The Outer Chloroplast Envelope Protein OEP16-1 for Plastid Import of NADPH:Protochlorophyllide Oxidoreductase A in *Arabidopsis thaliana*

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The outer plastid envelope protein OEP16-1 was previously identified as an amino acid-selective channel protein and translocation pore for NADPH:protochlorophyllide oxidoreductase A (PORA). Reverse genetic approaches used to dissect these mutually not exclusive functions of OEP16-1 in planta have led to descriptions of different phenotypes resulting from the presence of several mutant lines in the SALK_024018 seed stock. In addition to the T-DNA insertion in the *AtOEP16-1* gene, lines were purified that contain two additional T-DNA insertions and as yet unidentified point mutations. In a first attempt to resolve the genetic basis of four different lines in the SALK_024018 seed stock, we used genetic transformation with the *OEP16-1* cDNA and segregation analyses after crossing out presumed point mutations. We show that *AtOEP16-1* is involved in PORA precursor import and by virtue of this activity confers photoprotection onto etiolated seedlings during greening.

Keywords: Chlorophyll biosynthesis • Photooxidative damage • Porphyrin-regulated plastid protein import • Singlet oxygen.

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; TUNEL, deoxynucleotidyl transferase-mediated dUTP nick end labeling; YFP, yellow fluorescent protein.

Introduction

Plastids accomplish key metabolic functions in higher plants. Because of their endosymbiotic origin, plastids are surrounded by two envelope membranes, called the outer and inner envelope, that are involved in the exchange of metabolites and the uptake of cytosolic precursor proteins (Cavalier-Smith 2006). Plastids need to import the majority of their protein constituents from the cytosol, requiring specialized protein translocon complexes to operate in the outer and inner envelope membranes, termed the TOC and TIC machinery (Bedard and Jarvis 2005, Hofmann and Theg 2005, Kessler and Schnell 2006). Current concepts indicate that multiple versions of the TOC and TIC machinery exist which differ by an interchange of components, allowing the adjustment of precursor import in time and space and according to the developmental and environmental conditions (Jarvis et al. 1998, Bauer et al. 2000, Ivanova et al. 2004, Kubis et al. 2004). Bauer et al. (2000) reported on different TOC receptor proteins designated TOC159, TOC132 and TOC120, sharing conserved acidic domains and transmembrane anchors but differing in the length of their cytosolically exposed pre-sequence-binding domains. A large number of cytosolic precursor proteins contain cleavable N-terminal transit sequences that are involved in the recognition and binding as well as translocation of the precursors across the limiting membranes of chloroplasts (Bedard and Jarvis 2005, Hofmann and Theg 2005, Kessler and Schnell 2006).

Bauer et al. (2000) identified a mutant deficient in TOC159 that is impaired in the import of photosynthetic proteins but not of non-photosynthetic proteins. Smith et al. (2004) observed that transit peptide fusions consisting of the transit peptide of NADPH:protochlorophyllide oxidoreductase A (PORA) and a cytosolic dihydrofolate reductase (DHFR) reporter protein of mouse was not precipitated in pull-down assays using a soluble form of TOC159 whose transmembrane segments had been removed. This finding confirmed studies of competition, antibody blocking and cross-linking which had

revealed that the PORA precursor (pPORA) does not interact with TOC75 during import (Reinbothe et al. 2000, Reinbothe et al. 2004a, Reinbothe et al. 2004b). Instead, an ortholog of the outer plastid envelope protein OEP16, originally discovered in pea chloroplasts (Pohlmeyer et al. 1997), was isolated that cross-linked pPORA and respective transA–DHFR fusions in plastids of barley, wheat, pea and *Arabidopsis thaliana* (Reinbothe et al. 2004a, Reinbothe et al. 2004b).

OEP16-1 from barley is closely related to OEP16-1 from pea (PsOEP16) (62% sequence identity) and AtOEP16-1 from *A. thaliana* (52% sequence identity) (Drea et al. 2006). In *A. thaliana* a small gene family encodes three members, designated *AtOEP16-1* (At2g28900), *AtOEP16-2* (At4g16160) and *AtOEP16-4* (At3g62880) (Reinbothe et al. 2004b, Drea et al. 2006, Murcha et al. 2007). A fourth relative exists (*AtOEP16-3*; encoded by At2g42210) that appears not to belong to this group since its encoded product is localized in mitochondria (Murcha et al. 2007).

Two non-exclusive functions have been indicated for the OEP16-1 protein: (i) a voltage-gated, amino acid-selective channel (Pohlmeyer et al. 1997, Philippar et al. 2007) and (ii) a translocation pore for pPORA (Pollmann et al. 2007). Knock-out mutants for *AtOEP16-1* have provided different results (Philippar et al. 2007, Pollmann et al. 2007). We found in an *AtOEP16-1* mutant with absence of OEP16 a lack of import of pPORA, aberrant etioplast ultrastructures and the accumulation of free, photoexcitable protochlorophyllide (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 in the mutant they investigated. Based on these results, we (Samol et al. 2011) and Pudelski et al. (2009) re-screened the original seed stock SALK_024018. Pudelski et al. (2009) showed that two additional T-DNA insertions and at least one point or footprint mutation are present in mutants of the original seed stock SALK_024018 (Alonso et al. 2003) that can affect the establishment of the cell death phenotype. In our recent study (see accompanying paper by Samol et al. 2011), pure lines of four OEP16-deficient mutants with different cell death properties were identified (Samol et al. 2011). All four mutants showed only one T-DNA band on Southern blots (Samol et al. 2011). Two of the mutants overproduced free Pchlide in the dark and died after illumination (Samol et al. 2011). The other two mutants avoided excess Pchlide accumulation and greened normally (Samol et al. 2011). One of these mutants, *Atoep16-1;6*, imported pPORA by a pathway which did not permit PORA to attain a functional state conferring photoprotection on etiolated seedlings during greening, as concluded from low temperature pigment fluorescence measurements and photobleaching/seed viability tests (Samol et al. 2011). Mutant *Atoep16-1;6* was used in the present study to readdress our previous results indicating that OEP16-1 is causally related to the substrate-dependent plastid import of pPORA and efficient seedling de-etiolation. We demonstrate that mutant *Atoep16-1;6* was restored to normal greening by transformation introducing synthesis of OEP16-1 from its cDNA or by cDNA encoding a fusion of OEP16-1 with green fluorescent protein (GFP). GFP::OEP16-1 was localized to chloroplasts, as demonstrated by confocal laser scanning microscopy. In the generated transgenic lines, OEP16-1 operated in the substrate-dependent import of pPORA. As a result, larger PORA:PORB complexes and photoactive Pchlide were produced that allowed for efficient seedling de-etiolation. Together, these results demonstrate a functional role for AtOEP16-1 in pPORA import.

Results

Age-dependent expression of the cell death phenotype in mutant *Atoep16-1;6*

Mutant F6-4a that was described by Pudelski et al. (2009) corresponds to mutant *Atoep16-1;6* (Samol et al. 2011). Pudelski et al. (2009) used young, 2.5-day-old seedlings to examine the de-etiolation phenotype, i.e. the lack of greening and cell death. Consistent with the results of Pudelski et al (2009) and Samol et al. (2011), mutant *Atoep16-1;6* showed strong red Pchlide fluorescence in the dark (**Fig. 1A**). However, we noted that pigment autofluorescence under our experimental conditions appeared only after 3.5–4 d of growth in darkness and was hardly detectable under a fluorescence microscope after 2 d (**Fig. 1A**). Pchlide accumulated in a free, non-POR-bound form and operated as a photosensitizer when the seedlings were irradiated. This is evident from the correlation between Pchlide accumulation and cell death during dark growth after 4–5 d and subsequent illumination (**Fig. 1C**).

Developmental expression of OEP16-1 and PORA

Pudelski et al. (2009) have contested the role of OEP16-1 during greening, using several different arguments. They argued, for example, that there would be no sufficient overlap in the expression of OEP16-1 and PORA during seed germination. To address this point, an antiserum was raised against bacterially expressed and purified OEP16-1 protein (Supplementary Fig. S1a, b) and tested for its reactivity with OEP16-1 and other plant proteins. The results summarized in Supplementary Fig. S1c showed that one protein band was detectable on Western blots of separated plastid proteins. When time course experiments were conducted for 5-day-old, etiolated seedlings that had been illuminated for variable periods, the expression of OEP16-1 remained almost constant and declined only transiently (**Fig. 2B**). These results were consistent with our previous observation obtained by chemical cross-linking in barley, demonstrating a light-dependent reduction in OEP16-1 expression during the transition of etioplasts to chloroplasts (Reinbothe et al. 2004b). When the Western blots were probed with antiserum against POR, three bands were seen, of which the uppermost representing PORA declined in irradiated seedlings (**Fig. 2B**). Thus, OEP16 and PORA are

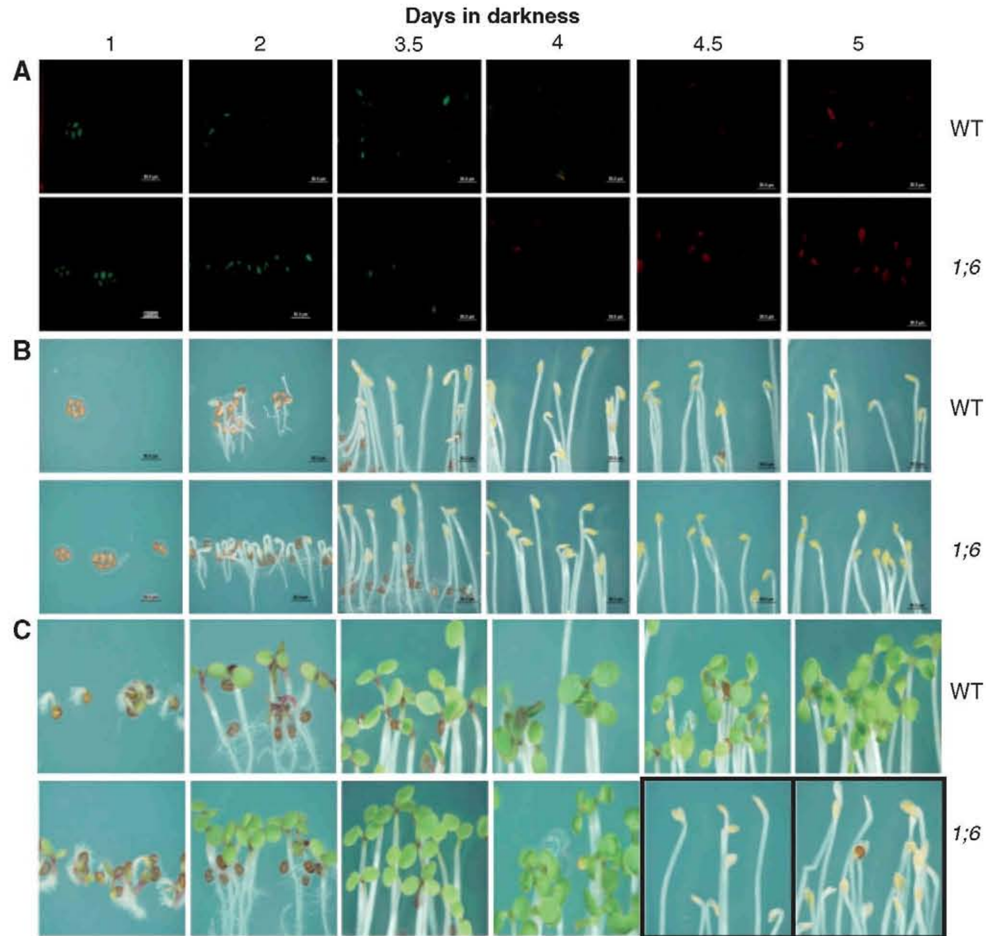

Fig. 1 Photobleaching as a function of Pchlide overaccumulation in mutant *Atoep16-1;6*. *Atoep16-1;6* (*1;6*) and wild-type (WT) seeds were germinated for different periods in darkness (A and B) and subsequently exposed to strong white light (210 μE m^{-2} s^{-1}) for 24 h (C). (A) Red Pchlide autofluorescence monitored under blue light (400–450 nm). (B) The same as in A, but showing the seedlings under normal daylight. (C) Phenotypes after irradiating etiolated seedlings with white light for 24 h.

expressed simultaneously in etiolated seedlings, in line with the proposed function of OEP16 in pPORA import.

Genetic complementation of mutant *Atoep16-1;6*

Gateway technology was used to assemble the following binary T-DNA overexpression destination vectors (Invitrogen). *OEP16* cDNA provided with the 35S cauliflower mosaic virus promoter (*35S::GFP::OEP16*) was cloned into vector pB7WG2 containing the Bar gene providing resistance to ammonium glufosinate. For placement of the GFP sequence tag between the 35S promoter and the N-terminus of OEP16, the vector pK7WGF2 containing the kanamycin resistance gene was used, while fusion of the yellow fluorescent protein (YFP) sequence tag to the C-terminus of OEP16 was carried out with vector pB7YWG2.

The cloned vector DNAs were transformed into *Agrobacterium tumefaciens*, strain GV3121, via electroporation. The presence of the correct T-DNA was verified by PCR (Innis et al. 1990), before proceeding with the in planta transformation (Clough and Bent 1998). After transformation, selection of the first, second and third generation of transgenic plants (T_1, T_2 and T_3) was performed on media with kanamycin (for plants containing *35S::GFP::OEP16*) and with ammonium glufosinate (for plants containing *35S::OEP16* and *35S::OEP16::YFP*).

Nine transgenic T_3 lines were obtained with vector pB7WG2 containing *35S::OEP16*, and five of these showed rescue from bleaching. Six transgenic lines resulted from transformation with vector pK7WG2 containing *35S::GFP::OEP16*. One of these provided rescue from bleaching. Four transgenic lines were obtained with plasmid pB7YWG2 containing *35S::OEP16::YFP*, but none provided rescue from bleaching.

A compelling example of a successful complementation is provided by line *Atoep16-1;6:35S::OEP16 E_6* which was composed of 100% homozygous, herbicide-resistant plants (**Fig. 3b**). After growth in the dark for 5 d and subsequent high light exposure (210 μE m^{-2} s^{-1}) for 24 h, seedlings of *Atoep16-1;6:35S::OEP16 E_6* looked like the wild type and

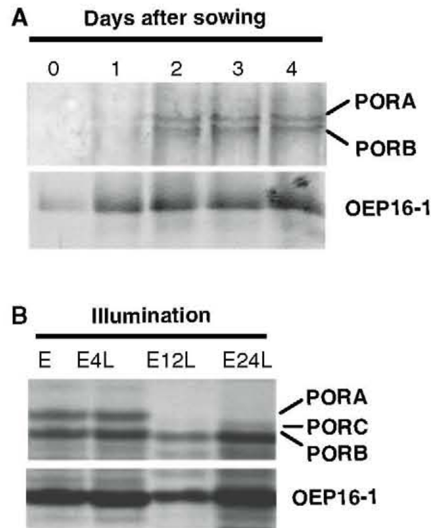

Fig. 2 OEP16-1 and POR expression during post-germination development in the dark (A) and after subsequent irradiation (B). Seeds were sown on agar medium containing 1% sucrose and germinated for variable periods in darkness (A). In a parallel experiment, seedlings were allowed to grow for 4.5 d in the dark (E) before being exposed to strong white light (210 $\mu E\ m^{-2}\ s^{-1}$) for 4 h (E4L), 12 h (E12L) and 24 h (E24L) (B). After extraction and resolution of total leaf protein on an SDS-containing 10–20% polyacrylamide gel, OEP16-1 and POR expression was assessed by Western blotting using the respective monospecific antisera. The three detected POR protein bands represent PORA, PORB and PORC.

were fully viable (**Fig. 3d**). This result indicated that the introduced *AtOEP16-1* gene had restored normal greening. Indeed, no red fluorescence indicative of the presence of free Pchlide molecules was seen in etiolated *Atoep16-1;6:35S::OEP16 E_6* seedlings, but was easily detectable in seedlings of the *Atoep16-1;6* mother generation (**Fig. 3c**). Exposure of the etiolated seedlings to white light for 48 h or longer periods did not affect seedling viability (data not shown). Immunoblotting proved the high expression level of AtOEP16-1 protein (**Fig. 3e**).

Fig. 4 shows results obtained for line T_3 *Atoep16-1;6:35S::GFP::OEP16 D_8* expressing the GFP::OEP16 fusion protein. Similar to the results for *Atoep16-1;6:35S::OEP16 E_6*, both the reduction of red Pchlide fluorescence during plant etiolation (**Fig. 4c**) and normal green appearance without detectable signs of photooxidative damage after illumination (**Fig. 4d**) emphasized that the introduced transgene was active in restoring normal greening. GFP fused to AtOEP16-1 at its N-terminus thus did not affect the function of AtOEP16-1 in planta.

In contrast to these results, transformation of mutant *Atoep16-1;6* with the YFP fusion protein gene did not regenerate plants that were viable during de-etiolation ($n = 4$). In spite of the presence of the AtOEP16-1 fusion protein in transformed T_3 *Atoep16-1;6* mutant seedlings (Supplementary Fig. S2e), all analyzed transgenic plants accumulated high levels of red-fluorescing Pchlide and died after illumination, as did the untransformed *Atoep16-1;6* mutant (Supplementary Fig. S2c, d). Together, these findings showed that AtOEP16-1 with the YFP tag either could not enter the outer envelope of chloroplasts or attained a non-functional conformation with the fusion to the YFP reporter protein.

Confocal laser scanning microscopy was used to study the localization of the GFP–OEP16-1 and OEP16-1–YFP fusion proteins in the regenerated transgenic lines. **Fig. 5** depicts representative images taken for line T_3 *Atoep16-1;6:35S::GFP::OEP16 D_8*. Clearly, GFP fluorescence in the 35S::GFP::OEP16 transformants co-localized with the red autofluorescence of Chl in chloroplasts of mesophyll cells and guard cells of stomata (**Fig. 5a, b**). This result is in accordance with previous localization data of OEP16-1 (Pohlmeyer et al. 1997, Reinbothe et al. 2004a, Reinbothe et al. 2004b). In contrast, no chloroplast import was detectable for transformants expressing OEP16-1–YFP (Supplementary Fig. S2f).

In vitro import of transA–DHFR into plastids of line *Atoep16-1;6:35S::OEP16 E_6*

Fusion proteins consisting of the first 67 N-terminal amino acids of the pPORA from barley (henceforth referred to as transA) and the DHFR reporter protein of mouse were activated with Ellman's reagent [5,5′-dithiobis(2-nitro)benzoic acid (DTNB)] (Reinbothe et al. 2004b). Then the precursor was imported into etioplasts or chloroplasts that had been isolated from 5-day-old, dark-grown and light-grown *Atoep16-1;6* mutant, *Atoep16-1;6:35S::OEP16 E_6* and wild-type seedlings, respectively. Import was assessed under standard conditions in assays containing 2.5 mM Mg-ATP and 0.1 mM Mg-GTP (Reinbothe et al. 2005). **Fig. 6** shows that mutant *Atoep16-1;6* imported a fraction of DTNB-activated precursor into both their etioplasts and chloroplasts. In either case, a fraction of the precursor (31 kDa) was shifted into a larger, 106 kDa cross-linked product which cross-reacted with TOC75 (75 kDa) in pull-down assays (Supplementary Fig. S3). In plastids from line *Atoep16-1;6:35S::OEP16 E_6*, however, no 106 kDa cross-linked product appeared and a smaller, 46 kDa product was formed (**Fig. 6**). This cross-linked product consisted of transA–DHFR and OEP16-1, as demonstrated by co-immunoprecipitation experiments (Supplementary Fig. S3). At time zero, some artificial processing occurred. However, the greater proportion of mature protein (DHFR) in etioplasts vs. chloroplasts in line *Atoep16-1;6:35S::OEP16 E_6* suggests that the introduced OEP16-1 had restored the Pchlide-dependent import of transA–DHFR. Pchlide needed for the substrate-dependent import of transA–DHFR is present only in etioplasts, while the pigment level in chloroplasts is too low for the import step in vitro (Reinbothe et al. 1995a, Reinbothe et al. 1995b, Reinbothe et al. 1996). When import assays were carried out for transB–DHFR, consisting of the transit sequence of pPORB and the DHFR, no differences were detectable for plastids isolated from mutant *Atoep16-1;6* and line *Atoep16-1;6:35S::OEP16 E_6* (Supplementary Fig. S4).

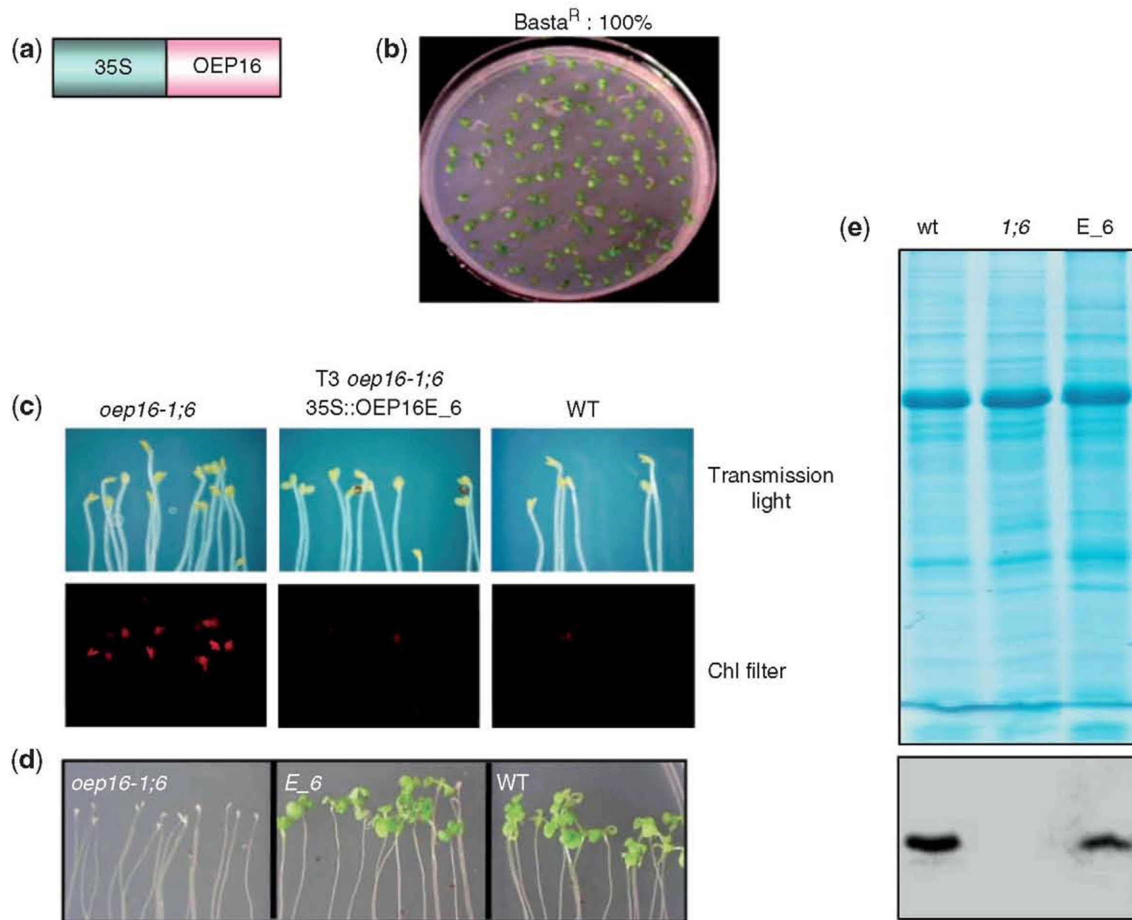

Fig. 3 Genetic complementation of *Atoep16-1;6* by *35S::OEP16*. (a) Structure of the generated 35S::OEP16 construct. (b) Selection of plants corresponding to line T_3 *Atoep16-1;6:35S::OEP16 E_6* on MS medium containing ammonium glufosinate (BASTA, 5 μg ml^{-1}). (c) Phenotypic analysis of T_3 seedlings of *Atoep16-1;6:35S::OEP16 E_6* compared with mutant *Atoep16-1;6* and the wild type after growth in darkness for 5 d without (c) or with (d) a subsequent exposure to strong white light (210 μE m^{-2} s^{-1}) for 48 h. Note the strong Pchlide fluorescence in mutant *Atoep16-1;6* and the lack of such fluorescence in line *Atoep16-1;6:35S::OEP16 E_6* and the wild type. (e) Detection of AtOEP16-1 in line T_3 *Atoep16-1;6:35S::OEP16 E_6* but not in untransformed *Atoep16-1;6* seedlings by Western blotting. Total protein was extracted from mature, green plants, separated on a 15% SDS–polyacrylamide gel and probed with an antibody that had been raised against the bacterially expressed and purified AtOEP16-1 protein. The Coomassie stain (upper panel) and Western blot (lower panel) show leaf protein corresponding to 40 μg of bovine serum albumin.

Low temperature fluorescence analysis of pigments in line *Atoep16-1;6:35S::OEP16 E_6*

On the basis of the results presented thus far it seemed likely that re-expressing AtOEP16-1 in mutant *Atoep16-1;6* restored the Pchlide-dependent import of pPORA. As a result, PORA–Pchlide *b*–NADPH complexes would be formed that could further assemble with PORB–Pchlide *a*–NADPH complexes to establish photoactive Pchlide in the prolamellar body of etioplasts (Reinbothe et al. 1999, Reinbothe et al. 2003, Buhr et al. 2008). Pchlide is normally present in two spectral pigment forms in low temperature in planta fluorescence measurements: Pchlide-F631 and Pchlide-F655 (Lebedev et al. 1995, Lebedev and Timko 1998). Pchlide-F655 has been named photoactive Pchlide because it can be converted to chlorophyllide upon a 1 ms flash of white light (Lebedev et al. 1995, Lebedev and Timko 1998); its establishment is an indicator of the presence of functional PORA:PORB–pigment complexes in the prolamellar body of etioplasts (Lebedev et al. 1995, Lebedev and Timko 1998). These complexes dubbed LHPPs, light-harvesting POR:Pchlide complexes, are involved in light trapping and excess light energy dissipation during greening (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 et al. 1995, Lebedev and Timko 1998). Pchlide-F631 is a mixture of POR–pigment–NADPH ternary complexes and free, non-POR bound Pchlide molecules (Reinbothe et al. 1999, Reinbothe et al. 2003, Buhr et al. 2008).

In order to examine the functional state of PORA in planta, low temperature fluorescence spectroscopy was carried out according to standard procedures (Lebedev et al. 1995).

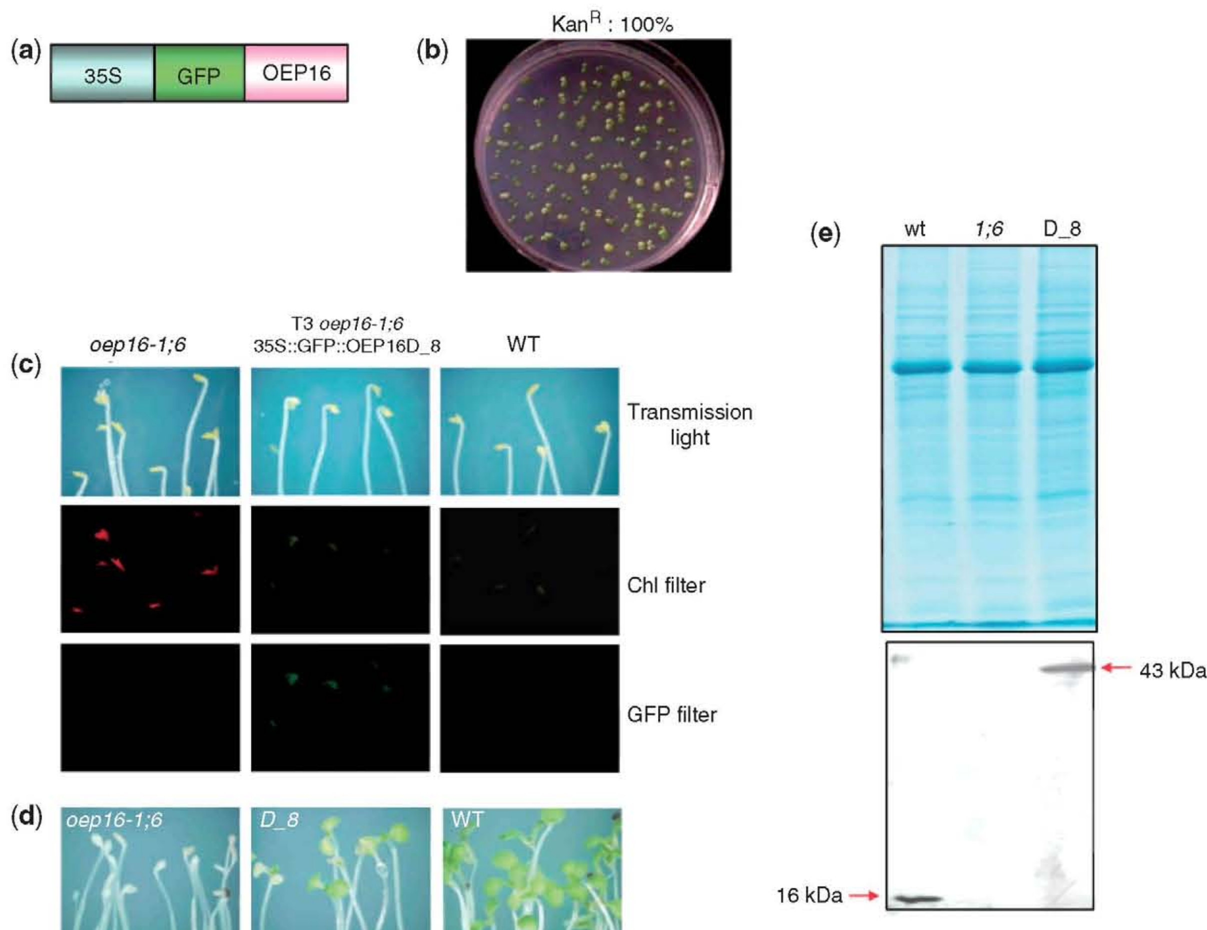

Fig. 4 Genetic complementation of *Atoep16-1;6* by *35S::GFP::OEP16*. As in **Fig. 3**, but depicting the results obtained for line T_3 *Atoep16-1;6:35S::GFP::OEP16 D_8* expressing the GFP::OEP16 fusion protein. (a) Structure of the generated construct. (b) Antibiotic resistance of the produced T_3 line. Note that all of the generated plants are resistant to kanamycin sulfate (50 μg ml^{-1}). (c and d) Phenotype of seedlings after growth in darkness (c) and after exposure to strong white light (210 μE m^{-2} s^{-1}) for 48 h (d). (e) Expression of GFP–OEP16-1 in total leaf protein extracts. Note the molecular mass shift of OEP16-1 relative to the wild type for line T_3 *Atoep16-1;6:35S::GFP::OEP16 D_8*. Protein equivalent to 40 μg of bovine serum albumin was loaded per lane and the blot was probed with AtOEP16-1 antibody.

Fig. 7A shows that mutant *oep16-1;6* contained large amounts of photoinactive Pchlide-F631 but little photoactive Pchlide-F655. In fact, only a shoulder appeared in the fluorescence emission spectrum at around 650 nm. In line T_3 *Atoep16-1;6:35S::OEP16 E_6* expressing OEP16-1, a substantial fraction of the pre-existing Pchlide was shifted into the photoactive state, establishing Pchlide-F655. When protein extracts were prepared from detergent-solubilized prolamellar bodies of isolated etioplasts and subjected to non-denaturing PAGE, larger PORA:PORB complexes were detectable in line T_3 *Atoep16-1;6:35S::OEP16 E_6*, but not in the *Atoep16-1;6* mutant (**Fig. 7B**). This result indicates that OEP16-1 is involved in pPORA import.

Cell death rescue in *Atoep16-1;6:35S::OEP16 E_6*

Three approaches were used to assess the mechanism of cell death rescue in line *Atoep16-1;6:35S::OEP16 E_6*. First, singlet oxygen measurements were carried using the DanePy method. The DanePy reagent is a dansyl-based singlet oxygen sensor that undergoes quenching of its fluorescence upon reacting with singlet oxygen (Hideg et al. 1998, Kálai et al. 2002). **Fig. 8A** shows the fluorescence emission spectra of DanePy for mutant *Atoep6-1;6*, line *Atoep16-1;6:35S::OEP16 E_6* and wild-type seedlings. Confirming our previous observations (Samol et al. 2011), DanePy fluorescence quenching and thus singlet oxygen production was readily detectable in seedlings of mutant *Atoep6-1;6* that had been grown in the dark for 5 d and illuminated for 30 min. In contrast, no comparable fluorescence quenching and thus singlet oxygen evolution was detectable for line *Atoep16-1;6:35S::OEP16 E_6* and the wild type (**Fig. 8A**).

Secondly, we carried out terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) measurements (Gavrieli et al. 1992). This method permits detection of the breakage of nuclear DNA that is a hallmark of cell death and apoptosis in animals and plants. In addition, DNA

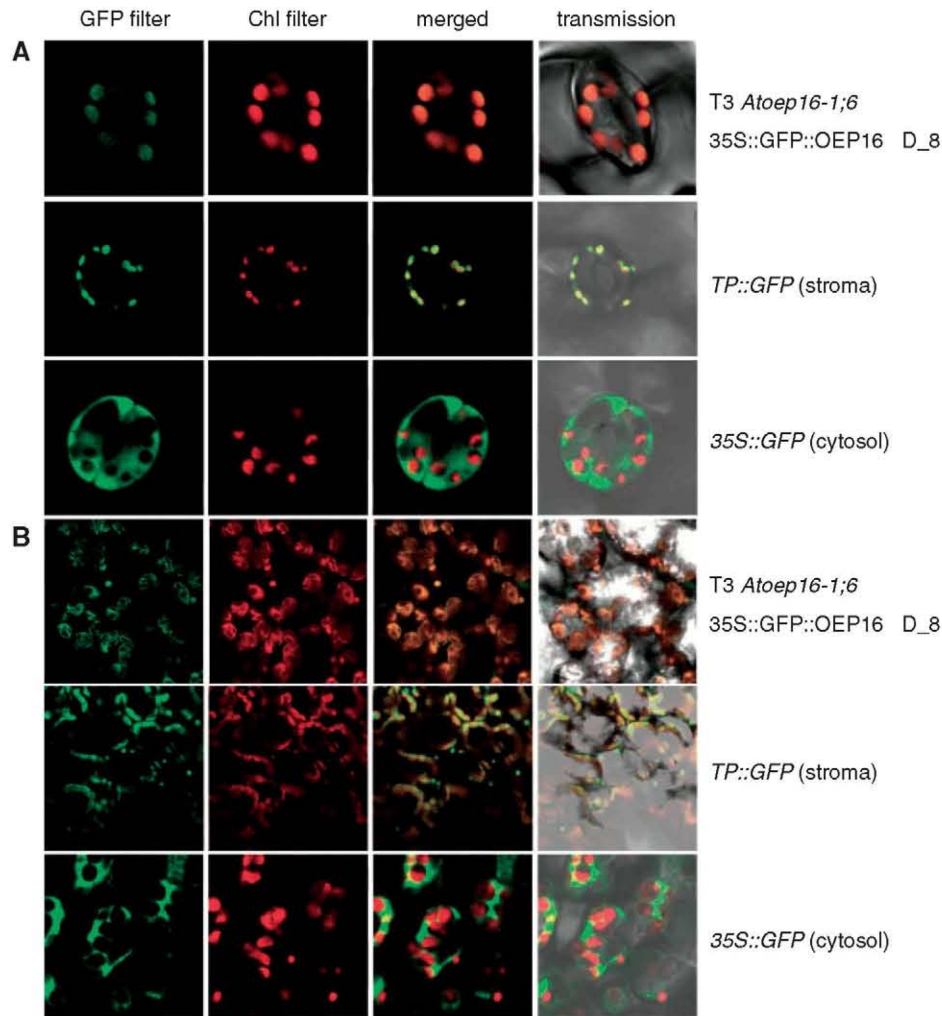

Fig. 5 Cytolocalization of GFP in mutant *Atoep16-1;6* transgenic for *35S::GFP::OEP16*. Plants of line T_3 *Atoep16-1;6:35S::GFP::OEP16 D_8* were grown for 14 d in continuous white light and the localization of the GFP reporter proteins was examined by confocal laser scanning microscopy. (a and b) Co-localization of GFP and Chl fluorescences in guard cells of stomata (a) and in mesophyll cells (b) in plants of line T_3 *Atoep16-1;6:35S::GFP::OEP16 D_8*. For imaging acquisition, GFP and Chl were excited at a wavelength of 488 nm, in combination with 493–573 nm (GFP) and 650–800 nm (Chl) emission filters. As a negative control, transgenic plants expressing *35S::GFP* were used which showed cytosolic and nuclear localizations of GFP. As a positive control, transgenic plants expressing a chimeric precursor consisting of the transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase and GFP (*TP::GFP*) were used (kindly provided by Dr. Norbert Rolland, CEA, Grenoble, France). In this case, almost all of the detected GFP fluorescence was found in the plastid compartment.

fragmentation was assessed by conventional agarose gel electrophoresis and ethidium bromide staining. **Fig. 8B** showed that while high molecular mass DNA accumulated in seedlings of the wild type and line *Atoep16-1;6:35S::OEP16 E_6*, seedlings of mutant *Atoep16-1;6* contained drastically reduced amounts of higher molecular mass DNA and instead contained lower mass DNA species. Similar to line *Atoep16-1;6:35S::OEP16 E_6*, the other analyzed transformed lines also contained increased levels of uncleaved DNA (**Fig. 8B**).

Thirdly, we performed pulse labeling studies with $[^{35}S]$methionine. Singlet oxygen is a powerful cytotoxin and potent signaling compound that causes changes in gene expression including effects on translation (Miller et al. 2007, Khandal et al. 2009; summarized in Reinbothe et al. 2009). Confirming previous results (Samol et al. 2011), protein synthesis was hardly detectable in dark-grown *Atoep16-1;6* mutant seedlings after 24 h of illumination (**Fig. 8C**). However, protein synthesis was unchanged in seedlings of line *Atoep16-1;6:35S::OEP16 E_6* and the wild type (**Fig. 8C**). Whereas mutant *Atoep16-1;6* failed to react to singlet oxygen with the synthesis of stress proteins, the previously described *flu* mutant (Meskauskiene et al. 2001) did so and translated key enzymes of jasmonic acid synthesis such as allene oxide synthase (AOS) (Wasternack 2007; summarized in Reinbothe et al. 2009) (**Fig. 8d**). *flu* is impaired in the negative feedback loop inhibiting excess Pchlide accumulation in the dark and suffers from photooxidative damage and eventually dies during greening (Meskauskiene et al. 2001, op den Camp et al. 2004).

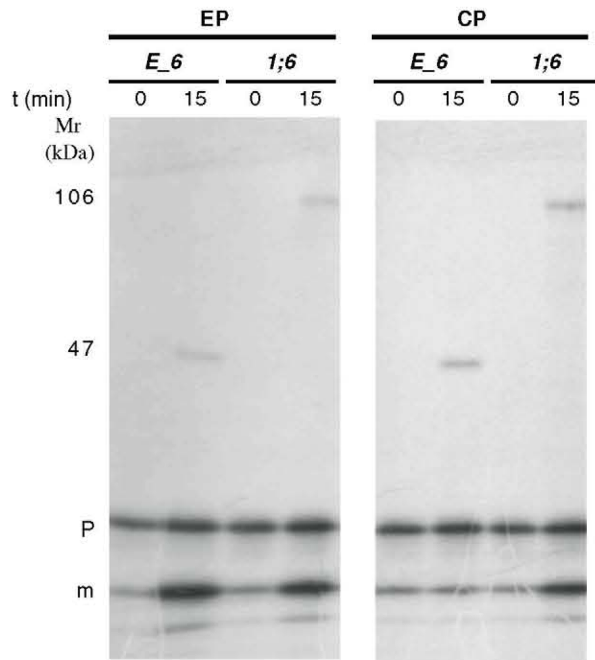

Fig. 6 Cross-linking of DTNB-activated [^{32}S]transA–DHFR in etioplasts (EP) and chloroplasts (CP) isolated from mutant *Atoep16-1;6* (*1;6*) and line T_3 *Atoep16-1;6:35S::OEP16 E_6* (*E_6*). The autoradiogram shows precursor (P) and mature (m) proteins as well as cross-linked products of 47 and 106 kDa at time zero (0 min) and after 15 min of import (15 min). The 47 kDa cross-linked product is caused by the formation of a disulfide bond between transA–DHFR and OEP16-1; this product is only detectable in plastids of line *Atoep16-1;6:35S::OEP16 E_6*. The 106 kDa cross-linked product results from the formation of a disulfide bond between transA–DHFR and TOC75; this product is found for plastids from *Atoep16-1;6* mutants.

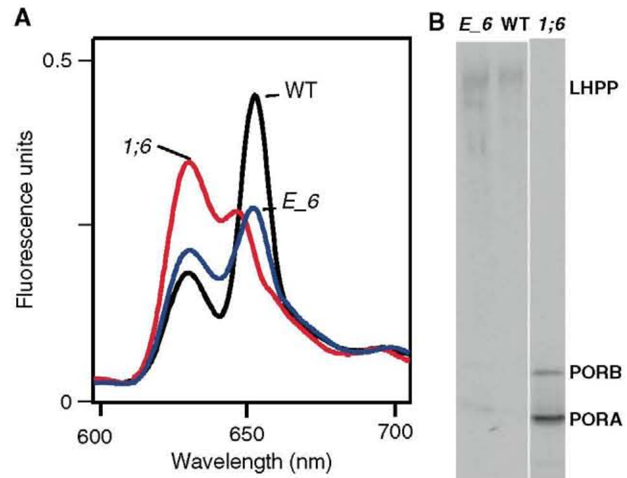

Fig. 7 Presence of PORA:PORB supracomplexes in line T_3 *Atoep16-1;6:35S::OEP16 E_6*. (A) Low temperature fluorescence analysis at 77 K of pigments in 5-day-old, etiolated seedlings of mutant *Atoep16-1;6* (*1;6*), line *Atoep16-1;6:35S::OEP16 E_6* (*E_6*) and the wild type (WT). Note the presence of photoactive Pchlide (Pchlide-F655) in line *Atoep16-1;6:35S::OEP16 E_6* and its almost total absence in mutant *Atoep16-1;6*. Spectral intensities refer to an equal cotyledon surface area. (B) Non-denaturing PAGE to detect PORA:PORB supracomplexes in mutant *Atoep16-1;6*, line *Atoep16-1;6:35S::OEP16 E_6* and the wild type.

Backcrosses of mutant *Atoep16-1;6* with the wild type

Mutant F6-4a studied by Pudelski et al. (2009) corresponds to mutant *Atoep16-1;6* (Samol et al. 2011) and contains only one detectable T-DNA insertion (Pudelski et al. 2009, Samol et al. 2011). Pudelski et al. (2009) suggested that mutant F6-4a may contain an additional point or footprint mutation that caused the cell death phenotype. If this point mutation is unlinked to the *Atoep16-1;6* mutation, it should be separable by a backcross with the wild type. In the F_2 of such a backcross, the plants segregated in 40 wild-type seedlings, 89 seedlings with a weak bleaching phenotype and 41 with a strong cell death phenotype. This fits to a monohybrid, semi-dominant expected ratio of 42.5 : 85.0 : 42.5 ($\chi^2 = 0.21$; $P = 0.975$) and excluded that the second mutation present in mutant *oep16-1;6* caused the bleaching phenotype. The homozygous F_2 plants with the strong bleaching phenotype lacked PORA (Fig. 9A) and contained exclusively photoinactive Pchlide-F631 (Fig. 9B); they may correspond to mutant *oep16-1;5*. In fact, no seedlings were obtained that were wild type with respect to the *OEP16-1* gene but showed the cell death phenotype. Thus, the cell death phenotype in mutant *oep16-1;5* is causally related to the import defect of pPORA, the lack of Pchlide sequestration and the production of singlet oxygen. Interestingly, a similar phenotype was observed for the *porA* knock-out mutant. Furthermore, Lebedev et al. (1995) described that seedlings of the *det340* mutant of *A. thaliana* are extremely susceptible to photooxidative damage and accumulate Chl only at extremely low light intensities. Dark-grown seedlings of the *det340* mutant lack PORA (and photoactive Pchlide) due to the *det* mutation (Lebedev et al. 1995).

To support further the conclusion that OEP16-1 is essential for pPORA import, we generated transgenic plants expressing pPORA–GFP fusion proteins consisting of the full-length PORA precursor (pPORA) of *A. thaliana* (Armstrong et al. 1995) and GFP. In four parallel batches, transgenic plants were generated for the wild type, mutant *Atoep16-1;6*, line *Atoep16-1;6:35S::OEP16 E_6* and the backcross of mutant *Atoep16-1;6*, and assessed for the presence of GFP fluorescence by confocal laser scanning microscopy.

As shown in Fig. 10A, etioplasts of transgenic wild-type plants synthesizing pPORA–GFP readily imported the chimeric precursor protein. Similarly, the pPORA–GFP fusion protein was imported into etioplasts of mutant *Atoep16-1;6*, although the amount of the fluorescent protein per plastid seemed reduced and some discontinuous distribution indicative of aggregation of unimported precursor occurred (Fig. 10B). Line *Atoep16-1;6* transgenic for *35S::OEP16 E_6* and expressing OEP16-1 imported pPORA–GFP into their plastids, and GFP fluorescence was sharply focused in etioplasts (Fig. 10C).

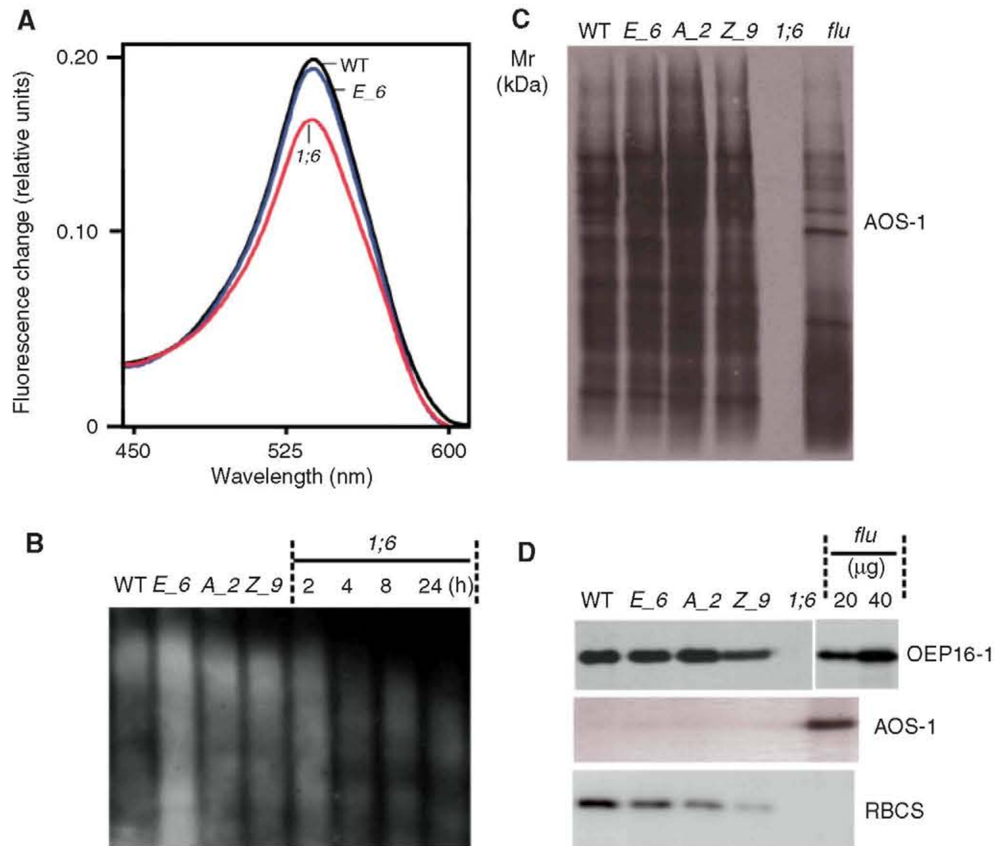

Fig. 8 Cell death rescue in line T_3 *Atoep16-1;6:35S::OEP16 E_6*. (A) Singlet oxygen evolution in 5-day-old, etiolated seedlings of mutant *Atoep16-1;6* (*1;6*), line *Atoep16-1;6:35S::OEP16 E_6* (*E_6*) and the wild type (WT) after a 30 min white light exposure. The curves show DanePy fluorescence emission spectra collected between 450 and 600 nm at an excitation wavelength of 331 nm. (B) DNA laddering in mutant *Atoep16-1;6*, line *Atoep16-1;6:35S::OEP16 E_6* and the wild type after a 24 h white light exposure. For comparison, DNA laddering was tested for two additional *Atoep16-1;6:35S::OEP16*-expressing lines, designated A_2 and Z_9. The last four lanes show a time course of DNA degradation in mutant *Atoep16-1;6*. (C) Protein synthesis in etiolated seedlings of mutant *Atoep16-1;6*, lines *Atoep16-1;6:35S::OEP16 E_6*, *A_2* and *Z_9*, as well as the wild type after a 24 h white light exposure. Total leaf protein was labeled with [^{35}S]methionine for 2 h before seedling harvest. After extraction and SDS–PAGE on a 15% polyacrylamide gel, ^{35}S-labeled proteins equivalent to 20 µg of bovine serum albumin were detected by autoradiography. For comparison, protein extracts were prepared form irradiated *flu* seedlings and processed identically. (D) Western blot analysis to detect OEP16-1, allene oxide synthase (AOS) and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RBCS) in etiolated seedlings of mutant *Atoep16-1;6*, lines *Atoep16-1;6:35S::OEP16 E_6*, *A_2* and *Z_9*, as well as the wild type and *flu* after a 24 h white light exposure.

In contrast, no GFP fluorescence was detectable in plastids and the precursor was most probably degraded in *Atoep16-1;6* mutant plants backcrossed with the wild type and selected for plants that were homozygous for the *Atoep16-1* mutation. This is illustrated for line *Atoep16-1;6 C_107* (**Fig. 10D**). That the introduced transgene was expressed in line *Atoep16-1;6 C_107* was proven by Western blotting using etioplast protein extracts from seedlings in which degradation of pPORA–GFP was inhibited by a protease inhibitor cocktail (Supplementary Fig. S5). Together with the in vitro import and cross-linking experiments, these results demonstrated that the 'hidden' mutation present in *Atoep16-1;6* caused the TOC75-dependent import of pPORA–GFP and presumably also of pPORA in planta. In contrast, crossing out this mutation rendered import of pPORA–GFP and pPORA OEP16-1 dependent, and, therefore, import was undetectable in seedlings of line *Atoep16-1;6 C_107*.

Discussion

OEP16-1 was previously identified as partner of pPORA during its Pchlide-dependent import into barley, wheat, pea and Arabidopsis plastids (Reinbothe et al. 2004a, Reinbothe et al. 2004b, Pollmann et al. 2007). Reverse genetic approaches carried out in two laboratories led to different results (Philippar et al. 2007, Pollmann et al. 2007). Pudelski et al. (2009) recently maintained that OEP16-1 in Arabidopsis is not involved in pPORA import and seedling de-etiolation. Arguments for this conclusion were (i) lack of overlap in the expression of OEP16-1

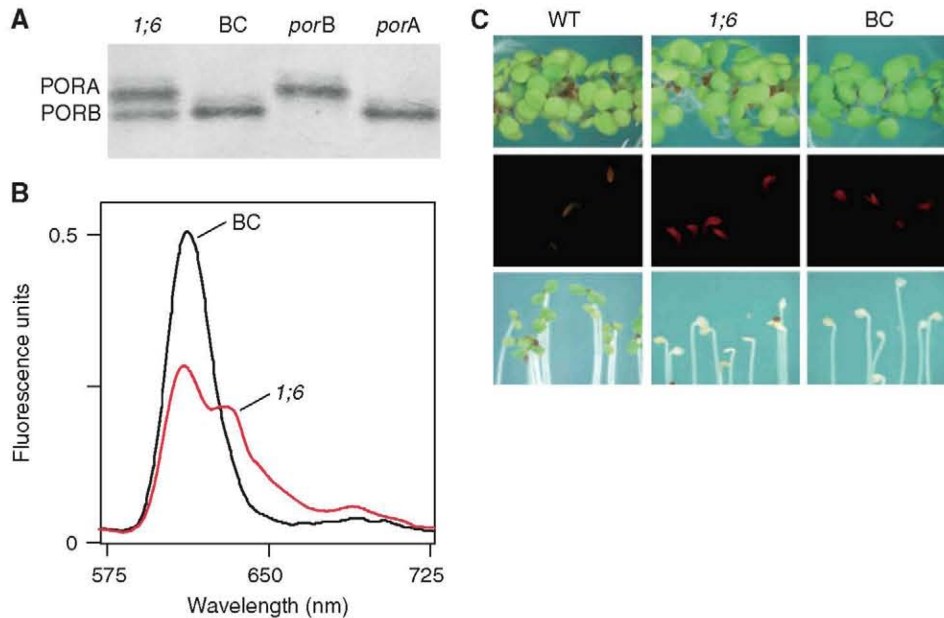

Fig. 9 Phenotypes of homozygous F_2 seedlings from the cross of homozygous mutant *Atoep16-1;6* with the wild type. (A) Western blot of PORA and PORB in mutant *Atoep16-1;6* and homozygous backcross plants (BC). For comparison, protein extracts were prepared from etiolated seedlings of *porA* and *porB* knock-out plants. (B) Low temperature fluorescence spectrum of photoactive Pchlide-F655 and photoinactive Pchlide-F631 in mutant *Atoep16-1;6*, and in homozygous backcross plants containing only photoinactive Pchlide-F631. (C) Phenotype of wild-type, homozygous *Atoep16-1;6* mutant and homozygous backcross seedlings after growth for 5 d in either continuous white light (top) or darkness (middle), and after 24 h of illumination with 210 $\mu E\ m^{-2}\ s^{-1}$ white light following dark growth (bottom).

and PORA during seed germination and seedling growth in the dark; (ii) lack of genetic complementation assays needed to demonstrate a causal relationship between the absence of AtOEP16-1, pPORA import, Pchlide sequestration and cell death progression; and (iii) lack of co-segregation between the *AtOEP16-1* mutation and the cell death phenotype (Pudelski et al. 2009). With the present study, answers are provided to all of these questions.

Overlapping developmental expression patterns of OEP16-1 and PORA

We show that AtOEP16-1 expression is not confined to chloroplasts. With a monospecific antibody raised against the bacterially expressed and purified AtOEP16-1 protein we demonstrate that AtOEP16-1 is also abundant in etiolated plants and that its expression transiently declines in illuminated seedlings. Western blot analyses with POR antibody confirmed high PORA protein levels in the dark and decreasing PORA protein levels in illuminated seedlings. These results are in agreement with previous findings (Armstrong et al. 1995, Reinbothe et al. 2004b) and support the conclusion that there is a significant overlap in the expression of AtOEP16-1 and PORA. Recently, an interesting observation was made for two barley mutants, *albina-e*^{16} (*alb-e*^{16}) and *alb-e*^{17} (Campoli et al. 2009). Both of them are disturbed in porphyrin biosynthesis although at different steps (a block of upstream Mg-protoporphyrin IX biosynthesis in *alb-e*^{16} and a block before chlorophyllide biosynthesis in *alb-e*^{17}). In both mutants, the down-regulation of PORA mRNA was associated with the up-regulation of OEP16 mRNA (Campoli et al. 2009). This observation may suggest a compensatory mechanism to avoid PORA deficiency through higher import rates of the cytosolic precursor protein into the plastids.

Essential role of OEP16-1 as assessed by genetic transformation

Genetic transformation was used to explore whether or not a causal relationship exists between the absence of AtOEP16-1, pPORA import, Pchlide sequestration and cell death progression. Transformation of *oep16-1;6* plants was carried out with OEP16-1, GFP::OEP16-1 and OEP16-1::YFP transgenes driven by the 35S cauliflower mosaic virus promoter, using established procedures. As demonstrated for numerous other transgenes, plant transformation is a hazardous process that produces a vast range of different insert lines. Depending on the site where the foreign DNA has integrated into the genome, a large range of different transgenic lines is normally obtained. These lines differ with respect to the temporal and spatial pattern where the transgene's encoded product is expressed. For example, some of the produced lines may exhibit transgene expression that is too low to allow the detection of the encoded product. In other cases, strong expression of the transgene may occur but the encoded product may aggregate and thus not reach its final destination, therefore not permitting restoration of the

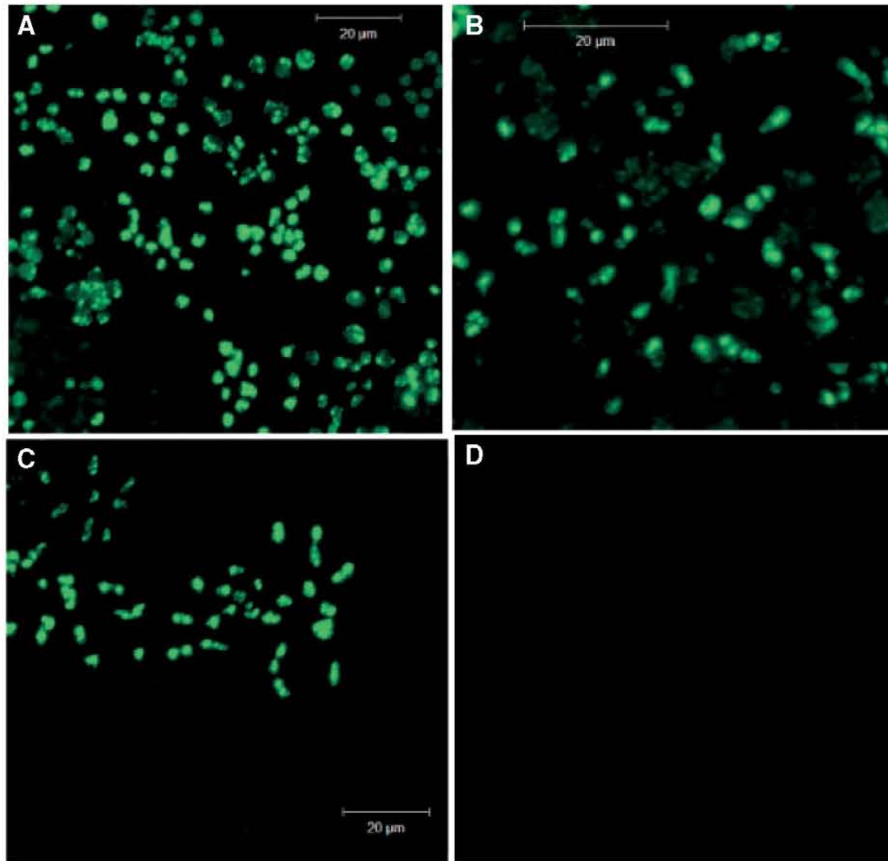

Fig. 10 In planta import of the pPORA–GFP fusion protein into plastids. Transgenic *A. thaliana* plants stably expressing pPORA–GFP under the control of the 35S cauliflower mosaic virus promoter were generated and assessed by confocal laser scanning microscopy for import of the introduced reporter protein. Images show GFP fluorescence in 5-day-old etiolated seedlings of the transgenic wild type (A), mutant *Atoep16-1;6* (B), line *Atoep16-1;6:35S::OEP16 E_6* expressing OEP16-1 (C) and the generated backcross of mutant *Atoep16-1;6*, designated *Atoep16-1;6 C_107* (D).

wild-type phenotype of the transformed mutant. Only in those transgenic lines where an almost wild-type expression level of the product of interest is found will restoration of the wild-type phenotype occur. This would, however, require that the trangene is expressed in the right developmental window and in the correct tissue, two processes that may be prone to variations from line to line. Even though the 35S promoter used in the present study should drive strong constitutive overexpression of the transgene, different lines were obtained, displaying significant variations in the expression level of the OEP16-1, GFP::OEP16-1 and OEP16-1::YFP transgenes. Nine transgenic T_3 lines were obtained containing *35S::OEP16*, of which five showed rescue from photobleaching. In contrast, only one of six T_3 lines, *35S::GFP::OEP16*, complemented the phenotype. In the case of the T_3 line *35S::OEP16::YFP*, none of the produced lines could restore normal greening. Those lines that displayed a cell death rescue showed normal greening and no longer died as a result of Pchlide overaccumulation and excitation, triggering the release of singlet oxygen. No red pigment fluorescence indicative of the presence of free Pchlide molecules was detectable in etiolated (dark-grown) seedlings of the regenerated transgenic plants. In line with this observation, low temperature pigment measurements identified photoactive Pchlide F-655 that was restored in dark-grown seedlings. Moreover, confocal laser scanning microscopy confirmed that the introduced AtOEP16-1 protein was localized in chloroplasts. Non-denaturing PAGE detected higher molecular mass PORA: PORB complexes in the regenerated transgenic lines. Taken together, these results show that synthesis of AtOEP16-1 protein from the transgene transformed into the mutant restored pPORA import, Pchlide sequestration and greening.

A critical factor that may influence the capability of a transgene to restore the wild-type phenotype of a given mutant (e.g. *oep16-1;6*) is the structure of the encoded protein. To establish its functional state, the protein must fold correctly and attain the natural conformation, thereby interacting with other cellular components, such as, for example, membrane lipids and membrane proteins. In the case of OEP16-1, the protein needs first to be directed to the respective target membrane, i.e. the outer envelope membrane of the chloroplast, before it can form cation-selective pores involved in amino acid and polypeptide transport (Pohlmeyer et al. 1997, Steinkamp

et al. 2000, Linke et al. 2004, Reinbothe et al. 2004b). OEP16-1 is a transmembrane protein which is anchored to the membrane presumably via four α-helices; its exact topology is very controversially discussed (see Pohlmeyer et al. 1997, Steinkamp et al. 2000, Linke et al. 2004). OEP16-1 additionally interacts with the other components of the Pchlide-dependent translocon comprising at least 10 different proteins to be operational in the substrate-dependent import of pPORA (Reinbothe et al. 2004a, Reinbothe et al. 2004b).

Whatever the exact insertion mechanism and topology of OEP16-1 might be, it is obvious that the presence of the reporter that was fused to the OEP16-1 protein affected the functionality of the resulting fusion protein. Any of the intermediate steps in the long and complicated pathway of this transmembrane protein may be impaired in some of the regenerated transgenic lines. For example, the folding of OEP16-1 may not occur correctly in the case of the OEP16-1::YFP fusion protein. On the other hand, the fusion protein may correctly fold but, due to the presence of the bulky reporter, not be able to insert properly into the outer chloroplast envelope membrane. Last, but not least, the OEP16-1::YFP protein may correctly fold and properly bind to the plastids but may not attain a functional, pore-forming and interactive state in the outer envelope membrane. In this case it could not be operational in the Pchlide-dependent import pathway of pPORA. For all of these reasons, the transgenic approach may have failed in the case of the T_3 35S::OEP16::YFP lines. In the case of one of the lines studied, T_3 *oep16-1;6:35S::OEP16::YFP G_4*, we noted that YFP fluorescence is detectable but not associated with chloroplasts, explaining why no cell death rescue occurred. In contrast, we found that at least one out of six T_3 lines, *oep16-1;6:35S::GFP::OEP16*, expressed correctly folded and properly targeted as well as functional fusion protein, permitting the Pchlide-dependent import of pPORA and thereby restoring normal greening. Because five of the nine produced *oep16-1;6:35S::OEP16* lines were capable of restoring normal greening we conclude that OEP16-1 is essential for pPORA import, Pchlide sequestration and normal greening.

Evidence for the presence of additional mutations in the SALK_024018 seed stock

Because of the existence of four different *Atoep16-1* mutants containing the same, single T-DNA insertion in the OEP16-1 gene, an additional mutation has been postulated to be present in mutant *Atoep16-1;6* (Samol et al. 2011), which corresponds to the mutant F6-4a used by Pudelski et al. (2009). Pudelski et al. (2009) discussed the possible presence of such a mutation in the SALK_024018 seed stock but offered no explanation for its role. Because mutant *Atoep16-1;6* contains a single T-DNA band of the expected size on Southern blots, we suggested that the additional mutation is a point mutation left over after an independent T-DNA insertion event followed by loss of the T-DNA (Samol et al. 2011). Such effects have been reported (Latham et al. 2006). Either cytosolic targeting factors operating in the post-translational import of pPORA or the plastid envelope protein translocon complexes themselves could be affected in mutant *Atoep16-1;6*, giving rise to TOC75-dependent import. However, the imported and processed enzyme did not assemble into larger complexes with PORB and, thus, no photoactive Pchlide-F655 was formed.

Previous biochemical approaches have identified cytosolic HSP70 and 14-3-3 proteins as key players for the targeting of pPORA to different import pathways (Schemenwitz et al. 2007). A guidance complex was formed similar to the one reported by May and Soll (2000). Binding of HSP70 and 14-3-3 proteins to the mature region of pPORA provoked a substrate-independent import of pPORA in vitro (Schemenwitz et al. 2007). Also in vivo, such an import route was observed by Kim and Apel (2004) in true leaves. Because no HSP70 and 14-3-3 protein complexes detectably bound to pPORA in protein extracts of etiolated seedlings (Schemenwitz et al. 2007), an explanation was offered as to why the Pchlide dependency of pPORA import is developmentally regulated and tied to seedling growth in darkness (Kim and Apel 2004).

The existence of a third import pathway of pPORA can be deduced from the results of the present study. We used transA–DHFR to demonstrate substrate-independent import of this model precursor into plastids isolated from mutant *Atoep16-1;6*. Because transA–DHFR does not contain the 14-3-3 recognition motif present in the mature region of the PORA, the import pathway followed in mutant *Atoep16-1;6* must rely on factors other than HSP70 and 14-3-3 proteins forming the guidance complex (May and Soll 2000, Schemenwitz et al. 2008). Qbadou et al. (2006) provided evidence for a HSP90-mediated pathway that could also be involved in directing pPORA to the TOC and TIC import machinery in mutant *Atoep16-1;6*.

For at least two reasons it seems unlikely that the conditional cell death phenotype observed in mutant *Atoep16-1;6* is caused by the additional mutation. First, independent transgenic lines expressing OEP16-1 either alone or as a fusion with GFP were obtained, which showed the same cell death rescue and greened normally. During in planta transformation, the generated vector DNA inserts randomly into the genome. It is unlikely that different transformants integrated the T-DNA at exactly the same site in the genome and that the additional mutation was thereby disrupted. Secondly, our segregation analysis on backcrossed *Atoep16-1;6* mutant plants did not provide seedlings that were both homozygous for the *Atoep16-1* mutation and lacked the cell death phenotype. On the other hand, no seedlings were obtained that were wild type for the *AtOEP16-1* gene but displayed the cell death phenotype. Finally, the transformants obtained for the *35S::OEP16::YFP* construct showed that insertion of the foreign DNA on the one hand and expression of the OEP16 protein without its correct localization (integration) in the plastid envelope membrane on the other hand were insufficient to prevent cell death. It is likely that the different position of the reporter in the OEP16–YFP and GFP–OEP16 fusion proteins affected their folding pathways

and topologies, leading to different plastid import properties. On the basis of these and the aforementioned results, we conclude that *AtOEP16-1* is involved in the Pchlide-dependent import of pPORA into etioplasts. Due to the coupling of pPORA import to Pchlide synthesis in the plastid envelope, an efficient mechanism is established to sequester the pigment in a protein-bound form and, thereby, avoid photooxidative damage during subsequent greening.

Materials and methods

Plant growth

Mutant *Atoep16-1;6* (At2g28900) has been isolated from SALK_024018 (Alonso et al. 2003) as described (Samol et al. 2011). Seeds of homozygous plants were surface-sterilized and germinated on agar medium containing half-strength Murashige and Skoog (MS) medium in the dark (Samol et al. 2011). For standard cultivation, the medium contained additionally 1% (w/v) sucrose. For photobleaching tests, media lacked sucrose in most cases. After variable periods of cultivation, the seedlings were exposed to white light of the indicated fluence rates of 210 $\mu E\, m^{-2}\, s^{-1}$. For chloroplast isolation experiments, seeds were sown on soil and cultivated in continuous white light for appropriate periods.

Genetic complementation assay

DNA cloning was performed following Gateway Technology (Invitrogen). cDNA for the AtOEP16-1 protein of *A. thaliana* was integrated into the binary T-DNA destination vectors pB7WG2, pK7WGF2 and pB7YWG2 to generate the following cDNA constructs: 35S::OEP16, 35S::GFP::OEP16 and 35S::OEP16::YFP. In the case of 35S::OEP16::YFP, the stop codon of the *AtOEP16-1* cDNA was eliminated during PCR cloning using appropriate primers (Innis et al. 1990). These different constructs were then used to transform *Atoep16-1* mutant plants by the floral dip method (Clough and Bent 1998). Plant transformants were identified by PCR (Innis et al. 1990) and Southern blotting (Sambrook et al. 1989), using appropriate primers and probes, respectively.

Pigment fluorescence analyses

Low temperature fluorescence spectroscopy was performed at 77 K at an excitation wavelength of 440 nm and collecting fluorescence emission between 575 and 725 nm (Lebedev et al. 1995) (spectrometer model LS50B, Perkin Elmer Corp.). Fluorescence microscopy was carried out using an excitation filter from 400 to 450 nm and an emission filter from 600 to 650 nm.

Seedling viability tests

Seedling viability was assessed by the previously described greening test (Meskauskiene et al. 2001, Pollmann et al. 2007) and confirmed by tetrazolium staining (Nortin 1966). While viable seedlings exhibit a strong red staining, dead seedlings are yellow to white. For statistic assessment, pools of about 250 seeds were analyzed in three independent experiments.

Singlet oxygen and TUNEL measurements

Singlet oxygen evolution was measured with the DanePy method (Hideg et al. 1998, Kálai et al. 2002). Fluorescence emission of DanePy, which is quenched by singlet oxygen, was collected between 425 and 625 nm, using an excitation wavelength of 330 nm (Life Sciences spectrometer, model LS50 Perkin Elmer Corp.). DNA laddering during cell death was followed by conventional agarose gel electrophoresis and TUNEL (Gavrieli et al. 1992).

Protein import in vitro and in planta

Construction of chimeric precursors consisting of the N-terminal targeting sequences of pPORA (transA) and pPORB (transB) of barley and DHFR has been described (Reinbothe et al. 1997, Reinbothe et al. 2000). In vitro import reactions were carried out using 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* (Schemenwitz et al. 2007). Chemical cross-linking of DTNB-activated [^{35}S]precursors was conducted as described (Reinbothe et al. 2004b).

For studying import in vivo, transgenic lines stably expressing fusion proteins of the pPORA and pPORB of *A. thaliana* (Armstrong et al. 1995) and the jellyfish GFP were produced. Transformed plantlets were grown in the dark or with white light under sterile conditions for appropriate periods and analyzed by confocal laser scanning microscopy (LSM 510 Meta microscope, Zeiss), using 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.

Purification of bacterially expressed (His)$_6$-AtOEP16-1 protein and antibody production

The presence of codons for the N-terminal hexa-His tag in pDEST^{TM}17 allowed the affinity purification of recombinant fusion protein using Ni-NTA agarose chromatography. The procedure of batch purification under denaturing conditions was adapted from the protocol of the QIAexpressionist (Qiagen). Bacterial pellets of *Escherichia coli* strain BL21AI grown with L-arabinose (to induce protein expression) for 4 h were lysed in lysis buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 8.0) using a French press. Cellular debris was removed by centrifugation at 10,000 × *g* for 30 min. The cleared lysate was mixed with 1 ml of suspension of 50% (w/v) Ni-NTA in buffer B and incubated on a rotary shaker at 200 r.p.m. overnight at 4°C. After transfer into a Biorad minispin column, the Ni-NTA

resin was washed twice with 4 ml of buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 6.3). Recombinant $(His)_6$-OEP16 protein was eluted with 4× 0.5 ml of buffer D (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 5.9), followed by 4× 0.5 ml of buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–HCl, pH 4.5). The fractions eluted with buffer D and E were separated by SDS–PAGE. Then, the polyacrylamide gel was stained with Coomassie brilliant blue G250 to identify the recombinant $(His)_6$-AtOEP16-1 protein. A 2 mg aliquot of each of the recombinant, SDS-denatured and electrophoretically resolved, Coomassie-stained proteins was used for the primary immunization and subsequent boost injections of two independent rabbits. Positive antisera were identified by enzyme-linked immunosorbent assays and conventional Western blotting using SDS–PAGE-resolved recombinant and native protein (Towbin et al. 1979).

Protein analyses

Protein was extracted and precipitated with trichloroacetic acid and analyzed by SDS–PAGE on 10–20% gradients (Laemmli 1970). For resolution of POR–pigment complexes, etioplasts were isolated from 4.5-day-old dark-grown plants and fractionated into prolamellar bodies, envelopes and stroma (Reinbothe et al. 2004a). Prolamellar body membranes were subsequently solubilized with detergent and subjected to electrophoresis on non-denaturing, analytical 7.5% polyacrylamide gels (Buhr et al. 2008). POR–pigment complexes were detected by their Pchlide autofluorescence under blue light and Western blotting using POR antiserum, respectively (Towbin et al. 1979, Buhr et al. 2008).

Segregation analyses

Reciprocal segregation analyses were carried out according to standard procedures (Weigel and Glazebrook 2002). Anthers were removed from the female parental flowers under a dissecting microscope, while the carpels were left intact. After 48 h, a flower from the male parent was dissected, squeezed, and the anthers were separated from the other flower organs mechanically. F_2 seeds were collected after appropriate periods following fertilization from independent plants.

Supplementary data

Supplementary data are available at PCP online.

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