid homeostasis and the identification of signaling factors involved in translating this disturbance into responses that change the physiological status of the whole plant will remain a major challenge for our future work.

EXPERIMENTAL PROCEDURES

Plant material

All experiments were performed with the *Arabidopsis thaliana* ecotypes L*er* and Col-0. Seeds were surface-sterilized with 70% (v/v) ethanol and plated on MS medium with 0.8% agar. Seeds were stratified at +4°C for 3 days in the dark and grown under continuous light (80–100 μmol m^{-2} sec^{-1}) at 20–21°C. Light was provided by white light tubes (Philips Master TDL 36W, Philips Electronics, http://www.philips.co.uk/ and Sylvania Gro Lux F36W, SLI Lichtsysteme, http://www.havells-sylvania.com/). In some of the experiments different light regimes were used as described under 'Results'.

Identification and complementation of the *soldat10* mutation

The *SOLDAT10* locus was mapped on chromosome II using cleaved amplified polymorphic sequence (CAPS) or simple sequence length polymorphism (SSLP) markers listed in the Arabidopsis Information Resource database (TAIR; http://www.arabidopsis.org/). Additional markers used for mapping were designed based on the collection of predicted Arabidopsis single nucleotide polymorphisms (SNP) and small insertions/deletions (INDELs) in the publicly available Columbia and Landsberg *erecta* sequences generated by Monsanto (http://www.arabidopsis.org/Cereon/).

Nine ORFs in the mapped genomic region of 45 kb were amplified by PCR from genomic DNA of the *soldat10/flu* double mutant and *flu*, which served as a control. The amplified DNA fragments were sequenced by Microsynth (http://www.microsynth.ch/). Sequences were compared using the SeqViewer tool from TAIR.

For complementation, specific primers (5′-CCCGGAATTCCTTCC-CATCCTGTTAATCCC-3′) and (5′-CCCGGAATTCGAGGATCGTG-TATTCGCTTC-3′) flanking *SOLDAT10* with its own promoter

region and downstream sequence were used for amplification of *SOLDAT10* from wild-type genomic DNA. The amplified DNA fragment was inserted into pCambia 3300 vector using the *Eco*RI restriction site and sequenced. Plasmid DNA of selected clones was transferred to *Agrobacterium tumefaciens* C58C1 cells. Homozygous *soldat10*/*flu* double mutant plants grown under continuous light on soil were transformed as described (Clough and Bent, 1998). Primary transformants were selected on Basta-containing media (25 μg Basta ml^{-1}). T_2 seeds were sown on Basta-containing media and the percentage of Basta-resistant seedlings per transgenic line was calculated. Lines with a 3:1 ratio of segregating Basta-resistant seedlings, corresponding to one copy of the inserted transgene, were selected for complementation analysis, which is described in the Results.

Isolation of the *soldat10* mutant

To identify *soldat10* mutants without the *flu* mutation, *soldat10*/*flu* double mutants were crossed to wild-type plants (L*er*). Among the F_2 progeny, putative homozygous *soldat10* mutants were pre-selected based on their slightly pale green color. Gene-specific primers for the *soldat10* mutation (5′-CAAAGCTCTCCGAGTAAACCT-3′) or for wild type (5′-CAAAGCTCTCCGAGTAAACCC-3′) in combination with a common reverse primer (5′-GATTTAGGTATGTCTTG CTCTG-3′) were used to confirm this identification by PCR analysis. In a second step, the same samples were tested for the presence of the wild-type *FLU* gene using gene-specific primers for the *flu* mutation (5′-CCAAGGGAAGTATAGGGAAGT-3′) or for the *FLU* wild-type gene (5′-CCAAGGGAAGTATAGGGAAGC-3′) and a common reverse primer (5′-GGCAATTGGCACTTAAGATGGC-3′).

Transient expression of GFP fusion proteins in *A. thaliana* protoplasts

Specific primers to amplify the *SOLDAT10* ORF from wild-type cDNA were used (CATGCCATGGTAGCAAGGTGTTCTC and CATGCCATGGCTCTTCTTTCAGCAGACCTAAGAC). The PCR fragment was inserted into modified pCambia 3300 vector, which contains the GFP sequence (Kim and Apel, 2004) using *Nco*I restriction site and sequenced. *Arabidopsis thaliana* plants were grown for 4 weeks on 0.5× MS medium under long-day (16-h light/8-h dark) conditions. Two different transient protoplast transformation assays were performed. First, protoplasts were isolated and transformed as described by Jin *et al.* (2001), except that cellulase and macerozyme (Serva, http://www.serva.de/enDE/index.html) concentrations were reduced to 1% and 0.25% (w/v), respectively. A Leica TCS 4D confocal laser scanning microscope (CLSM) (Leica Microsystems, http://www.leica-microsystems.com/) was used to monitor the fluorescence in protoplasts 48 h after transformation. The fluoresceinisothiocyanate (FITC) laser line (488 nm) was used to detect GFP. Chlorophyll autofluorescence was monitored using the tetramethylrhodamine isothiocyanate (TRITC) excitation wavelength (568 nm). The FDH::GFP construct (Ambard-Bretteville *et al.*, 2003) was used as a control for mitochondrial localization. In a second experiment, protoplast isolation and transformation were performed according to Yoo *et al.* (2007). Fluorescence images were taken 18 h after transformation. MitoTracker® Red CMXRos (Molecular Probes, http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) was added to protoplasts 5 min before monitoring the fluorescence under a Leica SP5 CLSM. The Chl, GFP and MitoTracker signals were taken from the same protoplast preparation by selecting different excitation and emission wavelengths (excitation 488 nm Chl and GFP; 578 nm MitoTracker; emission 507–520 nm GFP; 620–700 nm Chl; 590–620 nm MitoTracker).

RNA isolation and real-time PCR analysis

Total RNA was isolated with TRIZOL® reagent (Invitrogen, http://www.invitrogen.com/) according to the manufacturer's protocol. To assess the quality of RNA, an aliquot containing 2 μg of RNA was loaded on a 2% agarose gel. Reverse transcription and real-time PCR were performed as described previously by Danon *et al.* (2005).

Protein analysis

Proteins were isolated from seedlings according to Goslings *et al.* (2004). Sodium dodecyl sulfate (SDS)–PAGE was carried out as described by Laemmli (1970). Western analysis was carried out according to the protocol provided with the Immun-StarTM HRP chemiluminescence detection kit (Bio-Rad, http://www.bio-rad.com/). For immunodetection of FLU, LHCP, D1 and RbcL, specific antibodies were used at dilutions of 1:3000, 1:5000, 1:5000 and 1:10000, respectively. Antibodies specific for D1 and RbcL were purchased from AgriSera AB (http://www.agrisera.com/). Secondary antibodies were anti-chicken or anti-rabbit IgG with conjugated horseradish peroxidase (HRP; Bio-Rad).

In vivo labeling of plastid proteins

In vivo labeling and immunoprecipitation were performed as described in Kim and Apel (2004). Ten 3-day-old seedlings were vacuum infiltrated and incubated for 1 h in 40 μl of 0.5× MS medium (pH 5.7) containing 100 μmol cycloheximide. After addition of 0.555 MBq of ^{35}S-methionine, plants were incubated under dim light for another 2 h. Proteins were isolated in 300 μl of extraction buffer containing 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)–HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 2 mM PMSF and 2.5 mM DTT. Samples were heated for 5 min at 55°C and centrifuged at 16 200 ***g***. Ten to twenty microliters of supernatant (equal radioactivity per sample) was used for SDS–PAGE and radioactively labeled proteins were detected by autoradiography. The rest of supernatant was used for immunoprecipitation with D1 specific antibody (AgriSera, antibody produced in rabbit).

In vivo measurement of photosynthetic activity

Chlorophyll fluorescence was measured with closed FluorCam System (Photon Systems Instruments, http://www.psi.cz/) The maximum quantum efficiency of PSII was calculated using the standard quenching analysis protocol provided by Photon Systems Instruments.

Stress experiments

Details of how stress experiments were performed with *soldat10* and wild-type seedlings of Arabidopsis are given in the Supporting Information (Appendix S1).

Array hybridization, evaluation and microarray data analysis

Details of microarray analysis of global gene expression changes are given in the Supporting Information (Appendix S1).

Other methods

Chemical mutagenesis of seeds of the *flu* mutant with EMS was performed as described previously (Runge *et al.*, 1995; Wagner *et al.*, 2004). Etiolated seedlings of wild type, *flu* and *soldat10*/*flu* were illuminated with blue light and examined under the Leica MZ12 fluorescence microscope with a Leica FM blue 10446146 filter (Leica Microsystems).

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers *EXECUTER1* (*At4g33630*), *SOLDAT10* (*At2g03050*) and *FLU* (AT3g14110).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of mTERF motifs in mTERF, MOC1, SOLDAT10 and PTAC15.

Figure S2. Embryos in wild-type siliques and a plant heterozygous for the T-DNA insertion in the *SOLDAT10* gene (*SOLDAT10/soldat10*).

Figure S3 The chlorophyll *a* and *b* content in 5-day-old wild-type and *soldat10* seedlings grown at 100 μmol m^{-2} sec^{-1} under continuous light.

Table S1. List of genes up-regulated at least two-fold in 6-day-old *soldat10* seedlings grown under low light versus wild-type seedlings grown under the same conditions.

Appendix S1. Stress experiments, array hybridization and evaluation, and microarray data analysis.

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