PCP
PLANT & CELL PHYSIOLOGY

Characterization of *soldat8*, a Suppressor of Singlet Oxygen-Induced Cell Death in *Arabidopsis* Seedlings

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The *flu* mutant of *Arabidopsis thaliana* overaccumulates in the dark the immediate precursor of chlorophyllide, protochlorophyllide (Pchlide), a potent photosensitizer, that upon illumination generates singlet oxygen ($^1O_2$). Once $^1O_2$ has been released in plastids of the *flu* mutant, mature plants stop growing, while seedlings die. Several suppressor mutations, dubbed singlet *o*xygen-*l*inked *d*eath *a*ctivator (*soldat*), were identified that specifically abrogate $^1O_2$-mediated stress responses in young *flu* seedlings without grossly affecting $^1O_2$-mediated stress responses of mature *flu* plants. One of the *soldat* mutations, *soldat8*, was shown to impair a gene encoding the SIGMA6 factor of the plastid RNA polymerase. Reintroduction of a wild-type copy of the *SOLDAT8* gene into the *soldat8*/*flu* mutant restored the phenotype of the *flu* parental line. In contrast to *flu*, seedlings of *soldat8*/*flu* did not bleach when grown under non-permissive dark/light conditions, despite their continuous overaccumulation of the photosensitizer Pchlide in the dark. The activity of SIGMA6 is confined primarily to the very early stage of seedling development. Inactivation of SIGMA6 in *soldat8* mutants disturbed plastid homeostasis, drastically reduced the non-photochemical quenching capacity and enhanced the light sensitivity of young *soldat8* seedlings. Surprisingly, after being grown under very low light, *soldat8* seedlings showed an enhanced resistance against a subsequent severe light stress that was significantly higher than in wild-type seedlings. In order to reach a similar enhanced stress resistance, wild-type seedlings had to be exposed to a brief higher light treatment that triggered an acclimatory response. Such a mild pre-stress treatment did not further enhance the stress resistance of *soldat8* seedlings. Suppression of $^1O_2$-mediated cell death in young *flu*/*soldat8* seedlings seems to be due to a transiently enhanced acclimation at the beginning of seedling development caused by the initial disturbance of plastid homeostasis.

Keywords: Acclimation • *flu* mutant • Light stress • Singlet oxygen • SIGMA6 • *soldat8*.

Abbreviations: CAPS, cleaved amplified polymorphic sequence; LHC, light-harvesting complex; NEP, nuclear-encoded RNA polymerase; NPQ, non-photochemical quenching; Pchlide, protochlorophyllide; PEP, plastid-encoded RNA polymerase; ROS, reactive oxygen species; SSLP, simple sequence length polymorphism.

Introduction

Plants exposed to environmental stress show an increased production of reactive oxygen species (ROS) (Apel and Hirt 2004). ROS may react with various biomolecules such as lipids, proteins and nucleic acids, causing irreversible damage that can lead to tissue necrosis and ultimately may kill the plant (Rebeiz et al. 1988, Girotti 2001, Przybyla et al. 2008), but they may also act as signals that activate and control genetic stress response programs that confer an enhanced stress tolerance to an otherwise vulnerable plant (Karpinski et al. 1999, Bowler and Fluhr 2000, Przybyla et al. 2008).

Because in plants under stress different ROS are generated simultaneously, it is difficult to determine the biological activity and mode of action for each of these ROS separately. For instance, abiotic stress conditions such as high light, high or low temperatures, drought, high or low salt or nutrient stress may limit the plant's ability to use light energy for

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Plant Cell Physiol. 50(4): 707–718 (2009) doi:10.1093/pcp/pcp036, available online at www.pcp.oxfordjournals.org
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photosynthesis (Barber and Anderson 1992, Niyogi 1999, Dat et al. 2001). Under such stress conditions, hyper-reduction of the photosynthetic electron transport chain favors the direct reduction of O_2 by PSI to the superoxide radical and the subsequent production of hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH·) (Asada 1999, Foyer and Noctor 2000). At the same time, excited triplet Chl in PSII interacts with O_2 to generate singlet oxygen ($^1O_2$) by energy transfer (Macpherson et al. 1993, Ledford and Niyogi 2005). Recently the conditional *flu* mutant of *Arabidopsis thaliana* has been used to discern the biological activity and specificity of one of these ROS. The mutant releases $^1O_2$ in plastids in a controlled and non-invasive manner (op den Camp et al. 2003). FLU is a nucleus-encoded protein that plays a key role during the negative feedback control of Chl biosynthesis (Meskauskiene et al. 2001). Inactivation of this protein in the *flu* mutant leads to the accumulation of free protochlorophyllide (Pchlide) in plastids of plants kept in the dark. Once these plants are shifted to the light, Pchlide acts as a photosensitizer and by energy transfer generates $^1O_2$. Immediately after the release of $^1O_2$, mature *flu* plants stop growing, while seedlings bleach and die (op den Camp et al. 2003).

Growth inhibition and seedling lethality of *flu* do not result from physicochemical damage caused by $^1O_2$ during oxidative stress, but are due to the activation of genetically determined stress response programs (Przybyla et al. 2008). During an extensive second-site mutant screen, a larger number of suppressor mutations have been identified that abrogate either one or both stress responses of *flu*. One of the mutated genes, *EXECUTER1* (*EX1*), has been shown previously to be indispensable for $^1O_2$-mediated stress responses of both seedlings and mature plants (Wagner et al. 2004). Here we have analyzed a member of another group of suppressor mutations, dubbed *s*inglet *o*xygen-*l*inked *d*eath *a*ctivator (*soldat*), that block predominantly $^1O_2$-mediated cell death responses of seedlings. In contrast to EXECUTER1, inactivation of the SOLDAT8 protein does not impair $^1O_2$-mediated signaling, but instead enhances the light sensitivity, activates an acclimatory response, and in this way seems to suppress the subsequent $^1O_2$-mediated cell death of seedlings.

Results

Characterization of the soldat8/flu double mutant

Second-site mutations of *flu* were characterized that suppress the $^1O_2$-mediated cell death response of seedlings, but not the growth inhibition of mature plants. Among the suppressor mutants only those were selected for this study that still accumulated amounts of free Pchlide in the dark similar to the parental *flu* mutant and also ceased growth, when mature plants were exposed to a dark/light regime. Each of these selected mutants was back-crossed at least once to the original *flu* mutant. Segregation analysis of the F_2 generation of these crosses verified that in each case the phenotype of the second-site mutants was caused by a single recessive gene mutation. These mutant lines were dubbed *soldat*. A total of 19 *soldat* mutant lines were found, that comprise 17 different loci. In the present study, one of these mutants, *soldat8/flu*, was analyzed in greater detail.

Etiolated seedlings of *soldat8/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, even though these plants also contained some Pchlide (**Fig. 1A** and data not shown). In the wild type, Pchlide together with NADPH and the NADPH-Pchlide oxidoreductase (POR) forms a ternary photoactive complex that uses light energy absorbed by Pchlide for the reduction of Pchlide to Chlide (Griffiths 1978). When seedlings of *soldat8/flu* initially grown under continuous light for 5 d were shifted to the dark for 15 h the Pchlide level at the end of the dark period exceeded that of wild-type seedlings 8- to 9-fold (**Fig. 1A**). Upon re-illumination of these seedlings, the levels of free Pchlide in *soldat8/flu* were sufficient to generate $^1O_2$ that induced a drastic activation of nuclear genes previously shown to be rapidly up-regulated in response to $^1O_2$ (op den Camp et al. 2003, Danon et al. 2005). Within the

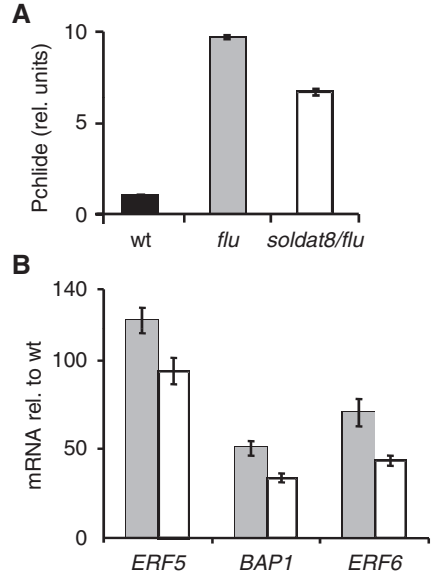

Fig. 1 The amounts of Pchlide (A) and $^1O_2$-mediated changes in gene expression (B) in wild-type (wt), *flu* and *soldat8/flu* seedlings. (A) Seedlings grown for 5 d under continuous light and shifted to 15 h of darkness. By the end of the dark period, Pchlide levels were determined. (B) Seedlings grown for 5 d under continuous light, shifted for 15 h to the dark and re-exposed for 30 min to light. Total RNAs were extracted and the expression levels of the three genes *ERF5*, *ERF6* and *BAP1* in *flu* (gray bars) and in *soldat8/flu* (white bars) were determined relative to those in the wild type.

first 30 min of re-illumination of 4-day-old *soldat8/flu* seedlings, transcripts of *Ethylene Responsive Factor5* and *6* (*ERF5* and *ERF6*), and *Bonsai1 Associated Protein* (*BAP1*) reached levels that were approximately 100-, 40- and 35-fold higher, respectively, than in the wild type and comprised approximately 80% of the corresponding transcript levels in seedlings of the parental *flu* mutant treated in the same way (**Fig. 1B**).

Identification of the *soldat8* gene by map-based cloning

We genetically mapped *SOLDAT8* in F_2 plants from a cross between the homozygous *soldat8/flu* mutant in ecotype Landsberg erecta (Ler) and the *flu* mutant in ecotype Columbia (Col-0). The *flu* line of ecotype Col had been obtained by seven backcrosses of *flu* Ler with Col wild type. Homozygous *soldat8/flu* F_2 seedlings were distinguished from the wild type on agar plates under non-permissive dark/light conditions by their lighter green color. The *soldat8* phenotype co-segregated with cleaved amplified polymorphic sequence (CAPS) and simple sequence length polymorphism (SSLP) markers in 120 F_2 individuals, placing *SOLDAT8* on chromosome 2 (**Fig. 2A**). For the subsequent fine mapping, the size of the mapping population was increased to a total of 470 F_2 homozygous *soldat8/flu* plants. *SOLDAT8* was located on a genomic fragment of approximately 55 kb (**Fig. 2A**). This fragment was sequenced and shown to contain 13 open reading frames. In one of the genes (At2g36990) encoding the chloroplast sigma factor SIGMA6, a single point mutation changing a thymine to a cytosine in the seventh exon of the gene was detected (**Fig. 2A**). This base pair exchange leads to a stop codon in amino acid position 354 within the conserved sigma 70-like r2 domain (Fujiwara et al. 2000). Transcripts of the mutated gene reached approximately 20–30% of the wild-type level (**Fig. 2D**).

The complementation of the soldat8 mutation

The identification of *soldat8* was confirmed by complementing the *soldat8/flu* double mutant. A genomic fragment of the wild-type *SOLDAT8* gene was stably integrated into the genome of the homozygous *soldat8/flu* double mutant by using *Agrobacterium tumefaciens* for transformation. Seeds of independent T_3 homozygous lines were collected and germinated under light/dark cycles. The *flu* phenotype could easily be scored by the bleaching of seedlings. Wild-type and *soldat8/flu* double mutants developed similarly except that the Chl content of the double mutant reached only about 60–70% of that of wild-type seedlings and the size of chloroplasts of young mutant seedlings was reduced (data not shown). Seedlings of the *soldat8/flu* double mutant that had been transformed with the genomic fragment containing the wild-type *SOLDAT8* gene were bleached and phenotypically indistinguishable from seedlings of the parental *flu* line

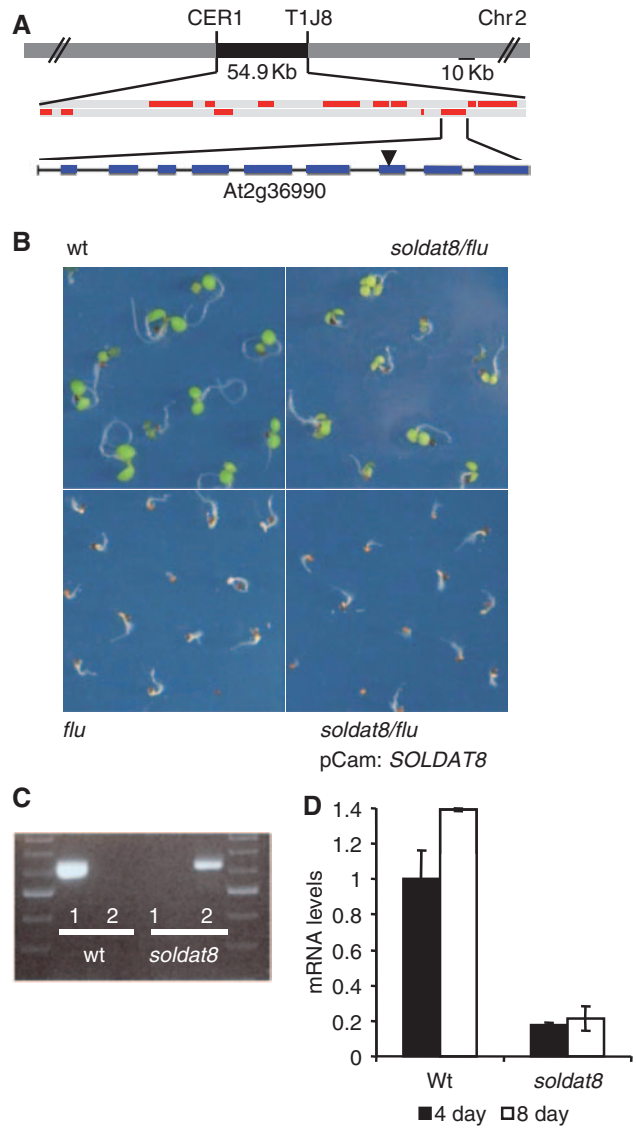

Fig. 2 Identification and isolation of the *SOLDAT8* gene by map-based cloning, sequencing and complementation. (A) Map-based cloning of the *SOLDAT8* gene. The gene was localized on a 54.9 kb fragment of chromosome 2. This fragment contained 14 open reading frames (red boxes). After sequencing, the gene At2g36990 encoding SIGMA6 was shown to carry a mutation in one of its nine exons (blue boxes) that leads to a premature stop codon (arrowhead). (B) Complementation of the mutation by the *SOLDAT8* wild-type gene. Seedlings of the *soldat8/flu* double mutant complemented with the genomic *SOLDAT8* gene behaved like the parental *flu* line, when grown under non-permissive dark/light conditions. (C) Selection of *soldat8* mutants in the wild-type background by PCR analysis. *soldat8/flu* was crossed with the wild type and, among the F_2 seedlings, *soldat8* homozygous mutants (2) were distinguished from *SOLDAT8* wild-type plants (1) and their DNAs were analyzed by using forward and wild-type-specific reverse (wt) or *soldat8*-specific reverse *soldat8* primers. In a similar PCR analysis, *flu*- and *FLU*-specific reverse primers were used to identify homozygous *soldat8*/*FLU* plants. (D) Transcript levels of *SIGMA6* in 4- and 10-day-old light-grown seedlings of *soldat8* and the wild type.

(Fig. 2B). Thus, suppression of the $^1O_2$-mediated cell death response of *flu* seedlings was caused by the impairment of the SIGMA6 subunit of plastid-encoded RNA polymerase (PEP).

The characterization of soldat8 in the wild-type background

The physiological consequences of the *soldat8* mutation and the assessment of its possible impact on $^1O_2$-mediated stress responses were determined in *soldat8* mutants lacking the *flu* mutation. The *soldat8/flu* double mutant was crossed with wild-type (Ler), and homozygous *soldat8* mutants in a wild-type background were selected in two steps from the segregating F_2 generation of this cross. First, seedlings with lighter green cotyledons were identified as putative homozygous *soldat8* mutants. In a second step, a PCR analysis was used to confirm the presence of two mutated alleles of the *SOLDAT8* locus (Fig. 2C).

There are six different sigma factors in *Arabidopsis* that confer different promoter specificities to the PEP during chloroplast gene transcription in response to various developmental and environmental cues (Allison 2000). Amongst them, SIGMA6 has been reported to act as a major sigma factor in chloroplasts during early development of light-grown seedlings (Ishizaki et al. 2005, Loschelder et al. 2006), but it may also be active in mature plants (Loschelder et al. 2006). Two null mutants had been described previously that lacked detectable transcript levels of *SIGMA6* and had an impaired chloroplast development in light-grown seedlings. Cotyledons of *sig6-1* were initially pale green, but reached Chl levels similar to those in the wild type after 8 d of seedling development (Ishizaki et al. 2005), whereas cotyledons of *sig6-2* turned yellowish and finally white (Loschelder et al. 2006). The following true leaves of both mutants developed similarly to those of the wild type. Transcript levels of most PEP-dependent genes were greatly reduced in 4-day-old seedlings of these mutants, whereas the accumulation of nuclear-encoded RNA polymerase (NEP)-dependent transcripts generally increased. As *soldat8* showed far less severe phenotypic changes than *sig6-1* and *sig6-2*, it was of interest to compare the impact of *soldat8* on plastid gene expression with those reported earlier for the two other *sigma6* mutants. Global plastid gene expression in *soldat8* and wild-type seedlings was first analyzed by using a DNA macroarray, containing 94 of the genes encoded by the plastid genome of *Arabidopsis*. Total RNA was extracted from 4- and 10-day-old seedlings of *soldat8* and the wild type. Radioactively labeled cDNA obtained by reverse transcription of this RNA was used as a hybridization probe. The cDNA of each plastid gene on the filter was present in three different concentrations and in each case had been spotted in duplicate. Hybridizations were repeated three times and carried out with cDNA probes derived from three independent experiments. Results of one of these hybridization experiments are shown in Fig. 3A. The intensities of hybridization signals representing the same gene were averaged. For most genes, no drastic differences in transcript levels between the wild type and *soldat8* mutant could be observed. Transcripts of only two of the genes were up-regulated at least 2-fold in 4-day-old mutant seedlings, encoding the D3 subunit of the NADPH chloroplast dehydrogenase complex (NDHC; 2.75 ± 0.06) and the chloroplast ribosomal protein RPL20 (2.72 ± 0.05) (Fig. 3B), and only one gene, encoding the proline-accepting tRNA (2.59 ± 0.23), was up-regulated in 10-day-old seedlings (Fig. 3C).

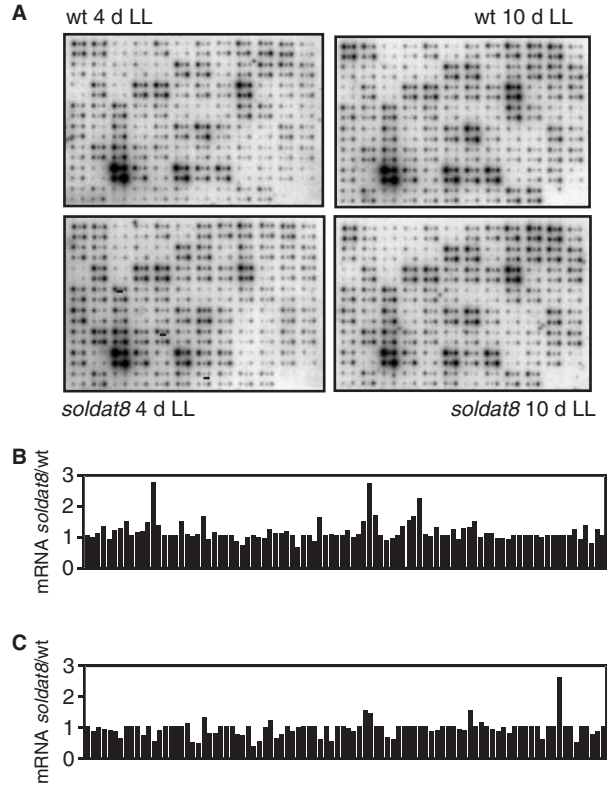

Fig. 3 A comparison of chloroplast DNA transcript levels in 4- and 10-day-old *soldat8* and wild-type (wt) seedlings of *Arabidopsis*. (A) Autoradiograms of chloroplast DNA arrays hybridized with *soldat8* and wild-type (wt) cDNA samples. RNA extracted from wild-type and *soldat8* 4- and 10-day-old seedlings was reverse transcribed into cDNA, using radioactively labeled dCTP. The labeled cDNA was hybridized with nylon filters containing probes corresponding to 94 plastid genes. (B, C) The chloroplast DNA macroarray shown in (A) was used to quantify the transcript levels of 94 plastid genes in seedlings of *soldat8* relative to those of the wild type on day 4 (B) and day 10 (C) of seedling development. Intensities of duplicate spots representing the same gene were averaged and expressed as percentages of the total signal on the array after subtracting the background. Each value represents the mean value of three independent experiments.

In order to exclude the possibility that a limit in sensitivity of the method used for quantification was the reason for the apparent lack of more drastic transcript changes in the *soldat8* mutant, transcript levels of 23 selected plastid genes, most of which had previously been shown to be up- or down-regulated in *sig6-1* and *sig6-2* mutants (Ishizaki et al. 2005, Loschelder et al. 2006), were analyzed by quantitative real-time PCR (**Fig. 4A, B**). For the majority of these selected plastid genes, transcript levels in 4- and 10-day-old *soldat8* mutant seedlings were identical or very similar to those in wild-type control seedlings of the same age (**Fig. 4A**). In 4-day-old *soldat8* seedlings, only transcripts of the two PEP-dependent genes, *psbA* encoding the D1 protein of the PSII reaction center and *rrn16* encoding the 16S rRNA, were slightly down-regulated in *soldat8* relative to the wild type, in contrast to the drastic down-regulation of these genes in *sig6-1* and *sig6-2*. Transcripts of other PEP-dependent genes that were strongly affected in *sig6-1* and *sig6-2* reached similar levels in *soldat8* and in the wild type. Transcripts of eight genes transcribed by the NEP of the plastid reached at least a ≥2-fold level in 4-day-old *soldat8* compared with the wild-type controls (**Fig. 4B**). A similar up-regulation of these NEP-dependent transcripts had been described earlier in *sig6-1* and *sig6-2* (Ishizaki et al. 2005, Loschelder et al. 2006), in PEP-deficient mutants (Legen et al. 2002) and in *sigma2* (*sig2*) knock-out mutants (Nagashima et al. 2004). Four of these genes encode the β subunit of acetyl-CoA carboxylase (*ACCD*), a caseinolytic protease (*CLPP*) and two hypothetical proteins (*YCF2.1* and *YCF2.2*), whereas the remaining four encode subunits of PEP (*RPOA*, *RPOB*, *RPOC1* and *RPOC2*) (Hess and Börner 1999) (**Fig. 4B**). Up-regulation of these genes occurred transiently in 4-day-old seedlings; in 10-day-old seedlings relative transcript levels, except for those of *CLPP*, had been reduced again and reached the levels of the wild-type controls (**Fig. 4A**). Hence, the mutant seems to compensate for the loss of SIGMA6 activity by enhancing the concentration of the PEP core enzyme. Since each of the remaining sigma factors may interact with PEP, transcript levels of the corresponding genes *SIG1*, *SIG2*, *SIG3*, *SIG4* and *SIG5* were determined in 4- and 10-day-old *soldat8* seedlings and compared with those of wild-type controls. Transcripts of all five genes reached similar relative levels that did not differ from those of the controls (**Fig. 5**).

The lower *rrn16* transcript level in *soldat8* may reduce the capacity of plastids to translate plastid-specific mRNAs and would be expected to affect primarily the concentration of chloroplast proteins with a high turnover rate. Immunodetection of several plastid-encoded proteins, such as the β and δ subunits of ATP synthase, the large subunit of ribulose 1,5 bisphosphate carboxylase and the D2 protein of the PSII reaction center, as well as major nuclear-encoded plastid proteins [light-harvesting complex II (LHCII), FLU protein, γ-subunit of ATP synthase, psbS subunit of PSII] revealed no

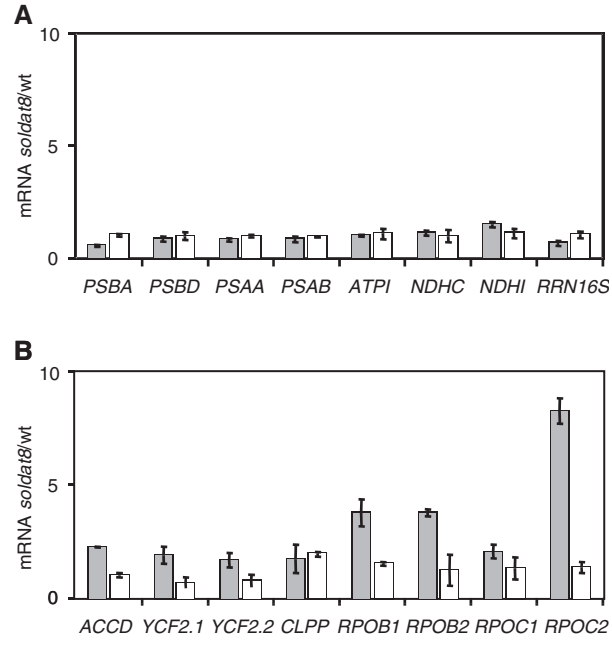

Fig. 4 A comparison of transcript levels of a selected subset of plastid genes analyzed in 4- (black bars) and 10-day-old (white bars) seedlings of *soldat8* relative to the corresponding wild-type levels by real-time PCR. (A) Most of the gene transcripts reached similar levels in 4- and 10-day-old seedlings of the wild type and *soldat8*. (B) For a total of eight genes, transcripts reached at least a 2-fold higher level in *soldat8* than in the wild type of 4-day-old but not of 10-day-old seedlings, except for *CLPP* with higher transcript levels also in 10-day-old *soldat8* seedlings.

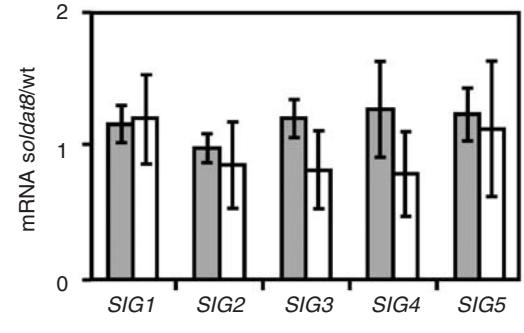

Fig. 5 Expression of *SIGMA* genes of *Arabidopsis* during early seedling development. Transcript levels of *SIGMA 1*, *2*, *3*, *4* and *5* in *soldat8* were expressed relative to wild-type levels in 4- (black bars) and 10-day-old (white bars) seedlings. The values represent means and standard deviations of three independent experiments.

obvious differences between *soldat8* and wild-type seedlings grown in the light for either 4 or 10 d (**Fig. 6**). However, the concentration of the D1 protein of PSII was strongly reduced in 4-day-old *soldat8* seedlings relative to the wild type, whereas in 10-day-old wild-type and *soldat8* seedlings this

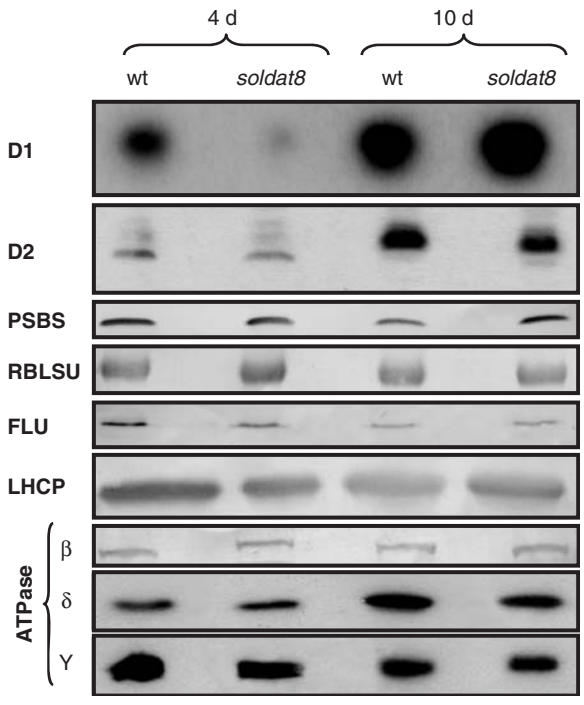

Fig. 6 The effect of the *soldat8* mutation on the levels of various plastid proteins in 4- and 10-day-old seedlings of the wild type and *soldat8*. Except for the D1 protein, which was hardly detectable in 4-day-old *soldat8* seedlings, all other proteins reached similar levels in 4- and 10-day-old wild-type and *soldat8* seedlings. Wild-type and *soldat8* seedlings were grown under continuous light. The blots were incubated with specific antibodies and the immunoreactive bands were detected with horseradish peroxidase or alkaline phosphatase.

protein reached similar levels that were significantly higher than in 4-day-old wild-type seedlings (**Fig. 6**). Among the proteins synthesized within chloroplasts, the D1 protein probably has the highest turnover rate. It forms part of the PSII core complex and plays a crucial role for photosynthetic electron transport and photoprotection of PSII (Andersson and Aro 2001). The deficiency of the D1 protein in 4-day-old seedlings of *soldat8* would be expected to impact the activity of PSII and to enhance the sensitivity of seedlings to light stress. The functional state of PSII in intact wild-type and *soldat8* mutant seedlings was assessed by measuring kinetics of Chl fluorescence induction with an imaging fluorometer in 3- to 7-day-old seedlings grown on agar plates under continuous light (**Fig. 7A, B**). The maximum quantum efficiency of PSII photochemistry, measured as the ratio of variable to maximum fluorescence (F_v/F_m), was reduced from 0.86 ± 0.009 in the wild type to 0.54 ± 0.004 in 3-day-old *soldat8* mutant seedlings (**Fig. 7A**). This value in mutants rapidly increased during the following days, and in 5-day-old seedlings had reached a similar value to that in wild-type controls (**Fig. 7A**). Also the non-photochemical quenching (NPQ) was strongly impaired in 3-and 4-day-old seedlings of *soldat8*, but reached wild-type levels in older seedlings (**Fig. 7B**).

Constitutive acclimation to light stress of soldat8 mutant seedlings

Inhibition of PSII activity and the reduced NPQ capacity should enhance the susceptibility of *soldat8* to light stress at the beginning of seedling development, even when grown under very low light. Minor stress is known to trigger an acclimatory response (Prasad et al. 1994, Chang et al., 2004, Ledford et al. 2007) that may enhance the resistance of *soldat8* seedlings to a subsequent more severe stress. This prediction was tested experimentally by growing *soldat8* and wild-type seedlings for 6 d at 20°C first under continuous very low light conditions (10 µmol m^{-2} s^{-1}) to minimize the impact of light stress on the development of wild-type seedlings (**Fig. 8A**). Afterwards the light was switched off and over the next 4 h the temperature of the growth chamber was lowered to 4°C, before seedlings were re-exposed to a continuous light of higher intensity (250 µmol $m^{-2}s^{-1}$) and kept at 4°C for the next 72 h (**Fig. 8A**). Responses of seedlings were registered by measuring changes in the maximum quantum efficiency of PSII that may be due to stress-induced photoinhibition. The F_v/F_m values, ranging from >0.8 for non-stressed plants down to 0 for fully bleached seedlings, were subdivided into seven groups (**Fig. 8C, D**). At the beginning of the combined low temperature/high light stress program, all mutant and wild-type seedlings were found in groups with F_v/F_m values between 0.65 and >0.85, with *soldat8* mutant seedlings having lower F_v/F_m values than the wild type. After 24 h of stress, F_v/F_m values of both the mutant and wild type were reduced, indicating that all seedlings suffered from light stress. Wild-type seedlings appeared to be more affected by light stress and the majority of them (80%) were found in the group covering F_v/F_m values between 0.35 and 0.45, whereas most of the *soldat8* seedlings (65%) showed higher F_v/F_m values (**Fig. 8C, D**). Seedlings of *soldat8* did not change their maximum quantum yield of PSII during the following 48 h and apparently were able to tolerate the continuous stress, whereas wild-type seedlings under the same conditions were severely affected and roughly 40% of them finally bleached and died. If this difference between the responses of wild-type and *soldat8* seedlings to the combined low temperature/high light stress was due to a lack of stress acclimation in wild-type seedlings, an additional higher light pre-stress preceding the following more severe high light/low temperature treatment would be expected to enhance significantly the stress resistance of these seedlings. This prediction was tested with seedlings subjected to the same light program as used in the previous experiment, except that on the fifth day the low light treatment was interrupted by a brief 6 h high light treatment of 250 µmol m^{-2} s^{-1} at room temperature (**Fig. 8B**). At the beginning of

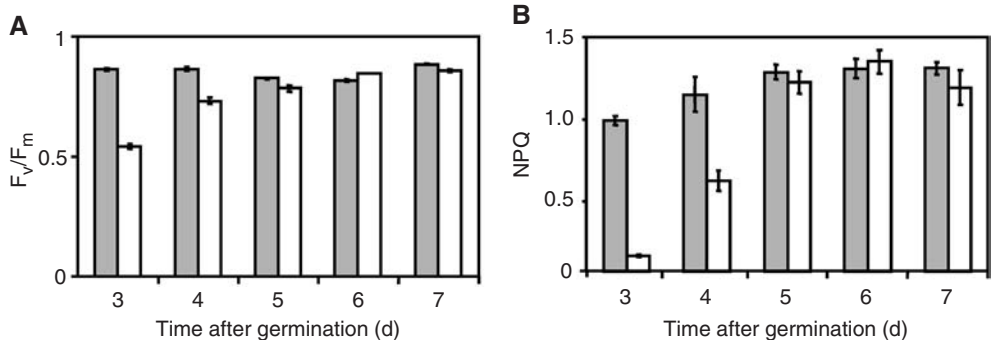

Fig. 7 In vivo measurements of the maximum quantum efficiency of PSII and non-photochemical quenching (NPQ) in wild-type and *soldat8* seedlings. (A) Changes of the maximum quantum efficiency of PSII, F_v/F_m, during seedling development of the wild type (gray bars) and *soldat8* (white bars). (B) Changes of the NPQ during seedling development of the wild type (gray bars) and *soldat8* (white bars).

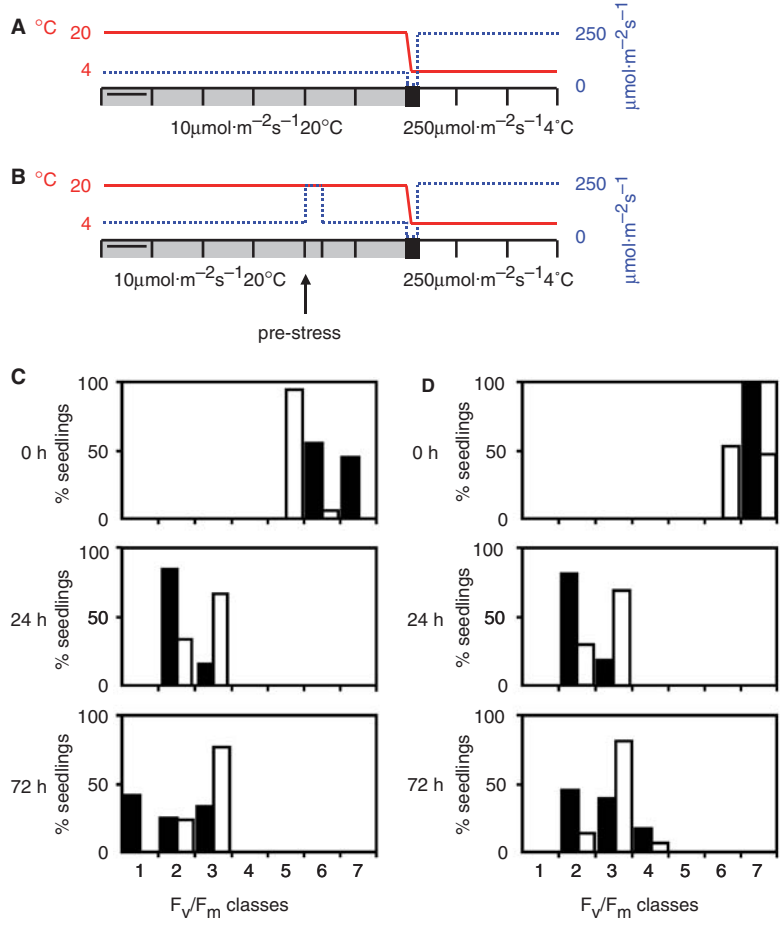

Fig. 8 The effect of a combined high light/low temperature stress on seedlings of the wild type and *soldat8*. (A, B) Seedlings were exposed to two different light/temperature regimes. During the first 6 d, seedlings were grown at room temperature under low light (10 µmol m^{-2} s^{-1}) without (A) or with (B) a brief 6 h acclimatory high light treatment (250 µmol m^{-2} s^{-1}) on day 5. On day 7, seedlings were shifted to the dark for 4 h and the temperature was lowered to +4°C. Afterwards seedlings were exposed to high light (250 µmol m^{-2} s^{-1}) and low temperature. (C, D) In seedlings without (C) and with (D) a pre-stress photoinhibition, a decline of the maximum quantum efficiency of PSII in wild-type (black bars) and *soldat8* (white bars) seedlings was measured. F_v/F_m values ranging from >0.8 (non-stressed seedlings) to 0 (bleached seedlings) were subdivided into seven classes (7 = ≥0.85, 6 = 0.75–0.85, 5 = 0.65–0.75, 4 = 0.55–0.65, 3 = 0.45–0.55, 2 = 0.35–0.45, 1 = 0). Values in (C, D) were taken from approximately 30 plants for each sample.

the seventh day, plants were shifted to the dark for 4 h and at the same time the temperature was lowered from 20°C down to 4°C. Afterwards seedlings were re-exposed to a 25-fold higher light intensity than before the dark period, and for the next 72 h stress responses of plants were monitored by measuring F_v/F_m values and the percentage of bleached seedlings. After 72 h of combined high light/low temperature treatment, none of the pre-stressed wild-type seedlings was bleached anymore and their F_v/F_m values were significantly higher than in non-pre-stressed wild-type seedlings (compare **Fig. 8C and D**). The brief high light pre-treatment of wild-type seedlings was clearly effective in improving the seedlings' ability to withstand the following more severe combined high light/low temperature stress. In *soldat8* mutants exposed to the same pre-stress light treatment, the F_v/F_m values were slightly higher than in non-pre-stressed mutant seedlings at the end of the low light period (**Fig. 8C, D**), but both plant groups were equally well protected during the 72 h of high light/low temperature stress, suggesting that *soldat8* plants without the pre-stress treatment were already acclimated.

Discussion

In the present work, the *flu* mutant of *Arabidopsis* has been used to study the biological activity of $^1O_2$. We have exploited the ability of the *flu* mutant to accumulate free Pchlide in the dark that upon illumination acts as a photosensitizer and generates $^1O_2$ by transferring light energy to ground-state, triplet molecular oxygen, thereby elevating it to the excited singlet state (Gollnick 1968). Previously, two stress responses of the *flu* mutant had been described, seedling lethality and growth inhibition of mature plants, which were triggered by the release of $^1O_2$ within the plastid compartment (op den Camp et al. 2003). Under the experimental conditions used, these stress responses were not caused by the cytotoxicity of $^1O_2$. This conclusion was based on the identification and characterization of the *executer1* mutation that in *flu* abrogates both stress responses (Wagner et al. 2004).

Here we have analyzed another group of second-site mutants of *flu* that suppress $^1O_2$-mediated death of seedlings, but not the growth inhibition of mature plants. Nineteen *soldat* mutants were identified during an extensive second-site mutant screen, most of which were non-allelic and represented different genes, very much in contrast to the 15 *executer/flu* mutants isolated during the same screen, that had been shown previously to represent only a single gene, *EXECUTER1* (Wagner et al. 2004). One of the *soldat* mutants, *soldat8*, encodes SIGMA6. Sigma factors are nuclear-encoded proteins that regulate promoter selectivity of PEP in chloroplasts of higher plants in response to different developmental and environmental cues (Allison 2000). In *Arabidopsis*, six different members of this protein family have been described (Isono et al. 1997, Tanaka et al. 1997, Fujiwara et al. 2000, Hakimi et al. 2000). Among these, SIGMA6 has been shown to act primarily during early seedling development (Ishizaki et al. 2005, Loschelder et al. 2006).

Young seedlings of *soldat8* showed a cotyledon-specific phenotype caused by slightly reduced amounts of Chl and a delay in chloroplast development. With increasing age, seedlings of *soldat8* rapidly restored normal development and after 10 d were no longer distinguishable from the wild type. These observations support the proposed specific role of SIGMA6 during early seedling development and explain why suppression of $^1O_2$-mediated stress responses by *soldat8* was mainly affecting the seedling stage.

There are at least two ways in which impairment of *SIGMA6* in *soldat8* could suppress $^1O_2$-mediated stress responses of the *flu* mutant. Inactivation of *SIGMA6* in the two null mutants *sig6-1* and *sig6-2* leads to a general severe disturbance of plastid development in light-grown seedlings (Ishizaki et al. 2005, Loschelder et al. 2006) due to the suppression of plastid genes essential for the assembly of photosynthetic membranes and Chl accumulation. Seedlings of *sig6-1* and *sig6-2* resemble other *xantha-* and *albino*-like mutants of *Arabidopsis* with a block of chloroplast development confined to cotyledons (Albrecht et al. 2006). Such a severe distortion of plastid development in young seedlings is likely to interfere with the overaccumulation of Pchlide in *flu* seedlings transferred from continuous light to the dark and $^1O_2$ production during re-illumination of such seedlings. However, in contrast to *sig6-1* and *sig6-2*, plastid development in young *soldat8* seedlings is far less disturbed. Four-day-old *soldat8* seedlings contained similar amounts to the wild type of major plastid proteins that form part of or are associated with photosynthetic membranes or accumulate within the stroma. Furthermore, after transfer to the dark, 4-day-old seedlings of *soldat8/flu*, similar to the parental *flu* line, overaccumulate excess amounts of free Pchlide and upon reillumination rapidly up-regulate $^1O_2$-responsive stress-related nuclear genes. Collectively, these results indicate that suppression of $^1O_2$-mediated stress responses in *soldat8/flu* seems not to be caused by a block in $^1O_2$ production and/or $^1O_2$-mediated control of nuclear genes involved in triggering $^1O_2$-dependent visible stress responses of *flu* seedlings.

Among the photosynthesis-related proteins whose concentrations were compared in seedlings of *soldat8* and the wild type, the D1 protein was strongly affected. Its concentration was drastically reduced in 4-day-old mutant seedlings, whereas other proteins reached similar levels to those in the wild type. D1 is one of the core proteins of the reaction center of PSII that plays a key role in the photoinhibition–repair cycle of PSII (Andersson and Aro 2001). Photoinhibition has been associated with the release of ROS that have been suggested to inactivate PSII by direct interaction with the D1 protein (Sharma et al. 1997, Trebst 2003)

or by inhibition of the repair of photodamaged PSII (Nishiyama et al. 2006). $^1O_2$ is the main ROS that is continuously produced by PSII even under low light conditions (Keren et al. 1995, Jansen et al. 1999). The reduced amounts of the D1 protein in young *soldat8* seedlings may be due to an increased degradation or a diminished synthesis of the protein, or a combination of both. In either case it would lead to an enhanced production of ROS by PSII that could cause photooxidative stress even at light intensities that have no apparent negative impact on wild-type seedlings. A possible impairment of PSII activity in young mutant seedlings grown under low light conditions was assessed by monitoring the Chl fluorescence changes in wild-type and mutant seedlings. The maximum quantum efficiency of PSII (F_v/F_m) of 3-day-old mutant seedlings was 30% lower than in the wild type. This reduced F_v/F_m value of young *soldat8* seedlings indicates a partial inhibition of photosynthesis that correlates with the decreased amounts of the D1 protein.

Chl fluorescence induction kinetics revealed higher levels of fluorescence in the mutant than in the wild type, indicating a reduced capacity of *soldat8* to use light energy for photochemistry (data not shown). The mutant showed the same maximum fluorescence after a saturating light pulse as the wild type, but the basal level of fluorescence (F_o) was higher, resulting in lower F_v/F_m values. An increase in F_o has been interpreted as a reduction of the rate constant of energy trapping by PSII centers (Havaux 1993), which may be due to either physical dissociation of LHCs from the PSII core or a damaged PSII core. The dissociation of antenna from the core complex has been observed during heat stress, leading to an impairment of PSII and a higher F_o value (Armond et al. 1980). The reduced levels of D1 in young seedlings of *soldat8*, however, suggest that a lower activity of PSII core rather than a disturbed interaction between core and antenna of PSII might be the cause of the higher F_o values of the mutant. Collectively, these data suggest that light per se is a constant stress factor for *soldat8* during early seedling development.

Excess light may evoke an acclimatory response that enhances the plant's ability to anticipate and resist a more severe subsequent stress. For instance, acclimation to low levels of $^1O_2$, medium intensity light, lower or higher temperatures and wounding have been shown to enhance the stress tolerance of the plants markedly (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). This acclimation may not only provide protection against the same stress that initially was 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, Ishikawa et al. 2005).

The sublethal 6 h light stress during the initial growth at room temperature rendered wild-type seedlings more resistant to the subsequent, normally lethal combination of low temperature and high light stress. In *soldat8* seedlings, however, this pre-stress treatment failed to improve the seedlings' stress tolerance further. Seedlings of *soldat8* without such a pre-stress treatment were already acclimated and reached a tolerance to the more severe stress similar to that of wild-type seedlings after they had been exposed to the sublethal pre-stress. This constitutive acclimation of *soldat8* seedlings to light stress seems to mitigate the $^1O_2$-mediated death response in the *soldat8/flu* double mutant.

Materials and Methods

Plant material

All experiments were performed with *A. thaliana* ecotypes Ler and Col-0. Seeds were surface-sterilized and plated on Murashige and Skoog medium with 0.8% agar, supplemented with sucrose (5 g l^{-1}). Light was provided by white light tubes (Philips Master TDL 36W, Philips Electronics NV, Eindhoven, The Netherlands and Sylvania Gro Lux F36W, SLI Lichtsysteme GmbH, Erlangen, Germany).

Isolation of the soldat8 mutant

To identify *soldat8* mutants without the *flu* mutation, *soldat8/flu* double mutants were crossed with wild-type plants (Ler). Among the F_2 progeny, putative homozygous *soldat8* mutants were pre-selected based on the slightly pale green color of their cotyledons. Gene-specific primers for the *soldat8* mutation (GCGAATCTGCATCCGGATTA) or for the wild type (GCGAATCTGCATCCGGATTG) in combination with a wild-type reverse primer (GATGATGCGC TTCAGCTCT) 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* genes using gene-specific primers for the *flu* mutation (CCAAGGGAAG TATAGGGAAGT) or for the *FLU* wild-type gene (CCAAGGG AAGTATAGGGAAGG) and a wild-type reverse primer (GGCAATTGGCACTTAAGATGGC).

Identification and complementation of the soldat8 mutation

The *SOLDAT8* locus was mapped on chromosome 2 using CAPS or SSLP markers listed in The *Arabidopsis* Information Resource database (TAIR, www.Arabidopsis.org). Additional markers used for the mapping were designed based on the collection of predicted *Arabidopsis* single nucleotide polymorphisms (SNPs) and small insertions/deletions (INDELs) in the publicly available Col-0 and Ler sequences generated by Monsanto (http://www.Arabidopsis.org/Cereon).

The 54.9 kb genomic fragment flanked by the two closest markers which could be mapped was sequenced. Overlapping primers covering the whole fragment were designed and PCR was performed on genomic DNA from the *soldat8/flu* double mutant and *flu*, which served as a control. The amplified

products were purified and approximately 100 ng of DNA per sample was sequenced by Microsynth (Balgach, Switzerland). Sequences were compared with those of the sequenced *Arabidopsis* genome, using the SeqViewer tool from TAIR.

Specific primers flanking the *SOLDAT8* genomic sequence, including the promoter regions, were used for amplification. The correct amplification was confirmed by sequencing the fragments. The genomic PCR product was cloned in the pCambia 3300 plasmid, and selected clones were transferred by electroporation to *A. tumefaciens* C58C1 cells. Homozygous *soldat8/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 medium (25 µg Basta ml^{-1}). Vernalized T_2 seeds were sown on MS-Basta medium 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.

Chloroplast gene macroarrays

A total of 94 plastid genes of *Arabidopsis* encoding photosynthetic proteins, rRNAs and tRNAs were amplified by PCR using gene-specific primer pairs. Genomic DNA of the wild type (Col) was used as a template. The genes were amplified using a standard PCR protocol with the following conditions: 30 cycles including 20 s denaturation at 94°C, 1 min annealing at 60°C and 1 min elongation at 72°C. The purified PCR products were diluted into three different concentrations (30, 7.5 and 1.87 g μl^{-1}, respectively). The 94 probes were spotted onto the 11.9 cm×7.8 cm positively charged nylon membrane (Hybond™-N+ Amersham Pharmacia Biotech, Freiburg, Germany) by using a 96-pin tool [0.4 mm pins with a BioGrid spotting Device-Roboter (BioRobotics, UK)]. Each probe was spotted 20 times in order to obtain final DNA quantities of 1.25, 5 or 20 ng per sample on the different spots. Three different concentrations of one gene were spotted in duplicate onto the filters. The spotted membranes were denaturated in 1.5 M NaCl, 0.5 M NaOH and afterwards neutralized in 0.5 M Tris–HCl pH 7.2, 1 M NaCl. The filters were subsequently cross-linked using 120 mJ of UV light (302 nm) (Stratalinker UV crosslinker 1800; Stratagene, La Jolla, CA, USA) and dried. Before hybridization, the filters were incubated at 65°C for 1 h in 10 ml of 0.25 M Na_2HPO_4 pH 7.2, 7% SDS.

Total RNA from the wild type and *soldat8* grown for either 4 or 10 d under continuous light was used to synthesize [α-^{32}P]dCTP-labeled DNAs with hexanucleotides (Roche, Mannheim, Germany) using the SuperScript T11 III RNase H– Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). After hybridization, the washed filters were exposed to phosphor image plates for at least 3 d. The radioactive images were scanned with a FLA-3000 phosphoimager (Fuji, Tokyo, Japan) and the obtained signals were imported to the AIDA Image Analyzer (3.25) software for background correction and normalization. The background noise was corrected after alignment and integration of the spots. For background subtraction, the mean value of three selected background dots within a subgrid containing six spots of a gene probe was calculated. Their intensity values were averaged and then used as a background value to all spots of the subgrid. Afterwards, all spots in a filter were used to calculate the normalization constant. The intensity value of the spots corresponding to a particular gene was compared between the wild type and the mutant using the software AIDA Array Compare 3.25 (raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

RNA isolation and real-time PCR analysis

Seedlings were frozen in liquid nitrogen and ground to a fine powder with a mortar and a pestle. A 50–100 mg aliquot of powder was transferred to a 2 ml Eppendorf tube, and 1 ml of TRIZOL® Reagent (Invitrogen AG, Basel, Switzerland) was added. The tubes were centrifuged at 11,400 r.p.m. for 15 min at 4°C (Eppendorf 5402) and the supernatants were transferred to a new tube. Chloroform was added (300 µl) and vortex was applied for 15 s. The samples were centrifuged again at 11,400 r.p.m. for 15 min at 4°C and the aqueous phase was transferred to a new tube. After the addition of 750 µl of isopropanol, the samples were incubated for 10 min at room temperature and centrifuged at 11,400 r.p.m. for 10 min at 4°C. After washing the pellet with 1 ml of 75% ethanol, the samples were centrifuged at 9,000 rpm for 5 min at 4°C. The pellet was air-dried and dissolved in 150 µl of RNase-free water. The RNA concentration was calculated according to Sambrook et al. (1989). To assess the quality of RNA, an aliquot containing 2 µg was loaded on a 2% agarose gel. The RNA was stored at −80°C. Real-time PCR was done as described previously by Danon et al. (2005).

In vivo measurement of photosynthetic activity

Chl *a* fluorescence measurements were used as a non-invasive technique to monitor photosynthetic processes in plants. The FluorCam System (Photon Systems Instruments, Brno, Czech Republic) is equipped with a sample chamber containing a CCD video camera and an irradiation system. The standard quenching analysis protocol provided by FluorCam calculates the maximum quantum efficiency of PSII and non-photochemical and photochemical quenching. For the quenching analysis of modulated fluorescence by the saturation pulse method, seedlings were first dark-adapted for 15 min. After applying a weak measuring beam for 2 s to obtain the minimum level of fluorescence (F_o), a first saturating pulse (0.8 s at 2,000 µmol m^{-2} s^{-1}) raised the fluorescence to its maximum level (F_m). After 1 min dark relaxation,

constant actinic light (350 µmol m^{-2} s^{-1} at 50% of intensity) was switched on and saturating pulses were applied during 1 min at intervals of 10 s. The minimum fluorescence value (F_o') was calculated after switching off the actinic light.

Other methods

Chemical mutagenesis of seeds from *flu* mutant plants with ethylmethanesulfonate (EMS) was performed as described previously (Wagner et al. 2004). For visualization of Pchlide accumulation, etiolated seedlings from the wild type, *flu* and *soldat8/flu* were illuminated with blue light and examined under a Leica MZ12 fluorescence microscope with a Leica FM blue 10446146 filter (Leica Microsystems AG, Wetzlar, Germany). The bright red fluorescence emitted by the mutant is caused by the excitation of free Pchlide. Pchlide determination by HPLC and separation and immunodetection of proteins were done as decribed in Goslings et al. (2004).

Funding

The Swiss Federal Institute of Technology (K.A.); the Swiss National Science Foundation (K.A.); the Deutsche Forschungsgemeinschaft SFB TR1 (J.M.).

Acknowledgments

We are indebted to Dr. Dieter Rubli for art work, to André Imboden for taking care of plants, and to members of our group for many helpful discussions during the completion of this work.

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(Received January 28, 2009; Accepted February 24, 2009)