BIOPHYSICS AND COMPUTATIONAL BIOLOGY


The authors request that Sebastian Peuker be added to the author list between Kálmán Hideg and Peter G. Schultz and be credited with designing research and performing research. The online version has been corrected. The corrected author and affiliation lines, author contributions, and related footnotes appear below.

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PLANT BIOLOGY


The undersigned authors wish to note that: “We have been associated with part of the experimental work described therein, yet now disagree with the title and main conclusion of the article. (i) Among the backcrossed lines homozygous for oep16-1 T-DNA insertion that we reanalyzed after publication, one did not show a bleaching phenotype during de-etiolation. Another line showed bleaching of etiolated seedlings upon light exposure, yet displayed wildtype levels of the PORA protein by Western blotting, in its processed form (e.g., mature, imported into etioplasts). The flu-like bleaching phenotype in part of the descent of this oep16-1 mutant stock therefore appears unlinked with the lack of OEP16-1 expression or with a defect in PORA import, in accordance with the recent report of Pudelski et al. (2009) [PNAS 106:12201–12206]. (ii) We were never granted access to the complete set of original data, in particular to those supporting Figs. 2G, 4C and D, and 6B. Therefore, we wonder about the quality of the underlying work and have expressed doubts concerning what is concluded from these pictures. It must also be made clear that the flu mutant used is not a published allele but contains a T-DNA insertion (SALK_002383) and displays a weak phenotype.”

Jean-Marc Bonnve
Gabrielle Tichtinsky

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The undersigned authors wish to note that: “We confirm that we are confident in the data and conclusions in the published article.”

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PHYSICS


The authors note that, due to a printer’s error, the affiliation for Stefano Zapperi appeared incorrectly. It should have appeared as Institute for Scientific Interface Foundation. The corrected affiliation line appears below. Additionally, the authors note that equation 8 appeared incorrectly in part. The corrected equation appears below.

“Consiglio Nazionale delle Ricerche, Istituto Nazionale per la Fisica della Materia–S3 and Dipartimento di Fisica, Università di Modena e Reggio Emilia, Via G. Campi 213/A, I-41100, Modena, Italy; and *Institute for Scientific Interface Foundation, Viale San Severo 65, 10133 Turin, Italy

\[
x = \frac{L}{2} = \frac{a \sqrt{2} B}{\pi \sqrt{V_0 \rho}} \frac{\log(\tan(\pi u/2a)) \sin(\pi u/a)}{\sqrt{1 - \cos(2\pi u/a)}}
\]

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A plant porphyria related to defects in plastid import of protochlorophyllide oxidoreductase A


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The plastid envelope of higher plant chloroplasts is a focal point of plant metabolism. It is involved in numerous pathways, including tetrapyrrole biosynthesis and protein translocation. Chloroplasts need to import a large number of proteins from the cytosol because most are encoded in the nucleus. Here we report that a loss-of-function mutation in the outer plastid envelope 16-kDa protein (oep16) gene causes a conditional seedling lethal phenotype related to defects in import and assembly of NADPH:protochlorophyllide (Pchlide) oxidoreductase A. In the isolated knockout mutant of Arabidopsis thaliana, excess Pchlide accumulated in the dark operated as photosensitizer and provoked cell death during greening. Our results highlight the essential role of the substrate-dependent plastid import pathway of precursor Pchlide oxidoreductase A for seedling survival and the avoidance of developmentally programmed porphyria in higher plants.

In plants, light provides an important environmental signal and trigger for the production of photosynthetically active chloroplasts (1, 2). In dark-grown angiosperms, plastid development is arrested at a state that leads only to the formation of so-called etioplasts. These organelles are devoid of chlorophyll and are incapable of photosynthetic function. Once dark-grown plants break through the soil after germination and reach the sunlight, they begin to synthesize chlorophyll and assemble the photosynthetic apparatus. The synthesis of chlorophyll from protochlorophyllide (Pchlide) is, in angiosperms, a light-dependent reaction and an essential step for the establishment of photosynthetically active chloroplasts. In barley, it is catalyzed by two closely related light-activated enzymes, NADPH:Pchlide oxidoreductases A and B (PORA and PORB) (3), which form supramolecular light-harvesting structures designated LHIPP in the prolaminellar body of etioplasts (4, 5). Dark-stable PORA:PORB-Pchlide-NADPH supracomplexes are poised such that absorption of a photon by Pchlide b band bound to PORA leads to energy transfer onto PORB-bound Pchlide a and its subsequent reduction, resulting in the formation of Chloride a (4, 5). Like other free tetrapyrroles, Pchlide not bound to POR could operate as a photosensitizer (6–8). Angiosperm plants, therefore, have evolved efficient mechanisms to keep the level of these potentially phototoxic compounds low and thereby avoid porphyrias (9–12). One such mechanism is feedback control of Pchlide synthesis by heme and Pchlide (10, 11). Another factor is the fluorescent (FLU) protein, which depresses Pchlide synthesis in darkness (12). After LHIPP’s dissociation, which is induced by light and correlates with the dispersal of the prolaminellar body (13), PORA gains activity as a Pchlide b-reducing enzyme such that another part of the light energy is quenched in a nonhazardous way (4, 5).

Being encoded in the nucleus, PORA and PORB are synthesized as larger precursors (pPOR A and pPORB) and are imported into the plastids through specific protein translocon complexes in the outer and inner envelope membranes (14–18). Whereas pPOR B enters the plastids via the general protein import apparatus comprising the presequence receptor TOC159 and translocation channel protein TOC75 (17, 18), pPOR A uses a Pchlide-dependent translocon named the PTC complex, which is distinctive from the general protein import site (14, 17). Among the identified PTC proteins was a 16-kDa protein related to a group of amino acid and preprotein transporters found in free-living bacteria and endosymbiotic mitochondria and chloroplasts (14, 17). Several lines of evidence verified that the identified barley PTC16 gene is an ortholog of the previously characterized pea and Arabidopsis OEP16 (Ato2g28900) gene (17).

In the present work a reverse genetic approach was taken to dissect the function of the Arabidopsis OEP16 (Ato2g28900) gene in planta. Using a knockout line of Arabidopsis thaliana that is deficient in the OEP16 gene we demonstrate that PTC16/OEP16 is involved in pPOR A import. Interestingly, dark-grown Atoep16 plants resembled etiolated flu plants and accumulated free Pchlide. After a dark-to-light shift, this pigment operated as photosensitizer and caused rapid bleaching and cell death. Our results underscore the essential role of the substrate-dependent import pathway of pPOR A, which couples protein translocation to pigment biosynthesis in the plastid envelope, for Pchlide homeostasis and cell viability during seedling de-etiolation.

Results and Discussion

Ato2g28900 (AtoEP16) contains six exons and five introns (Fig. 1A), and we used reverse genetics to determine its role in Arabidopsis. A respective Arabidopsis mutant was obtained from the Salk Institute Genomic Analysis Laboratory collection (19) carrying a T-DNA insertion SALK_024018 that disrupts the AtoEP16 locus (Fig. 1A and C). Sequencing of the T-DNA/AtoEP16 junction established that the insertion disrupted the gene 6 bp upstream of the 3′ end of exon 2. We refer to this line as Atoep16-1 throughout the rest of the article. DNA gel blot analyses yielded a single T-DNA hybridizing band in genomic DNA isolated from light-grown homozygous Atoep16-1 plants that had been digested with two different restriction enzymes (Fig. 1B), demonstrating that Atoep16-1 contained a single T-DNA insertion in the genome. Expression studies showed that...
neither OEP16 transcript nor OEP16 protein was detectable in Atoep16-1 plants, and thus the mutant was null with respect to the Atoep16-1:1 gene (Fig. 1D).

Depending on the growth conditions, Atoep16-1 seedlings exhibited different phenotypes (Fig. 2). If grown in darkness and exposed to white light, the mutant rapidly bleached and finally died (Fig. 2B). In plants kept under continuous white light right from the beginning of germination, no phenotype was detectable, and the plants looked like the wild type (Fig. 2, compare C and F). Etiolated Atoep16-1 plants examined under blue light with a Leica MZ12 fluorescence microscope showed a strong red fluorescence indicative of the presence of free porphyrin pigments in darkness (Fig. 2C, compare with the wild type shown in D). These results were reminiscent of findings reported for the flu mutant of Arabidopsis that contains elevated levels of red-fluorescing Pchlide in darkness (12). Pchlide is present in a free form in etiolated flu plants, which triggers singlet oxygen formation and cell death upon illumination (12). When dark-grown Atoep16-1 seedlings were exposed to white light, very similar survival rates of wild-type (open circles), Atoep16-1 (triangles), and flu (filled circles) plants were observed, including bleaching and cell death (Fig. 2B versus E). However, the time courses shown in Fig. 2G revealed some differences in cell death progression (Fig. 2G). We assumed that this difference may reflect the actual level and/or composition of free porphyrin pigments(s) in Atoep16-1 versus flu plants. Pigment analyses (20) indeed showed that whereas flu plants accumulate ~8.5-fold-higher levels of total Pchlide than wild-type plants, Atoep16-1 plants contained only ~4.5-fold-higher pigment levels. Interestingly, the composition of pigments was also altered in Atoep16-1 versus flu and wild-type plants (Fig. 2H). Whereas Atoep16-1 plants accumulated Pchlide a, flu plants contained elevated levels of Pchlide b (Fig. 2H; for details of pigment identification, see supporting information [SI] Fig. 8).

OEP16 is a transmembrane channel protein of the outer plastid envelope membrane implicated in amino acid transport (16) and/or polysaccharide transport (17). When we performed radioisotope labeling studies with ^14C-glutamate, ^14C-glutamine, and ^14C-glycine, and isolated chloroplasts and etioplasts (22), no difference in amino acid uptake was found for Atoep16-1 versus

Fig. 3. Amino acid uptake into Atoep16-1 and wild-type chloroplasts. (A) Isolated chloroplasts were incubated with ^14C-glutamate for various time intervals, and uptake of radioactivity was determined. (B) After labeling isolated chloroplasts with ^35S-Methionine for 5 min (lanes 1 and 2) or 20 min (data not shown), protein was extracted and precipitated with trichloroacetic acid, separated by SDS/PAGE, and detected by either autoradiography (lanes 1 and 2) or Coomassie staining (lanes 3 and 4). LSU and SSU, large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase; CAB, chlorophyll a/b-binding protein of photosystem II.
wild-type plants (Fig. 3A and data not shown). Also, no large differences were apparent in the patterns of plastid-encoded, [35S]methionine-labeled (23) proteins (Fig. 3B). Furthermore, the red fluorescence phenotype in darkness implies that Atoep16 plants were not impaired in glutamate- and 5-aminolevulinic acid (5-ALA)-dependent Pchlide synthesis. If OEP16 would be involved in amino acid uptake, an inhibition, but not an elevation, of glutamate-dependent synthesis of 5-ALA and Pchlide should have occurred in etiolated Atoep16-1 plants, which was obviously not observed (see Fig. 2). We thus concluded that Atoep16-1 was not affected in bulk amino acid uptake into isolated plastids.

Alternatively, OEP16 could be involved in polypeptide transport (17). This idea was tested by performing in vitro import experiments (24). Fig. 4 shows that no differences in import of the precursors to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU), ferredoxin (pFd), and the chlorophyll a/b-binding protein precursor of photosystem II (pCAB). Light and dark gray columns refer to precursor and mature protein levels after 15-min import reactions into 5-ALA-treated, Pchlide-containing plastids; nevertheless, the precursor could not enter a productive import pathway (Fig. 4F) indeed confirmed the import defect of Atoep16 plants. 

Fig. 5. Transient expression and plastid import of TransA-GFP in Arabidopsis leaf epidermis cells of Atoep16-1 and wild-type plants. DNA for transA-GFP, encoding transA and the GFP, was transformed into leaf epidermis cells of Arabidopsis wild-type and atoep16 plants. After 24 h in darkness, GFP (A and D) and chlorophyll (B and E) fluorescences were monitored between 505–530 nm and 575–650 nm, respectively, by using an excitation wavelength of 488 nm. (C) Merge of A and B. (F) Merge of D and E. As subcellular localization controls, the naked GFP moiety lacking a transit peptide attached to it (not imported into either a toep16 or wild-type plastids) and transB-GFP (imported into both plastid types) were used (data not shown).

That knockout in the Atoep16-1:1 gene did not pleiotropically affect all of the different cytosolic precursors that need to be taken up by the plastid compartment is further supported by the growth rescue of Atoep16-1 plants in continuous white light (compare Fig. 2C). Like pSSU, pFd, pCAB, and pPORB, the enzymes that are involved in 5-ALA synthesis and subsequent steps of Pchlide production were also obviously not affected by the lack of the OEP16 protein. Otherwise, no excess pigment would have accumulated in dark-grown Atoep16-1 plants. All enzymes of the C5 pathway leading to chlorophyll are nucleus-encoded and imported posttranslationally from the cytoplasm (1).
plants (Fig. 6 A and B). Electron microscopy highlighted a complete lack of the prolamellar body in *Atoep16-1* as compared with wild-type etioplasts (Fig. 6C). Together, these findings conclusively showed that PORA, as part of larger complexes in the prolamellar body, played an essential role for plant survival during seedling de-etiolation and greening. Free Pchlide not bound to the PORA, by contrast, operated as photosensitizer and, by triplet–triplet interchange, provoked singlet oxygen production and cell death. Similar observations have been made for photon bleached *flu* plants (12). In a sense, these findings are reminiscent of genetically inherited porphyrias in humans where perturbations in heme metabolism trigger cell death, including nuclear condensation, chromatin separation, DNA breakdown, and release of cytochrome c from mitochondria into the cytosol (25). However, porphyrin-dependent, plastid-dependent cell death in plants also exhibits unique properties, as illustrated by recent work of Wagner et al. (26) on the *executer* mutant of *Arabidopsis*.

Our results suggest the working model described in Fig. 7, explaining the possible interaction of OEP16, PORA, and FLU during greening. Accordingly, PORA-Pchlide b-NADPH ternary complexes not assembled into PORA:PORB suprastructures after import through OEP16 could provide the link to the FLU protein and hence fine-tune the size of the prolamellar body. In wild-type plants, PORA would sense the amount of Pchlide, and once enough LHPP is made, excess PORA-Pchlide b-NADPH ternary complexes could bind to and block the activity of the FLU protein, thereby inhibiting glutamyl-tRNA reductase. By contrast, this negative feedback would no longer operate in the OEP16-deficient *Atoep16-1* mutant line because of the lack of imported PORA. It is clear that the intimate interaction of all players is required for successful greening. Any change in Pchlide homeostasis has deleterious effects on plant growth.

**Materials and Methods**

**Plant Growth.** Seeds of the *Atoep16* line (SALK_024018) were obtained from the Salk Institute Genomic Analysis Laboratory collection (19). Seeds were germinated on half-concentrated Murashige–Skoog–agar medium on Petri dishes for 4.5 days. To trigger photooxidative damages, dark-grown seedlings were exposed to white light at 125 μE·m⁻²·s⁻¹. Alternatively, seeds were germinated on soil and grown to maturity in continuous white light.

**Pigment Measurements.** High-performance liquid chromatography of acetone-extracted pigments was carried out by using a C18 reverse-phase silica gel column (Hypersil ODS, 5 μm; Shandon HPLC, Cheshire, U.K.) and synthetic Pchlide a and b as standards (5, 20). Pigments were detected and quantified at 455 nm, the Soret band of Pchlide b (5, 20).

**Analysis of Amino Acid Uptake into Isolated Plastids.** Uptake of ¹⁴C amino acids into isolated chloroplasts was monitored by using the filter paper disk method of Mans and Novelli (22). Pulse-labeling of chloroplast protein with [³⁵S]methionine (1.87 MBq per 50-μl assay, 37 TBq/mmol; Amersham Pharmacia, Uppsala, Sweden) was carried out according to Mullet et al. (23). Protein was extracted with trichloroacetic acid, depleting of chlorophyll, and washed and run on 10–20% SDS/polyacrylamide gradients (27). Protein detection was made by Coomassie staining and autoradiography or by Western blotting (28).

**Protein Import Assay in Vitro and in Planta.** Protein import into isolated *Arabidopsis* chloroplasts and etioplasts was studied as described by using cDNA-encoded, wheat germ-translated ³⁵S precursors (24). Briefly, ³⁵S precursors were synthesized from corresponding cDNA clones by coupled transcription/translation and incubated with isolated, energy-depleted *Arabidopsis* plastids from wild-type and *Atoep16-1* plants. Plastids were treated with thermolysin (29) after import to degrade unimported precursors. Chemical cross-linking of 5,5′-dithiobis(2-nitrobenzoic acid)-derivatized ³⁵S-transA-DHFR, consisting of the transit peptide of pPORA (transA) and a cytosolic dihydro-
folate reductase (DHFR) reporter protein of mouse, was carried out as described (17).

TransA-GFP derivatives were produced as described in SI Methods. Transient expression of TransA-GFP derivatives in Arabidopsis leaf epidermis cells was performed after ballistic bombardment by using a pneumatic particle inflow gun according to Finer et al. (30). The conditions of bombardment were adjusted to helium pressure of 6.5 bar, at a 12-cm target distance, with a disperse grid at 7 cm, using 1 μm of gold microcarriers (Bio-Rad, Hercules, CA). After bombardment, the plantlets were kept under sterile conditions and incubated for 24 h in darkness. Confocal laser scanning microscopy was carried out by using a LSM 510 Meta microscope (Zeiss, Jena, Germany) with krypton/argon laser excitation at 488 nm and an emission wavelength window from 505 to 530 nm. LSM 510 Meta software release 3.2 (Zeiss, Oberkochen, Germany) and Photoshop 7 (Adobe Systems, San Jose, CA) were used for image acquisition and processing. Nondenaturing, analytical PAGE was carried out as described (31).

Miscellaneous. Electron microscopy was carried out by using ultrathin sections of leaf tissues prepared from etiolated plants using a Zeiss 109 electron microscope.

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