Supporting Information

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SI Materials and Methods

Plant Growth Conditions. Seeds were surface sterilized with 5% hypochloride and to synchronize germination, all seeds were kept at 4 °C for 3 days before transfer to growth chambers. Plants were grown either under a 16-h light (23 °C, photonflux density of 100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) and 8-h dark (16 °C) cycle or as specified for the respective analyses in darkness or continuous light. For survey of the de-etiolation phenotype, seedlings were kept in darkness for 2.5 days, followed by 3 days in continuous white light (350 $\mu mol \cdot m^{-2} \cdot sec^{-1}$). Pchlide fluorescence was monitored in the dark with a SteREO Lumar.V12 microscope (Zeiss), using the Lumar 05 filter (exication 395–440 nm, emission 470–750 nm). The solid medium for seedlings contained 0.3% Gelrite (Serva), 0.5× Murashige and Skoog salts at pH 5.8, and—except for the phenotype analysis—1% sucrose. For DNA extraction, reproduction, and segregation analyses, 2-week-old seedlings were transferred to soil and grown to maturity.

Protein Analysis. To prevent decomposition of PORA protein harvesting and protein extraction were performed under dim green safe light. For protein isolation, shoots of 500 etiolated, 7-day-old seedlings were homogenized in liquid nitrogen. Soluble and membrane proteins were extracted in 50 mM Tris·HCl (pH 8), 50 mM EDTA, 2% LDS, 10 mM DTT, and 100 mM PMSF on ice for 15 min. Cell debris was pelleted at 4 °C for 15 min by centrifugation at 14,000 × *g*. Equal amounts of total cellular proteins from each plant line (30, 90, and 100 μg for detection of PORA/B and LSU, and OEP16.1 and OEP16.2 proteins, respectively) were separated by SDS/PAGE followed by immunoblot analysis using antibodies in 1:200 to 1:2,000 dilutions. All antisera were raised in rabbit against heterologously expressed proteins from *Arabidopsis* (OEP16.1, OEP16.2, and PORB) and pea (LSU) (Pineda Antibody Service).

For mass spectrometry, the protein band corresponding to mature POR (36 kDa) was excised from the SDS gel and subjected to trypsin digestion followed by peptide mass fingerprint analysis (LC-MSMS; Zentrallabor für Proteinanalytik, Adolf-Butenandt Institut, Ludwig-Maximilians-Universität, Munich, Germany).

Transcript Level Profiling. To monitor transcript abundance, total RNA was extracted from shoots of 300 etiolated, 7-day-old seedlings harvested under dim green safe light by using the Plant RNeasy extraction kit including DNase digestion (Qiagen). For quantitative real-time RT-PCR 1 μg RNA was reverse transcribed into cDNA as described in ref. 1. Detection and quantification of transcripts were performed as described previously (2), using a LightCycler (Roche). The mRNA content was quantified relative to the signal of actin cDNA fragments generated by the primers Actfw and Actrev, which amplified cDNA from actin 2 and 8 (At3g18780 and At1g49240). To prevent amplification of contaminating genomic DNA, the gene-specific primers for *OEP16.1* (OEP16.1-LCfw, -rev), *PORA* (PORA-LCfw, -rev), and *PORB* (PORB-LCfw, -rev) were selected to flank intron regions.

To provide optimal growth, seedlings for DNA microarray analysis were grown under a day/night regime with a photonflux density of 200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ during the day. In all lines tested seedling germination and growth were 95%. For each data point, total RNA from 20 individual, 8-day-old seedlings was isolated using the Plant RNeasy extraction kit. Three samples were harvested for each line to decrease biological variation. Subsequently RNA was processed and hybridized to Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays as described (1). Microarray signals were made comparable by scaling the average overall signal intensity of all probe sets to a target signal of 100. Signals from lines 5.4 and 4.2 were compared against Col-0 wild type and line 2.2 by Affymetrix Data Mining Tool software.

Molecular Characterization of the T-DNA Insertion Line SALK_024018. For the identification of T-DNA insertion sites in SALK_024018, genomic DNA was isolated from leaf material of 4-week-old plants. The tissue was homogenized in liquid nitrogen and incubated in DEB buffer [1.4 M NaCl, 20 mM EDTA, 0.1 M Tris·HCl (pH 8.0), 3% CTAB, 1% DTT] for 30 min at 65 °C. After chloroform extraction the DNA was precipitated by isopropanol and resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0. After RNase digestion, chloroform extraction was repeated, and the DNA was precipitated by NaOAc/EtOH at −20 °C for 2 h and resuspended in 10 mM Tris, pH 8.0.

For inverse PCR (see ref. 3) 5 μg of the genomic DNA were digested with 8 different enzymes: Hpy99I, NciI, BfaI, BanII, NspI, CviQI, BstYI, and BsiHKAI (New England Biolabs). After extraction with phenol/chloroform the DNA fragments were precipitated by NH_4Ac/EtOH at −20 °C for 20 min and resuspended in H_2O. Ligation of DNA fragments by T4 Ligase was performed overnight at 16 °C. Afterward, the DNA fragments were purified by phenol/chloroform for a second time. Two T-DNA-specific nested primer pairs for the right (RBkp1fw, -rev and RBkp0fw, -rev) and the left border (LBkp0.5fw, -rev and LBkp0fw, -rev) of pROK2 (4) were used to PCR amplify the unknown *Arabidopsis* DNA at the T-DNA borders, using the Advantage 2 Polymerase mix (Clontech Laboratories). All products of the inverse PCR were purified and sequenced.

Thermal asymmetric interlaced (TAIL)-PCR was performed according to the protocol by Liu et al. (5) on 1 μg of genomic DNA with 9 degenerated random primers, AD1–9. Each AD primer was used in 3 subsequent PCR reactions in combination with 3 nested T-DNA-specific primers, LBT0a–c [(*i*) PCR, AD1–9 and LBT0a; (*ii*) PCR, AD1–9 and LBT0b; and (*iii*) PCR, AD1–9 and LBT0c]. The first PCR included 5 cycles with an annealing temperature of 60 °C, followed by 1 cycle at 25 °C and 16 repeats of 2× 60 °C and 1× 44 °C primer annealing. The second PCR was performed in 13 repeats of 2× 62 °C and 1× 44 °C, while the third PCR passed through 31 cycles with 54 °C for primer annealing. All TAIL-PCR reactions were catalyzed by Taq polymerase (New England Biolabs) for 2.5 min at 68 °C for each cycle. Nested PCR products amplified by the second and third PCRs were purified and sequenced.

To identify the presence of the T-DNA pROK2 in different lines of SALK_024018, plants were grown on kanamycin (100 μg/mL)-containing plates. Furthermore, a pROK2 fragment of 1209 bp was PCR amplified on genomic DNA, using the oligonucleotide primers M13fw and M13rev. For PCR genotyping and segregation analysis of the T-DNA insertions *oep16.1–1*, *arogp1–1*, and *cm1–1*, gene-specific primers in combination with T-DNA-specific left and right border primers were used (compare with Fig. 3). On heterozygous and homozygous *oep16.1–1* mutants the primers 16.1_T-DNAfw and 16.1_T-DNArev in combination with LBkp0fw generated fragments of 413 and 750 bp, respectively. For genotyping of *arogp1–1*, the primers GP1_T-DNAfw in combination with LBkp0fw (265 bp) and GP1_T-DNArev with RBkp0fw (473 bp) were used, while the primer

pairs CM1_T-DNArev and LBa1 (431 bp) and CM1_T-DNAfw and M13fw (3,509 bp) generated fragments on heterozygous and homozygous *cm1–1* mutants. To identify plants with the T-DNA insertion in both alleles of *OEP16.1*, *AroGP1*, and *CM1* we used gene-specific primers (16.1–4018fw, -rev; GP1_T-DNAfw, -rev; and CM1_T-DNAfw, -rev) flanking the T-DNA insertion sites. DNA from homozygous mutant lines gave no amplification product, whereas the amplified regions on wild-type and heterozygous DNA were 635 bp (*oep16.1–1*), 431 bp (*arogp1–1*), and 553 bp (*cm1–1*). For positions and orientations of the T-DNA inserts and oligonucleotide primers and for PCR products see Fig. 3 and Table S1. To verify PCR products and T-DNA insertion sites, all amplified DNA fragments were sequenced.

SI Discussion

Pchlide-Dependent or -Independent Import of prePORA? On the one hand in vitro import of prePORA was described by Reinbothe and coworkers to depend strictly on its substrate Pchlide (6–11), thereby using a so-called Pchlide-dependent translocon complex (PTC). Further, they identified the outer envelope channel OEP16 and the precursor protein receptor TOC33 as proteinaceous subunits of the PTC. These results were obtained by chemical cross-linking of the prePORA precursor during in vitro import into isolated plastids of barley and *Arabidopsis* (8–10). All import studies used to evaluate the Pchlide-dependent import route were based on urea-denatured prePORA protein translated in nonplant in vitro systems (6–12) and could not be verified by independent research of different groups (13–15). On the other hand, using nondenatured prePORA in vitro translated by a wheat germ lysate, Reinbothe and coworkers recently disproved their own hypothesis by showing that prePORA when complexed by the chaperone proteins 14:3:3 and HSP70 was transported into Pchlide-free chloroplasts through the TOC75-containing standard translocon (16). Although a 14:3:3 binding site was identified in the mature PORA protein and non-urea-pretreated precursors clearly are closer to in vivo conditions than denatured proteins, it was suggested by the authors that the Pchlide-independent translocation represents only a default, in vitro import pathway for prePORA. However, in vivo analyses on TOC-receptor and OEP16 mutant plants performed by other groups unequivocally support a Pchlide-independent, TOC75-mediated translocation of prePORA (15, 17). The mutant analysis for the proposed function of OEP16.1 in Pchlide-dependent import of prePORA was performed not only on the OEP16.1 single-knockout line (SALK_024018) but also on single and double mutants of all plastid-localized OEP16 isoforms in *Arabidopsis* (i.e., OEP16.1, OEP16.2, and OEP16.4; see ref. 15). In this analysis we could clearly demonstrate that prePORA import into plastids is not Pchlide dependent and that none of the OEP16 proteins is involved.

The T-DNA line SALK_024018 contains at least 3 different T-DNA insertions. In the current study, we could show that besides the T-DNA mutation of *OEP16.1* the line SALK_024018 is characterized by 2 additional insertions within the genes *At-AroGP1* (At1g70370) and *At-CM1* (At3g29200). Furthermore, it became evident that the T-DNA insertion in OEP16.1 contains a concatemeric, back-to-back arrangement of the T-DNA pROK2 with the left border sequence at the 5′- and 3′-ends of the T-DNA/OEP16.1 borders (compare with Fig. 3*A*). Reinbothe and coworkers (12) show a Southern blot to prove that their *oep16.1–1* line contains only 1 T-DNA insertion. Prior to blotting, they digested DNA of their *oep16.1–1* line by the restriction enzymes EcoRI and BamHI and detected only a single band with a T-DNA probe on both DNA digestions. However, the size of these signals is not given. When calculating the expected signal size of the back-to-back T-DNA we identified in *OEP16.1*, we expect to detect 2 bands for EcoRI (4,632 and 3,024 bp) and 2 bands for BamHI (7,513 and 2,452 bp)-digested genomic DNA. Thus, the results by Pollmann et al. (12) can be explained only by incomplete digestion, blotting, and/or hybridization of their DNA. The existence of 2 additional T-DNA insertions in SALK_024018 highlights the importance and absolute requirement to analyze different alleles of T-DNA mutations or at least to include the respective wild-type genotypes when correlating phenotype and gene function.

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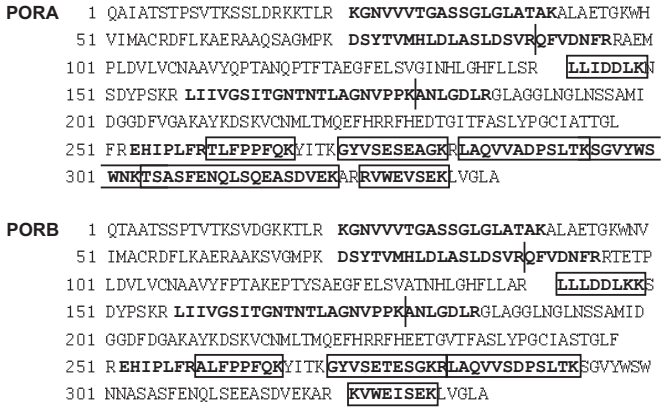

Fig. S1. Detection of specific PORA and PORB peptides in etiolated cotyledons of different SALK_024018 lines. Peptide mass fingerprint analysis of POR proteins is shown in protein extracts of 7-day-old etiolated cotyledons of Col-0 and different SALK_024018 lines (F6−4a, 5.2, 4.1, 4.2, 19.3, and 2.2) as depicted in Fig. 2*B*. The protein band corresponding to mature POR (36 kDa, compare with Fig. 2*B*) was excised from the SDS gel and subjected to trypsin digestion followed by LC-MSMS analysis. Sequences of oligopeptides detected in all lines of SALK_024018 are depicted in boldface type and separated by vertical lines. Oligopeptides specific for mature PORA (*Upper* sequence) or PORB (*Lower* sequence) are boxed.

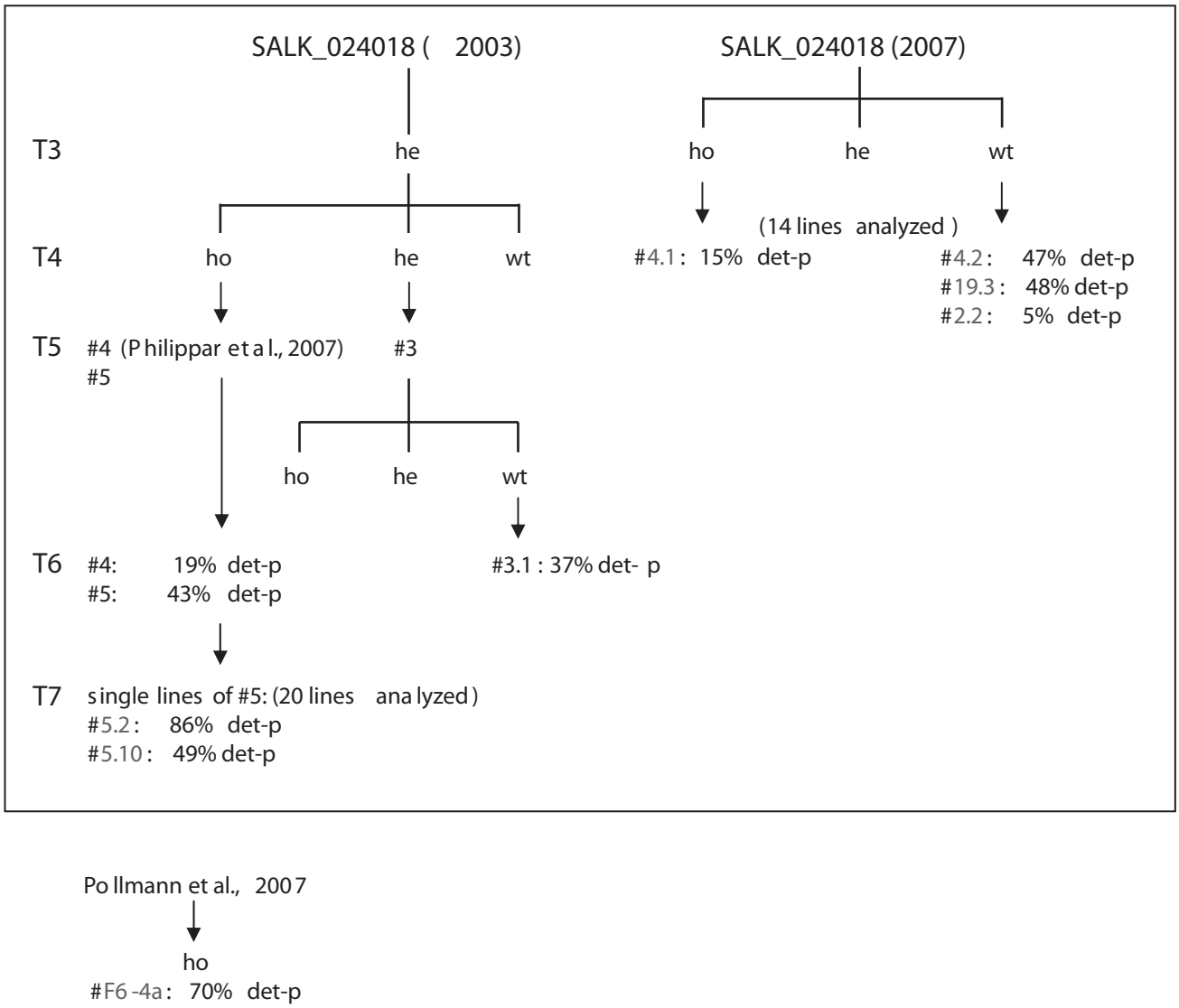

Fig. S2. Segregation and origin of SALK_024018 lines used in this study. Seeds of the T-DNA insertion line SALK_024018 were ordered in 2003 and 2007 and segregated into progeny that was homozygous (ho), heterozygous (he), and wild type (wt) for the *oep16.1–1* allele. To simplify tracking of generations we consider all seeds directly received from SALK to be the T3 generation. Line 4 of SALK_024018 (T5 generation, ordered in 2003), which is homozygous for *oep16.1–1*, was used for the previous mutant analysis in Philippar et al. (1). In the current study this line displayed no de-etiolation phenotype (det-p), while its sister line 5 and a corresponding wild-type allele (line 3.1) showed defects in etioplast to chloroplast transition. In the direct progeny (T4 generation) of SALK_024018 (ordered in 2007) the de-etiolation phenotype was detectable in variable quantities. Lines described in Table 2 of the current analysis (lines 5.2 and 5.10, T7 generation, originally received in 2003; lines 4.1, 4.2, 19.3, and 2.2, T4 generation ordered in 2007) are depicted in blue. Line F6–4a, which represents progeny of SALK_024018 homozygous for the *oep16.1–1* allele and was published as *Atoep16–1* in Pollmann et al. (2), was donated by Diter von Wettstein (Washington State University, Pullman, WA).

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Table S1. Oligonucleotide primers used in this study

Primer name	Primer sequence
Actfw	5′-GGTGATGGTGTGTCT-3′
Actrev	5′-ACTGAGCACAATGTTAC-3′
OEP16.1LCfw	5′-TGTTAGCACGCCGAAG-3
OEP16.1LCrev	5′-CTTACCAACCGCTGAG-3′
PORALCfw	5′-GTTACGTCTCCGAGTCAG-3′
PORALCrev	5′-GCCAAAACACAACTACTAAATC-3′
PORBLCfw	5′-TTCACAGGCGTTTCCA-3′
PORBLCrev	5′-GTACCGAGAGGTGTCAT-3′
RBkp1fw	5′-AGCTCATTAAACTCCAGAAACCCGCG-3′
RBkp1rev	5′-GCTCCACTGACGTTCCATAAATTCCC-3′
RBkp0fw	5′-ACGTTGCGGTTCTGTCAGTTCCAAAC-3′
RBkp0rev	5′-ATCTGCACCGGATCTGGATCGTTTC-3′
LBkp0.5fw	5′-ACTGGAACAACACTCAACCCTATCTCGG-3′
LBkp0.5rev	5′-TAAATCGGAACCCTAAAGGGAGCCC-3′
LBkp0fw	5′-CGATTTCGGAACCACCATCAAACAGG-3′
LBkp0rev	5′-TGGCGAGAAAGGAAGGGAAGAAAGC-3′
AD1	5′-NTCGASTWTSGWGTT-3′
AD2	5′-NGTCGASWGANAWGAA-3′
AD3	5′-WGTGNAGWANCANAGA-3′
AD4	5′-WGGWANCWGAWANGCA-3′
AD5	5′-WCGWWGAWCANGNCGA-3′
AD6	5′-WGCNAGTNAGWANAAG-3′
AD7	5′-AWGCANGNCWGANATA-3′
AD8	5′-SSTGGSTANATWATWCT-3′
AD9	5′-CGSATSTCSAANAAWAT-3′
LBT0a	5′-GGTTCCGATTTAGTGCTTTACG-3′
LBT0b	5′-AACTTGATTTGGGTGATGGTTC-3′
LBT0c	5′-CCCTATCTCGGGCTATTCTTTT-3′
16.1_T-DNAfw	5′-GCCTTCAAGCACATTCTCCGGGACTGTTA-3′
16.1_T-DNArev	5′-CCAATCTCTGCTGCCACGGATAC-3′
16.1–4018fw	5′-GACTGTTAGCACGCCGAAGCTG-3′
16.1–4018rev	5′-CCGCATGCTCCAAAGTGCTC-3′
GP1_T-DNAfw	5′-ACACAATCCGCTCTCAAACCCAAAC-3′
GP1_T-DNArev	5′-GCTGAGTCTACGGCGTTGAGTGGTG-3′
CM1_T-DNAfw	5′-GTTTGATTGTTTCTATGTATCGACC-3′
CM1_T-DNArev	5′-GAAGAGAACAACGAACAGAGAAAG-3′
LBa1	5′-TGGTTCACGTAGTGGGCCATCG-3′
M13fw	5′-GTAAAACGACGGCCAG-3′
M13rev	5′-CAGGAAACAGCTATGAC-3′

Primers were used for quantitative RT-PCR, iPCR, TAIL-PCR and for PCR genotyping and verification of *oep16.1–1*, *arogp1–1*, and *cm1–1* mutants. W, A/T-; S, C/G-; N, A/C/G/T-wobbles.