Ubiquitin facilitates a quality-control pathway that removes damaged chloroplasts

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Abstract

Energy production by chloroplasts and mitochondria causes constant oxidative damage. A functioning photosynthetic cell requires quality-control mechanisms to turn over and degrade chloroplasts damaged by reactive oxygen species (ROS). Here, we generated a conditionally lethal *Arabidopsis* mutant that accumulated excess protoporphyrin IX in the chloroplast and produced singlet oxygen. Damaged chloroplasts were subsequently ubiquitinated and selectively degraded. A genetic screen identified the plant U-box 4 (PUB4) E3 ubiquitin ligase as being necessary for this process. *pub4-6* mutants had defects in stress adaptation and longevity. Thus, we have identified a signal that leads to the targeted removal of ROS-overproducing chloroplasts.

In chloroplasts, an electron transport chain allows energy from sunlight to be used. When the chloroplast's capacity to transfer electrons is exceeded (owing to high irradiance, drought, or extreme temperatures) reactive oxygen species (ROS) (e.g., singlet oxygen (¹O₂) or peroxides) are generated (1). ROS can then damage DNA, proteins, lipids, and other cellular components. Thus, safeguards have evolved that allow for rapid cellular responses to these injuries.

Information about ROS damage is relayed by chloroplast-to-nucleus (retrograde) signals that broadly regulate nuclear genes involved in chloroplast function and stress adaptation (2, 3).

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SUPPLEMENTARY MATERIALS

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Retrograde signals involve chloroplast-localized tetrapyrroles (hemes and chlorophylls) (4), chloroplast gene expression (5), chloroplast-produced ROS (6, 7), and metabolites (8–10). These signals are assumed to affect the function of all 50 to 100 chloroplasts in a cell. Here, we looked for other quality-control mechanisms that could work at the level of the individual chloroplast.

Plastid ferrochelatases 1 and 2 (FC1 and FC2) are conserved enzymes at the heme-chlorophyll branch point of the chloroplast-localized tetrapyrrole biosynthetic pathway (fig. S1). They convert protoporphyrin IX (Proto) to heme and may play a role in the quality control of individual chloroplasts (11–13). To test this hypothesis, we monitored *Arabidopsis thaliana* *fc1* and *fc2* mutants (fig. S2, A to C) during de-etiolation. De-etiolation involves development of nongreen plastids into mature chloroplasts and requires chloroplast signaling. When this process is disrupted, photoautotrophic growth can be inhibited (14). Seedlings were grown in the dark for 4 days and then moved to light. After 1 day of light, wild-type (wt) and *fc1-1* plants became green, whereas *fc2-1* and *fc2-2* mutants failed to do so (Fig. 1, A and B), and photosynthetic cells in cotyledons died (Fig. 1C).

To determine the cause of this phenotype, we profiled the transcriptomes of wt and *fc2-1* seedlings at the start of de-etiolation. RNA profiles were similar between these genotypes before the dark-to-light shift (Fig. 1D, table S1), but diverged after 2 hours of light. Genes enriched for plastid function (group 3) were repressed, whereas genes enriched for heat stress (group 1) and chitin response (group 2) (table S2) were more highly expressed in *fc2-1* than in wt. Thus, the dark-to-light transition appeared to cause photooxidative stress in the *fc2-1* mutant, possibly within chloroplasts.

To test this, we grew seedlings in 24-hour light/dark cycling periods (Fig. 1E and fig. S3, A and B). Wt and *fc2* seedlings turned green when grown in constant light (24 hours) or 16 hours of light (8 hours of dark)/day. In shorter light periods [8 hours of light (16 hours of dark)/day or 4 hours of light (20 hours of dark)/day], *fc2* seedlings, unlike wt, did not become green; transgenic complementation confirmed this was an *fc2* mutant defect. For plants provided with 8 hours of light/day, the third dawn blocked their ability to turn green, and stress marker genes were induced (fig. S3, C and D, and table S3). This condition was used for most of the subsequent seedling experiments. These phenotypes were not dependent on EXE1, which mediates chloroplast-induced cell death (7) (fig. S4, A to I, and supplementary text).

We used transmission electron microscopy (TEM) to monitor the development of *fc2* chloroplasts. In constant light conditions, the ultrastructure of *fc2-1* chloroplasts was similar to that of wt (Fig. 1F). In 8 hours of light/day, *fc2-1* chloroplasts were severely damaged, with swollen thylakoid membranes, large plastoglobule structures, and disrupted outer envelope membranes. This loss of chloroplast integrity was confirmed using *fc2-1* seedlings expressing chloroplast-localized yellow fluorescent protein (YFP) (Fig. 1G and fig. S3H). When these lines were grown in 8 hours of light/day, YFP leaked into the cytoplasm after 1 hour. After 8 hours, YFP and chlorophyll fluorescence were barely detectable. Thus a chloroplast stress signal induced fast chloroplast degradation.

Because tetrapyrroles can cause photooxidative damage (15), we monitored the levels of five chlorophyll intermediates. In 8 hours of light/day growth conditions, Proto was the only one that accumulated in *fc2* mutants (Fig. 2A and fig. S5A). Genetic complementation experiments of *fc2* mutants showed that expression of *FC1* could not prevent this accumulation (Fig. 2A), but did restore wt levels of heme (Fig. 2B) and chlorophyll (Fig. 2C and fig. S5B) in constant light and protochlorophyllide (fig. S5C) in the dark. In 8 hours of light/day growth conditions, *FC1* expression did not restore the ability to turn green (Fig. 2C and fig. S5B), block chloroplast degradation (fig. S4I), or block stress marker gene induction (fig. S4H) in *fc2-1* plants. Expressing maize FC1 or a catalytically dead variant of FC2 (H295A) (12) also did not restore an ability to turn green to *fc2-1* plants (figs. S6 and S7, A and B). Finally, reducing excess tetrapyrrole accumulation in *fc2-1* by introducing *hema1* and *hema2* mutations that reduce the first step of tetrapyrrole synthesis (fig. S1) allowed seedlings to avoid chloroplast degradation when provided with 8 hours of light/day (Fig. 2C and fig. S4I). Together these findings suggested that Proto accumulation correlated with chloroplast degradation and confirmed that FC2 has a specialized function in plastids (11, 12, 16).

Proto is a photosensitizing molecule that generates $^1\text{O}_2$ in cells (17). As expected, *fc2-1* mutants provided with 8 hours of light/day accumulated $^1\text{O}_2$ (Fig. 2D) and induced $^1\text{O}_2$ and oxidative stress-responsive genes within 2 hours (fig. S8A). Feeding the $^1\text{O}_2$ scavenger vitamin B6 (18) blocked chloroplast degradation in *fc2-1* seedlings and restored their ability to become green (Fig. 2E and fig. S8B). Thus, a burst of $^1\text{O}_2$, owing to Proto accumulation in *fc2* mutants, was likely to be responsible for chloroplast degradation and an inability to turn green.

To identify genes required for $^1\text{O}_2$ -induced chloroplast degradation, we screened for second site mutations in *fc2-1* mutants that restored a wt ability to green when provided with 4 hours of light/day. By one-step mapping and whole-genome sequencing, we identified 24 *ferrochelatase-two-suppressor* (*fts*) mutants affecting 17 independent loci (Fig. 3A). Four of these loci were characterized; three were genes that affect tetrapyrrole and $^1\text{O}_2$ accumulation (fig. S9A; fig. S10, A to E; and table S4) (19).

The fourth loci, *fts29* (called *pub4-6* hereafter), had a missense mutation in *Plant U-Box 4* (*PUB4*) *E3 ubiquitin ligase*, a broadly expressed gene encoding an active cytoplasmic-localized (Fig. 3B and fig. S11) E3 ubiquitin ligase involved in cell death and development (20, 21). E3 ubiquitin ligases catalyze the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to a protein substrate. A wt copy of *PUB4* complemented the *pub4-6* phenotype (Fig. 3C and fig. S12, A and B) and *pub4-6* was allelic with *pub4*T-DNA lines (fig. S12, C to D). *fc2-1/pub4-6* had no reduction in tetrapyrrole synthesis [protochlorophyllide, chlorophyll, and ALA levels remained elevated (fig. S10, A to C)]. When provided with 8 hours of light/day, Proto and $^1\text{O}_2$ (Fig. 3, D and E) accumulated in *fc2-1/pub4-6*, but chloroplasts were not degraded. Instead, chloroplasts appeared stressed with irregular shapes and angular membranes (Fig. 3F). *fc2-1/pub4-6* mutants still induced many nuclear stress-associated genes (fig. S13), which suggested that chloroplast degradation was not because of $^1\text{O}_2$ damage per se but was because of a $^1\text{O}_2$ -generated signal involving PUB4.

Because E3 ligases ubiquitinate protein substrates, we tested whether the ubiquitination of chloroplast proteins was linked to chloroplast degradation. Chloroplast fractions of *fc2-1* plants provided with 16 hours of light/day exhibited a PUB4-dependent increase in polyubiquitinated protein(s) compared with wt (Fig. 3G and fig. S14, A and B). Immunoelectron microscopy also showed a PUB4-dependent increase of ubiquitin on the chloroplast envelopes in *fc2-1* mutants (Fig. 3H and fig. S14C) (19), which suggested that ubiquitination was responsible for the chloroplast degradation in *fc2-1* plants.

Next, we tested if chloroplast degradation happened under permissive conditions without cell death. In constant light conditions, chloroplast degradation occurred in wt, but *fc2* mutants had more chloroplasts being degraded (Fig. 4, A to E) (19). These aberrant chloroplasts often had starch granules (indicating that they had been photosynthetic) (Fig. 4D), large plastoglobules, and compressed grana/thylakoid membranes (Fig. 4E and fig. S15, A and B). Sometimes these chloroplasts were observed to be interacting with a globular vacuole (Fig. 4, B and C, and fig. S15, C and D). Nearby organelles appeared normal, which suggested that specific chloroplasts had been selected for degradation. Degradation depended on PUB4; *pub4-6* and *fc2-1/pub4-6* plants had less chloroplast degradation than their parent lines wt and *fc2-1*, respectively (Fig. 4A). *pub4-6* plants also accumulated more chloroplasts/cell area and chlorophyll (Fig. 4, F and G, and fig. S16, A to C). Thus, PUB4 may control chloroplast degradation in healthy cells.

To test if chloroplast degradation is important for long-term maintenance of photosynthetic cells during stress (22, 23), we characterized the phenotypes of *pub4-6* single mutants. *pub4-6* mutants senesced early (Fig. 4H and fig. S16, D and E) and were stunted in excess light that causes long-term chloroplast oxidative damage (Fig. 4I). Thus, a ubiquitination-dependent chloroplast degradation pathway and/or PUB4 may be important for stress adaptation and longevity.

Indiscriminate chloroplast degradation can occur during osmotic stress (24) or used to recycle nutrients during starvation or senescence (25, 26). Here, we found that plant cells also use a selective chloroplast degradation system to remove ROS-damaged chloroplasts. When chloroplasts accumulated $^1\text{O}_2$, proteins in their envelope membranes became ubiquitinated by the direct or indirect action of PUB4, and were degraded (fig. S17). $^1\text{O}_2$ is the major ROS species generated during photosynthesis (27), but its short half-life ($\sim 4 \mu\text{s}$) ensures that it is confined only to chloroplasts in which it was generated (28) so that healthy chloroplasts are not accidentally degraded.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. *fc2* mutants suffer from chloroplast degradation and photooxidative stress during dark-to-light transitions

(A) *fc2* mutant seedlings are unable to turn green after 4 days of growth in the dark. (B) The survival rate (the ability to turn green in 48 hours of light) of 100 seedlings first grown for the indicated number of days in the dark. (C) *fc2-1* cotyledons undergo cell death, as shown by SYTOX green staining of dead nuclei. Scale bars, 200 μ m. (D) A microarray analysis of transcripts induced (at least twofold, $P < 0.05$) in 4-day-old dark grown seedlings during the first 2 hours of de-etiolation. (E) Five-day-old seedling phenotypes under different day lengths. (F) Representative TEM micrographs of chloroplasts in 3-day-old cotyledon mesophyll cells 2 hours after dawn. Scale bars, 2 μ m. (G) Confocal images of 3-day-old *fc2-1* seedling cotyledons grown in 8 hours of light/day expressing plastid-targeted YFP

(RBCS-YFP) before (time 0) or after light exposure. White arrows indicate cells with cytoplasmic YFP. Scale bars, 20 μ m.

Fig. 2. *fc2* mutants accumulate Proto and $^1\text{O}_2$

(A) Time course (starting at dawn) of Proto levels in 3-day-old seedlings provided with 8 hours of light/day ($n = 2$ replicates). (B) Free heme levels of seedlings ($n = 3$ replicates). (C) Phenotypes of transgenic seedlings grown for 5 days in the indicated conditions. (D) Representative confocal images of singlet oxygen (as shown by singlet oxygen sensor green) accumulation in cotyledon mesophyll cells (2 hours after dawn) of 3-day-old seedlings provided with 8 hours of light/day. Scale bars, 20 μ m. (E) Effect of feeding vitamin B₆ on chlorophyll levels ($n = 3$ replicates) and survival. All values are means $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$, two-tailed Student's t test.

Fig. 3. A mutant screen to identify genes involved in chloroplast stress and degradation
 (A) Locations of 24 independent *fts* (*ferrochelatase two suppressors*) mutations representing 17 loci. Boxed mutants are allelic. (B) Subcellular localization of stably expressed PUB4-YFP in hypocotyl cells. (C) Complementation of the *fts29* (*pub4-6*) phenotype with a wt copy of *PUB4* grown for 5 days in 8 hours of light/day. (D) Proto levels in 3-day-old seedlings provided 8 hours of light/day 10 min after dawn ($n = 3$ replicates). (E) Representative confocal images of $^1\text{O}_2$ accumulation in cotyledon mesophyll cells (2 hours after dawn) of 3-day-old seedlings provided with 8 hours of light/day. Scale bars, 20 μm . (F)

Representative TEM micrographs of chloroplasts in 3-day-old cotyledon mesophyll cells 6 hours after dawn. Scale bars, 2 μm . **(G)** Antiubiquitin immunoblot of whole chloroplast fractions isolated from leaves 3 hours after dawn. Plants were grown for 2 weeks in constant light and then transferred to 16 hours of light/day or kept in constant light for 2 days. **(H)** Quantification of chloroplast envelope-associated ubiquitin by immunoelectron microscopy (gold labeling) (19) in 3-day-old seedlings ($n = 3$ seedlings, 10 cells each) 1 hour after dawn. All values are means $\pm$ SEM. *** $P < 0.001$, two-tailed Student's t test.

Fig. 4. PUB4 is part of a chloroplast quality-control pathway

(A) Quantification of degrading chloroplasts using scanning electron microscopy micrographs (19) of cotyledon mesophyll cells of 5-day-old seedlings grown in constant light ($n = 54$ cells). (B to E) TEM images of chloroplasts undergoing various levels of degradation in *fc2-1* seedlings. Scale bars, 1 μ m. (F) Quantification of chloroplasts in cotyledon palisade mesophyll cells ($n = 6$ seedlings) and (G) chlorophyll levels ($n = 3$ replicates) of 6-day-old seedlings grown in constant light. (H) Senescing rosette leaves of 5-week-old plants grown in constant light. (I) Phenotypes and fresh weight (FW) of seedlings ($n = 7$ groups of 10) grown for 1 week in low light ($30 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 22°C) and then transferred to excess light ($275 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ at 15°C) for 1 week. All values are means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-tailed Student's t test.