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:protochlorophyllide oxidoreductase A (pPORA) and died after illumination. The other two mutants avoided excess Pchlide accumulation. Using pulse labeling and polysize 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 (Mühlbock 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 BONZA1, 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, Reinhothe 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 executor 1 (exel) mutant of A. thaliana, but only if low levels of singlet oxygen accumulate and trigger limited cytotoxic effects. EXECUTOR 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 At2g62210) 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 (Reinhothe et al. 2004a, Reinhothe 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 pPPORA (Reinhothe et al. 2004a, Reinhothe 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 pPPORA, 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 pPPORA, 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 POR, consistent with our previous findings (Pollmann et al. 2007), and another subclass with wild-type POR 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 POR-containing and POR-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. 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
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 (t50 = 4 h) in mutant *oep16-1;5* and a delayed event (t50 = 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.

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**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 (D + 4hL) exposure to white light of approximately 75 µE m⁻² s⁻¹ for 4 h (D + 4hL). (B) Seedling viability (% in %) as a function of the time of irradiation.
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 μE 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 POR, suggested that import of pPOR 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 (Schmelewitz et al. 2007). 35S-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) (Schmelewitz 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 (Schmelewitz et al. 2007).

Fig. 5 demonstrates that a significant fraction of 35S]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,
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 [35S]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 over-accumulates 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. 2006), such treatment was expected to activate the genetic component of singlet oxygen-dependent signaling but without provoking cytotoxic effects. Pulse labeling and polypeptide 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

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...
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 $\mu$E 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.
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. 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 oepl6-1;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, Schemenowitz et al. 2007) that could be prone to modifications in mutant oepl6-1;6. Mutant oepl6-1;7 did not show a cell death phenotype. Even without detectable levels of PORA, etiolated seedlings greened normally. The level of Pchlde-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 oepl6-1;7. Mutant oepl6-1;8 is similar to the line isolated by Philipp et al. (2007). It contained normal levels of PORA and wild-type levels of Pchlde-F655. Upon illumination, etiolated oepl6-1;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 oepl6-1;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 Pchlde 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 oepl6-1;5 and oepl6-1;6 was as yet undetermined, 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 oepl6-1;5 and oepl6-1;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 oepl6-1;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 oepl6-1;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 oepl6-1;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 oepl6-1;6 plants, which are virtually identical with regard to their phenotypic properties (Pchlde overproduction and presence of PORA), therefore represent tools to explore these mechanisms.

**Materials and Methods**

**Plant growth**

Seeds of the oepl6-1 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 (125µE m⁻² s⁻¹) or low light (25µE 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 oepl6-1 knock-out plants by PCR (Innis et al. 1990). High stringency Southern hybridization was performed on DNA filters containing 10µg of DNA that had been digested with ClaI or BsrGI 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'-CAATATCGGTTGAGACCA-3'; NPTII-F2, 5'-CG GTTCTTTTGTGAGACCA-3'; LBGT1, 5'-ACCTAAACACT GCGGACG-3'; and LBGT2, 5'-CTTATCGCTTGCAGCAC 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).

**Seeding viability tests**

Seeding viability was assessed by tetrazolium staining (Norton 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, Káli 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.

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


