Implication of the *oep16-1* Mutation in a *flu*-Independent, Singlet Oxygen-Regulated Cell Death Pathway in *Arabidopsis thaliana*

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Singlet oxygen is a prominent form of reactive oxygen species in higher plants. It is easily formed from molecular oxygen by triplet–triplet interchange with excited porphyrin species. Evidence has been obtained from studies on the *flu* mutant of *Arabidopsis thaliana* of a genetically determined cell death pathway that involves differential changes at the transcriptome level. Here we report on a different cell death pathway that can be deduced from the analysis of *oep16* mutants of *A. thaliana*. Pure lines of four independent OEP16-deficient mutants with different cell death properties were isolated. Two of the mutants overproduced free protochlorophyllide (Pchlide) in the dark because of defects in import of NADPH:Pchlide oxidoreductase A (pPORA) and died after illumination. The other two mutants avoided excess Pchlide accumulation. Using pulse labeling and polysome profiling studies we show that translation is a major site of cell death regulation in *flu* and *oep16* plants. *flu* plants respond to photooxidative stress triggered by singlet oxygen by reprogramming their translation toward synthesis of key enzymes involved in jasmonic acid synthesis and stress proteins. In contrast, those *oep16* mutants that were prone to photooxidative damage were unable to respond in this way. Together, our results show that translation is differentially affected in the *flu* and *oep16* mutants in response to singlet oxygen.

Keywords: Chlorophyll biosynthesis • Porphyrin-regulated plastid protein import • NADPH:protochlorophyllide oxidoreductase A (PORA) • Reactive oxygen species • Translation.

Abbreviations: DHFR, dihydrofolate reductase; DTNB, 5,5′-dithiobis(2-nitro)benzoic acid; GFP, green fluorescent protein; MS, Murashige and Skoog; Pchlide, protochlorophyllide; pPORA, NADPH:protochlorophyllide oxidoreductase A precursor; ROS, reactive oxygen species.

Introduction

Reactive oxygen species (ROS) are prominent by-products of aerobic metabolism and potent signaling compounds. They are involved in plant–pathogen interactions and also accumulate in response to abiotic stress (Apel and Hirt 2003, Miller et al. 2008). Singlet oxygen is one form of ROS that has gained wide interest because it is generated during photosynthesis (Mühlenbock et al. 2008). On the other hand, a role for singlet oxygen has been demonstrated for mutants that are impaired in sequestering Chl and its precursor and degradation products in a protein-bound form. This is illustrated by studies on the *flu* mutant of *Arabidopsis thaliana* that is defective in the negative feedback loop inhibiting excess protochlorophyllide (Pchlide) synthesis in the dark (Meskauskiene et al. 2001). Etiolated *flu* plants rapidly die when illuminated because of singlet oxygen production (Meskauskiene et al. 2001). Light-adapted *flu* plants respond to non-permissive dark to light shifts with a marked growth inhibition and/or cell death, depending on the amount of singlet oxygen produced by Pchlide operating as a photosensitizer (op den Camp et al. 2004, Danon et al. 2005).

Two major effects have been proposed to explain the singlet oxygen-mediated cell death phenotype of *flu* plants: a cytotoxic effect including lipid peroxidations and membrane destruction, and a genetic effect including specific signaling cascades (op den Camp et al. 2004, Wagner et al. 2004, Danon et al. 2005; see Kim et al. 2008, Reinbothe et al. 2010, for a review). Transcriptome analyses identified a large number of genes that differentially respond to singlet oxygen (op den Camp et al.

2004). Genes that were up-regulated by singlet oxygen include *BONZAI1*, the enhanced disease susceptibility (*EDS*) 1 gene, and genes encoding enzymes involved in the biosynthesis of ethylene and jasmonic acid, two key components of stress signaling in higher plants (Wasternack 2007, Kendrick and Chang 2008, Reinbothe et al. 2009). On the other hand, genes encoding components of the photosynthetic apparatus were rapidly down-regulated in response to singlet oxygen (op den Camp et al. 2004). Wagner et al. (2004) demonstrated that cell death execution is suppressed in the *executer 1* (*exe1*) mutant of *A. thaliana*, but only if low levels of singlet oxygen accumulate and trigger limited cytotoxic effects. EXECUTER 1 is a membrane protein of chloroplasts of unknown function (Wagner et al. 2004).

It is as yet undetermined how *flu* plants translate the rapid, singlet oxygen-dependent changes at the transcriptome level into protein synthesis. It is also unresolved whether there is one cell death pathway that is activated by porphyrin excitation and singlet oxygen production or whether there are more. In our previous work, we described a conditional cell death mutant of *A. thaliana* that is defective in the *OEP16* gene (Pollmann et al. 2007). *OEP16* forms a small gene family comprising three members, designated *AtOEP16-1* (At2g28900), *AtOEP16-2* (At4g16160) and *AtOEP16-4* (At3g62880), that are all plastid proteins that lack a transit sequence (Murcha et al. 2007, Drea et al. 2006). A fourth relative exists (*AtOEP16-3*; encoded by At2g42210) that appears not to belong to this group and is, unlike the other members, localized in mitochondria (Philippar et al. 2007). AtOEP16-1 shows the highest protein sequence identity (62%) to OEP16 from pea (Pohlmeyer et al. 1997, Murcha et al. 2007) and to HvOEP16-1;1 from barley (52%), which was identified as partner of the cytosolic precursor of NADPH:protochlorophyllide oxidoreductase A (pPORA) during its Pchlide-dependent plastid import (Reinbothe et al. 2004a, Reinbothe et al. 2004b). Two non-exclusive functions currently being considered for the OEP16-1 protein in the outer envelope of chloroplasts are (i) a voltage-gated, amino acid-selective channel (Philippar et al. 2007) and (ii) an import channel of pPORA (Reinbothe et al. 2004a, Reinbothe et al. 2004b). Knock-out mutants in *A. thaliana* for AtOEP16-1 (designated *oep16-1;1*, corresponding to At2g28900) have provided different results (Philippar et al. 2007, Pollmann et al. 2007). We found that the absence of OEP16 correlates with the lack of import of pPORA, aberrant etioplast ultrastructures and the accumulation of free, photoexcitable Pchlide molecules that triggered cell death upon irradiation of dark-grown seedlings (Pollmann et al. 2007). In contrast, Philippar et al. (2007) observed no import defects of pPORA, normal etioplast ultrastructures and unimpaired greening.

The phenotype of the *oep16-1;1* mutant we studied (Pollmann et al. 2007) is very similar to that of *flu* plants (Meskauskiene et al. 2001; op den Camp et al. 2004, Wagner et al. 2004, Danon et al. 2005). We therefore compared the molecular events leading to cell death in etiolated *oep16-1;1* and *flu* plants after illumination, with a focus on translation as the site of active protein synthesis. For comparison, three additional *oep16-1* mutants were isolated from the original seed stock provided by the Salk Institute that displayed different cell death properties. Two of these mutants have phenotypes with different singlet oxygen production and/or signaling patterns. Together, our results provide evidence for the existence of a second, *flu*-independent singlet oxygen-dependent cell death pathway in *oep16-1* plants.

Results

Identification of *oep16-1* knock-out mutant plants

A PCR-based approach using previous primer combinations (Philippar et al. 2007, Pollmann et al. 2007) was employed to re-screen the original seed stock of the Salk Institute (At2g28900, corresponding to SALK_018024) (Alonso et al. 2001) for *oep16-1* knock-out plants (**Fig. 1A**). Several independent homozygous *oep16-1* knock-out plants were obtained that were backcrossed once with the wild type. Seeds from individual plants of the offspring of these crosses were propagated further to establish seed stocks. Aliquots from these seed stocks were sown on Murashige and Skoog (MS) medium and germinated in the dark. After 5 d, the seedlings were inspected under blue light using a microscope.

Some seedlings exhibited a strong red Pchlide fluorescence while other seedlings were, like the wild type, not fluorescent (**Fig. 1B**). Western blotting using isolated etioplasts and POR antiserum showed that among the fluorescent seedlings two subclasses of *oep16-1* mutant plants were present: one subclass lacking PORA, consistent with our previous findings (Pollmann et al. 2007), and another subclass with wild-type PORA protein levels (**Fig. 1C**, panel a). When etioplasts from seedlings that had not shown pigment autofluorescence under blue light were tested by Western blotting, the same segregation into PORA-containing and PORA-free lines was observed (**Fig. 1C**, panel a), leading all together to four types of *oep16-1* mutant plants that were designated *oep16-1;5*, *oep16-1;6*, *oep16-1;7* and *oep16-1;8*, and characterized further. Western blotting confirmed that all four mutant types were devoid of OEP16 protein (**Fig. 1C**, panel b). In Southern blot analyses, all four lines gave rise to only a single T-DNA band (**Fig. 1D**).

Low temperature fluorescence analysis of pigments in the different *oep16-1* mutants

The red pigment fluorescence present in etiolated *oep16-1;5* and *oep16-1;6* plants suggested the presence of free, non-photoconvertible Pchlide molecules not bound to POR in planta. In situ fluorescence spectroscopy at 77 K (Lebedev et al. 1995) was used to determine the functional state of Pchlide in the four different *oep16-1* mutants. Pchlide normally gives rise to two spectral pigment species: Pchlide-F631 and Pchlide-F655 (Lebedev and Timko 1998). Pchlide-F655 has been named photoactive Pchlide because it can be converted into chlorophyllide upon a 1 ms flash of white light (Lebedev

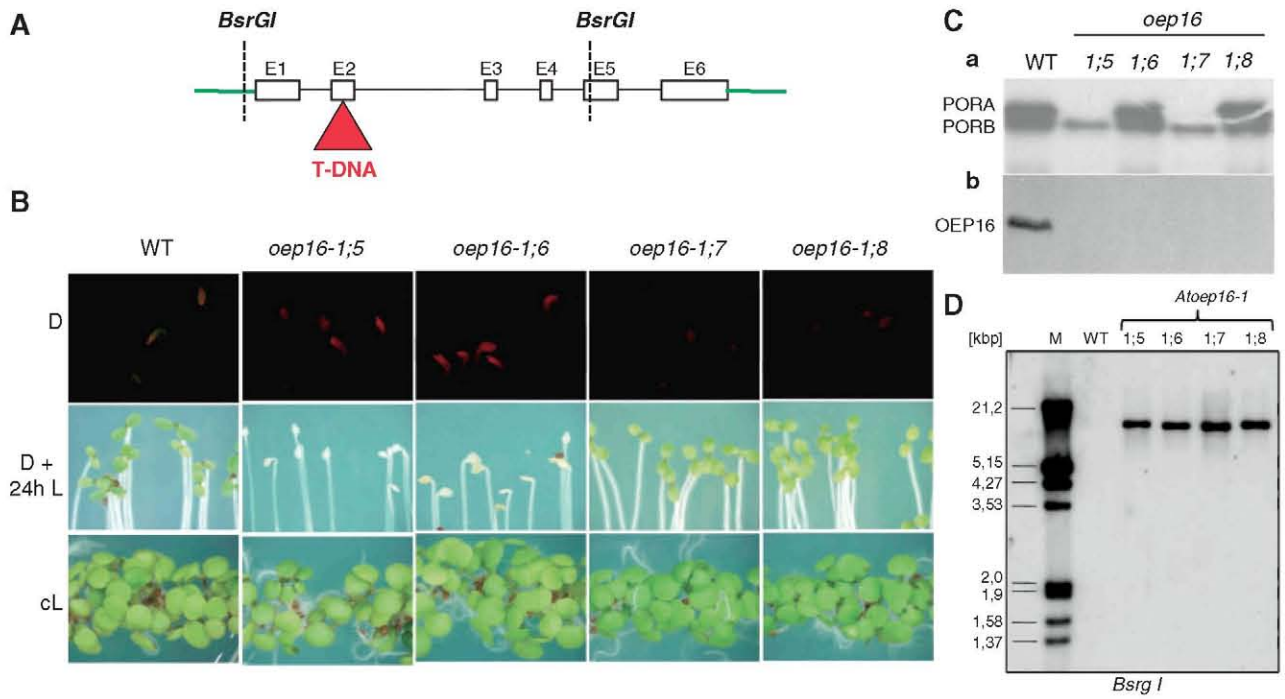

Fig. 1 Identification of *oep16-1* mutants. (A) Schematic presentation of the *OEP16-1* gene and T-DNA insertion. *Bsr*GI marks the position of the restriction sites used for the subsequent Southern blot analyses. (B) Phenotypic analysis of four different *OEP16-1* mutants isolated from the SALK seed stock, designated *oep16-1;5*–*oep16-1;8*, and the wild type (WT) after growth in the dark for 5 d (D), after a non-permissive dark to light shift (D + 24 h L) and after growth in continuous white light (cL). The upper panels show fluorescence images after exciting dark-grown seedlings with blue light (400–450 nm) and collecting the emitted fluorescence between 600 and 650 nm. The middle and lower panels depict seedling phenotypes viewed under white light. (C) Western blot analysis of POR-related polypeptides (a) and OEP16 (b) in etioplasts of *oep16-1* mutant seedlings. (D) Southern blot analysis to detect T-DNA insertions in the *OEP16-1* gene in mutants *oep16-1;5*, *oep16-1;6*, *oep16-1;7* and *oep16-1;8*. Positions of size markers are given in kbp.

and Timko 1998); its establishment is an indicator for the presence of functional PORA:PORB–pigment complexes in the prolamellar body of etioplasts (Reinbothe et al. 1999, Reinbothe et al. 2003, Buhr et al. 2008). These complexes are involved in light trapping and energy dissipation once dark-grown seedlings break through the soil after germination (Reinbothe et al. 1999, Reinbothe et al. 2003, Buhr et al. 2008). Pchlide-F631, in contrast, is called photoinactive Pchlide because it cannot be converted immediately to chlorophyllide (Lebedev and Timko 1998). Pchlide-F631 is a mixture of PORA-bound Pchlide *b* and free, non-protein-bound Pchlide molecules (Reinbothe et al. 1999, Reinbothe et al. 2003, Buhr et al. 2008).

Fig. 2 shows that mutant *oep16-1;5* contained large amounts of Pchlide-F631 but no photoactive Pchlide-F655. Etiolated seedlings of mutant *oep16-1;6* contained lower amounts of photoactive Pchlide and reduced levels of photoinactive Pchlide, if compared with mutant *oep16-1;5*. For mutant *oep16-1;7*, a small amount of Pchlide-F631 was present but no Pchlide-F655 was detected, whereas mutant *oep16-1;8* contained both pigment species in ratios that were similar to those in the wild type (**Fig. 2**). Taking into account previous findings, we concluded that mutant *oep16-1;5*

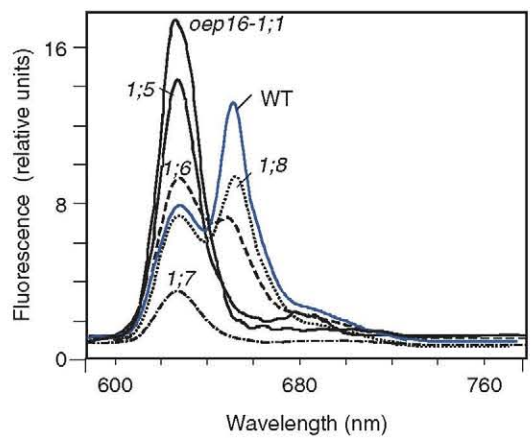

Fig. 2 Low temperature fluorescence analysis at 77K of pigments in 5-day-old, etiolated *oep16-1;5*, *oep16-1;6*, *oep16-1;7* and *oep16-1;8* seedlings. The excitation wavelength was at 440 nm. The two peaks correspond to photoinactive Pchlide (Pchlide-F631) and photoactive Pchlide (Pchlide-F655). For comparison, respective fluorescence spectra are included for etiolated wild-type seedlings and seedlings of line *oep16-1;1* isolated by Pollmann et al. (2007). Spectral intensities refer to an equal cotyledon surface area.

may correspond to the line originally described by Pollmann et al. (2007), which was obtained after two subsequent backcrosses with wild-type plants, whereas mutant *oep16-1;8* could be identical to the line identified by Philippar et al. (2007).

Viability of *oep16-1* mutant seedlings

Tetrazolium staining was used to assess seedling viability (Nortin 1966). Seedlings of all four *oep16-1* mutants and of the wild type were grown in the dark for 5 d and subsequently exposed to white light. Whereas all mutant plants were similarly viable in the dark, they responded differentially to illumination. The microscopic images and established seedling survival curves shown in **Fig. 3A and B** demonstrate that cell death was a rapid event (t_{50} = 4 h) in mutant *oep16-1;5* and a delayed event (t_{50} = 8 h) in mutant *oep16-1;6*. Similar to wild-type seedlings, almost no cell death occurred in mutants *oep16-1;7* and *oep16-1;8* (**Fig. 3A, B**).

The establishment of the cell death phenotype in mutants *oep16-1;5* and *oep16-1;6* is dependent on seedling age and the growth conditions used. Consistent with results reported for the *pif1* mutant of *A. thaliana* (Huq et al. 2004), younger *oep16-1;5* and *oep16-1;6* seedlings were less prone to cell death upon illumination than older seedlings, presumably because of the lower levels of free, non-POR-bound Pchlide accumulated (Supplementary Fig. S1C). The severity of the cell death phenotype in *oep16-1;5* and *oep16-1;6* seedlings was thus also dependent on the light intensity (Supplementary Fig. S1A, B). Inclusion of sucrose in the growth medium partially negated the high light effects (data not shown). We explain this finding by the growth-promoting effect of the sugar and the resulting greater capability of the seedlings to sustain a semi-heterotrophic state once the nutrient reserves of the seed have been consumed. On the other hand, sucrose has been reported to affect the polysomal binding of transcripts (Nicolaï et al. 2006) as well as the structure and integrity of membranes (Crowe and Crowe 1984). All of these effects could allow for a better stress accommodation.

Singlet oxygen production in *oep16-1* mutant seedlings

The cell death phenotype in *oep16-1;5* and *oep16-1;6* seedlings after irradiation suggested that free, non-photoconvertible Pchlide molecules not bound to POR operated as a photosensitizer and caused singlet oxygen production. To prove this hypothesis, singlet oxygen measurements were performed with the DanePy reagent which is a dansyl-based singlet oxygen sensor undergoing quenching of its fluorescence upon reacting with singlet oxygen (Hideg et al. 1998, Kálai et al. 2002). DanePy has a broad emission peak at around 530 nm. Upon reacting with singlet oxygen, this peak is reduced, the drop in the amount of fluorescence reflecting the amount of singlet oxygen produced. Thus the greater the amount of fluorescence change, the greater the amount of singlet oxygen

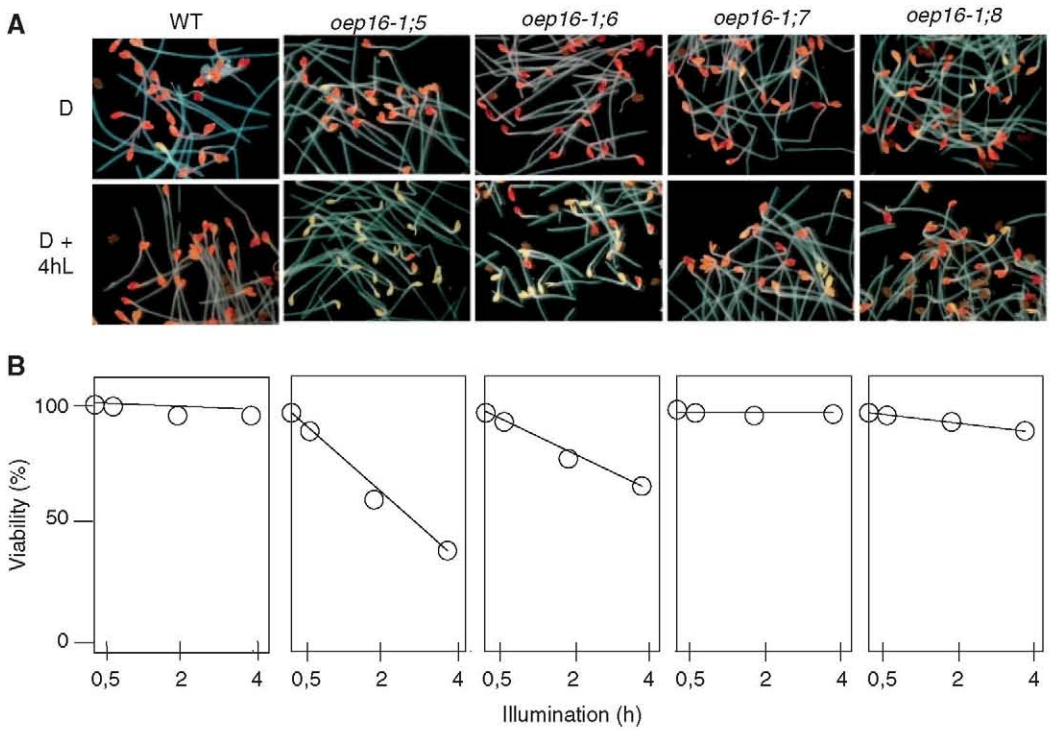

Fig. 3 Seedling viability test on the four types of *oep16-1* mutants and the wild type (WT). (A) Tetrazolium staining of 5-day-old, etiolated *oep16-1;5*, *oep16-1;6*, *oep16-1;7* and *oep16-1;8* seedlings before (D) and after exposure to white light of approximately 75 $\mu E\, m^{-2}\, s^{-1}$ for 4 h (D + 4hL). (B) Seedling viability (in %) as a function of the time of irradiation.

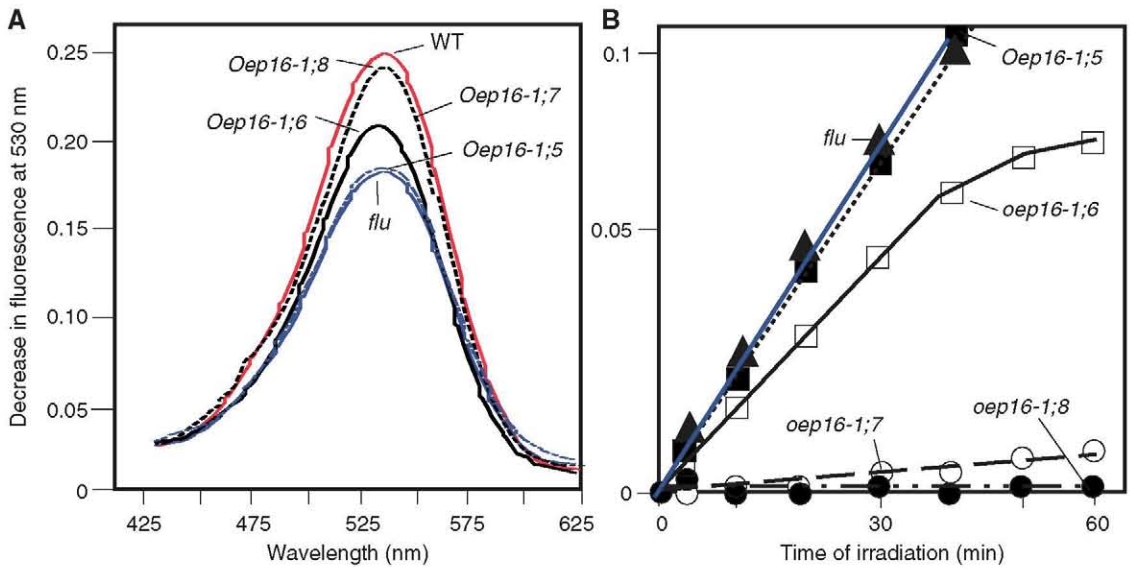

Fig. 4 Singlet oxygen evolution in dark-grown *oep16-1*, *flu* and wild-type seedlings after a 30 min white light shift. (A) DanePy fluorescence emission spectra between 425 and 625 nm at an excitation wavelength of 330 nm obtained for *oep16-1;5* (blue dotted line), *oep16-1;6* (black solid line), *oep16-1;7* (black dashed line) *and oep16-1;8* (black hatched line) vs. *flu* (blue solid line) and wild-type seedlings (red solid line). (B) Time courses of DanePy fluorescence quenching in mutants *oep16-1;5* (dotted line), *oep16-1;6* (solid line), *oep16-1;7* (dashed line) and *oep16-1;8* (hatched line) vs. *flu* seedlings (blue solid line). Spectral intensities refer to an equal cotyledon surface area.

generated in the mutant seedlings. **Fig. 4** shows DanePy fluorescence spectra derived from three independent experiments each comprising 120 etiolated plants that had been infiltrated with DanePy and subsequently exposed to white light of 125 $\mu E\, m^{-2}\, s^{-1}$ for 30 min. As a reference, we used 5-day-old *flu* plants that had been treated identically. **Fig. 4** revealed that mutants *oep16-1;5* and *oep16-1;6* produced significant amounts of singlet oxygen, as evidenced by the quenching of DanePy fluorescence that was collected between 425 and 625 nm. Kinetic measurements demonstrated that singlet oxygen production in mutant *oep16-1;5* was almost indistinguishable from that in *flu*, whereas that in *oep16-1;6* was slightly lower (**Fig. 4A, B**). In wild-type seedlings and seedlings of mutant *oep16-1;7*, there was little DanePy fluorescence quenching, indicative of the generation of a very tiny amount of singlet oxygen. In *oep16-1;8* seedlings, some minor decrease in DanePy fluorescence was observed in two out of three independent experiments, suggesting that some low amounts of singlet oxygen accumulated.

Chloroplast protein import

The finding that mutant *oep16-1;6* did not contain wild-type levels of Pchlide-F655, despite the presence of PORA, suggested that import of pPORA may not proceed via the Pchlide-dependent translocon complex described previously (Reinbothe et al. 2004a, Reinbothe et al. 2004b) but may proceed via another import machinery. To test this hypothesis, in vitro import and cross-linking experiments were carried out (Schemenewitz et al. 2007). ^{35}S-labeled transA–DHFR precursor molecules, consisting of the first 67 N-terminal amino acids of pPORA (henceforth referred to as transA) and a cytosolic dihydrofolate reductase (DHFR) reporter protein of mouse, were activated with 5,5′-dithiobis(2-nitro)benzoic acid (DTNB) (Schemenewitz et al. 2007). Then the precursor was added to chloroplasts that had been isolated from 14-day-old, light-grown *oep16-1;6* plants and energy depleted. Import was assessed under standard conditions in the dark in the presence of 2.5 mM Mg-ATP and 0.1 mM Mg-GTP with chloroplasts that lacked Pchlide (Schemenewitz et al. 2007).

Fig. 5 demonstrates that a significant fraction of [^{35}S]transA–DHFR was imported into Pchlide-free chloroplasts of mutant *oep16-1;6*. Cross-linking gave rise to an ~110 kDa product that consisted of transA–DHFR and TOC75, as demonstrated by immunoprecipitations (data not shown, but see accompanying paper by Samol et al. 2011). Similar to the plastids from mutant *oep16-1;6*, a fraction of transA–DHFR was taken up and processed via a TOC75-dependent pathway by chloroplasts isolated from mutant *oep16-1;8*. In contrast, drastically less precursor import was detectable for chloroplasts of mutants *oep16-1;5* and *oep16-1;7*, and higher molecular mass cross-linked products were not detected (**Fig. 5**). The presence or absence of Pchlide produced by 5-aminolevulinic acid pre-treatment of isolated chloroplasts did not affect this result (Supplementary Fig. S2A). All four types of mutant plastids imported indistinguishable levels of a precursor consisting of the transit peptide of pPORB (transB) and the DHFR (Supplementary Fig. S2B).

To support further the hypothesis that chloroplasts from line *oep16-1;6* take up transA–DHFR by a Pchlide-independent pathway not involving the previously characterized Pchlide-dependent translocon complex (PTC; Reinbothe et al. 2004a,

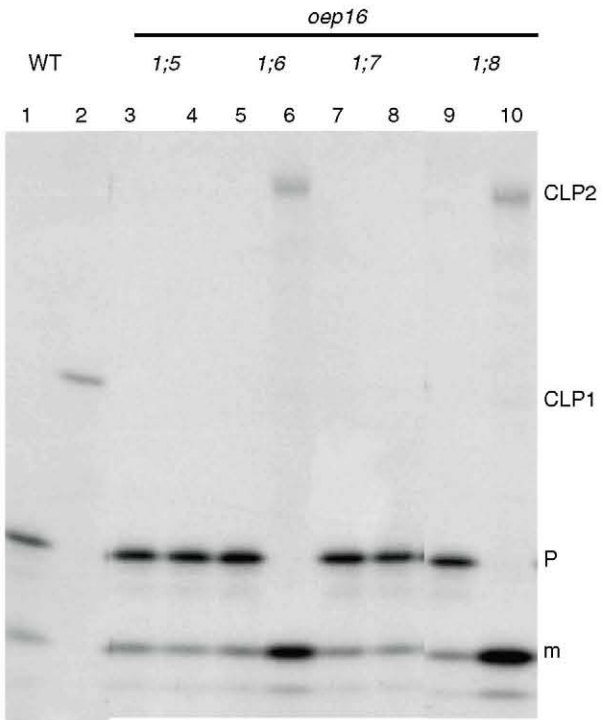

Fig. 5 In vitro import and cross-linking of DTNB-activated [^{35}S]transA–DHFR determined for chloroplasts isolated from wild-type and *oep16* mutant plants. The autoradiogram shows precursor (P) and mature (m) proteins as well as cross-linked products (CLP1 and CLP2) at time zero (lanes 1, 3, 5, 7 and 9) and after 15 min of import (lanes 2, 4, 6, 8 and 10). CLP1 is caused by the formation of a disulfide bond between transA–DHFR and OEP16; this product is only detectable for wild-type chloroplasts. CLP2 resulted from formation of a disulfide bond between transA–DHFR and TOC75; this CLP is found for chloroplasts from mutants *oep16-1;6* and *oep16-1;8*.

Reinbothe et al. 2004b), transient expression and import studies were conducted as described (Finer et al. 1992, Reinbothe et al. 2008). After ballistic bombardment of leaf pavement cells of wild-type and mutant *A. thaliana* plants, the localization of expressed transA–green fluorescent protein (GFP) was followed by confocal laser scanning microscopy. **Fig. 6** depicts fluorescence images for all of the four *oep16-1* mutants described in this study and also shows the respective controls obtained for wild-type plants and seedlings of the *oep16-1;1* mutant described by Pollmann et al. (2007). According to the results, mutants *oep16-1;5* and *oep16-1;7* imported only small amounts of transA–GFP into their plastids, whereas mutants *oep16-1;6* and *oep16-1;8* imported significant levels of the precursor. Confirming previous findings (Pollmann et al. 2007, Reinbothe et al. 2008), the plastids from wild-type plants imported transA–GFP well, whereas the plastids from the *oep16-1;1* mutant were import incompetent.

Protein synthesis during greening and in response to photooxidative stress

op den Camp et al. (2004) have shown that mature *flu* plants accumulate a large number of stress-responsive transcripts after non-permissive dark to light shifts. At the same time, irradiated *flu* plants rapidly depress photosynthetic gene expression (op den Camp et al. 2004). Whether similar changes would occur in *oep16* plants belonging to the genotypes *1;5* and *1;6* was obviously undetermined and motivated us to perform the following experiments. Pulse labeling of proteins was carried out with [^{35}S]methionine in 5-day-old dark-grown seedlings that had been irradiated for 4 or 24 h. Furthermore, total RNA was extracted from dark-grown and 2 h-irradiated plants and used for in vitro translation and Northern hybridization. When the patterns of polypeptides synthesized in a wheat germ lysate were compared for the four different *oep16* mutants, no major differences were found. No new polypeptide species were detected in mutants nor were any detectably absent (**Fig. 7A** and Supplementary S3A), as would be expected if *oep16-1;5* and *oep16-1;6* followed the same cell death pathway as *flu* (op den Camp et al. 2004).

Fig. 7B and Supplementary Figs. S3B and S4 show that all four *oep16* mutants synthesized very similar protein patterns in the dark but responded differentially to illumination. Seedlings of mutants *oep16-1;5* and *oep16-1;6* began synthesizing photosynthetic proteins after a few hours of illumination but stopped translating these proteins after approximately 24 h of illumination. Instead of accumulating Chl, the seedlings died (cf. **Figs. 1** and **3**). In contrast, seedlings of mutants *oep16-1;7* and *oep16-1;8* carried on translating photosynthetic proteins at the later stages of light exposure (**Fig. 7B**) and greened normally (cf. **Figs. 1** and **3**). Remarkably, no synthesis of mass stress proteins was detectable in the cotyledons of plants from mutants *oep16-1;5* and *oep16-1;6* (**Fig. 7B**) that would have occurred if cell death triggered in these *oep16-1* mutant types followed the same course as in *flu* plants (Supplementary Fig. S3B).

Mutant *oep16-1;6* phenotypically strongly resembles the *flu* mutant isolated by Meskauskine et al. (2009). Like *flu*, mutant *oep16-1;6* contains normal levels of PORA protein but overaccumulates free photoexcitable Pchlide molecules in the dark that cause singlet oxygen production and cell death upon illumination. However, in marked contrast to *flu*, etiolated *oep16-1;6* seedlings do not synthesize stress proteins upon illumination (Supplementary Fig. S3). In order to characterize the differences between *oep16-1;6* and *flu* further, light-adapted, mature plants were used. Plants were grown for 14 d in continuous white light; then the plants were transferred to darkness for 8 h and re-illuminated for variable periods. According to previous work on *flu* (op den Camp, 2004, Wagner et al. 2004, Danon et al. 2005), such treatment was expected to activate the genetic component of singlet oxygen-dependent signaling but without provoking cytotoxic effects. Pulse labeling and polysome profiling experiments revealed that *flu* plants indeed react to singlet oxygen production with the rapid synthesis of stress proteins and the selective depression of synthesis of photosynthetic proteins (**Fig. 8A**, panel a). After 24 h, a drastic decrease in protein synthesis was observed in illuminated *flu* plants (**Fig. 8B**) which

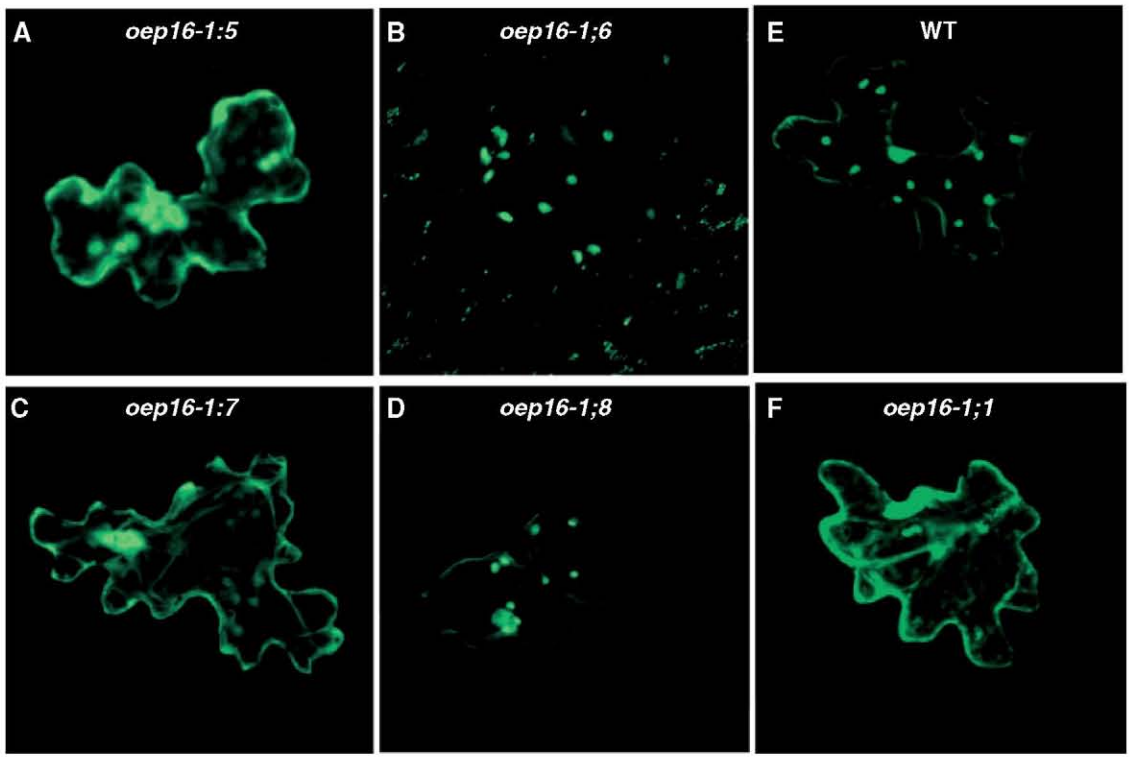

Fig. 6 In vivo import of transA–GFP into plastids of mutants *oep16-1;5* (A), *oep16-1;6* (B), *oep16-1;7* (C) and *oep16-1;8* (D) as well as plastids from the wild-type (WT) (E) and mutant *oep16-1;1* described by Pollmann et al. (2007) (F) after ballistic transformation of leaf pavement cells of *A. thaliana*. GFP fluorescence was collected between 505 and 530 nm, using an excitation wavelength of 488 nm.

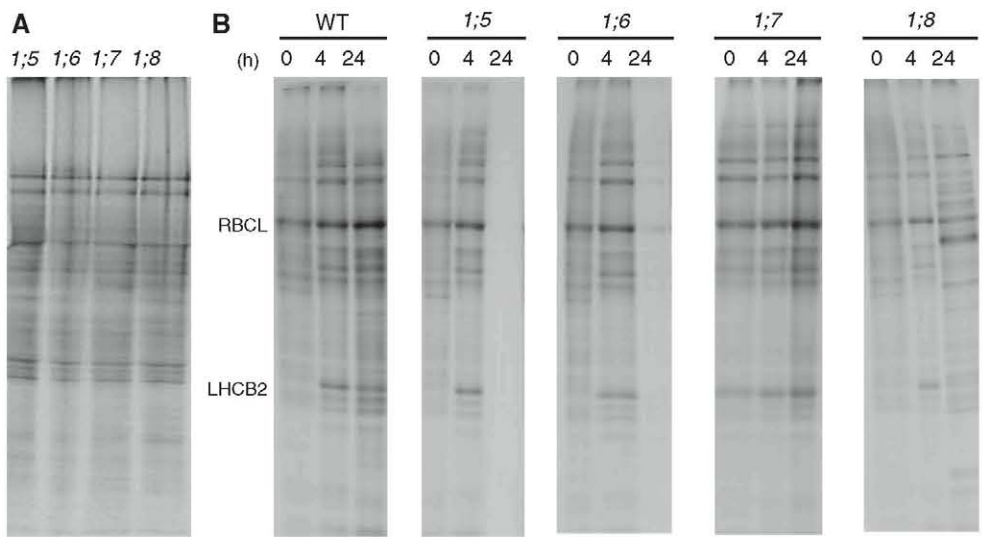

Fig. 7 Protein expression in the different *oep16-1* mutants. (A) Pattern of proteins synthesized in a wheat germ lysate with RNA from mutants *oep16-1;5*, *oep16-1;6*, *oep16-1;7* and *oep16-1;8* after growth in the dark for 5 d and exposure to white light for 2 h. (B) Patterns of ^{35}S-labeled total proteins in wild-type seedlings and seedlings of mutants *oep16-1;5*, *oep16-1;6*, *oep16-1;7* and *oep16-1;8* in the dark (0 h) and after 4 h and 24 h, respectively, of exposure to white light of 125 μE m^{-2} s^{-1}. After extraction and SDS–PAGE, proteins were detected by autoradiography. RBCL and LHCB2 mark the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and the major light-harvesting chlorophyll *a*/*b*-binding protein of PSII.

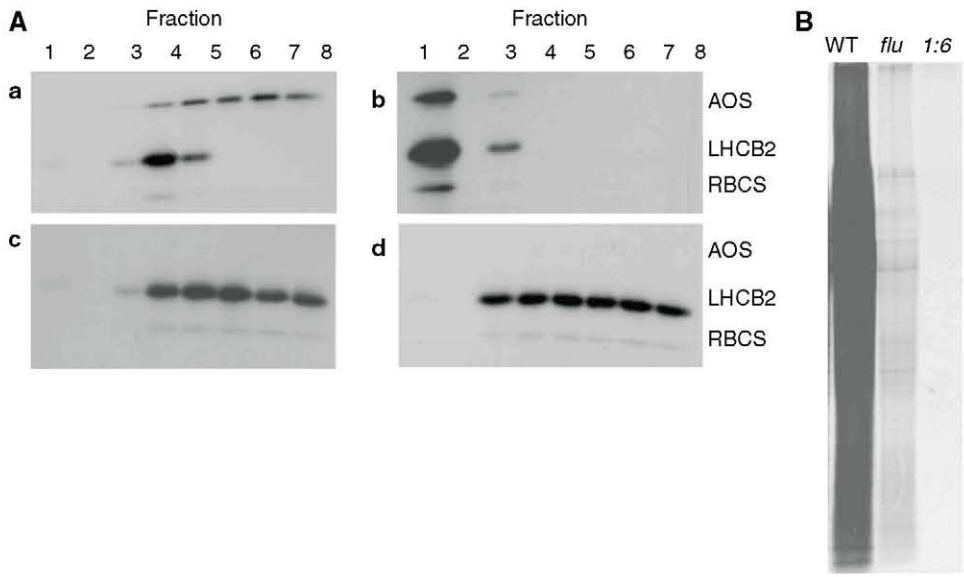

Fig. 8 Protein synthesis in leaf tissues of 14-day-old light-grown wild-type, *flu* and *oep16-1;6* plants after transfer to darkness for 8 h and subsequent illumination. (A) Polysomal synthesis of light-harvesting chlorophyll *a/b*-binding protein of PSII (LHCB2) and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RBCS), as well as allene oxide synthase (AOS) in *flu* (a and b), wild-type (c) and *oep16-1;6* (d) plants detected 4 h (a, c and d) and 24 h (b) after the non-permissive dark to light shift. Fraction 1 contained the 40S ribosomal subunit, fraction 2 contained the 60S ribosomal subunit, and fractions 3–8 contained cytoplasmic polysomes of increasing size. Protein from each of the different fractions was separated by SDS–PAGE and subjected to Western blotting using the indicated antisera. (B) Patterns of ^{35}S-labeled total leaf proteins in wild-type, *flu* and *oep16-1;6* plants detected 24 h after the non-permissive dark to light shift.

correlated with a decay of cytoplasmic polysomes (**Fig. 8A**, panel b). In marked contrast to *flu*, *oep16-1;6* plants reacted to non-permissive dark to light shifts with no early synthesis of stress proteins (**Fig. 8A**, panel d), although protein synthesis later declined to an even greater extent than in *flu* plants (**Fig. 8B**). Together, these findings highlighted that only the early reprogramming of translation in response to singlet oxygen is different in *flu* and *oep16-1;6* plants.

Discussion

In the present work, two major questions were addressed. What is the reason for completely different phenotypes described for two *oep16-1* knock-out mutants derived from the T-DNA insertion SALK_024018 (Philippar et al. 2007, Pollmann et al. 2007)? Is the cell death regulation in the *oep16-1* mutant we identified (Pollmann et al. 2007) comparable with the singlet oxygen-dependent pathway reported for the *flu* mutant (Meskauskiene et al. 2001, op den Camp et al. 2004, Wagner et al. 2004, Danon et al. 2005)?

We discovered that the original seed stock provided by the Salk Institute contains several independent knock-out mutants. Four *OEP16-1*-deficient mutants were discovered that all showed a single T-DNA insertion on Southern blots. Obviously, these mutants are different from those described recently by Pudelski et al. (2009) containing additional T-DNA insertions. Presumably because of the prior backcrosses, mutants *oep16-1;5*–*oep16-1;8* were homozygous for the *oep16-1* locus and did not contain additional T-DNA fragments on Southern blots. Mutants *oep16-1;5*–*oep16-1;8* suffered or did not suffer from photooxidative damage under high light conditions and contained or lacked PORA. Mutant *oep16-1;5* had the strongest phenotype. It lacked PORA and rapidly died upon non-permissive dark to light shifts. PORA's function is to bind Pchlide and to assemble with PORB into larger POR:Pchlide complexes involved in light harvesting and energy dissipation during greening (Reinbothe et al. 1999, Reinbothe et al. 2003, Buhr et al. 2008), explaining its phenotype. The characteristics of mutant *oep16-1;5* are virtually identical to those of mutant *oep16-1;1* isolated by Pollmann et al. (2007).

Mutant *oep16-1;6* displayed a weaker phenotype. Despite the presence of PORA, almost no Pchlide-F655 was found. Import and cross-linking studies demonstrated that transA–DHFR entered both Pchlide-free and Pchlide-containing mutant plastids. However, the precursor did not seem to interact with the pigment and also it did not establish larger complexes with PORB in etioplasts. Otherwise, Pchlide-F655 should have accumulated to the same extent as in wild-type plants, which was not the case. In vitro and in planta import assays showed that pPORA is likely to enter the plastids via an import pathway involving TOC75. As shown previously, multiple versions of the TOC machinery exist that differ by an interchange of receptor and regulatory components and exhibit different precursor specificities (Jarvis et al. 1998, Bauer et al. 2000, Ivanova et al. 2004). It is attractive to hypothesize about the presence of an exogenic, presumably point or footprint mutation outside the *AtOEP16-1* gene. It has been reported that

T-DNA insertion lines often contain multiple T-DNA copies of which some or all are lost in subsequent generations, provoking secondary effects (Latham et al. 2006). We hypothesize that the 'hidden' mutation in *oep16-1;6* may affect the composition and/or activity of the plastid envelope protein translocon complexes or the state of cytosolic targeting factors involved in import. Evidence has been obtained for the operation of different cytosolic targeting pathways of nucleus-encoded plastid precursors involving 14-3-3, HSP70 and HSP90 proteins (May and Soll 2000, Qbadou et al. 2006, Schemenewitz et al. 2007) that could be prone to modifications in mutant *oep16-1;6*.

Mutant *oep16-1;7* did not show a cell death phenotype. Even without detectable levels of PORA, etiolated seedlings greened normally. The level of Pchlide-F631 was drastically reduced in this mutant as compared with wild-type seedlings. It has previously been shown by Lebedev et al. (1995) that greening in the absence of PORA can occur via a pathway involving only PORB. This pathway is likely to operate in line *oep16-1;7*. Mutant *oep16-1;8* is similar to the line isolated by Philippar et al. (2007). It contained normal levels of PORA and wild-type levels of Pchlide-F655. Upon illumination, etiolated *oep16-1;8* mutant seedlings greened like the wild-type and without any sign of photooxidative damage. It is likely that in this line an additional suppressor mutation is present that affected the cell death phenotype. DNA arrangements provoked by the insertion and loss of multiple T-DNA copies (Latham et al. 2006) may be the reason for the complex genetic background observed in mutant *oep16-1;8*. The identity of these mutations remains to be determined by map-based cloning and whole-genome sequencing approaches.

FLU is an important regulator of Pchlide accumulation in *A. thaliana* and other angiosperms (Meskauskiene et al. 2001, op den Camp et al. 2004, Wagner et al. 2004, Danon et al. 2005). Its absence causes cell death (Meskauskiene et al. 2001, op den Camp et al. 2004, Wagner et al. 2004, Danon et al. 2005). To what extent *flu* would be involved in controlling cell death in mutants *oep16-1;5* and *oep16-1;6* was as yet undermined, and is explored in the second part of the present study. *flu* plants respond to non-permissive dark to light shifts with growth arrest and/or cell death (Meskauskiene et al. 2001, op den Camp et al. 2004, Wagner et al. 2004, Danon et al. 2005). While etiolated *oep16-1;5* and *oep16-1;6* seedlings share similar cell death symptoms with *flu* seedlings, no growth inhibition was observed for mature green plants. In etiolated plants one might attribute cell death to the cytotoxic effect of singlet oxygen. This explanation, however, does not seem very likely for light-adapted plants. A major clue for understanding the differences in cell death regulation in *oep16-1;6* and *flu* plants was provided by the pulse labeling and polysome profiling studies which revealed a lack of stress protein synthesis both for etiolated and light-adapted *oep16-1;6* plants after non-permissive dark to light shifts. In either case, the normal reprogramming of translation detected for *flu* plants and leading to stress protein synthesis was abrogated. Nevertheless, at later stages of the singlet oxygen-dependent stress response, protein synthesis declined to similar extents in *flu* and *oep16-1;6* plants. In the *flu* orthologous mutant of barley, *tigrina d12*, the same early as well as later effects on translation have been observed as reported here for *flu* plants (Khandal et al. 2009). Our results show that both effects are separable, implying two different mechanisms of translational control in which singlet oxygen is involved. The *flu* and *oep16-1;6* plants, which are virtually identical with regard to their phenotypic properties (Pchlide overproduction and presence of PORA), therefore represent tools to explore these mechanisms.

Materials and Methods

Plant growth

Seeds of the *oep16-1* mutant (At2g28900; SALK_024018) and *flu* mutant (At3g14110; SALK_002383) were obtained from the Salk Institute Genomic Analysis Laboratory collection (Alonso et al. 2001) and germinated in the dark on half-strength MS–agar medium containing or lacking 1% (w/v) sucrose. For comparison, the *flu* mutant isolated by Meskauskiene et al. (2001) was used. After variable periods, the seedlings were exposed to high light (125 $\mu E\,m^{-2}\,s^{-1}$) or low light (25 $\mu E\,m^{-2}\,s^{-1}$). For dark to light transfer and plastid isolation experiments, seeds were sown on soil and cultivated in continuous white light for appropriate periods.

Mutant identification

Previously described primer combinations (Philippar et al. 2007, Pollmann et al. 2007) were used to identify *oep16-1* knock-out plants by PCR (Innis et al. 1990). High stringency Southern hybridization was performed on DNA filters containing 10 μg of DNA that had been digested with *Cla*I or *Bsr*GI and probes corresponding to either the ROK2 vector or the left border (LB) and right border (RB) of the T-DNA (Sambrook et al. 1989). LB and RB primer combinations were as follows: NPTII-R2, 5′-CAATATCACGGGTAGCCAAC-3′; NPII-F2, 5′-CG GTTCTTTTTGTCAAGACC-3′; LBGT1, 5′-ACTTAATAACACAT TGCGGACG-3′; and LBGT2, 5′-CTTAATCGCCTTGCAGCAC ATC-3′.

Pigment fluorescence analyses

Fluorescence microscopy was performed using excitation filters from 400 to 450 nm and emission filters from 600 to 650 nm. Low temperature fluorescence measurements were performed at 77 K at an excitation wavelength of 440 nm (spectrometer model LS50B, Perkin Elmer Corp.) (Lebedev et al. 1995).

Seedling viability tests

Seedling viability was assessed by tetrazolium staining (Nortin 1966). Whereas vital seedlings show a strong red staining, dead seedlings are unable to produce the dye and look whitish. For

statistic assessment, pools of about 250 seeds were analyzed in three independent experiments.

Singlet oxygen measurements

Singlet oxygen generation was measured with the DanePy method (Hideg et al. 1998, Kálai et al. 2002). Fluorescence emission of DanePy was collected between 425 and 625 nm, using an excitation wavelength of 330 nm (Life Sciences spectrometer, model LS50 Perkin Elmer Corp.).

Protein import in vitro and in planta

Construction of chimeric precursors consisting of the N-terminal targeting sequences of pPORA (transA) and pPORB (transB) and GFP or mouse DHFR has been described (Schemenewitz et al. 2007, Reinbothe et al. 2008). To study the import in vivo, leaf pavement cells of *A. thaliana* were transformed by ballistic bombardment according to Finer et al. (1992), using a helium pressure of 6.5 bar, 12 cm target distance, a disperse grid at 7 cm and 1 μM of gold microcarriers (Bio-Rad). Transformed plantlets were kept under sterile conditions for 24 h in darkness. Confocal laser scanning microscopy was carried out using an LSM 510 Meta microscope (Zeiss) with argon laser excitation at 488 nm. GFP and Chl were detected at emission wavelengths of 505–530 and 650–750 nm, respectively. LSM 510 Meta software release 3.2 (Zeiss) and Adobe Photoshop 7 (Adobe Systems) were used for image acquisition and processing, respectively.

In vitro import reactions were carried out using cDNA-encoded, wheat germ-translated, urea-denatured [^{35}S]precursors produced by coupled transcription/translation of the respective clones and Percoll/sucrose-purified chloroplasts from *A. thaliana* (Schemenewitz et al. 2007). Chemical cross-linking of DTNB-derivatized [^{35}S]precursors was carried out as described (Reinbothe et al. 2004b).

Protein analyses

Pulse labeling of protein was performed with [^{35}S]methionine (37 TBq mmol^{-1}, Amersham-Pharmacia) for 2 h prior to seedling harvest. Protein was extracted with 80% (v/v) acetone, and protein pellets obtained after centrifugation were boiled for 5 min in SDS sample buffer [2.9% SDS, 68 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 0.1 M β-mercaptoethanol] (Laemmli 1970) (**Fig. 7**). Alternatively protein was extracted with buffer A [50 mM Tris–HCl, pH 7.8, 25 mM KCl, 10 mM MgCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 0.5% (v/v) mercaptoethanol, 1% (v/v) Triton X-100, 250 mM sucrose] (Scharf and Nover 1984) (Supplementary Fig. S3), followed by further homogenization in a Branson Sonifier (model B-12, microtip, 80 W, 1 min) and precipitation with 5% (v/v) trichloroacetic acid (Reinbothe et al. 1990). Protein that had been extracted with acetone and boiled in SDS sample buffer was cleared by centrifugation and only the clear supernatant was used for SDS–PAGE. Protein that had been extracted with buffer A and precipitated with trichloroacetic acid was washed with acetone, ethanol and ether, dried and resuspended in SDS sample buffer (Laemmli 1970). SDS–PAGE was carried out on 10–20% polyacrylamide gradients (Scharf and Nover 1984). Isolation, fractionation and analysis of polysomes and polysomal messengers were conducted as described (Reinbothe et al. 1993). Immunodetection was carried out using an enhanced chemiluminescence system (Amersham-Pharmacia) and the indicated antisera (Towbin et al. 1979).

Supplementary data

Supplementary data are available at PCP online.

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