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The chloroplast division mutant *caa33* of *Arabidopsis thaliana* reveals a crucial impact of chloroplast homeostasis on stress acclimation and retrograde plastid-to-nucleus signaling

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SUMMARY

Retrograde plastid-to-nucleus signaling tightly controls and coordinates nuclear and plastid gene expression that is required for plastid biogenesis and chloroplast activities. As chloroplasts act as sensors of environmental changes, plastid-derived signaling also modulates stress responses of plants by transferring stress-related signals and altering nuclear gene expression. Various mutant screens have been undertaken to identify constituents of plastid signaling pathways. Almost all mutations identified in these screens have in common that they target plastid-specific but not extra-plastidic functions. They have been suggested to define either genuine constituents of retrograde signaling pathways or components required for the synthesis of plastid signals. Here we report the characterization of the *caa33* (*constitutive activator of AAA-ATPase*) mutant, which reveals another way of how mutations that affect plastid functions may modulate retrograde plastid signaling. *caa33* disturbs a plastid-specific function by impeding plastid division thereby perturbing plastid homeostasis. This results in pre-conditioning plants by activating the expression of stress genes, enhancing pathogen resistance and attenuating the plant's capacity to respond to plastid signals. Our study reveals an intimate link between chloroplast activity and the plant's susceptibility to stress and emphasizes the need to consider the possible impact of pre-conditioning on retrograde plastid-to-nucleus signaling.

Keywords

chloroplast homeostasis; chloroplast division mutant; retrograde signaling; stress acclimation; singlet oxygen; *Arabidopsis thaliana*

INTRODUCTION

Plants are exposed to environmental changes that may adversely affect their development, growth or productivity. Adverse conditions are caused by changes in the physical and chemical environment or may be due to pathogens or herbivores attacking plants (Bray *et al.*, 2000). Chloroplasts play a key role in sensing these environmental changes (Liu *et al.*, 2007; Li *et al.*, 2009; Padmanabhan and Dinesh-Kumar, 2010). Stress conditions such as

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pathogen attack, drought, high light or high or low temperature interfere with the photosynthetic electron transport and lead to an enhanced production of reactive oxygen species (ROS) (Apel and Hirt, 2004). A major difficulty in elucidating the physiological significance of these enhanced ROS levels during stress stems from the fact that several chemically distinct ROS with different biological activities are generated simultaneously within chloroplasts, thus making it almost impossible to link a particular stress response to a specific ROS (Apel and Hirt, 2004; Hideg and Schreiber, 2007). This problem has been alleviated by using the conditional *flu* (*fluorescent*) mutant of *Arabidopsis* (Meskauskiene *et al.*, 2001). In the dark the *flu* mutant accumulates protochlorophyllide (Pchl_{id}), a potent photosensitizer that upon illumination generates singlet oxygen (¹O₂) (op den Camp *et al.*, 2003). ¹O₂ is highly reactive and potentially toxic. By modifying the length of the dark period, conditions can be established that suppress the cytotoxic effect of ¹O₂ and reveal its signaling role. Under these optimized conditions enhanced levels of ¹O₂ within plastids of the *flu* mutant trigger responses such as nuclear gene expression changes and rapid death of seedlings that depend on the two nuclear-encoded chloroplast proteins EXECUTER 1 (EX1) and EXECUTER 2 (EX2) (Wagner *et al.*, 2004; Lee *et al.*, 2007; Kim *et al.*, 2009). Inactivation of both EX proteins in an *ex1/ex2/flu* triple mutant abrogates seedling lethality and abolishes almost completely the up-regulation of ¹O₂-responsive gene expression. This block of ¹O₂-mediated responses in an *ex1/ex2* genetic background has been used to define a signaling pathway through which plastids under stress convey information to the nucleus.

Plastid-to-nucleus signaling has not only been shown to modulate stress responses of plants, as in the case of ¹O₂-mediated signaling, but also to control plastid biogenesis and chloroplast activity (Nott *et al.*, 2006; Pogson *et al.*, 2008). As plastid proteins are encoded by plastid and nuclear genomes, gene expression in these separate compartments needs to be coordinated. Multiple factors have been proposed to be generated within plastids and to affect nuclear gene expression (Pogson *et al.*, 2008). Collectively, they have been referred to as retrograde signals (Nott *et al.*, 2006). Different retrograde signals do not seem to act independently, but to form part of a complex signaling network in which their activity and specificity may be modulated by other intra- or extra-plastidic signals (Sullivan and Gray, 2002; Koussevitzky *et al.*, 2007; Larkin and Ruckle, 2008; Cottage *et al.*, 2010).

Thus far it is not known, whether other plastid-derived signals may interact with ¹O₂-mediated signaling. A second-site mutagenesis of the *flu* mutant has been employed to search for components of the ¹O₂ signaling network (Baruah *et al.*, 2009a). During this genetic screen a mutant with a defect in chloroplast division has been isolated that identifies perturbation of chloroplast homeostasis as a major cause for plastid signaling. This signaling pre-conditions plants and modulates their capacity to respond to ¹O₂ by inducing stress acclimation and suppressing ¹O₂-mediated cell death.

RESULTS

Characterization of the *caa33* mutant

The *flu/AAA:LUC+/caa33* mutant was found in a screen for mutants that show a constitutive high expression of the singlet oxygen (¹O₂)-responsive AAA-ATPase (At3g28580) and a AAA-ATPase promoter-LUCIFERASE reporter gene (Baruah *et al.*, 2009a,b). The *caa33* mutant was back-crossed to the original *flu/AAA:LUC+* line. The F₂ segregation analysis of this cross verified that a single recessive mutation causes the *caa* phenotype (Baruah *et al.*, 2009a). *flu/AAA:LUC+/caa33* displayed a very high luciferase activity in 10-d-old seedlings as well as in 21-d-old rosette plants grown under continuous light (Figure 1a). In 10-d-old seedlings, the luciferase activity was confined predominantly to cotyledons (Figure 1a) (Baruah *et al.*, 2009a). Transcript levels of both the endogenous AAA-ATPase (AAA) gene and the LUCIFERASE reporter gene (LUC) were constitutively up-regulated (Figure 1b),

demonstrating that *caa33* acts in *trans*. Seedlings of *flu/AAA:LUC+/caa33* exhibited a reduced size and a pale green leaf color (Figure 1a; Figure S1). The growth defect of *flu/AAA:LUC+/caa33* became even more obvious in older plants (Figure 1a). The chlorophyll content of *flu/AAA:LUC+/caa33* at different stages of seedling development was lower than in wild type (Figure S1b). It could not be restored to wild type level by growing seedlings on MS media supplemented with 0.5 % sucrose (Figure S1c) or under low light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Figure S4a).

***caa33* modulates, but is not part of the $^1\text{O}_2$ -dependent signaling pathway**

The screen for *caa* mutations in the *flu* parental line was based on the constitutive expression of the $^1\text{O}_2$ -responsive *AAA-ATPase* gene in the absence of enhanced $^1\text{O}_2$ production. Following a dark-to-light shift expression of the endogenous *AAA-ATPase* and the *AAA:LUC+* reporter gene in the *flu* mutant is strongly up-regulated and the transcripts of these genes reach high levels that exceed those in the *flu/caa33* mutant prior to re-illumination (Figure S2a). After shifting light-grown *flu/caa33* seedlings to the dark for 8 h, their Pchl_a reached a 5-fold higher level than in wild-type controls (Figure S2b). During re-illumination generation of $^1\text{O}_2$ in these mutant seedlings triggered a further strong up-regulation of *AAA-ATPase* and *AAA:LUC+* expression (Figure S2a). Hence, *caa33* does not perturb $^1\text{O}_2$ -mediated signaling. This conclusion was confirmed by analyzing *AAA-ATPase* expression in EX1- and EX2-deficient seedlings. The plastid proteins EX1 and EX2 are required for the transfer of $^1\text{O}_2$ -dependent signals from the plastid to the nucleus and the up-regulation $^1\text{O}_2$ -responsive genes. If the constitutive up-regulation of *AAA-ATPase* in *caa33* occurs via a different signaling pathway, inactivation of EX1/EX2 should not interfere. The *ex1/ex2* mutations were introduced into the *flu/caa33* background by crossing *flu/ex1/ex2* with *flu/caa33*. Homozygous *flu/caa33/ex1/ex2* quadruple mutants were selected from the segregating F₂ population of this cross. In *ex1/ex2* double mutants normal plastid differentiation during seedling development occurred only when immature seeds were allowed to develop in the dark (Kim *et al.*, 2009). Hence, for the subsequent analysis of the quadruple mutant only seeds were used from siliques that had been kept in the dark (Kim *et al.*, 2009). Seedlings were grown for 5 d under continuous light and the visible phenotype and transcript level of the *AAA-ATPase* gene were scored. Seedlings of the *flu/ex1/ex2* mutant and *flu* were indistinguishable, whereas *flu/caa33* and *flu/caa33/ex1/ex2* displayed a pale-green phenotype (Figure S3). Transcripts of *AAA-ATPase* reached a 6- to 7-fold higher level in *flu/caa33* and *flu/caa33/ex1/ex2* than in *flu* and *flu/ex1/ex2* (Figure 2), indicating that constitutive up-regulation of *AAA-ATPase* expression in *flu/caa33* was retained in the absence of EX1 and EX2.

The *caa33* mutant shows spontaneous light-intensity-dependent cell death formation

Major consequences of $^1\text{O}_2$ generation in the *flu* mutant after a D/L shift are rapid changes in nuclear gene expression followed by growth inhibition and cell death (op den Camp *et al.*, 2003; Kim *et al.*, 2008). The constitutive activation of the $^1\text{O}_2$ -responsive *AAA-ATPase* gene (Figure 1b) and growth inhibition of *flu/AAA:LUC+/caa33* (Figure 1a, Figure S1a) suggested that also cell death might be constitutively activated in the *flu/AAA:LUC+/caa33* mutant. The onset of cell death was analyzed in seedlings grown under continuous light. In cotyledons of 5-d-old *flu/AAA:LUC+/caa33* mutant seedlings only a few dead cells could be detected by Trypan Blue (TB) staining (Figure S1d). In 10-d-old seedlings the number of dead cells in cotyledons strongly increased (Figure 1c). In 21-d-old *flu/AAA:LUC+/caa33* plants a similar pattern of dead cells could be seen in rosette leaves as in cotyledons of 10-d-old seedlings (Figure S1d). The results of TB staining were confirmed by treating seedlings with propidium iodide (PI) (Figure 1c). PI is excluded from intact cells but penetrates dying or dead cells and intercalates double-stranded nucleic acids (Oparka and Reed, 1994).

Similar results were obtained after *flu/AAA:LUC+/caa33* was first backcrossed twice with Col-0 and homozygous *caa33* mutants (Col-0) lacking the *flu* mutation were analyzed.

Induction of cell death may result from exposure of plants to excess excitation energy (Mullineaux and Baker, 2010). However, in *caa33* mutants the appearance of cell death and other stress responses, such as the constitutive activation of the $^1\text{O}_2$ -responsive *AAA-ATPase* gene (Figure 1b) and reduced growth (Figure 1a), occur already under moderate light conditions ($90 \mu\text{mol m}^{-2} \text{s}^{-1}$). To analyze the possible light-intensity-dependency of these stress responses, seedlings were grown under continuous low light ($10 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 13 d, when seedlings reach a similar developmental stage as 10-d-old seedlings grown under $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ (compare Figure S4a with Figure 1a). Cell death was strongly suppressed in low light-grown seedlings, which contained only a few randomly distributed dead cells (Figure S4a). A comparable suppression of the growth-inhibition phenotype was observed (compare Figure S4b with Figure S1a). Similarly, the *AAA-ATPase* gene, but also other genes, such as *BAP1* and *ZAT12*, were expressed to a much lower extent in *caa33* grown for 13 d under low light (compare Figures 1b and S4c). Collectively, these results suggest that spontaneous cell death formation, constitutive activation of *AAA-ATPase* and growth inhibition in *caa33* are light-intensity-dependent and increase with higher light intensities.

caa33* is a new allele of *CRUMPLED LEAF

The mutated gene causing constitutive activation of the *AAA:LUC+* reporter gene in the *flu/AAA:LUC+/caa33* line was identified by map-based cloning using SSLP and CAPS polymorphic markers. The homozygous *flu/AAA:LUC+/caa33* mutant was backcrossed with *flu* in Ler ecotype. From the F2 mapping population of this cross homozygous *flu/AAA:LUC+/caa33* seedlings were selected that showed strong luciferase activity co-segregating with a reduced size and a pale green leaf color (Figure 1a). A bulk-segregant procedure (Michelmore *et al.*, 1991) was used to determine the approximate chromosomal position of the *caa33* mutation. PCR analysis of bulk DNA originating from 86 F2 recombinant *flu/AAA:LUC+/caa33* plants established a genetic linkage for the K20J1 and MAC9 polymorphic markers located on the lower arm of chromosome V (Figure S5).

763 F2 *flu/AAA:LUC+/caa33* plants of the segregating mapping population were analyzed for genetic linkage to polymorphic markers localized in the region between the K20J1 and MAC9 markers. *caa33* was localized in a 80 kb-region covered by two BAC clones, K7B16 and K3K7, which carry 23 open reading frames (Figure 3a). Three of these genes were predicted to encode chloroplast proteins. As the pale green phenotype of *flu/AAA:LUC+/caa33* linked to the constitutive luciferase activity is likely due to an impairment of chloroplast activity, genes predicted to encode chloroplast proteins were considered to be prime candidates for the *caa33* mutation. In one of these genes, At5g51020, a single point mutation at position 92 after the predicted start codon was detected that resulted in an amino acid exchange from Gly to Asp at position 31 (Figure 3b). Gly31 is located in the N-terminal hydrophobic region of the predicted protein that constitutes a putative trans-membrane domain (Figure S6). Previously, a mutant with an aberrant function of At5g51020 had been identified during a T-DNA library screen for plants with an altered leaf lamina that was named *crl* for *crumpled leaf* (Asano *et al.*, 2004). Hence, *caa33* represents a second, EMS-mutagenesis-derived allele of *crl* that was dubbed *crl-2*.

To confirm that the mutation in the *CRL* gene was responsible for constitutive luciferase expression and the morphological characteristics of *caa33*, two approaches were used. First, an allelism test was performed. *crl* and *flu/caa33* were crossed and F1 plants of this cross were analyzed. They resembled closely the parental *flu/caa33* plants, except that they were slightly paler (Figure 3c). Second, a plasmid containing the coding region of At5g51020 under the control of the cauliflower mosaic virus (CaMV) 35S promoter (*35S:CRL*) was

introduced into *flu/caa33* (Figure 3d–g). Complemented *flu/caa33* plants were phenotypically indistinguishable from *flu*, reverting the reduced size and pale phenotype of *flu/caa33* (Figure 3d). Transcripts of the AAA-ATPase gene (AAA) reached similar low levels in the T3 complementing line and in *flu* (Figure 3g), very much in contrast to the transcript level of this gene in *flu/caa33*. The complemented line also did no longer show a defect in chloroplast division as seen in *flu/caa33* (Figure 3f; see below). TB staining of the complemented T3 line demonstrated that spontaneous cell death in *flu/caa33* was no longer detectable (Figure 3e). Hence, constitutive AAA-ATPase expression, stunted growth, reduced Chl content, disturbed chloroplast division and spontaneous cell deaths in *caa33* are due to the mutation of a single gene, *CRL*.

Enlarged chloroplasts in mesophyll cells of *crl-2*

CRL forms part of the outer envelope membrane of plastids (Figure 4a) (Asano *et al.*, 2004). As shown previously mesophyll cells of the *crl* mutant contain only few, extremely enlarged chloroplasts (Asano *et al.*, 2004). Also in *crl-2/caa33* plastid division was severely impaired (Figures 3f and 4b,c). Mesophyll cells of *crl-2* (Col-0) contained a highly variable population of different-sized plastids, ranging from cells with approximately 12 enlarged plastids to cells with a single extremely large chloroplast (Figure 4b,c), whereas in wild type approx. 100 chloroplasts per mesophyll cell are present (Lopez-Juez and Pyke, 2005). The wide range of different plastid sizes in mesophyll cells of *crl-2* suggests that in *crl-2* plastids were divided unevenly as previously already reported for the *crl* mutant (Asano *et al.*, 2004). The plastid division defect in *crl-2* was also observed in other cell types like guard cells (Figure 4b). *crl-2* guard cells contained only two or one plastid per cell, or were completely devoid of plastids (Figure 4b). Guard cells lacking plastids were previously reported for other plastid division mutants such as *arc6* and *arc12* (Robertson *et al.*, 1996) and have been described in detail in *crl* (Chen *et al.*, 2009).

To test its light dependency the plastid division phenotype was also studied in etiolated seedlings. *crl-2* was crossed with a *SSU-GFP* transgenic line in a *flu* background (Kim and Apel, 2004). *flu/crl-2/SSU-GFP* plants were grown on MS media in the dark for 4 d and cotyledons were analyzed under a confocal microscope for GFP fluorescence and red autofluorescence of free Pchl_{ide} that over-accumulates in dark-grown *flu* seedlings (Meskauskiene *et al.*, 2001; op den Camp *et al.*, 2003). As shown in Figure 4d, mesophyll cells of dark-grown *flu/crl-2/SSU-GFP* seedlings contain etioplasts that are significantly enlarged relative to etioplasts of the parental *flu/SSU-GFP* line. The loss of a functional CRL protein thus results in an impairment of plastid division also in the absence of light.

Other plastid division mutants display a *crl-2*-like phenotype

In the *crl-2* mutant a defect in plastid division is linked to a change in the expression of the ¹O₂-responsive marker gene AAA-ATPase (Figure 1b) (Baruah *et al.*, 2009a), growth reduction (Figure 1a) and spontaneous cell death (Figure 1c). This suggests that an impairment of plastid division by other mutations also evokes a similar pleiotropic response as *crl-2*. To address this question known plastid division mutants were analyzed that either contain similar numbers of enlarged chloroplasts in mesophyll cells as *crl-2* or are affected in chloroplast envelope proteins with a similar localization pattern as the CRL protein (Asano *et al.*, 2004). Based on these criteria *arc6-1*, *ftsZ1-1* and *pdv2-2* mutant lines were selected (Pyke *et al.*, 1994; Osteryoung and Vierling, 1995; Osteryoung *et al.*, 1998; Marrison *et al.*, 1999; Stokes and Osteryoung, 2003; Miyagishima *et al.*, 2006) (Table S1). Seeds of T-DNA-tagged lines were obtained for all mutants from the SALK collection (Alonso *et al.*, 2003). Homozygous T-DNA mutant lines were identified by genotyping (Table S1) and/or by microscopic identification of enlarged chloroplasts in mesophyll cells

of young seedlings. Similar patterns of enlarged chloroplasts were observed in mesophyll cells of *arc6-1*, *ftsZ1-1*, *pdv2-2* and *crl-2* (Figure 5).

In each of these lines initiation of cell death was analyzed by TB staining. Positive TB staining was detected in cotyledons of *arc6-1* and *ftsZ1-1* 10-d-old seedlings (Figure 5c). In cotyledons of *pdv2-2* only a few randomly distributed dead cells were observed. True leaves of all selected mutants displayed positive TB staining primarily in palisade parenchyma cells (Figure 5c). Such a staining was not seen in wild type. A similar result was obtained after staining dead cells with PI (data not shown). As in *crl-2*, light-intensity-dependent cell death initiation was also observed in cotyledons and true leaves of *arc6-1*, *ftsZ1-1* and *pdv2-2* showing attenuated cell death under low light conditions (Figure S7).

Finally, the expression of ROS marker genes was analyzed in cotyledons of chloroplast division defect mutants. Similar to *crl-2*, de-repression of *AAA-ATPase* gene expression occurred also in *arc6-1*, *pdv2-2* and, to a lesser extent, *ftsZ1-1* (Figure 5d). Another $^1\text{O}_2$ -responsive gene, *BAP1*, was even more de-repressed in *arc6-1* and *pdv2-2* than in *crl-2*. The general oxidative stress marker gene *ZAT12* was constitutively activated in all mutant lines with transcript levels higher in *pdv2-2* and *arc6-1* than in *crl-2*, whereas the H_2O_2 -marker gene *FER1* (At5g01600) was hardly affected in any of the mutant lines.

The possible impact of the light avoidance response of chloroplasts

Impairment of plastid division in *crl-2* and other plastid division mutants activates a stress response that results in the up-regulation of $^1\text{O}_2$ -responsive and general oxidative stress marker genes and in spontaneous microlesion formation in cotyledons and true leaves. The light-intensity dependency of these stress responses may be due to the reduced ability of plastid division mutants to avoid potentially harmful higher light by moving chloroplasts to the cell periphery and orienting them in columns parallel to the plane of the incoming light (Glynn *et al.*, 2007; Königer *et al.*, 2008). Chloroplast movement is thought to alleviate photo-damage of photosystem II under high light conditions (Kasahara *et al.*, 2002; Ii and Webber, 2005). Enlarged chloroplasts have a reduced capacity to utilize light energy and to minimize photo-damage under varying light conditions (Jeong *et al.*, 2002). To verify this possible cause of stress responses in plastid division mutants, the avoidance response mutants *phot2* and *chup1* (Kasahara *et al.*, 2002) were grown under the same light conditions as *crl-2*. *AAA-ATPase* transcript levels in these mutants were compared to those in wild-type control seedlings. In contrast to *crl-2*, *phot2* and *chup1* seedlings did not up-regulate *AAA-ATPase* relative to wild type (Figure S8a) and they did not form microlesions (Figure S8b). Hence, constitutive stress responses in *crl-2* do not seem to be primarily due to a perturbed light avoidance response of chloroplasts.

Cross-acclimation in the *crl-2* mutant

The *crl-2* mutation does not perturb the $^1\text{O}_2$ -dependent signaling pathway (Figures S2 and 6a,b). Thus, a $^1\text{O}_2$ -mediated cell death response after a dark-to-light shift may be expected not only in *flu* but also in *flu/crl-2* seedlings. Surprisingly, the presence of the *crl-2* mutation in *flu* seedlings suppresses the onset of $^1\text{O}_2$ -mediated cell death (Figure 6c).

Previously, other second-site mutations have been identified in *flu* that like *crl-2* do not perturb $^1\text{O}_2$ -mediated signaling but lead to a suppression of $^1\text{O}_2$ -mediated cell death (Coll *et al.*, 2009; Meskauskiene *et al.*, 2009; Baruah *et al.*, 2009a,b). Suppression of $^1\text{O}_2$ -mediated cell death in *caa13/flu*, *soldat8/flu* and *soldat10/flu* has been attributed to a constitutive minor stress response that activates acclimation prior to the release of $^1\text{O}_2$ after a dark-to-light shift. Two criteria have been used to define such a change in stress susceptibility of the two *soldat* mutants and *caa13*: First, the constitutive up-regulation of stress-responsive

genes and second, an enhanced resistance against a combined low temperature/high light stress. *crl-2* seedlings also up-regulate the expression of stress-responsive genes but they do not show an enhanced resistance against the combined low temperature/high light stress (Baruah *et al.*, 2009a). If indeed constitutive activation of acclimation suppresses $^1\text{O}_2$ -mediated cell death in *crl-2/flu*, an acclimatory response in *crl-2* must differ from that in the other second-site mutants of *flu*. The cell death response pattern in *crl-2* seedlings recapitulates closely microlesion formation in Arabidopsis plants in response to pathogens (Alvarez *et al.*, 1998). The possible constitutive activation of a pathogen defense response in *crl-2* was first tested by measuring transcript levels of the *PR1* (**P**athogen-**R**elated protein 1) gene in 10-d-old seedlings of *crl-2*, *flu* and wild type. An enhanced expression of the pathogen defense marker gene *PR1* was confined to light-grown *crl-2* seedlings and to *flu* seedlings exposed to a dark/light shift. In wild type and *flu* seedlings grown under continuous light *PR1* transcripts were not detectable (Figure 7a). To test the possible physiological impact of stress pre-acclimation in *crl-2*, wild type and *crl-2* seedlings were spray-inoculated with virulent *Pseudomonas syringae* DC3000 that expresses constitutively a *LUCIFERASE* reporter gene (Fan *et al.*, 2007). The bacterial growth was determined after different lengths of incubation by measuring the luciferase activity in extracts of inoculated plants. In *crl-2* growth of the virulent pathogen was strongly suppressed compared to wild type seedlings (Figure 7b).

DISCUSSION

In the present work we have characterized one of the *caa* mutants isolated previously during a screen aimed at identifying constituents of the $^1\text{O}_2$ -dependent plastid-to-nucleus signaling pathway (Baruah *et al.*, 2009a). Mutations of *CAA* genes in the *flu* mutant result in the constitutive up-regulation of the $^1\text{O}_2$ -responsive *AAA-LUCIFERASE* reporter gene and the endogenous *AAA-ATPase* in the absence of enhanced $^1\text{O}_2$ production. Two types of *caa* mutants have been distinguished. *caa* mutants that genetically form part of the $^1\text{O}_2$ signaling pathway and contain the *flu* mutation have already strongly activated the $^1\text{O}_2$ -dependent signaling pathway prior to the release of $^1\text{O}_2$ and are unable to further enhance the expression of $^1\text{O}_2$ -responsive genes after a dark-to-light shift. In the second group activation of $^1\text{O}_2$ -responsive genes by enhanced levels of $^1\text{O}_2$ is not impaired. Following a dark-to-light shift the release of $^1\text{O}_2$ leads to a similar up-regulation of these genes as in the parental *flu* line, suggesting that these *caa* mutations modulate $^1\text{O}_2$ -mediated signaling more indirectly by affecting other signaling pathways that happen to co-regulate the expression of $^1\text{O}_2$ -responsive genes (Baruah *et al.*, 2009a).

caa33 was identified by map-based cloning as a weak allele of the *crumpled leaf* mutation (*crl*) characterized earlier by Asano *et al.* (2004). Hence, *caa33* was renamed *crl-2*. The *CRL* gene encodes a plastid envelope protein that is required for normal plastid division. Unlike other plastid division mutants that phenotypically closely resemble wild type, the *crl* mutant is disturbed in its growth and development. Similar to *crl* also *crl-2* shows such a pleiotropic effect. Besides the impairment of plastid division *crl-2* seedlings and mature plants have less Chl and grow more slowly than wild type. Because of these pleiotropic consequences of the *crl* mutation, *CRL* was not only associated with plastid division but was also implicated with the transduction of signals that control plant development (Maple and Moller, 2007). In accordance with this proposed signaling activity it would have been conceivable that *CRL* forms part of the $^1\text{O}_2$ -dependent signaling pathway by transferring $^1\text{O}_2$ -dependent signals across the plastid envelope to the surrounding cytoplasm. However, our work clearly rules out such a direct involvement of *CRL* in $^1\text{O}_2$ signaling.

Firstly, after a dark-to-light shift *crl-2/flu* mutants retain the ability of the parental *flu* line to strongly up-regulate the expression of $^1\text{O}_2$ -responsive genes in response to enhanced $^1\text{O}_2$

production. Hence, *crl-2* does not seem to perturb directly the $^1\text{O}_2$ -dependent signaling pathway. Secondly, constitutive up-regulation of AAA-ATPase expression in *flu/caa33* was retained in a *flu/caa33/ex1/ex2* quadruple mutant. Thus, constitutive up-regulation of AAA-ATPase in *caa33* must occur via a signaling pathway that is different from EX1/EX2-dependent $^1\text{O}_2$ -mediated signaling. Finally, constitutive up-regulation of $^1\text{O}_2$ -responsive genes and spontaneous microlesion formation as seen in *crl-2* seedlings kept under continuous light were also found in other plastid division mutants. These data suggest that the defect in proper plastid division *per se* is a factor that impacts normal plastid function and modulates $^1\text{O}_2$ -mediated signaling.

Various mutant screens have been carried out to identify constituents of retrograde signaling (Susek *et al.*, 1993; Gray *et al.*, 2003; Ball *et al.*, 2004; Wagner *et al.*, 2004; Rossel *et al.*, 2006; Ruckle *et al.*, 2007; Heiber *et al.*, 2007; Baruah *et al.*, 2009a; Saini *et al.*, 2011; Woodson *et al.*, 2011). Several of these screens have been undertaken using carotenoid-deficient seedlings grown in the presence of norflurazon (NF), an inhibitor of phytoene desaturase. NF-treated seedlings are bleached and suppress the expression of nuclear genes encoding chloroplast proteins. Several *gun* (*genomes uncoupled*) and *hon* (*happy-on-norflurazon*) mutants have been isolated that de-repress the expression of these genes (Susek *et al.*, 1993; Gray *et al.*, 2003; Saini *et al.*, 2011; Woodson *et al.*, 2011). Suppressor screens with the *flu* mutant have led to the identification of *executer* (*ex*) and *soldat* mutants that either fully (*ex*) or partially (*soldat*) suppress responses to $^1\text{O}_2$ -mediated signaling (Wagner *et al.*, 2004; Meskauskiene *et al.*, 2009). Most mutations isolated during these various screens have in common that they impede plastid-specific functions. They have been suggested to define either genuine constituents of retrograde signaling pathways (*ex1*, *gun1*) (Wagner *et al.*, 2004; Koussevitzky *et al.*, 2007) or components required for the synthesis of putative plastid signals such as Mg protoporphyrin IX or heme (*gun2 – gun6*) (Mochizuki *et al.*, 2001; Strand *et al.*, 2003; Woodson *et al.*, 2011). The analysis of *soldat* and *hon* mutants, however, suggests another way to explain modulation of retrograde signaling by mutations. *soldat* and *hon* mutants suffer from defects in protein synthesis or degradation within the plastid compartment that perturb plastid protein homeostasis (Coll *et al.*, 2009; Meskauskiene *et al.*, 2009; Saini *et al.*, 2011). As plastids may act as sensors of stress and evoke stress responses of plants via plastid-to-nucleus signaling, it is not surprising that the perturbation of plastid homeostasis in *soldat* and *hon* mutants is perceived as stress and triggers an acclimatory response. Exposing plants to $^1\text{O}_2$, higher light, lower or higher temperatures and wounding may also initiate acclimatory responses. These treatments usually do not visibly damage the plant but markedly enhance their resistance against a subsequent more severe stress (Prasad *et al.*, 1994; Karpinski *et al.*, 1999; Iida *et al.*, 2000; Chang *et al.*, 2004; Ledford *et al.*, 2007; Kim *et al.*, 2008). Acclimatory responses may also confer cross-resistance against stress factors different from those that were initially perceived (Bowler and Fluhr, 2000; Mateo *et al.*, 2004; Mühlenbock *et al.*, 2008). In *soldat8* and *soldat10* mutants constitutive acclimation to light stress results in the suppression of $^1\text{O}_2$ -mediated cell death (Coll *et al.*, 2009; Meskauskiene *et al.*, 2009). Seedlings of *hon5* and *hon23* suffering from a minor constitutive stress are able to green in the presence of NF (Saini *et al.*, 2011). In *flu/crl-2* $^1\text{O}_2$ -mediated cell death was strongly attenuated after a dark-to-light treatment, whereas activation of $^1\text{O}_2$ -dependent AAA-ATPase gene expression was not affected. These results suggest that *crl-2* likewise activates an acclimatory response prior to the release of $^1\text{O}_2$ that reduces the mutant's stress sensitivity. However, in contrast to *soldat* and *hon* mutants resistant to a combined harsh low temperature/high light treatment (Coll *et al.*, 2009; Meskauskiene *et al.*, 2009; Saini *et al.*, 2011), *crl-2* subjected to the same photo-inhibitory condition still bleached and died as wild type. At the same time *crl-2* seedlings were more resistant against a virulent strain of the plant pathogen *Pseudomonas syringae*. Hence, the term “disturbance of plastid homeostasis” does not describe a well-

defined physiological condition that activates a uniform acclimatory response. Instead, depending on the initial perturbation, the impact of acclimation may vary.

Activation of stress acclimation by a disturbance of plastid homeostasis has important physiological consequences. For instance, as shown in the present work, the impact of $^1\text{O}_2$ -mediated plastid signaling in *flu* seedlings leading to cell death is suppressed under conditions that pre-acclimate *flu/crl-2*. Drastic stress responses of plants kept under well-defined experimental conditions may not occur under field conditions that impede plastid activity and may attenuate these responses due to acclimation. Studies of retrograde signaling in plants and the identification of putative retrograde signaling constituents by mutant screens do not always consider the possible de-repressing effect of pre-conditioning plants. Hence, a major challenge for future studies will be the identification of signaling pathways that respond to a perturbation of plastid homeostasis and activate acclimation, thereby attenuating the impact of retrograde plastid-to-nucleus signaling.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

All experiments were performed with *Arabidopsis thaliana* ecotype *Columbia* (Col-0), if not stated differently. The *flu* Col-0 line used in this work had been obtained by five backcrosses of *flu1-1* (Meskauskiene *et al.*, 2001) in *Landsberg erecta* (*Ler*) with wild-type Col-0. Other lines used in this study were: *flu/AAA:LUC+* (Baruah *et al.*, 2009a); *flu/AAA:LUC+/caa33*, *flu/caa33* and *caa33* obtained by three backcrosses of *flu/AAA:LUC+/caa33* with *flu* Col-0 and Col-0, respectively; *crl*, kindly provided by Dr. Yasushi Yoshioka, Riken Institute, Japan; *phot2* and *chup1*, kindly provided by Dr. Masamitsu Wada, National Institute for Basic Biology, Okazaki, Japan; *arc6-1* (N286), *ftsZ1-1* (SALK_073878), *pdv2-2* (SAIL_875_E10), *ex1* (SALK_002088) and *ex2* (SALK_012127). Sequences of primers used for genotyping the mutant lines are listed in supplementary Table S1. Primers used to identify homozygous *ex1/ex2* double mutants have been described by Lee *et al.* (2007). Seeds were either surface sterilized and grown on Murashige and Skoog medium (without sucrose) including vitamins and MES buffer (M0255; Duchefa, Haarlem, The Netherlands) and 0.8% (w/v) agar (Sigma-Aldrich, Buchs, Switzerland) at 20°C in continuous light (80–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or, for cultivation of mature plants, sown on soil (Klasmann Substrat 2, Klasmann-Deilmann, Geeste, Germany) and grown for 20 d under the same conditions.

Luciferase imaging

Luciferase imaging in plants was performed as described previously (Baruah *et al.*, 2009a).

Identification and complementation of the *caa33* mutation

A segregating F2 mapping population was generated from a cross of *flu/AAA:LUC+/caa33* in Col-0 with *flu/AAA:LUC+* in *Ler*. Out of 3000 F2 plants, homozygous *flu/AAA:LUC+/caa33* mutants were selected based on high constitutive luciferase expression in continuous light. The *CAA33* locus was mapped using CAPS (cleaved amplified polymorphic sequence) or SSLP (simple sequence length polymorphism) markers listed in “The Arabidopsis Information Resource database” (TAIR, www.arabidopsis.org). Additional markers used for mapping were designed based on the collection of predicted Arabidopsis single nucleotide polymorphisms (SNP) and small insertions/deletions in the publicly available Col-0 and *Ler* sequences generated by Monsanto (<http://www.arabidopsis.org/Cereon>) and are provided in Table S2. For complementation, the coding region of the *CRL* gene in pSK1 under the control of the *CaMV* 35S promoter (*p35S::CRL*, kindly provided by Prof. Yasushi Yoshioka, Nagoya University, Japan) was introduced into *flu/caa33* via *Agrobacterium*-mediated transformation as described (Clough and Bent, 1998). Primer information and details are

described in Asano *et al.* (2004). Positive transformants were selected on hygromycin-containing media.

Extraction and measurement of chlorophyll

For pigment analysis of seedlings approximately 20 mg fresh weight per sample was harvested. The plant material was immediately frozen in liquid nitrogen and total pigment extracted in 1 ml of 96% ethanol. The absorbance of light by pigments in the supernatant was measured at 663, 645 and 480 nm. The total chlorophyll content was calculated as described by Hendry and Price (1993).

Confocal laser scanning microscopy

The green fluorescence of GFP and the red fluorescence of chlorophyll were monitored using a confocal laser scanning microscope (CLSM) (TCS-NT; Leica Microsystems, Heidelberg, Germany) with Kr/Ar laser excitation. GFP and chlorophyll fluorescence were induced at an excitation wavelength of 488 nm. GFP and chlorophyll were detected at emission wavelengths of 507 to 520 nm and 620 to 700 nm, respectively. TCS-NT software version 1.6.587 (Leica Microsystems) was used for image acquisition and processing. For measurements of plastid size, the sections of mesophyll cells in cotyledons and leaves were digitally scanned by using a Confocal Laser Scanning Microscope (Leica Microsystems). Plastid size measurements were performed for three independently selected areas. The Image program software (TCS NT, version 1.6.587, Leica Microsystems) was used to trace plastid outlines and to determine the diameter of each plastid.

Determination of Cell Death

The absence or presence of cell death was analyzed using 10-d-old wild type and *caa33* seedlings grown under continuous light ($90 \mu\text{mol m}^{-2} \text{s}^{-1}$). To identify the onset of cell death, seedlings were incubated with $50 \mu\text{M}$ of propidium iodide (PI, Molecular Probes, Eugene, USA) diluted with water. After removing the excess of dye with water, samples were observed under the CLSM. The DNA and PI complexes in cells undergoing cell death were visualized with the 561 DPSS and the emission was captured using 613–630-nm bandpass filter. Trypan blue staining of seedlings was performed as previously described (Keogh *et al.*, 1980).

RNA extraction and RT-PCR

Total RNA was extracted from seedlings by using an RNeasy plant mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from 0.6 μg of RNA, treated with DNase (Promega) by using random primers (Promega) and Improm II reverse transcriptase (Promega) according to the manufacturer's instructions. RT-PCR was performed with equal amounts of cDNAs by using the GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed as described previously (Baruah *et al.*, 2009b). *AAA-ATPase* was selected as an early singlet-oxygen responsive gene (GEO accession number for microarray data, GSE10509; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE10509>). Sequences of primers used for quantitative RT-PCR are listed in Table S3.

Pathogen treatment and determination of susceptibility

To analyze stress susceptibility of plants, 10-d-old wild type and *caa33* seedlings were challenged with 5×10^8 cfu/ml of *luxCDA- BE*-tagged virulent *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) (Fan *et al.*, 2007). The relative luminescence of bacteria of individual seedlings following D-luciferin (2mM) treatment was determined using a luminescence reader (Modulus™ II Reader, TUNRNER BIOSYSTEM, Sunnyvale, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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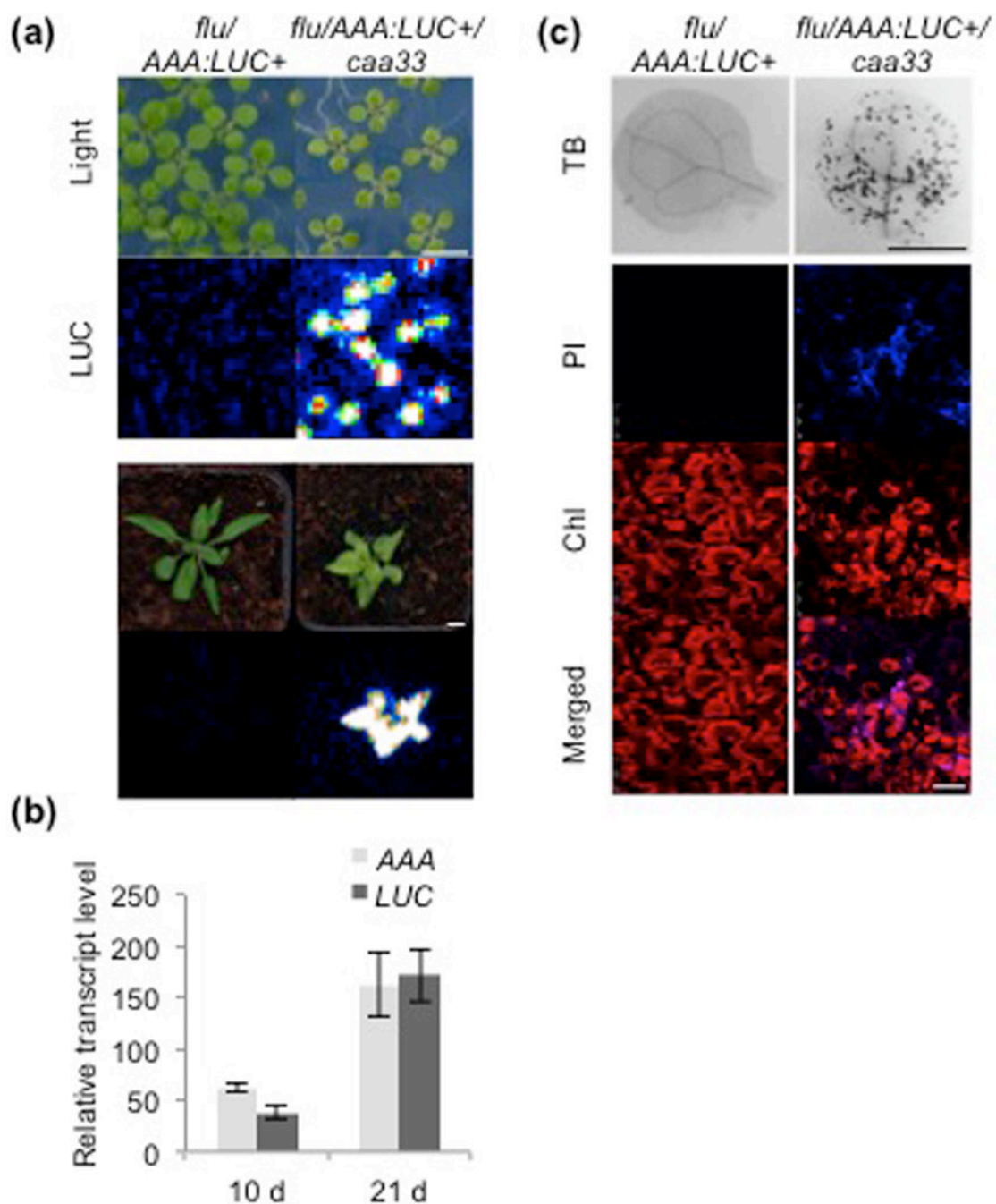


Figure 1. Characterization of the *caa33* mutant

(a) Phenotype and luciferase activity of the *flu/AAA:LUC+/caa33* mutant and the *flu/AAA:LUC+* parental line grown under continuous light (CL) ($90 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) on MS agar plates for 10 d (top panel) and in soil for 21 d (bottom panel). Bioluminescence images (LUC) and corresponding visible pictures (Light) are presented. Scale bars represent 0.5 cm.

(b) Relative transcript levels of *AAA-ATPase* (*AAA*) and the *AAA:LUC+* reporter gene (*LUC*) in cotyledons of 10-d-old seedlings and rosette leaves of 21-d-old *flu/AAA:LUC+/caa33* mutant plants. Transcript levels were determined by qRT-PCR and are expressed relative to the parental *flu/AAA:LUC+* line. Results represent mean values of two independent experiments \pm SD.

(c) Spontaneous cell death formation in the *caa33* mutant. Staining with Trypan Blue (TB) and propidium iodine (PI) was done using 10-d-old light-grown seedlings of *flu/AAA:LUC+* and *flu/AAA:LUC+/caa33*.

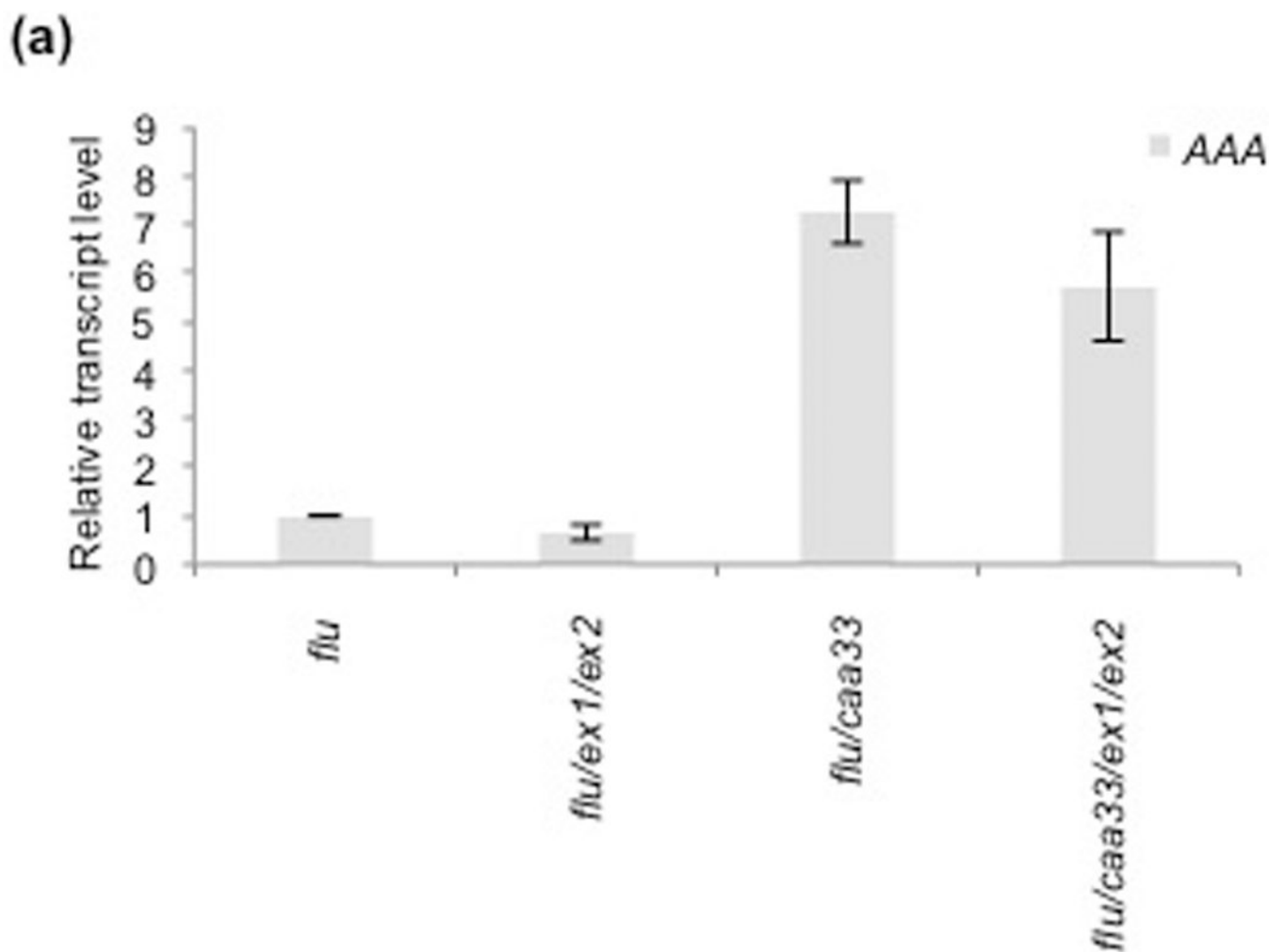


Figure 2. The expression of $^1\text{O}_2$ -responsive genes in 5-d-old seedlings of *flu*, *flu/ex1/ex2*, *flu/caa33* and *flu/caa33/ex1/ex2* grown under continuous light
Relative AAA-ATPase (AAA) transcript levels as determined by qRT-PCR. The transcript level in *flu* was set as “1”. Results represent mean values of two independent experiments \pm SD.

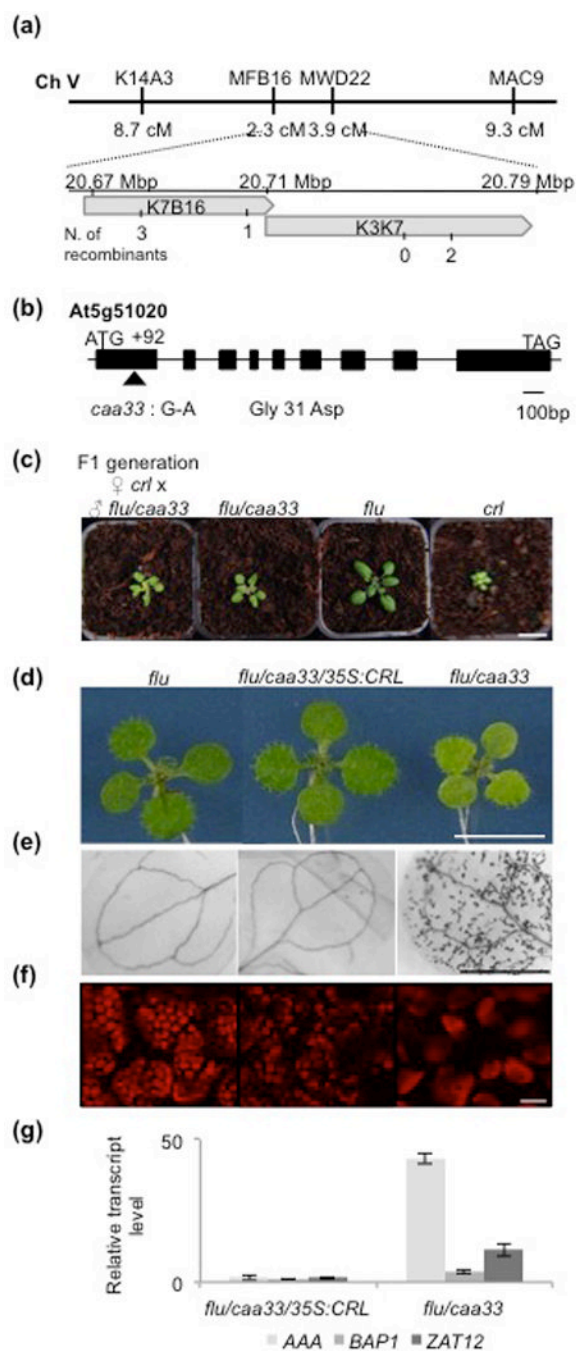


Figure 3. Identification and complementation of the *caa33* mutation

(a) Mapping of *CAA33*. Initial mapping analysis revealed genetic linkage of *CAA33* to markers located on the lower arm of chromosome V. Fine mapping localized the *caa33* mutation in an 80 kb region covered by BAC clones K7B16 and K3K7.

(b) A single G to A nucleotide substitution was found in the first exon of the At5g51020 locus in the *caa33* mutant, resulting in a Gly31 to Asp amino acid exchange. Filled boxes indicate exons, lines indicate transcribed regions.

(c) Allelism test of *flū/caa33* with the *crl* mutant. The *flū/caa33* mutant was crossed with *crl* and the F1 generation was raised. Phenotypes of 2-week-old F1, *flū/caa33* and *flū* and 4-week-old *crl* plants grown under continuous light. Scale bar represents 1 cm.

- (d) Complementation of *flu/caa33*. Phenotypes of 10-d-old seedlings of *flu*, *flu/caa33/35S:CRL* and *flu/caa33*. Scale bar represents 0.5 cm.
- (e) Trypan Blue staining of cotyledons of 10-d-old seedlings of *flu*, *flu/caa33/35S:CRL* and *flu/caa33* grown under continuous light. Scale bar represents 0.3 cm.
- (f) Confocal image of chlorophyll autofluorescence in mesophyll cells of 10-d-old seedlings of *flu*, *flu/caa33/35S:CRL* and *flu/caa33* grown under continuous light. Scale bar represents 30 μm .
- (g) Relative transcript levels of *AAA-ATPase*, *BAP1* and *ZAT12* in cotyledons of 10-d-old seedlings of *flu/caa33/35S:CRL* and *flu/caa33* grown under continuous light. Expression levels were analysed by qRT-PCR and expressed relative to *flu*. Results represent mean values of two independent experiments \pm SD.

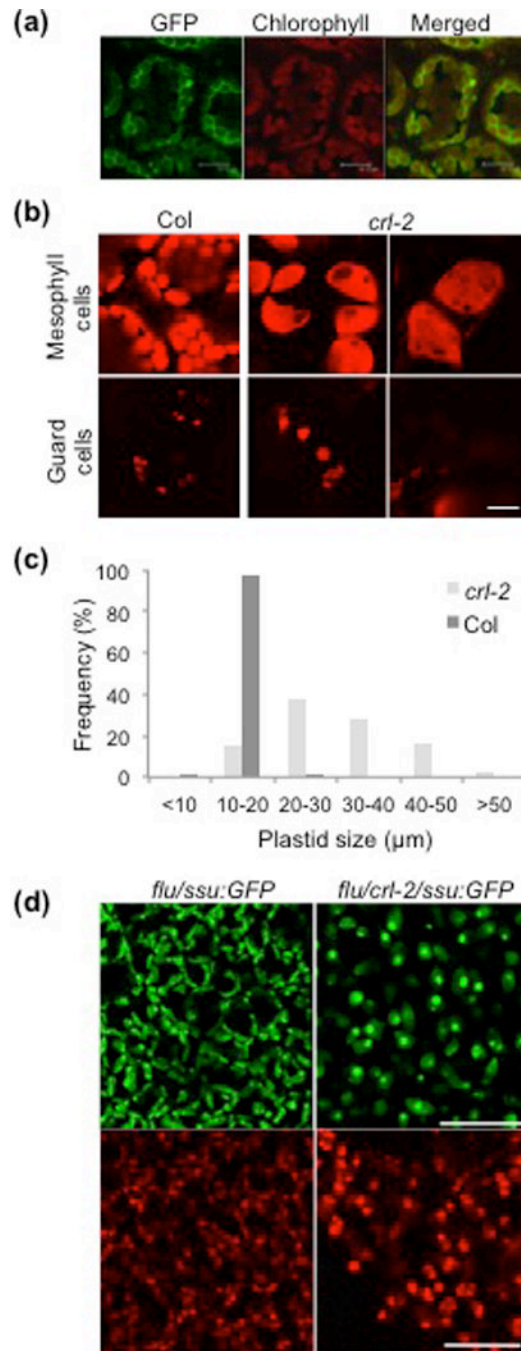


Figure 4. Mesophyll cells of *crl-2* mutant contain enlarged chloroplasts

(a) GFP fluorescence (left panel), chlorophyll autofluorescence (middle panel) and merged image (right panel) showing mesophyll cells of transgenic plants expressing CRL-GFP under the control of 35S promoter. Scale bars represent 18 μm .

(b) Chlorophyll autofluorescence confocal laser scanning microscopy images of chloroplasts in mesophyll and guard cells of 5-d-old *crl-2* and wild type (Col) seedlings grown under continuous light. Scale bar represents 20 μm .

(c) Frequency distribution of chloroplast sizes in cotyledons of 5-d-old seedlings of *crl-2* and wild type (Col). Percentage of plastids of different sizes measured from 100 Col (dark

grey bars) and *caa33* (light grey bars) chloroplasts are presented. Average of two experiments is presented.

(d) GFP fluorescence (upper panel) and red autofluorescence of free protochlorophyllide (lower panel) in etioplasts of 4-d-old dark grown *flu/crl-2/SSU-GFP* and *flu/SSU-GFP* seedlings. Scale bar represents 30 μm .

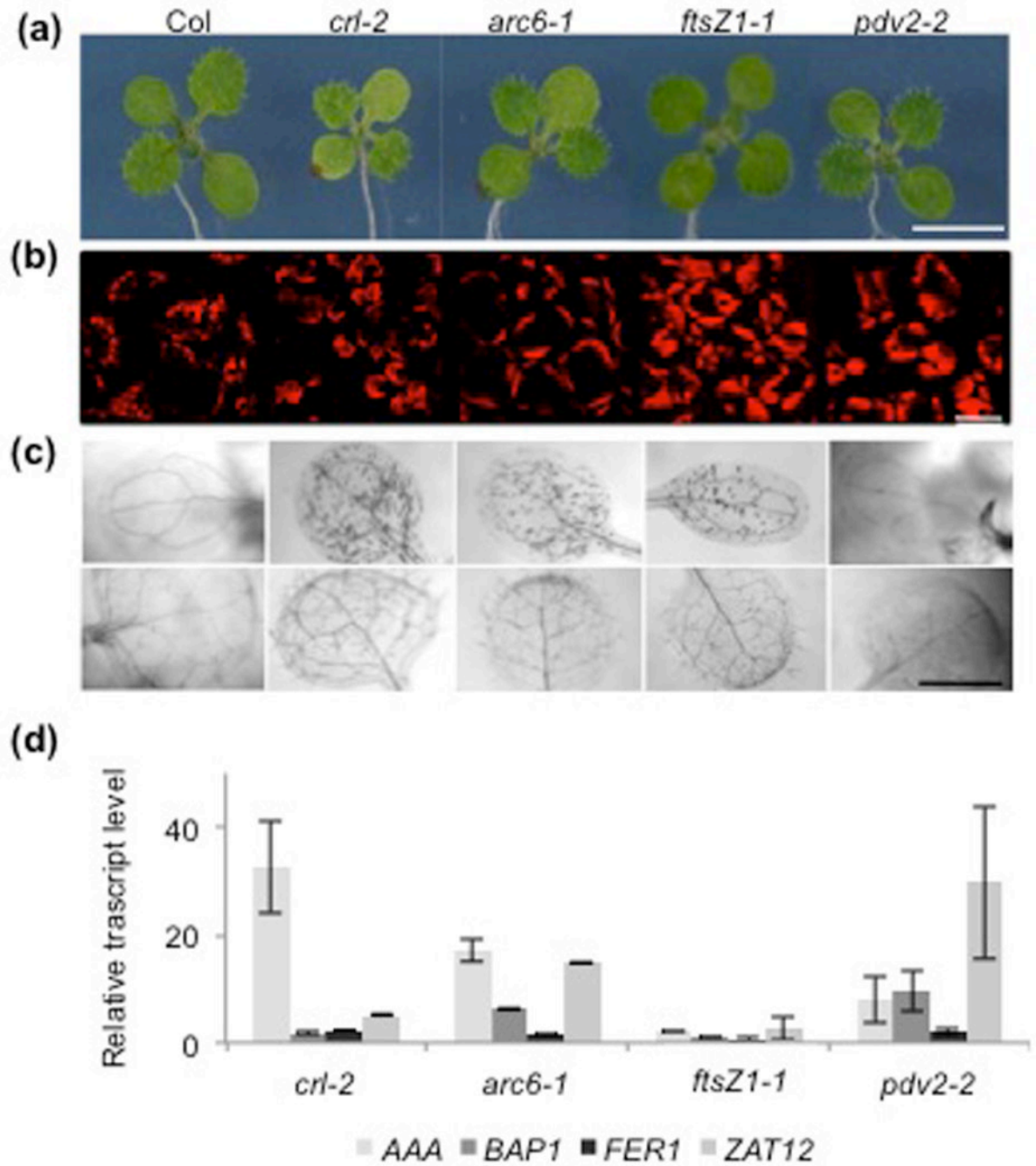


Figure 5. A comparison of *crl-2*, *arc6-1*, *ftsZ1-1* and *pdv2-2* plants

Seedlings were grown for 10 d under continuous light.

(a) Morphological phenotypes of the selected mutant lines. Scale bar represents 0.5 cm.

(b) Chlorophyll autofluorescence confocal laser scanning microscopy images of mesophyll cells of the selected mutant lines. Scale bar represents 30 μ m.

(c) Trypan Blue staining of cotyledons (upper panel) and true leaves (lower panel) of the selected mutant lines. Scale bar represents 0.25 cm.

(d) Relative transcript levels of *AAA-ATPase*, *BAP1*, *FER1* and *ZAT12* marker genes in cotyledons of 10-d-old *crl-2*, *arc6-1*, *ftsZ1-1* and *pdv2-2* seedlings. Transcript levels were determined by qRT-PCR relative to the corresponding wild type line (*Col-0* for *crl-2*,

ftsZ1-1 and *pdv2-2*, and WS-2 for *arc6-1*). Results represent mean values of two independent experiments \pm SD.

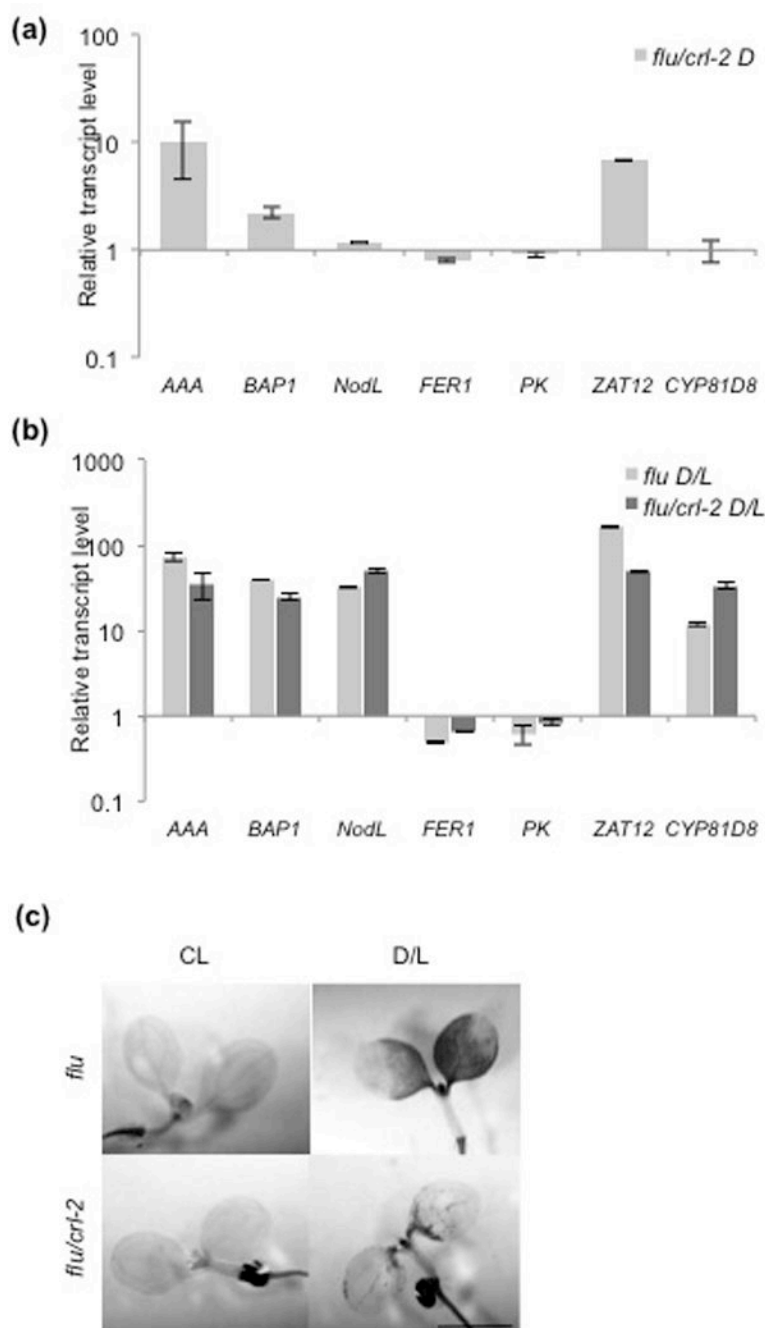


Figure 6. The response of *flu/crl-2* to the release of singlet oxygen

Relative transcript levels of ROS-marker genes were analyzed in 5-d-old seedlings grown under continuous light and transferred to the dark for 8 h without or with a subsequent exposure to light.

(a) Transcript levels in *flu/crl-2* at the end of the 8 h dark period as determined by qRT-PCR relative to *flu*. Results represent mean values of two independent experiments \pm SD.

(b) Fold-inductions in *flu* (*flu* D/L; light grey bars) and *flu/crl-2* (*flu/crl-2* D/L; dark grey bars) after 1 h re-illumination. Transcript levels were determined by qRT-PCR relative to transcript levels at the end of the dark period. Results represent mean values of two independent experiments \pm SD.

(c) Trypan Blue staining of 5-d-old seedlings of *flu* and *flu/crl-2* grown under continuous light (CL) or following an 8h-D/16 h-L shift (D/L).

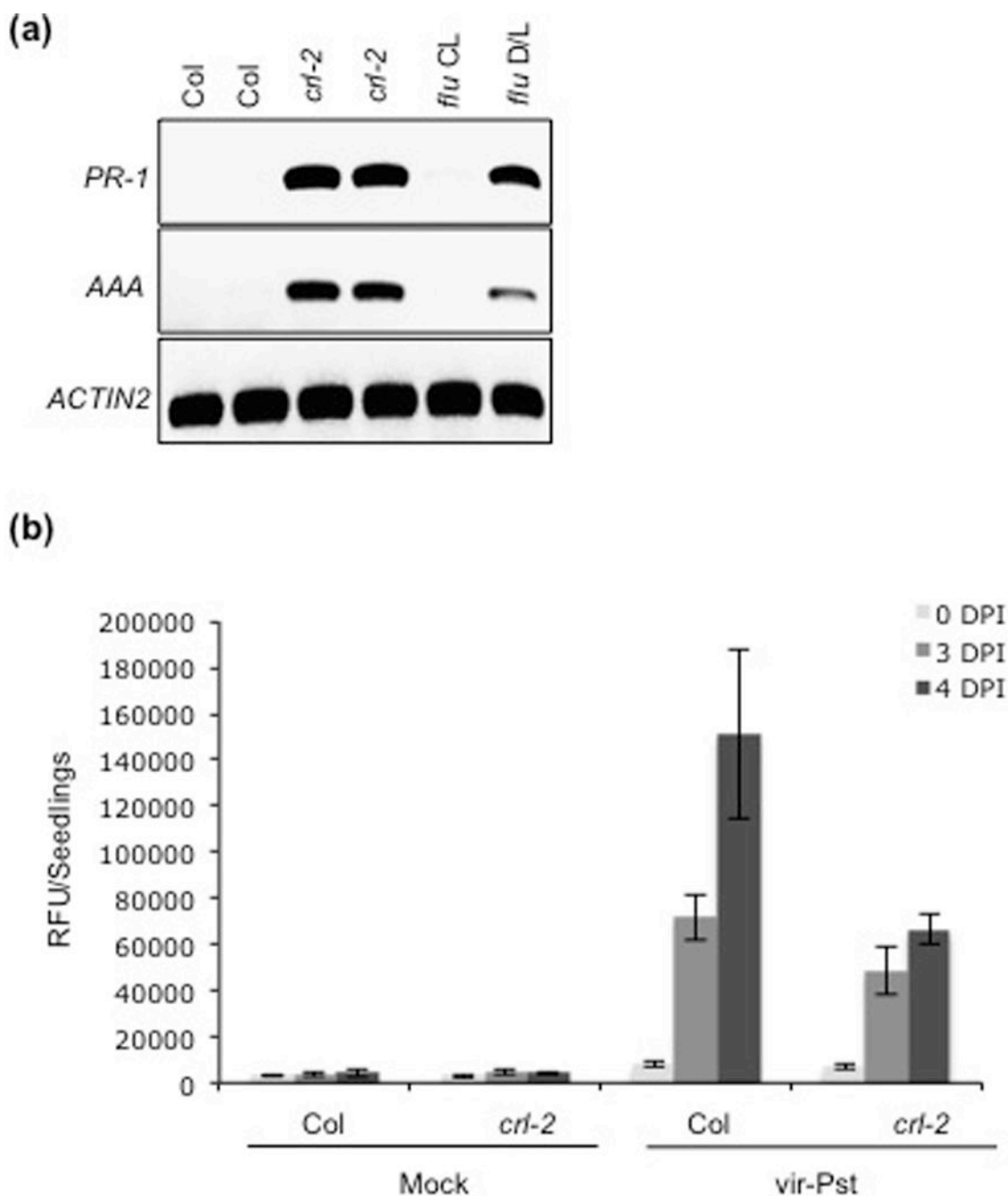


Figure 7. Constitutive stress acclimation in the *crl-2* mutant

(a) Transcript levels of the *PATHOGEN-RELATED PROTEIN1* (*PR1*), *AAA-ATPase* and *ACTIN2* genes in 10-d-old seedlings of wild type (Col), *crl-2* and *flu* grown under continuous light (wt, *crl-2*, *flu* CL) or transferred to the dark for 8 h and re-exposed to light for 1 h (*flu* D/L).

(b) Constitutive, enhanced pathogen resistance of *crl-2*. 10-d-old seedlings of wild type (Col) and *crl-2* seedlings were spray-inoculated with virulent *Pseudomonas syringae* pv *tomato* DC3000 (vir-Pst) expressing a *LUCIFERASE* reporter gene (Fan *et al.*, 2007). Seedlings were inoculated for 0, 3 and 4 d (DPI) and the bacterial growth was determined by measuring the luciferase activity in plant extracts as relative fluorescence units (RFU).

Mock-treated plants sprayed with a solvent without bacteria were used as controls. Values represent the average and standard deviation of three experiments.