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# Arabidopsis mutants reveal multiple singlet oxygen signaling pathways involved in stress response and development

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**Abstract** Shortly after the release of singlet oxygen ($^1O_2$) in chloroplasts drastic changes in nuclear gene expression occur in the conditional *flu* mutant of Arabidopsis that reveal a rapid transfer of signals from the plastid to the nucleus. Factors involved in this retrograde signaling were identified by mutagenizing a transgenic *flu* line expressing a $^1O_2$-responsive reporter gene. The reporter gene consisted of the luciferase open reading frame and the promoter of an *AAA-ATPase* gene (At3g28580) that was selectively activated by $^1O_2$ but not by superoxide or hydrogen peroxide. A total of eight second-site mutants were identified that either constitutively activate the reporter gene and the endogenous *AAA-ATPase* irrespectively of whether $^1O_2$ was generated or not (*c*onstitutive *a*ctivators of *A*AA-ATPase, *caa*) or abrogated the $^1O_2$-dependent up-regulation of these genes as seen in the transgenic parental *flu* line (*n*on-*a*ctivators of *A*AA-ATPase, *naa*). The characterization of the mutants strongly suggests that $^1O_2$-signaling does not operate as an isolated linear pathway but rather forms an integral part of a signaling network that is modified by other signaling routes and impacts not only stress responses of plants but also their development.

**Keywords** *Arabidopsis* · Oxidative stress · Singlet oxygen · Signaling · *flu* mutant · AAA-ATPase

## Introduction

In plants under environmental stress enhanced levels of reactive oxygen species (ROS) are generated in various intracellular compartments that may cause oxidative damage or act as signals (Apel and Hirt 2004; Gechev et al. 2006; Laloi et al. 2004). Chloroplasts and peroxisomes have been shown to be major sites of ROS production (Foyer and Noctor 2000, 2003). The enhanced generation of ROS in these cellular compartments has been attributed to the disturbance of the light-driven photosynthetic electron transport by a variety of environmental factors such as high light, low temperature or drought (Long et al. 1994; Niyogi 1999). Under these conditions plants are exposed to light intensities that exceed their capacity to assimilate $CO_2$ and that lead to the hyperreduction of the electron transport chain and ultimately may result in the photoinactivation of photosystem II (PSII). Plants may activate alternative electron sinks to prevent the stress-induced inhibition of photosynthesis. Such a photochemical quenching may be achieved in chloroplasts through the direct reduction of oxygen by reduced electron transport components associated with PSI (Asada 1999; Rizhsky et al. 2003) and by reactions linked to the photorespiratory cycle that result in the enhanced production of hydrogen peroxide ($H_2O_2$) in peroxisomes (Kozaki and Takeba 1996). Additionally, various non-photochemical processes, such as chlorophyll fluorescence or thermal dissipation, participate in the

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dissipation of excess excitation energy (Muller et al. 2001). When these quenching mechanisms are not sufficient to maintain the PSII acceptor site in a partially oxidized state, photoinhibition occurs that stimulates further the production of another ROS, singlet oxygen ($^1O_2$). $^1O_2$ is continuously produced by PSII through energy transfer from excited chlorophyll to oxygen (Krieger-Liszkay et al. 2008), as shown by a basal lipid peroxidation mediated by $^1O_2$ (Triantaphylides et al. 2008) and the rapid turnover of the D1 protein at low light due to oxidative processes (Keren et al. 1997).

The enhanced production of ROS in chloroplasts and peroxisomes has been correlated with drastic changes in nuclear gene expression (Gadjev et al. 2006). Since several chemically distinct ROS are generated simultaneously during stress, it is not possible in wild type plants to attribute these stress-induced changes of nuclear gene expression to enhanced levels of a particular ROS within the plastid compartment. This problem has been partially alleviated by using the conditional *flu* mutant of Arabidopsis that generates $^1O_2$ in plastids in a controlled and non-invasive manner (Meskauskiene et al. 2001; op den Camp et al. 2003). In the dark the *flu* mutant of Arabidopsis accumulates excess protochlorophyllide (Pchlide) that upon illumination acts as a photosensitizer and generates $^1O_2$ (Flors et al. 2006; Gollnick 1968; Hideg et al. 2006; op den Camp et al. 2003). A major consequence of this $^1O_2$ generation is a rapid change in nuclear gene expression that reveals the transfer of $^1O_2$-derived signals from the plastid to the nucleus (Laloi et al. 2006; op den Camp et al. 2003). Many of the $^1O_2$-responsive genes are different from those activated by superoxide ($O_2^{\bullet-}$) or $H_2O_2$, suggesting that $O_2^{\bullet-}$/$H_2O_2$- and $^1O_2$-dependent signaling occurs via distinct pathways (Laloi et al. 2006; op den Camp et al. 2003). These pathways could act independently or may interact with each other. Evidence supporting the latter possibility was obtained after modulating the $H_2O_2$ concentration in *flu* plants non-invasively by overexpressing the thylakoid ascorbate peroxidase (Murgia et al. 2004). The reduction of the $H_2O_2$ concentration accelerated the intensity of $^1O_2$-mediated stress responses, suggesting that $H_2O_2$ either directly or indirectly antagonizes $^1O_2$-mediated signaling (Laloi et al. 2007). Since $^1O_2$ is very reactive and unlikely to leave the plastid compartment (Gorman and Rodgers 1992; Sies and Menck 1992), its physiological impact has been attributed to the generation of more stable second messengers within the plastid that are assumed to activate a signaling cascade outside of the plastid compartment. Thus far, virtually nothing is known about the identity of these postulated messengers and the way they may affect nuclear gene expression.

In our present work we have set up genetic screens to identify mutants impaired in their response to $^1O_2$-mediated signals. The characterization of these mutants strongly suggests that $^1O_2$-signaling does not operate as an isolated linear pathway but rather forms an integral part of a signaling network. The present work forms an important first step towards penetrating the complexity of this network and dissecting interactions of $^1O_2$-mediated signals with other signaling routes.

## Materials and methods

### Plant material and growth conditions

Seeds were either surface sterilized and grown on Murashige and Skoog medium (without sucrose; Murashige and Skoog 1962) including vitamins and MES buffer (M0255; Duchefa, Haarlem, The Netherlands) and 0.8% (w/v) agar (Sigma®, St. Louis, USA) at 20°C in continuous light (80–100 μmol $m^{-2}$ $s^{-1}$) or, for cultivation of mature plants, sown on soil (Klasmann Substrat 2, Klasmann-Deilmann, Geeste, Germany) and grown for 20 days under the same conditions. Prior to the dark-to-light shift treatment, plants were kept in the dark for 8 h and then transferred to the light. The mutant used in this work was a *flu* Col-0 line that had been obtained by five backcrosses of *flu1-1* in Landsberg erecta with wild type Col-0.

### Construction of singlet oxygen-responsive *flu AAA:LUC+* lines

The −2,405 to −4 bp fragment up-stream of the start codon of the *AAA-ATPase* gene was obtained by PCR using primers 5′-AGGAATTCGCGATGCTCTGTAAAGTTG-3′ and 5′-AAAGATCTCTTGAAGCTTTTGGCTTAGG-3′. *EcoRI* and *BglII* restriction enzymes were used for the insertion of the fragment into the binary vector NOS-pPCV containing the luciferase (*LUC+*) gene (Koncz et al. 1994; Toth et al. 2001). *flu* Col-0 mutant plants were transformed with the *AAA:LUC+* construct and plants homozygous for the *AAA:LUC+* construct were selected from the T3 generation. A line homozygous for a single copy of the *AAA:LUC+* transgene, as revealed by segregation analysis of LUC activity and hygromycin resistance in T2 and T3 generations, and showing a low basal luciferase activity and a high induction after the release of $^1O_2$, was selected for further experiments and referred to as parental line (*flu AAA:LUC+*). In order to prevent possible artifacts inherent to the reporter system, the expression of the endogenous *AAA-ATPase* gene was analyzed in parallel.

### Luciferase imaging

An intensified charge-coupled device video camera (model C2400 47; Hamamatsu Photonics, Hamamatsu City, Japan)

in conjunction with an Image Intensifier Controller (model M4314; Hamamatsu Photonics) and Image Processor (Argus 50; Hamamatsu Photonics) was used to image plants within a Hamamatsu Photonics imaging chamber (model A417) mounted with a Xenon CM 120 lens (Schneider, Bad Kreuznach, Germany). Fifteen minutes before luciferase imaging, plants were sprayed with 1 mM luciferin dissolved in 0.1% Tween-20. Images were viewed as photon counting using ARGUS-50 image processing software. The images were always acquired with the same sensitivity level 5 using the slice/gravity mode.

### Treatment of the *flu AAA:LUC+* plants with ROS producing agents

The different ROS producing agents such as Rose Bengal (RB) and paraquat were prepared in 0.1% Tween-20. RB is a potent photosensitizer and produces $^1O_2$ under light conditions. Paraquat is an herbicide, which produces $O_2^{\bullet -}$ in chloroplasts. $O_2^{\bullet -}$ is then rapidly disproportionated to $H_2O_2$ either spontaneously or via catalysis by superoxide dismutases (SOD) (Asada et al. 1974). The concentration of each agent (RB 500 µM, paraquat 50 µM and $H_2O_2$ 2 mM) was adjusted on the basis of the visible intensity of the stress response shown by the plants after 48 h of treatment. The solutions were applied to 8-day-old plants grown on MS plates by spraying with a fine mist sprayer. After 2 h of treatment luciferin was sprayed and images of luciferase activity were acquired for 2 min.

### Isolation of *flu caa* and *flu naa* mutants

Chemical mutagenesis of seeds from the selected *flu AAA:LUC+* line with ethyl methanesulfonate (EMS) was performed as previously described (Runge et al. 1995). Seeds from M1 plants were pooled into 80 different batches and used for the two subsequent mutant screenings.

#### *Screening for flu caa mutants*

Approximately 200 M2 seedlings from each batch (about 16,000 plants) were cultivated by germinating surface-sterilized seeds on MS agar medium, and grown under continuous light (80–100 µmol·photons $m^{-2}{\cdot}s^{-1}$) for 10 days. Fifteen minutes prior to luminescence imaging, luciferin was applied to the seedlings. Mutant seedlings showing constitutive high luciferase activity in comparison to wild type plants were selected and transferred to soil. After the first round of screening 43 putative *flu caa* mutants could be isolated. The M3 seeds of putative *flu caa* mutants were re-screened in two rounds under the same conditions. Finally 6 mutants, *flu caa4, flu caa5, flu caa12, flu caa13, flu caa33* and *flu caa39*, showing robust and reproducible high constitutive luciferase activity, were selected and used for further studies. These 6 mutants were backcrossed twice to the *flu AAA:LUC* + Col-0 parental line and Landsberg erecta (Ler) wild type for further genetic analysis and crude mapping.

#### *Screening for flu naa mutants*

Approximately 200 M2 seedlings from 42 different M2 batches (about 8,500 plants) were grown on soil under continuous light (80–100 µmol·photons $m^{-2}{\cdot}s^{-1}$) for 20 days. M2 plants were then transferred for 8 h to the dark and subsequently re-exposed to light. Seventy five minutes after re-illumination, luciferin was sprayed and 15 min later luminescence imaging was performed. Mutants that did not show any induction of luciferase activity and were able to induce cell death response in rosette leaves were selected. The M3 generation of putative *flu naa* mutants was re-screened under the same conditions. Selected *flu naa* mutants were backcrossed twice to the *flu AAA:LUC+* Col-0 parental line for further analyses.

### RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated according to the method described by Melzer et al. (1990). RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and reverse-transcribed using random hexamers and SuperScript II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Quantitative RT-PCR was performed using the ABI Prism® 7700 Sequence Detection System together with SYBR®Green PCR Master Mix (Applied Biosystems, Foster City, USA). Relative mRNA abundance was calculated by using the comparative $C_T$ method and normalized to the *Profilin 1* (At2g19760) gene levels (Pfaffl 2001). The sequences of primers used in the quantitative RT-PCR are listed in Supplementary Table S7.

### Trypan blue staining

Dead cells were identified by staining with lacto-phenol trypan blue as previously described (Keogh et al. 1980).

### Measurement of the maximum quantum yield of PSII

The *flu AAA:LUC+* parental line, *flu caa* and *flu naa* mutants were grown for 6 days under continuous low light (20 µmol $m^{-2}$ $s^{-1}$) at 22°C in a growth chamber (CLF plant Climatics, Percival Scientific, E-66HO/2). The low-light grown plants were then subjected to photoinhibitory conditions either combining moderate high light and low temperatures (300 µmol $m^{-2}$ $s^{-1}$ at 12°C) or consisting of

higher light intensity (1,000 μmol $m^{-2}$ $s^{-1}$ at 22°C). At the indicated times, the maximum quantum yield of PSII (Fv/Fm) was measured after 15 min of dark adaptation with a Fluor-Cam 700MF (Photon Systems Instruments, Brno, Czech Republic). The length of the 15 min dark adaptation period at 22°C prior to the Fv/Fm measurement was too short to allow a detectable accumulation of free Pchlide.

## Results

### Generation of Arabidopsis transgenic reporter lines expressing a singlet oxygen-inducible *AAA-ATPase* promoter-Luciferase construct

For the identification of factors involved in $^1O_2$-mediated retrograde signaling from the plastid to the nucleus, a forward genetic mutant screen was used. This approach was based on first mutagenizing a transgenic *flu* line that expresses a $^1O_2$-responsive reporter gene and then identifying second-site mutations that lead either to a constitutive up-regulation of the reporter gene irrespectively of whether $^1O_2$ was generated or not, or abrogate the $^1O_2$-dependent up-regulation of the reporter gene as seen in the transgenic parental *flu* line. The reporter gene consisted of the luciferase open reading frame and the promoter of a $^1O_2$-responsive nuclear gene of Arabidopsis. Special emphasis was placed on selecting a suitable $^1O_2$-responsive gene. As shown previously rapid changes in nuclear gene expression occur following the transfer of the conditional *flu* mutant from the dark to the light (Laloi et al. 2006; op den Camp et al. 2003). Our search for a suitable candidate gene was confined to $^1O_2$-responsive genes that were upregulated as early as 30 min after reillumination of predarkened *flu* plants, but not after treatment with paraquat, a terminal oxidant of PSI that in the light generates $O_2^{\bullet-}$ which rapidly dismutates to $H_2O_2$ (Babbs et al. 1989; Laloi et al. 2006; op den Camp et al. 2003). In addition, we took into account that the concentration of several phytohormones, in particular salicylic acid, 12-oxo-phytodienoic acid (OPDA) and jasmonic acid, rapidly increased after the release of $^1O_2$ (Danon et al. 2005; Ochsenbein et al. 2006; Przybyla et al. 2008), that might be responsible for secondary gene inductions. Therefore, genes were excluded that had been shown previously to be also under the control of various phytohormones. This selection was based on a comparison with expression data produced by the AtGenExpress Consortium in which the effects of plant hormones, including salicylic acid, abscisic acid, gibberellin, brassinosteroid, cytokinin, auxin, ethylene, and jasmonate were surveyed with Affymetrix ATH1 GeneChips (http://arabidopsis.org/info/expression/ATGenExpress.jsp) and partially analyzed by Chory et al. (Nemhauser et al. 2006). This analysis revealed that the expression of approximately half of the $^1O_2$-responsive genes was also affected by some of these phytohormones, in particular auxins, salicylic acid, abscisic acid and jasmonic acid (Fig. 1). Among the remaining genes, only those that were reported to be specific to $^1O_2$ (Laloi et al. 2006) and whose expression level in wild type or in *flu* mutant kept in the dark or under continuous light were very low, were selected. Among three genes that fulfilled these criteria, an *AAA-ATPase* gene (At3g28580) was chosen. Its expression level in the *flu* mutant grown under continuous light and exposed to a dark-to-light (D/L) shift was upregulated approximately 10-fold within the first 30 min of illumination (Supplementary Fig. S1a). In addition, transcripts of this gene accumulated after treatment of wild type seedlings with the $^1O_2$-specific photosensitizer RB, but not after paraquat treatment (Supplementary Fig. S1b). AAA-ATPases (ATPases associated with various cellular activities) are chaperone-like proteins that play crucial roles in controlling both the folding status of proteins and the refolding or degradation of aberrant proteins (Mogk et al. 2008). Oxidative stress caused by the release of $^1O_2$ may perturb the folding state of proteins and lead to protein inactivation and the accumulation of misfolded protein species that can affect cellular processes. In this context, the selected $^1O_2$-induced AAA-ATPase protein, which has recently been detected in the endoplasmic reticulum (Dunkley et al. 2006), could play a role in endoplasmic reticulum-associated protein degradation.

The 2,405 bp region upstream of the start codon of the *AAA-ATPase* gene was fused to the *LUC+* luciferase gene (Fig. 2a) and *flu* mutant plants were transformed with this reporter gene construct. Several *flu AAA:LUC+* transgenic reporter lines were obtained in this way and characterized. In each of them the luciferase activity rapidly increased following the release of $^1O_2$ during a D/L shift, but not in the dark (Fig. 2b) or under continuous light (data not shown). The time course of *LUC+* transcript accumulation was very similar to that of the endogenous *AAA-ATPase*, starting as early as 15 min after the D/L shift and reaching a maximum at 60 min within the time period monitored (Fig. 2c). This strong correlation indicates that all *cis*-elements necessary for the transcriptional induction of *AAA-ATPase* by $^1O_2$ seem to be present in the 2,405 bp upstream region of the selected $^1O_2$-responsive gene. To confirm the $^1O_2$ specificity of the reporter line, *flu AAA:-LUC+* plants were analyzed under other ROS-producing stress conditions that, based on the visible intensity of the stress response shown by the plants after 48 h of treatment (data not shown), provoke stress responses of similar intensities: in light-grown *flu AAA:LUC+* plants treated with 500 μM RB, a singlet oxygen-specific photosensitizer (Kochevar et al. 1994), the luciferase activity increased

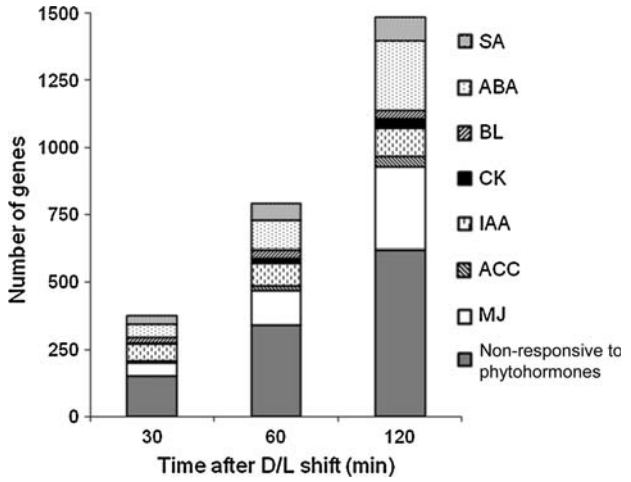


**Fig. 1** Selection of $^1O_2$-responsive genes in the *flu* mutant. $^1O_2$-mediated changes in gene expression were monitored in *flu* mutant plants that had been grown under continuous light, transferred to the dark for 8 h and re-exposed to light for 0.5, 1 and 2 h. At each time point the total number of genes that were up-regulated more than threefold was determined on Affymetrix ATH1 gene chips. Among the $^1O_2$-responsive genes those reported previously to be also up-regulated in response to salicylic acid (*SA*), abscisic acid (*ABA*), brassinolide (*BL*), the cytokinin zeatin (*CK*), indole acetic acid (*IAA*), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (*ACC*) and methyl jasmonate (*MJ*) (Nemhauser et al. 2006) are indicated. The *AAA-ATPase* gene selected for the construction of the luciferase reporter gene was found among the remaining genes that did not respond to any of these hormones and was upregulated in the *flu* mutant within the first 30 min of reillumination, following 8 h in the dark

(Fig. 2d); when *flu AAA:LUC+* reporter plants grown under continuous light were treated with 50 μM paraquat, the reporter gene was not affected (Fig. 2d). In *35S:LUC+* control plants, luciferase activity was unaffected by the different treatments (Fig. 2d).

## Isolation of mutants altered in singlet oxygen-inducible *AAA-ATPase* expression

Seeds of a selected transgenic *flu AAA:LUC+* reporter line (hereafter designated parental line; see "Materials and methods" for the selection criteria) were mutagenized with ethyl methanesulfonate (EMS) and M1 plants were grown under continuous light. M2 seedlings were grown under the same conditions and the luciferase activity was monitored with a CCD camera in order to find mutants that either showed a constitutive high luciferase activity under continuous light (*c*onstitutive *a*ctivator of *A*AA-ATPase, *caa*) or did not show any increase of luciferase activity after the release of $^1O_2$ during a D/L shift (*n*on *a*ctivator of *A*AA-ATPase, *naa*). Screening for *flu AAA:LUC+ caa* and *flu AAA:LUC+ naa* mutants (hereafter designated *flu caa* and *flu naa*), respectively, was performed at two different developmental stages (Fig. 3a). For the identification of *flu*

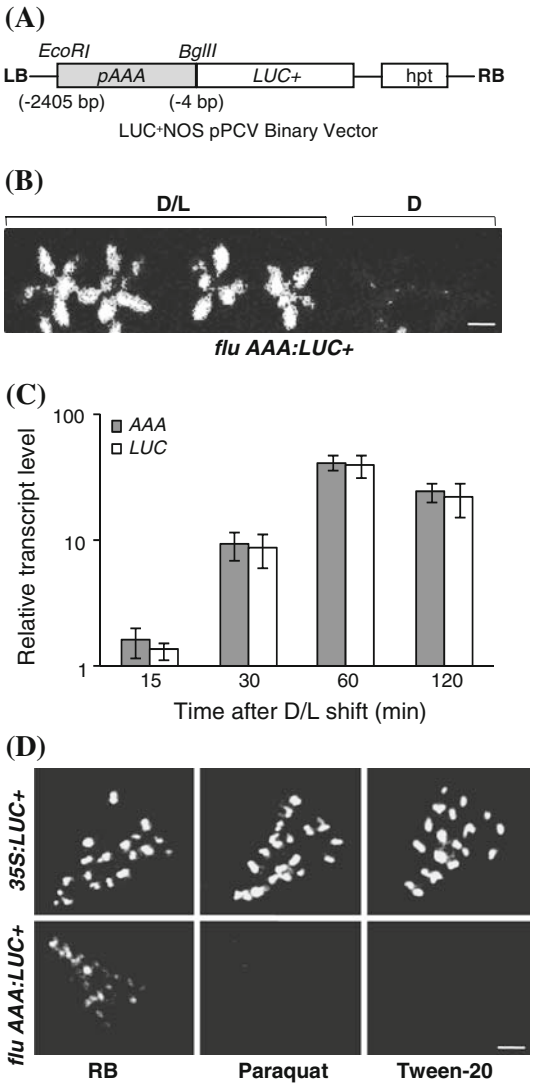


**Fig. 2** Generation of transgenic Arabidopsis plants expressing a singlet oxygen-inducible *AAA-ATPase* (*AAA*) promoter-luciferase reporter construct. **a** Schematic illustration of the *AAA-ATPase* promoter and luciferase reporter construct (*AAA:LUC+*). **b** Luciferase activity detected in 14-day-old *flu* mutant plants transformed with the *AAA:LUC+* construct. *flu AAA:LUC+* plants first grown under continuous light for 14 days were subjected to a 8 h dark and 1 h light (D/L) treatment. Luciferase activity was captured at the end of the 8 h dark period (D) and after the D/L shift. The *scale bar* represents 1 cm. **c** Transcript levels of the endogenous *AAA-ATPase* gene correlate with the expression of *LUC* gene in the *flu AAA:LUC+* reporter line subjected to a D/L shift. Rosette leaves of 14-day-old *flu AAA:LUC+* plants were harvested after a D/L shift at the indicated time points and used for total RNA isolation. Transcript levels were determined by quantitative RT-PCR and expressed relative to the levels at the end of the dark period. **d** Induction of luciferase activity in the *AAA:LUC+* reporter line by the singlet oxygen-producing photosensitizer Rose Bengal (*RB*, 500 μM), but not by paraquat (50 μM). Images were captured 2 h after treatment. Concentrations of the chemicals were established based on a similar level of necrosis that could be observed after 48 h. Mock treatment consisted of Tween-20 (0.1%). The *scale bar* represents 0.5 cm

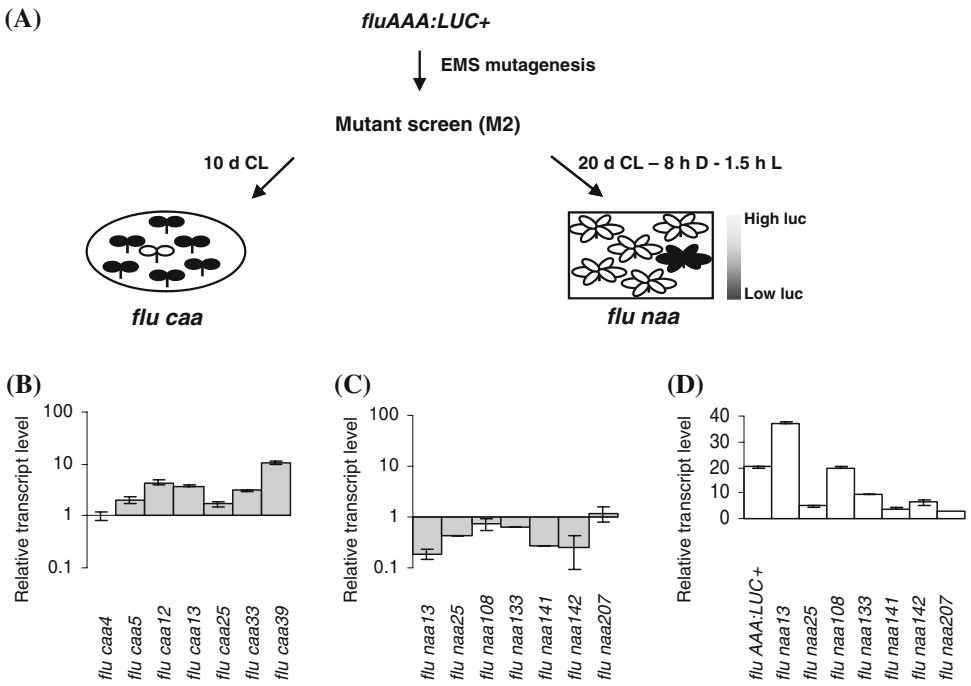


**Fig. 3** Isolation of EMS mutants altered in the expression of the singlet oxygen-inducible *AAA-ATPase* gene. **a** Schematic representation of the screening conditions used to identify *flu caa* and *flu naa* EMS mutants. *flu AAA:LUC+* M2 plants were grown under continuous light (*CL*) and either directly screened for mutants with constitutive luminescence (*flu caa*) after 10 days, or, after 20 days in CL, subjected to a 8 h dark period (*D*) and screened for mutants that are no longer able to induce luciferase activity (*flu naa*) 1.5 h after re-illumination (20 days CL- 8 h D –1.5 h L). **b** Relative *AAA-ATPase* transcript levels in shoots of 10-day-old CL grown *flu caa* mutants compared to the *flu AAA:LUC+* parental line as determined by quantitative RT-PCR. **c** and **d** Relative *AAA-ATPase* transcript levels in rosette leaves of 20-day-old *flu naa* mutants subjected to 8 h dark and 1.5 h re-illumination, as determined by quantitative RT-PCR. **c** *AAA-ATPase* transcript levels relative to the *flu AAA:LUC+* parental line 1.5 h after D/L shift. **d** Fold-induction of the endogenous *AAA-ATPase* in *flu naa* mutants after a D/L shift relative to the expression level at the end of the dark period

*caa* mutants, 10-day-old M2 seedlings were utilized that had been grown under continuous light on agar plates. In this way large numbers of M2 seedlings could be monitored and putative mutants could easily be rescued by transferring them from agar plates to soil. For the identification of *flu naa* mutants, we could not use 10-day-old M2 seedlings as *flu* plants, and potentially also *flu naa* plants, suffer from severe cell death following the 8 h D/L shift. However, 20-day-old *flu* plants at the rosette stage grown under continuous light simply stopped growing after a D/L shift, but did not die and could be rescued by transferring them to continuous light (op den Camp et al. 2003). Hence, for the identification of putative *flu naa* mutants, M2 plants were grown on soil under continuous light for 20 days, before the D/L shift.

Out of 16,000 10-day-old M2 seedlings a total of 43 putative *flu caa* mutants were initially identified that as a result of second-site *caa* mutations seemed to constitutively up-regulate the *AAA:LUC+* reporter gene. After a second screening of the progenies of the selected plants, seven mutants (*flu caa4*, *flu caa5*, *flu caa12*, *flu caa13*, *flu caa25*, *flu caa33*, and *flu caa39*) were confirmed and used for a subsequent detailed characterization. In order to distinguish between *cis*- and *trans*-acting mutations, the transcript levels of the endogenous *AAA-ATPase* gene were determined in the selected *flu caa* mutants grown under continuous light. *flu caa5*, *flu caa12*, *flu caa13*, *flu caa25, flu caa33*, and *flu caa39* mutants constitutively upregulated not only the *AAA:LUC+* reporter gene, but also the endogenous *AAA-ATPase* gene, indicating that these mutations acted in *trans.* (Fig. 3b). In contrast, *flu caa4* was identified as a putative *cis*-acting mutant and was excluded from further analysis. The extent of constitutive up-regulation varied greatly among the selected *trans*-acting mutant lines with *flu caa39* giving the strongest *AAA-ATPase* expression in 10-day-old shoots (Fig. 3b). *flu caa25*, which showed a moderately high luciferase activity and reduced accumulation of the endogenous *AAA-ATPase* transcripts, was not further characterized. The remaining mutants were back-crossed to the parental *flu* line. Segregation analysis of the resulting F2 generations revealed that, in each mutant, the constitutive up-regulation of the *AAA:LUC+* reporter gene was caused by a single recessive mutation (Supplementary Table S2). Allelism tests showed that the *caa5*, *caa12*, *caa13*, *caa33*, and *caa39* mutations represent five different loci (data not shown). Crude mapping confirmed that the mutated genes are located on very

distinct loci distributed on three different chromosomes (Supplementary Fig. S3).

Out of 8,400 20-day-old M2 plants, a total of 305 putative *flu naa* mutants were initially identified that, because of second-site *naa* mutations, seemed to fail to up-regulate the reporter gene in response to $^1O_2$ following a D/L shift. After two subsequent rescreening steps, seven of these mutants were selected for a detailed analysis (*flu naa13*, *flu naa25*, *flu naa108*, *flu naa133*, *flu naa141*, *flu naa142*, and *flu naa207*). To distinguish between *cis*- and *trans*-acting mutations, the transcript levels of the endogenous *AAA-ATPase* gene were determined before and after the D/L shift by quantitative RT-PCR. Two criteria were used for the confirmation of the selected mutants. First, *AAA-ATPase* transcript levels of the mutants were compared to levels in parental plants after 1.5 h of illumination. In four out of the seven selected mutants (*flu naa13, flu naa25, flu naa141* and *flu naa142*) transcript levels were considerably lower after reillumination than in *flu* plants (Fig. 3c). Second, the light-dependent induction of the *AAA-ATPase* mRNA accumulation was determined in mutant and parental plants by comparing transcript levels in plants re-exposed to light for 1.5 h relative to transcript levels in the dark prior to reillumination. *AAA-ATPase* transcript levels in *flu naa108* and *flu naa13* were similarly or even higher up-regulated during re-illumination than in parental *flu* plants (Fig. 3d). In the other *flu naa* mutants, up-regulation of *AAA-ATPase* transcripts during re-illumination was strongly attenuated.

Based on the results obtained, *flu naa13*, *flu naa108*, *flu naa133*, and *flu naa207* mutants were discarded from further analysis. *flu naa108* seemed to act in *cis* as it did not show a significant de-regulation of the endogenous *AAA-ATPase* gene relative to the parental *flu* line (Fig. 3c, d). In *flu naa133* and *flu naa207, AAA-ATPase* transcript accumulation during re-illumination was hardly induced (Fig. 3d), but at the end of re-illumination the transcript levels were just as high as in illuminated parental *flu* plants (Fig. 3c). Rather than blocking the up-regulation of *AAA-ATPase* transcript levels during illumination, these two mutations caused a constitutive up-regulation of the transcript level already in dark-treated plants. Finally, in *flu naa13* transcript levels of the *AAA-ATPase* gene were much lower than in the parental *flu* line after illumination (Fig. 3c), but the extent of up-regulation during re-illumination was even higher than in the parental plant (Fig. 3d). Hence, *flu naa13* seems to be a constitutively down-regulated mutant, in which the mutation affects the overall expression level of the *AAA-ATPase* gene rather than its inducibility by $^1O_2$.

In the remaining three *flu naa* mutants (*flu naa 25, flu naa141, flu naa142*), the expression level of the endogenous *AAA-ATPase* gene correlated with the luciferase activity, i.e. *AAA-ATPase* transcript levels as well as their fold-induction after the D/L shift were reduced compared to the parental *flu* line (Fig. 3c, d); the loss of responsiveness to $^1O_2$ was more pronounced in *flu naa25* and *flu naa141* than in *flu naa142*. *flu naa141* and *flu naa142* overaccumulated similar amounts of free Pchlide in the dark as the parental *flu* line (Supplementary Fig. S4a). In contrast, *flu naa25* showed a slightly reduced accumulation of the photosensitizer that might be responsible in part for the reduced induction of the *AAA-ATPase* gene (Supplementary Fig. S4a). The mutants were backcrossed to the parental *flu* line. Segregation analysis of the resulting F2 generations revealed that the failure to up-regulate the *AAA:LUC+* reporter gene was caused by single recessive mutations (Supplementary Table S2).

## The constitutive *flu caa* mutants reveal spatial regulation of *AAA-ATPase* gene expression by at least three different broad pathways

Based on the *LUC* expression patterns in 10-day-old seedlings grown under continuous light, the *flu caa* mutants were classified into three groups. The first group included the mutant lines *flu caa33* and *flu caa39*, which showed a very high luciferase activity in cotyledons whereas the activity in other parts of the plants was much lower (Fig. 4a, b). Trypan blue staining of *flu caa33* and *flu caa39* seedlings revealed extensive cell death in cotyledons, but only very few or no lesions in true leaves and other parts of the plants (Fig. 4b). In both mutant lines, cell death and constitutively high luciferase activity in cotyledons were linked, suggesting that the mutations were responsible for both phenomena (data not shown). In the case of *flu caa39*, the lesions were already detectable as chlorotic regions on cotyledons prior to trypan blue staining (Fig. 4b), whereas seedlings of *flu caa33* were slightly pale green and reduced in size, but did not show visible necrotic spots (Fig. 4b). Crude mapping of *caa33* and *caa39* mutations indicated that the mutated genes do not co-localize with mutations known to cause a constitutive activation of programmed cell death like *acd* and *cpr* (Supplementary Fig. S3).

The second group of *flu caa* mutants consisted of *flu caa5* and *flu caa13*, which showed luciferase activity distribution that was distinct from that of members of the first group. The constitutive, strong luciferase activity was restricted to true leaves and/or the apical region, while in cotyledons this activity was much lower (Fig. 4a, b). In both mutants, cell death occurred neither in cotyledons nor in leaves and apical regions, where the constitutive luciferase activity was strong (Fig. 4b). The different organ specificities of luciferase activity and the fact that the linkage of constitutive *LUC* expression and spontaneous cell death was confined to members of the first *flu caa* mutant group supports the view that *caa* mutations affect at least two different regulatory pathways. The complexity of

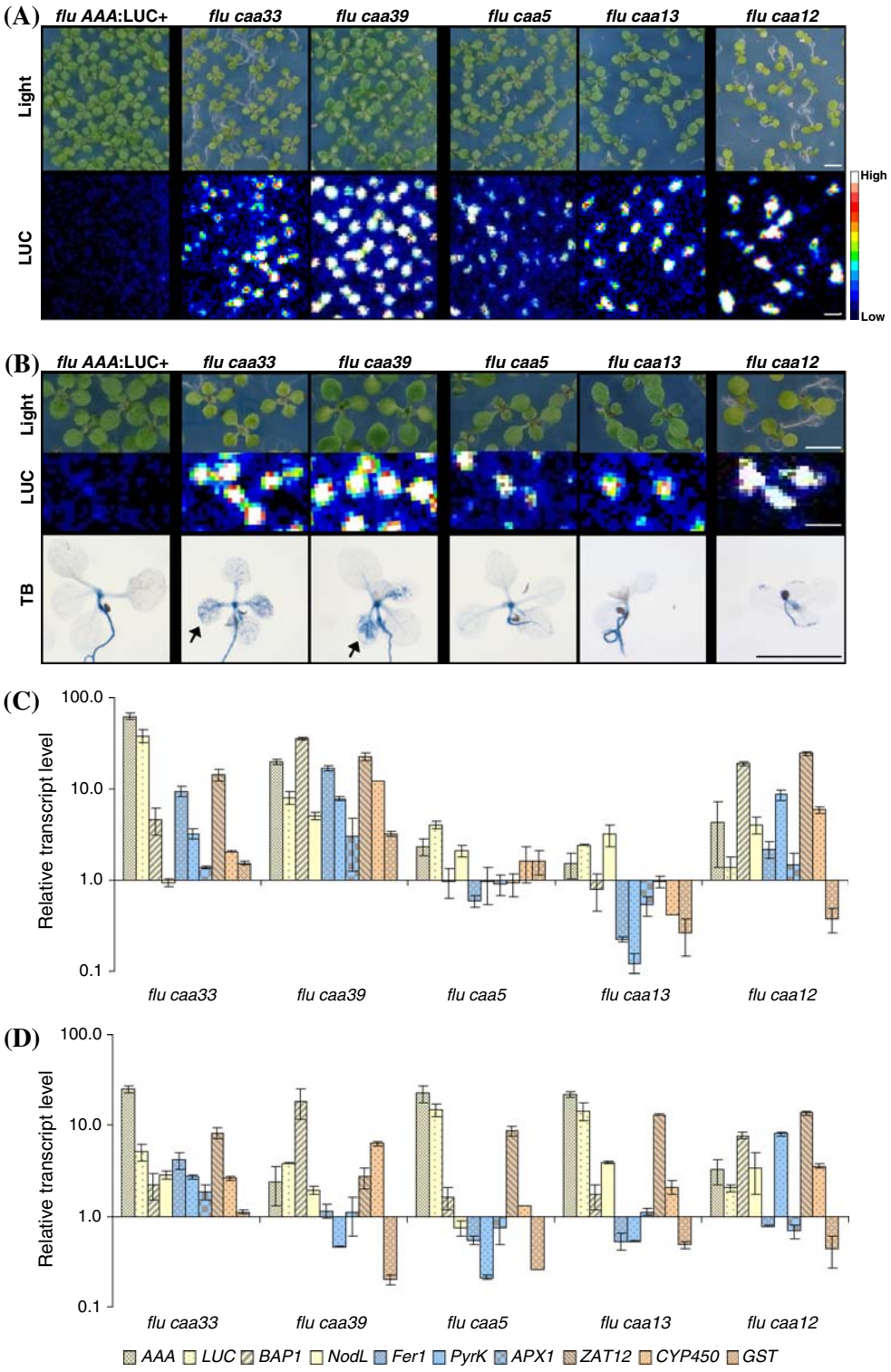


**Fig. 4** Characterization of selected *flu caa* mutants. **a** Light and bioluminescence images of 10-day-old CL grown *flu caa* mutants. *flu caa* mutants show constitutive strong luciferase activity under conditions that do not generate singlet oxygen. **b** Light and LUC image magnification and cell death analysis in *flu caa* mutants. Luciferase activity and spontaneous cell death as revealed by trypan blue (*TB*) staining in 10-day-old *flu caa* mutant plants display an organ-specific pattern; *arrows* indicate TB staining restricted to the cotyledons of *flu caa33* and *flu caa39* seedlings. The *scale bars* represent 0.5 cm. **c** and **d** ROS marker gene expression profiles in cotyledons (**c**) and shoots devoid of cotyledons (**d**) in 10-day-old *flu caa* seedlings grown in CL on MS agar plates. *AAA-ATPase (AAA)*, *luciferase (LUC)*, *BAP1* (At3g61190) and *NodL* (At5g64870) were used as $^1O_2$–marker genes (*yellow patterns*), *FER1* (At5g01600), *APX1* (At1g07890) and *PK* (At3g49160) as $O_2^-$/$H_2O_2$–marker genes (*blue patterns*) and *ZAT12* (At5g59820), *GSTU24* (At1g17170) and *CYP81D8* (At4g37370) as general oxidative stress response marker genes (*orange patterns*). Relative transcript levels compared to the *flu AAA:LUC+* parental line were determined by quantitative RT-PCR; average and SD of two repetitions are shown

signaling as revealed by the *caa* mutations increased even further, when *flu caa12* is also considered. This mutant did not show any specific enrichment of luciferase activity in particular organs of the seedling, but instead, it displayed a high constitutive luciferase activity homogeneously throughout the seedling (Fig. 4a, b).

## Expression of ROS-induced genes in the constitutive *flu caa* mutants

As part of the characterization of *flu caa* mutants, the expression of other genes known to be activated either selectively by different ROS, or more generally by

oxidative stress was also analyzed. In addition to *AAA-ATPase*, *BAP1* (At3g61190) and a gene encoding an unknown protein showing similarities with nodulin (At5g64870) were used as $^1O_2$ marker genes. Transcripts of all three genes have been shown to accumulate in the *flu* mutant in response to $^1O_2$, but not in response to $O_2^{\bullet-}$ and $H_2O_2$ (op den Camp et al. 2003). As $O_2^{\bullet-}$- and $H_2O_2$ marker genes *APX1* (At1g07890; Rizhsky et al. 2004), *FER1* (At5g01600; Petit et al. 2001) and a pyruvate kinase-like gene (*PK*, At3g49160) were analyzed. Transcripts of these genes accumulate under $O_2^{\bullet-}$- and $H_2O_2$-generating conditions, but not in response to $^1O_2$ (Laloi et al. 2006; op den Camp et al. 2003). As a third set, general oxidative stress response marker genes such as *AtGSTU24* (At1g17170), *CYP81D8* (At4g37370) and *ZAT12* (At5g59820) were analyzed. Their transcripts accumulate massively after the release of $^1O_2$ in the *flu* mutant, as well as in wild type plants treated with ozone, $O_2^{\bullet-}$- or $H_2O_2$-generating agents, such as paraquat and 3-aminotriazole, and the fungal *Alternaria alternata f. sp. lycopersici* toxin, or in transgenic plants with compromised levels of catalase or APX1 (Gadjev et al. 2006). Since most of the *flu caa* mutants showed very different spatial expression patterns of luciferase activity, expression studies using these various marker genes were done separately for cotyledons and shoots devoid of cotyledons (referred to as shoots from now on) from 10-day-old seedlings (Fig. 4c, d).

Similar to the *AAA-ATPase* transcripts, *BAP1* transcripts overaccumulated in all five *flu caa* mutants relative to the parental *flu* line, and the extent of accumulation of both transcripts varied greatly among the different mutants (Fig. 4c, d). However, *BAP1* transcripts did not overaccumulate in cotyledons of *flu caa5* and *flu caa13*. The nodulin-like protein (*NodL*) transcripts also overaccumulated in most of the *flu caa* mutants, except for the cotyledons of *flu caa33* and the shoots of *flu caa5*. Differences between transcript levels of these three $^1O_2$-responsive genes in different *flu caa* mutants and different plant parts suggest that additional factors, other than $^1O_2$, may contribute to the regulation of the expression of these genes. The analysis of other ROS marker genes revealed a striking similarity between *flu caa5* and *flu caa13* mutants, especially in shoots. For instance, the $H_2O_2$ marker genes *FER1* and *PK* are down-regulated in both mutants, in contrast to $^1O_2$ marker genes that are up-regulated. Another striking feature that these two mutants have in common is the organ-specific deregulation of *ZAT12*. Transcripts of this gene overaccumulated in shoots of both mutants, but not in cotyledons.

In contrast to *flu caa5* and *flu caa13*, in *flu caa33* and *flu caa39* transcript levels of almost all $^1O_2$-, $O_2^{\bullet-}$/$H_2O_2$- or general oxidative stress response-marker genes analyzed in this study were elevated in cotyledons, suggesting that these two mutations affect either a general communication between plastids and the nucleus, or the regulation of general oxidative stress response genes, rather than only a $^1O_2$–responsive signaling pathway. *flu caa33* and *flu caa39* mutants were distinguishable from each other, however, in that the gene expression signatures in shoots and cotyledons of *flu caa39* were significantly different ($H_2O_2$ marker genes are not up-regulated in shoots of 10-day-old *flu caa39* seedlings), whereas in *flu caa33* they were remarkably similar (Fig. 3c, d). Finally, in *flu caa12* both the expression signatures as well as the relative transcript levels in shoots and cotyledons were similar, suggesting that the gene product mutated in *flu caa12* is not, or only very poorly, spatially regulated.

## Response of *flu caa* mutants to the release of $^1O_2$ after a D/L shift

The screen for *flu caa* mutants has been based on the constitutive expression of *AAA-ATPase*. However, this selection scheme alone is not sufficient to identify unequivocally genuine $^1O_2$-signaling components. Therefore, we have used an additional selection step to distinguish between CAA proteins that genetically form part of $^1O_2$-signaling and other CAA factors that suppress the *AAA-ATPase* gene under steady state conditions but are not directly linked to $^1O_2$-signaling. In *caa* mutants that genetically form part of the $^1O_2$-signaling pathway and that contain the *flu* mutation, generation of $^1O_2$ by a D/L shift should not further enhance the expression of $^1O_2$-responsive genes.

As cotyledons of 10-day-old *flu* seedlings grown under continuous light are no longer able to accumulate significant amounts of Pchlide in the dark and hence do not release $^1O_2$ during reillumination (data not shown), this analysis was performed in 5-day-old seedlings. Also at this earlier developmental stage, all five *flu caa* mutants showed constitutive high levels of *AAA-ATPase* transcripts under continuous light compared to the parental *flu AAA:LUC+* line (Fig. 5a). Following a D/L shift, the *flu caa39* mutant did not show any further $^1O_2$–induced accumulation of *AAA-ATPase* transcripts (Fig. 5b), even though it accumulated similar excess amounts of Pchlide as the parental *flu AAA:LUC+* plants (Supplementary Fig. S4b). In contrast, in *flu caa33*, *flu caa5*, *flu caa13* and *flu caa12* mutants *AAA-ATPase* transcripts accumulated further in response to $^1O_2$ generation (Fig. 5b). In *flu caa5* and *flu caa12* lines, the fold induction of *AAA-ATPase* was slightly reduced relative to the parental line. In the case of *flu caa12* this could be attributed to a reduced Pchlide accumulation (Supplementary Fig. S4b). In *flu caa33* and *flu caa13* lines, the fold induction of *AAA-ATPase* was similar or even higher, respectively, to the one in the parental line

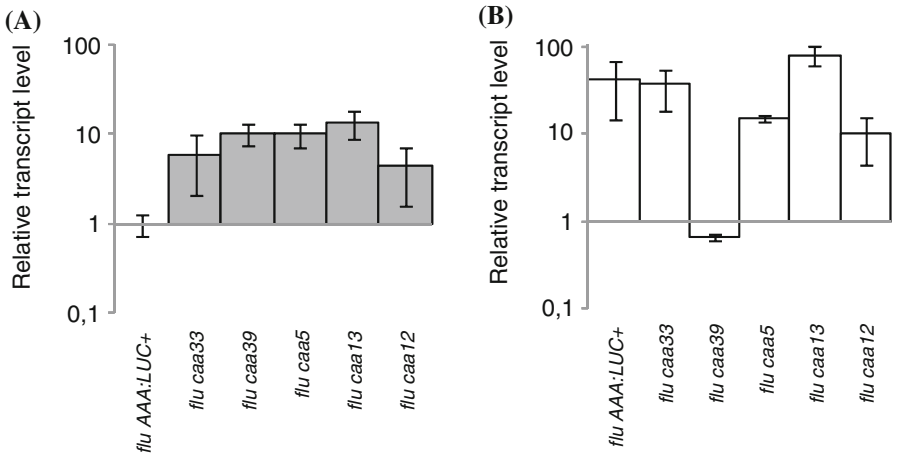


**Fig. 5** Response of the *flu AAA:LUC+* parental line and *flu caa* mutants to singlet oxygen. Relative transcript levels of the endogenous *AAA-ATPase* gene were analyzed in 5-day-old seedlings and determined by quantitative RT-PCR. **a** Relative *AAA-ATPase* transcript levels in 5-day-old seedlings grown under continuous light. The *flu AAA:LUC+* parental line is set to 1. **b** Fold-induction of *AAA-ATPase* in 5-day-old seedlings grown under continuous light and subjected to 8 h dark and 1 h re-illumination. Transcript levels are relative to the levels at the end of the 8 h dark period

(Fig. 5b), and eventually resulted in very high levels of *AAA-ATPase* transcripts after D/L shift. The full responsiveness of *flu caa33* and *flu caa13* plants to $^1O_2$ suggests that *caa33* and *caa13* mutations may affect negative regulators of $^1O_2$–signaling that are not directly part of the $^1O_2$–signaling cascade.

## Response of *flu naa* mutants to different ROS-producing stress conditions

The low level of luciferase activity and the reduced induction of the endogenous *AAA-ATPase* gene in *flu naa25, flu naa141* and *flu naa142* suggest that because of these second-site mutations the ability of the parental *flu* line to respond to $^1O_2$ has been impaired (Figs. 3c, 6a). To test this notion, the expression of other marker genes was analyzed in the three *flu naa* double mutants after a D/L shift. In *flu* mutants with the second-site mutations *naa25*, *naa141* or *naa142*, the expression of *BAP1*, similar to the expression of *AAA-ATPase*, was less induced in response to the release of $^1O_2$, than in the parental *flu* line (Fig. 6b). Also, the $^1O_2$-induced expression of *ZAT12* was strongly suppressed in all three mutants relative to the parental *flu* line, particularly in *flu naa141*. $H_2O_2$-marker gene transcript levels were hardly affected in *flu naa25* and *flu naa142*, and were slightly downregulated in *flu naa141* after a D/L shift.

The specificity of these mutational defects was further tested under other ROS-producing conditions. First, mutant plants were treated with the $^1O_2$-specific photosensitizer, RB. This experiment was particularly important for the characterization of *flu naa25*, because failure of this mutant to respond to $^1O_2$ may be due to a reduced accumulation of Pchlide (Supplementary Fig. S4a). Treatment of the parental *flu AAA:LUC+* line grown under continuous light with 0.5 mM RB, induced luciferase activity to a similar extent as after a D/L shift (Fig. 6a, c). Luciferase activity in all three mutants, however, remained very low (Fig. 6c), confirming that not only *flu naa141* and *flu naa142*, but also *flu naa25* has a reduced responsiveness to $^1O_2$.

The three *flu naa* mutants and the parental *flu* line were also treated with paraquat. In case *naa* mutations selectively affect only $^1O_2$-mediated signaling, these mutations should not impede the mutants' responses to $O_2^{\bullet-}$ or $H_2O_2$. Indeed, in *flu naa25* and *flu naa141* mutant plants, all three $H_2O_2$-responsive genes, *APX1*, *FER1* and *PK*, were induced to similar levels as in *flu AAA:LUC+* parental line plants treated with paraquat (Fig. 7). In *flu naa142*, *APX1* transcripts reached similar levels as in parental *flu* plants after paraquat treatment, but *FER1* and to a lesser extent *PK* expression were reduced relative to the parental *flu* plants. This suggests that the *naa142* mutation is less specific and does not only suppress responses to $^1O_2$, but also to some extent responses to $O_2^{\bullet-}$ and/or $H_2O_2$.

## Responses of *flu caa* and *flu naa* mutants to photoinhibitory conditions

During light stress, chloroplasts generate enhanced levels of $^1O_2$, $O_2^{\bullet-}$ and $H_2O_2$ that may act as signals or cause photooxidative damage. *caa* and *naa* mutations have been shown to interfere with ROS-dependent signaling by either constitutively up-regulating ROS-responsive genes or suppressing their responsiveness. The consequences of these mutations on the responses of plants to severe stress were tested by exposing *flu caa* and *flu naa* mutants together with parental *flu AAA:LUC+* control plants to photoinhibitory

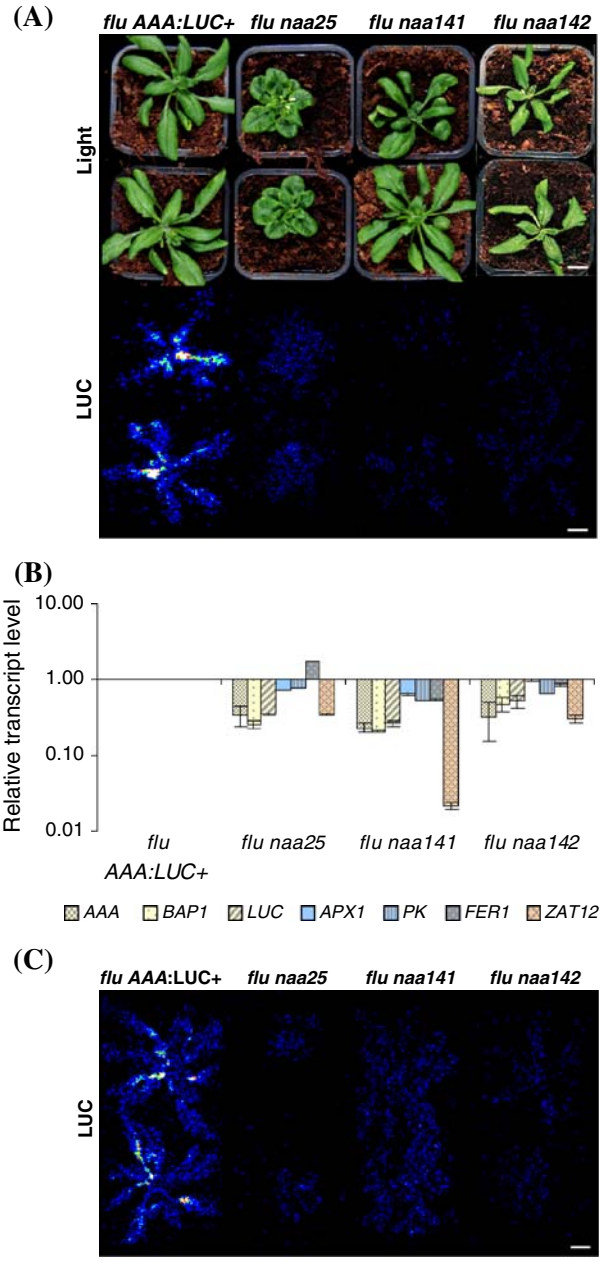


**Fig. 6** Characterization of selected *flu naa* mutants. **a** Light images of 20-day-old *flu naa* mutants grown in continuous light (*CL*) and bioluminescence images after 8 h of dark exposure and 1.5 h of re-illumination. In contrast to the *flu AAA:LUC+* parental line, *flu naa* mutants are no longer able to induce luciferase activity after the release of singlet oxygen in *flu*. **b** ROS marker gene expression profiles in rosette leaves of 20-day-old *flu naa* mutants subjected to 8 h of dark and 1.5 h of re-illumination. Results are expressed relative to the *flu AAA:LUC+* parental line after the D/L shift. Relative transcript levels were determined using quantitative RT-PCR, average and SD of two biological replicates are presented. **c** *flu naa* mutants show reduced responsiveness to treatment with the singlet oxygen-producing photosensitizer Rose Bengal (*RB*) compared to the parental *flu* line. 20-day-old *flu naa* mutants grown in CL were sprayed with 0.5 mM RB and luciferase activity was captured after 12 h. All pictures were taken at the same magnification and with the same exposure time; the *scale bars* represent 1 cm

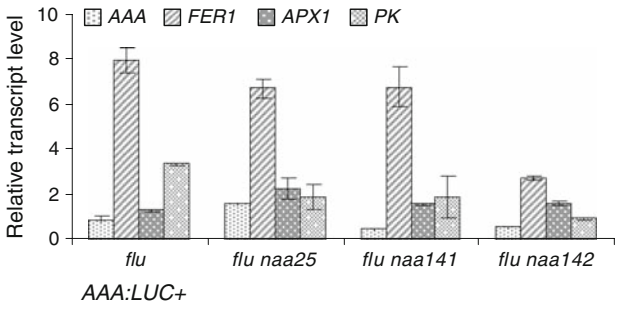


**Fig. 7** Response of the *flu AAA:LUC+* parental line and *flu naa* mutants to paraquat treatment. Transcript levels of the endogenous *AAA-ATPase* (*AAA*) gene and the three $O_2^{\bullet-}/H_2O_2$–marker genes *FER1*, *APX1* and *PK* were analyzed in rosette leaves of 20-day-old plants after 2 h of treatment with 20 μM paraquat. Relative transcript levels were determined by quantitative RT-PCR and are expressed relative to the values of mock treatment (0.1% Tween-20)

conditions. First, seedlings were grown for 6 days at 22°C under continuous low light (20 μmol $m^{-2}$ $s^{-1}$) and then transferred to 300 μmol $m^{-2}$ $s^{-1}$ at 12°C. Such a combination of moderate high light and low temperature has been shown previously to cause photoinhibition and to enhance the production of $^1O_2$ and $H_2O_2$ in plants (Hetherington et al. 1989; Hideg et al. 2000; Wise and Naylor 1987). Shortly after exposure of plants to this stress program the photosynthetic efficiency declined (Fig. 8b) and the expression of the $^1O_2$ marker genes *AAA-ATPase* and *BAP1* increased rapidly (Fig. 8a). The induction of the $H_2O_2$ marker genes *PK* and *FER1* was delayed and occurred only after 24 h, suggesting that it is mostly $^1O_2$ that is produced during the first hours of this photoinhibitory stress. Responses of seedlings to stress-induced photoinhibition were registered by measuring the reduction of the maximum quantum efficiency of PSII photochemistry (Fv/Fm). Under low light, prior to the transfer to photoinhibitory conditions, Fv/Fm values of control and mutant seedlings were very similar (Fv/Fm = 0.87–0.88), except for *flu caa12*, which has a pale phenotype and a significant lower content of total chlorophyll (data not shown). Exposure of seedlings to 300 μmol $m^{-2}$ $s^{-1}$ and 12°C led to a rapid reduction of the Fv/Fm values to about 0.6 within the first 24 h indicating that all seedlings suffered from photoinhibition (Fig. 8b). However, mutants seemed to be slightly more resistant than the parental *flu AAA:LUC+* line as indicated by their higher Fv/Fm values. After 72 h of stress treatment differences between the parental line and the different mutant seedlings became more obvious and different lines started to diverge greatly with respect to their stress susceptibility (Fig. 8b; Supplementary Fig. S5). In *flu AAA:LUC* + parental line, *flu caa5*, *flu caa33*, *flu caa39*, *flu naa141*, and *flu naa142* seedlings, the maximum efficiency of PSII was further reduced and finally after 7 days this stress treatment resulted

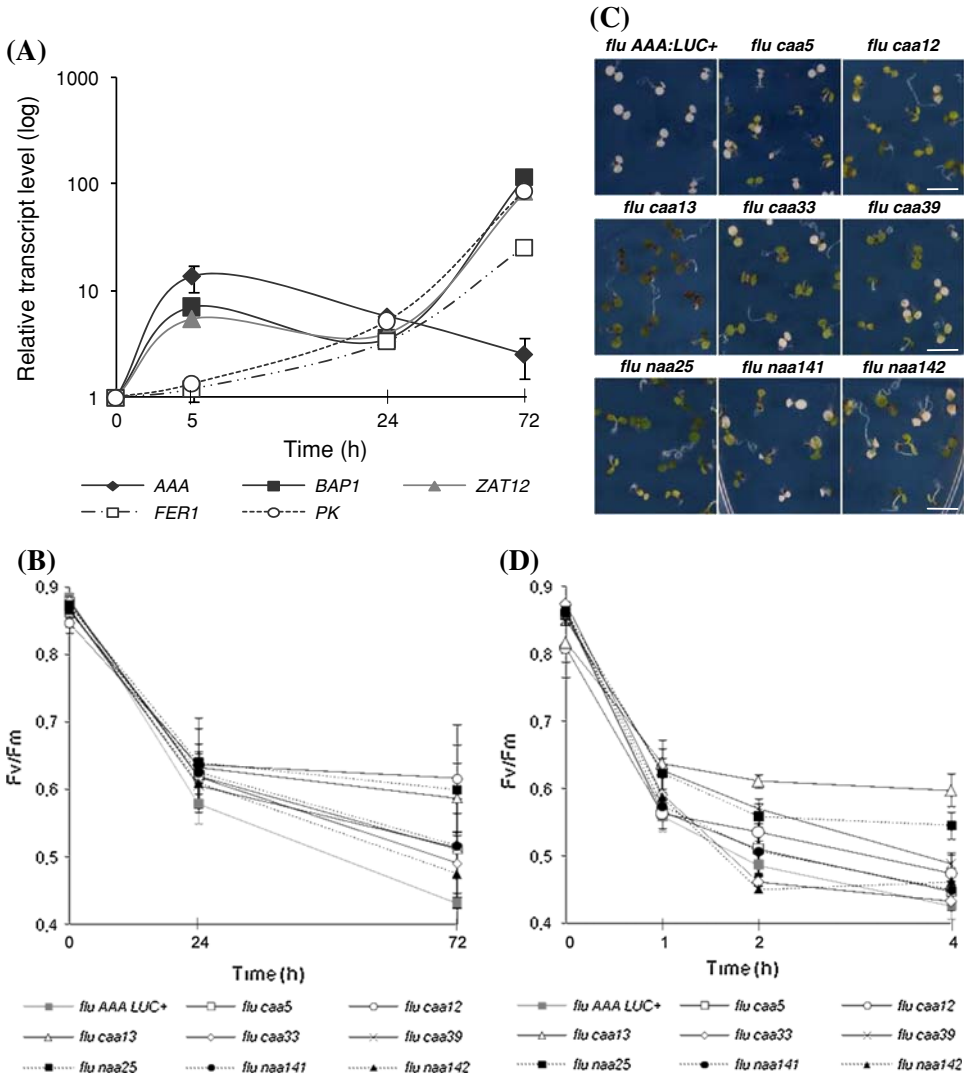


**Fig. 8** Responses of the *flu caa* and *flu naa* mutants to photoinhibitory stress conditions. **a** Transcript levels of the $^1O_2$-marker genes *AAA-ATPase (AAA)* and *BAP1*, the $H_2O_2$-marker genes *FER1* and *PK*, and the general oxidative stress response marker gene *ZAT12* were analyzed at the onset and 5, 24 and 72 h after the initiation of the combined moderate high light (300 µmol $m^{-2}$ $s^{-1}$) and low temperature (12°C) treatment. Relative transcript levels were determined by quantitative RT-PCR and expressed relative to the levels before the initiation of the stress treatment. **b** The maximum quantum yield of PSII photochemistry (Fv/Fm) that reflects the extent of photoinhibition was determined in *flu caa* and *flu naa* mutants and in the parental line (*flu AAA:LUC+*) before and 24 and 72 h after the onset of the combined high-light and low temperature treatment. **c** Visible phenotypes of the parental line and selected mutant lines 7 days after the initiation of photoinhibitory stress. All pictures were taken at the same magnification; the *scale bars* represent 0.5 cm. **d** The same lines were subjected to higher light stress (1,000 µmol $m^{-2}$ $s^{-1}$) at 22°C and their responses recorded as Fv/Fm decreases

for the parental line in the complete, and for the mutant seedlings in the partial bleaching and collapse (Fig. 8c). In contrast, *flu caa12*, *flu caa13* and *flu naa25* seedlings sustained the reduced maximum yield of PSII after 72 h (Fig. 8b; Supplementary Fig. S5) of stress and during the following days started to recover (data not shown). Even after 7 days of high light/low temperature stress, all these seedlings remained viable (Fig. 8c). Under different photoinhibitory conditions, i.e. higher light intensity (1,000 µmol $m^{-2}$ $s^{-1}$), but constant temperature (22°C), *flu caa13* and *flu naa25*, but hardly *flu caa12*, were markedly more resistant than the parental *flu* line (Fig. 8d; Supplementary Fig. S5).

## Discussion

### Singlet oxygen-mediated chloroplast-to-nucleus communication

Plastid-to-nucleus signaling has been shown to play a central role in controlling gene expression in the nucleus (Beck

2005; Mullineaux and Karpinski 2002; Nott et al. 2006; Rodermel 2001; Taylor 1989). At least three types of retrograde signaling have been distinguished: one that depends on the expression of plastid genes during early seedling development (Sullivan and Gray 1999), a second that is mediated through the perception of redox changes within the plastid compartment (Baier and Dietz 2005; Pfannschmidt 2003) and a third, which is subject to intensive debate, that implicates tetrapyrrole intermediates with the control of nuclear genes (Mochizuki et al. 2008; Moulin et al. 2008; Strand et al. 2003). Whether these pathways act independently (Heiber et al. 2007; Surpin et al. 2002) or converge within the plastid compartment and form part of a common signal transduction pathway that conveys information from the plastid to the nucleus is also under discussion (Koussevitzky et al. 2007). More recently, $^1O_2$ has emerged as a potential contributor to this retrograde signaling (Wagner et al. 2004; Woodson and Chory 2008).

One of the immediate consequences of $^1O_2$ generation in plastids of the *flu* mutant is an extensive change of nuclear gene expression that reveals a rapid transfer of signals from the plastid to the nucleus (op den Camp et al. 2003). Since $^1O_2$ is very reactive and unlikely to leave the plastid compartment (Redmond and Kochevar 2006), its physiological impact has been attributed to the generation of more stable second messengers within the plastid that are assumed to be exported and to activate plastid-to-nucleus signaling outside the plastid compartment. A genetic screen led to the identification of the plastid protein EXECUTER (EX) 1 as an essential signaling component involved in the translocation of $^1O_2$ signal from the plastids to the nucleus (Wagner et al. 2004). Inactivation of EX1 and the closely related EX2 nuclear encoded plastid proteins in a *flu/ex1/ex2* triple mutant almost completely abrogated $^1O_2$-mediated nuclear gene expression changes (Lee et al. 2007; Wagner et al. 2004). Therefore, the sensing of $^1O_2$ in the *flu* mutant of Arabidopsis seems to take place primarily within the plastid, in close vicinity to its production sites. The situation might be different, however, in Chlamydomonas cells under very high light-stress (3,500 μmol $m^{-2}$ $s^{-1}$), in which a small fraction of $^1O_2$ produced from the PSII reaction centre has been reported to leave the chloroplast and activate the nuclear gene *GPXH* (Fischer et al. 2007). Arabidopsis mutant screens aimed at identifying extraplastidic components involved in retrograde plastid-to-nucleus signaling have failed so far suggesting that retrograde signaling may not operate via isolated linear pathways, but rather merge in a signaling network that modulates and integrates various cues and relays these to the nucleus (Mittler et al. 2004; Overmyer et al. 2003). The stability and robustness of such a network has been ascribed to the interaction of numerous and redundant signaling routes with partially overlapping specificities (Stelling et al. 2004).

In our present work, we have used a reporter gene-based mutant screen for genetically dissecting the $^1O_2$-signaling network. We have utilized the promoter of an *AAA-ATPase* gene which is activated by $^1O_2$, but not by other ROS or phytohormones involved in well-characterized signaling cascades (Nemhauser et al. 2006). For this approach two strategies are possible that use either a single *cis*-element (Shao et al. 2007) or the full promoter (this work) of a $^1O_2$ responsive gene to drive the expression of the reporter. Both strategies have been shown to be successful for the characterization of other signaling pathways (Ball et al. 2004; Cao et al. 1994; Medina et al. 2005; Millar et al. 1995; Rama Devi et al. 2006). The single *cis*-element approach has the advantage of avoiding complications that may arise during the interaction of several different *trans*-acting factors with a full promoter. On the other hand using a single *cis* element most likely is not sufficient to trigger the full response under investigation. As details of the $^1O_2$-signaling network are largely unknown and no a priori assumption about the identity of factors responsible for $^1O_2$-specific signaling could be made, we have chosen the second strategy.

In order to identify signaling factors responsible for the activation of nuclear genes, EMS mutants affected in the regulation of the $^1O_2$ marker gene *AAA-ATPase* were isolated and characterized. Some of the *flu naa* mutants, notably *flu naa25* and *flu naa141* seemed to be specifically affected in their response to $^1O_2$, supporting the view that this approach is appropriate for the identification of signaling constituents that control the expression of $^1O_2$-responsive genes. The screen for *caa* mutations, however, is not sufficient to identify unequivocally genuine $^1O_2$-signaling components. Besides factors that genetically form part of $^1O_2$-signaling, this screen may also identify other CAA factors that suppress the *AAA-ATPase* gene under steady-state conditions but are not directly linked to $^1O_2$-signaling. An additional selection criterion was used to distinguish between these two types of *caa* mutants: in *caa* mutants that genetically form part of the $^1O_2$-signaling pathway and that contain also the *flu* mutation, generation of $^1O_2$ by a D/L shift should not further enhance the expression of $^1O_2$-responsive genes. In addition, it seems likely that these *flu caa* mutants constitutively show other, physiological responses, i.e. cell death and growth inhibition, that in the parental *flu* line are triggered by the release of $^1O_2$. Based on these assumptions, CAA39 seems to represent a likely candidate for a genuine $^1O_2$ signaling component.

## Crosstalk between different ROS signaling pathways

The selective activation of nuclear genes by $^1O_2$ that are not or far less responsive to other ROS, has been reported

for plants (Gadjev et al. 2006; Laloi et al. 2006; op den Camp et al. 2003), as well as mammals (Klotz et al. 2003), the unicellular alga Chlamydomonas (Leisinger et al. 2001) and the phototrophic bacterium *Rhodobacter sphaeroides* (Anthony et al. 2005). Moreover, we have shown previously that $H_2O_2$ may also either directly or indirectly antagonize $^1O_2$-mediated signaling. A similar interaction between these two ROS has been described in Chlamydomonas too (Ledford et al. 2007). This interaction between $^1O_2$-mediated signaling and $H_2O_2$-derived signals suggests that cellular factors might exist that do not only allow to discriminate between different ROS, but also to sense relative quantities of different ROS and eventually use this information to modulate ROS-mediated stress responses. In this respect, it is remarkable that the *flu caa5* and *flu caa13* mutations affect the expression of $^1O_2$-responsive and $H_2O_2$-responsive genes in opposite ways. Because of their highly similar expression signatures of ROS-responsive genes, *flu caa5* and *flu caa13* mutants are likely to affect factors acting either together or being part of a common pathway. Also *flu naa25* and *flu naa141* mutations seem to differentially affect the responsiveness of plants to distinct ROS. On the one hand, both mutants show a dramatically reduced response of the *AAA-ATPase* and other $^1O_2$-marker genes to $^1O_2$. On the other hand these mutations barely affect responses to the $O_2^{\bullet-}$ and $H_2O_2$-generating paraquat treatment. These four mutants (*flu caa5*, *flu caa13*, *flu naa25* and *flu naa141*) with differentially altered responses to different ROS should be valuable tools to uncover genetic determinants of cross-talk between $^1O_2$- and $H_2O_2$-dependent signaling described earlier in the *flu* mutant of Arabidopsis (Laloi et al. 2007).

## Most of the *flu caa* and *flu naa* mutants are affected in their sensitivity to photoinhibitory stress conditions and their development

Photoinhibitory conditions result primarily in the production of $^1O_2$ (Hideg et al. 2002, 1998). Therefore, we have tested responses of *flu caa* and *flu naa* mutants defective in $^1O_2$-signaling to such conditions by exposing them to a combination of moderately high light and cool temperature. Under these conditions rapid photoinhibition occurred, as revealed by the decrease of maximum quantum efficiency of PSII photochemistry shortly after the beginning of the stress treatment. The expression levels of different ROS marker genes indicated that $^1O_2$ production starts early on, whereas the onset of $O_2^{\bullet-}$ and $H_2O_2$ generation is delayed by approximately 24 h. Surprisingly, *flu caa* and *flu naa* mutants reacted similarly to these photoinhibitory conditions by showing a slightly increased resistance to photoinhibition. It has to be emphasized that all mutations, except *naa141,* that led to the constitutive activation of $^1O_2$–responsive genes in *flu caa* mutants and the disruption of $^1O_2$ signaling in *flu naa* mutants, also resulted in physiological responses that *flu* plants show after the release of $^1O_2$, i.e., cell death in *flu caa33* and *flu caa39* and growth inhibition in *flu caa* and *flu naa* mutants. Thus, *flu caa* and *flu naa* mutants seem to be under constitutive stress due to the disruption of their homeostasis that may activate an acclimatory response and increase their tolerance to a subsequent more severe stress. The fact that different mutants display distinct phenotypes under steady state conditions, prior to the photoinhibitory stress treatment, may account for the different levels of tolerance among the various *naa* and *caa* mutants.

Most of the *flu caa* and *flu naa* mutants, except for *flu naa141*, also display visible morphological alterations that co-segregate with the constitutive expression and the reduced up-regulation, respectively, of the *AAA:LUC+* reporter gene (Supplementary Fig. S6). This observation suggests that the mutated genes act pleiotropically and may affect $^1O_2$–mediated signaling of stress responses as well as the control of plant development. The analysis of crosstalk between development and stress responses in yeast cells showed that the transcript levels of more than 80% of the genes affected by the growth rate were also affected by different types of stress (Regenberg et al. 2006). In plants, $O_2^{\bullet-}$ and $H_2O_2$ are known to be involved in the regulation of many biological processes such as growth, development, programmed cell death and stress adaptation (Gechev et al. 2006). For instance, double mutants that lack the cytosolic and thylakoid $H_2O_2$ scavenging enzyme ascorbate peroxidase are not only affected in their response to abiotic stresses, but also in their development (Miller et al. 2007). In mutants defective in different $O_2^{\bullet-}$-producing plasma membrane-bound NADPH oxidase isoforms, not only resistance to pathogens (Yoshioka et al. 2003), but also regulation of stomatal closure (Kwak et al. 2003), cell expansion and plant development have been perturbed (Foreman et al. 2003). Morphological alterations, as seen in the majority of *flu caa* and *flu naa* mutants, support the view that also $^1O_2$-mediated signaling may play an important, but hitherto largely unexplored, role during the regulation of plant development.

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