Plant Cell Physiol. 44(10): 963–974 (2003)

Rapid Paper

Functional Analysis of Isoforms of NADPH:Protochlorophyllide Oxidoreductase (POR), PORB and PORC, in *Arabidopsis thaliana*

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NADPH:protochlorophyllide oxidoreductase (POR) catalyzes the light-dependent reduction of protochlorophyllide. To elucidate the physiological function of three differentially regulated POR isoforms (PORA, PORB and PORC) in *Arabidopsis thaliana*, we isolated T-DNA tagged null mutants of *porB* and *porC*. The mature seedlings of the mutants had normal photosynthetic competencies, showing that PORB and PORC are interchangeable and functionally redundant in developed plants. In etiolated seedlings, only *porB* showed a reduction in the photoactive protochlorophyllide and the size of prolamellar bodies (PLBs), indicating that PORB, as well as PORA, functioned in PLB assembly and photoactive protochlorophyllide formation in etiolated seedlings. When illuminated, the etiolated *porB* seedling was able to green to a similar extent as the wild type, whereas the greening was significantly reduced under low light conditions. During greening, high light irradiation increased the level of PORC protein, and the greening of *porC* was repressed under high light conditions. The *porB*, but not *porC*, etiolated seedling was more sensitive to the far-red block of greening than the wild type, which is caused by depletion of endogenous POR proteins resulting in photo-oxidative damage. These results suggest that, at the onset of greening, PLBs are important for efficient capture of light energy for photoconversion under various light conditions, and PORC, which is induced by high light irradiation, contributes to photoprotection during greening of the etiolated seedlings.

Keywords: *Arabidopsis thaliana* — NADPH:protochlorophyllide oxidoreductase — Prolamellar bodies — Protochlorophyllide — Plastid differentiation.

Abbreviations: Chl, chlorophyll; Col, Columbia; FR, far-red; HL, high light; LHPP, light-harvesting POR Pchlide complex; LL, low light; ML, medium light; PAM, pulse-amplitude-modulation; Pchlide, protochlorophyllide; PLBs, prolamellar bodies; POR, NADPH:protochlorophyllide oxidoreductase; *UBQ* gene, ubiquitin gene; Ws, Wassilewskija.

Introduction

Most angiosperms strictly couple chlorophyll (Chl) biosynthesis to the light environment by relying on the nuclear-encoded, plastid-localized, light-dependent NADPH:protochlorophyllide oxidoreductase (POR, EC 1.3.1.33). POR catalyzes the photoreduction of protochlorophyllide (Pchlide) *a* to chlorophyllide *a*, and is present at high levels as a ternary complex with Pchlide and NADPH forming prolamellar bodies (PLBs) in etioplasts of dark-grown seedlings (von Wettstein et al. 1995). Two structurally related but differentially regulated POR isozymes, PORA and PORB, were first identified in barley (Holtorf et al. 1995) and *Arabidopsis thaliana* (Armstrong et al. 1995). In both plants, *PORA* and *PORB* transcripts accumulate in young seedlings, but only the *PORB* transcript is detectable at later stages of development. In addition, whereas PORA expression is strongly down-regulated by light, PORB expression is less light sensitive in *Arabidopsis* and barley. The data collected from the *Arabidopsis* and barley systems have motivated recent hypotheses that PORA and PORB might have unique functions in etiolated seedlings and at the onset of greening. There have been reports of the abnormal greening of seedlings that do not detectably express PORA (Runge et al.

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1996), selective degradation of PORA upon illumination by putative light-induced protease (Reinbothe et al. 1995a), and unusual Pchlide-dependent transport of PORA into plastids (Reinbothe et al. 1995b, Reinbothe et al. 2000), although such a selective proteolysis and unusual substrate-dependent transport of PORA are strongly disputed by recent studies (Dahlin et al. 2000, Aronsson et al. 2001, Aronsson et al. 2003). Based primarily on the abundance of PORA in barley etioplasts and the Pchlide-dependent nature of its plastid import, specific functions for PORA have been hypothesized, including protection against photo-oxidative damage by the binding of free Pchlide, the structural organization of PLBs, and the assembly of photoactive Pchlide (Reinbothe et al. 1996, von Wettstein et al. 1995). Moreover, it was recently proposed that PORA and PORB might have specific functions at the onset of greening. Based on in vitro reconstitution experiments, it was suggested that in barley, the PORA–Pchlide *b* complex functions in light harvesting to transfer light energy to Pchlide *a* for photoreduction by PORB with a 5 PORA to 1 PORB stoichiometry (Reinbothe et al. 1999). This light-harvesting POR Pchlide (LHPP) complex is speculated to serve as the central structural determinant of the PLBs, and function in photoprotection by dissipating the light energy of Pchlide-induced photo-oxidative damage during greening. However, the LHPP model is now subject to discussion (Armstrong et al. 2000, Reinbothe et al. 2003a), since conflicting results about the accumulation of Pchlide *b* in barley etioplasts in vivo have been reported (Scheumann et al. 1999, Reinbothe et al. 2003b). Furthermore, recent work in our laboratory identified a novel isoform of POR, PORC, from *A. thaliana* (Oosawa et al. 2000). *PORC* mRNA accumulates only after illumination in etiolated seedlings and is predominantly detected in fully matured green tissues (Oosawa et al. 2000, Su et al. 2001). Thus, it has been clarified that the biosynthesis of Chl in *Arabidopsis* is controlled by three distinctly regulated POR isoforms.

A differential gene organization and expression of *POR* have also been reported in other plant species. Our previous studies identified two *POR* genes from tobacco, but the expression of both isoforms was not negatively regulated by light and persisted in matured green tissues (Masuda et al. 2002). In cucumber, only a single *POR* gene has been identified (Fusada et al. 2000), the expression of which was positively light regulated (Kuroda et al. 1995). Pea also contains only a single POR gene, and the level of its transcript was almost unchanged during greening (Sundqvist and Dahlin 1997). Thus, it is likely that there is a wide variety of gene family organizations and light- and development-dependent regulatory mechanisms for the *POR* gene among angiosperms, although in angiosperms, plastids generally differentiate into etioplasts, which accumulate PLBs, in dark grown seedlings (Masuda et al. 2002).

From these studies, we question whether a particular POR isoform in other plant species has distinct physiological functions, as suggested in the case of barley POR isoforms. Moreover, we address the questions of why angiosperms form massive PLBs which may be composed of differentially regulated POR proteins in etioplasts of dark grown seedlings, and whether PLBs are really responsible for only photoprotection in greening seedlings. To answer these questions, in this study, we have analyzed the physiological function of each POR isoform in *A. thaliana*, by isolating knockout mutants depleted of specific POR isoforms. The data presented here suggest that in *Arabidopsis*, three POR isoforms are functionally and enzymatically equivalent in mature leaves, but their differential gene regulations define the role of each POR protein during greening of etiolated seedlings. Our results further suggest that PLBs are important for efficiently capturing light energy for photoconversion of Pchlide under various light conditions, rather than protection against photo-oxidative damage.

Results

Isolation of T-DNA insertion mutants

A reverse genetic approach was used to isolate T-DNA mutants affected in the *POR* gene of *A. thaliana*. Over 20,000 independently T-DNA-mutagenized *Arabidopsis* lines of the Kazusa DNA Research Institute (Kisarazu, Japan) were screened by PCR with primers based on the sequences of *POR* genes and common primers based on the T-DNA border sequence. Since open reading frames of *PORA* and *PORB* possess identical sequences for the 20–40 bp length at both 5′- and 3′-ends, we designed PCR primers from these sequences for screening T-DNA mutants inserted in either the *PORA* or *PORB* gene. In the case of the *PORC* gene, primers specific to the 5′- and 3′-ends of the *PORC* gene sequence were designed for screening. Consequently, among T-DNA tagged lines, a single positive line was obtained after *PORA/PORB* screening. PCR screening with primers specific to *PORA* and *PORB* revealed that the T-DNA was inserted in the *PORB* gene in this line. Meanwhile, two lines were obtained after the screening of *porC* mutants. As predicted from the size of the obtained PCR fragments, sites of T-DNA insertion in these two lines were apparently different. We designated these lines *porC-1* and *porC-2*. The *porC-1* line had the Wassilewskija (Ws) background and hygromycin resistance, while *porC-2* was of the Columbia (Col) background and resistant to kanamycin. The *porB* line had the Col background and kanamycin resistance.

All of the mutants obtained were fertile, and self-pollinated seeds were selected for antibiotic resistance. We then selected homozygous mutants by PCR with primers specific to each *POR* gene and common primers based on the T-DNA border sequence. By Southern blot analysis, a homozygous line was revealed to possess a single T-DNA insertion in *porB* (data not shown). Then, we sequenced the PCR fragments by screening to identify the site of insertion of T-DNA into *PORB*. As shown in Fig. 1A, the T-DNA was inserted in the second exon of the gene. Likewise, we analyzed the two *porC* lines, and revealed a single and multiple insertion of T-DNA in *porC-1* and *porC-2*, respectively. In *porC-1*, the T-DNA was inserted

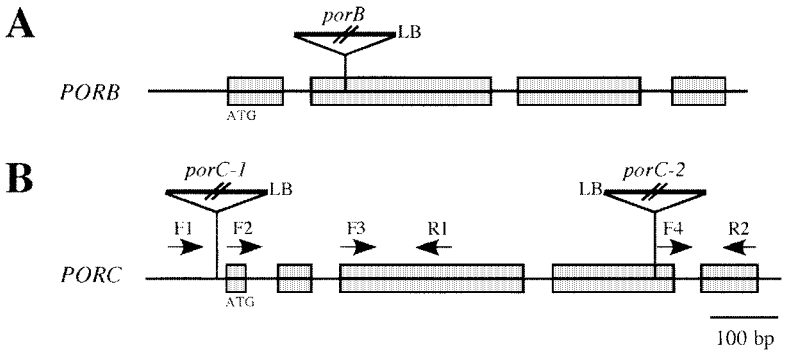

Fig. 1 Schematic representation of isolated T-DNA-tagged mutants of *POR* genes in *Arabidopsis*. (A) Schematic representation of the T-DNA insertion in the *PORB* gene. A single T-DNA was inserted in the 2nd exon of *PORB* (nucleotide position 333 from the initiation codon). (B) Schematic representation of the T-DNA insertion in the *PORC* gene. Single and multiple insertions of T-DNA were detected in *porC-1* and *porC-2*, respectively. In *porC-1*, the T-DNA was inserted 26 bp upstream of the initiation codon of *PORC*, while in *porC-2*, one of the T-DNA inserts existed in the fourth exon of the *PORC* gene (nucleotide position 1262 from the initiation codon). Arrows indicate relative positions of primers used for screening and RT-PCR.

26 bp upstream of the initiation codon of *PORC*, while in *porC-2*, one of the T-DNA inserts existed in the fourth exon of *PORC* (Fig. 1B).

Impact of T-DNA insertion on the expression of POR genes

To evaluate the impact of the T-DNA insertion on the expression of *POR* genes, total RNA was isolated from mature leaves of wild types (Col and Ws), and *porB*, *porC-1* and *porC-2* mutants. Fig. 2A shows the results of Northern blot analysis, using the *PORA*, *PORB* and *PORC* gene-specific probes (Armstrong et al. 1995, Oosawa et al. 2000). In Col, transcripts of *PORB* and *PORC* were readily detected, and only a trace level of *PORA* mRNA was detected, since *PORA* mRNA massively accumulates in etiolated seedlings and levels dramatically declined upon illumination (Armstrong et al. 1995). The same result was also obtained in Ws (data not shown). In *porB* mutants, the *PORB* mRNA was not detectable, indicating the disruption of *PORB* gene expression in *porB*. In *porC-1* and *porC-2*, however, very faint levels of *PORC* transcripts were detectable in both lines (Fig. 2A). In *porC-2*, two faint bands with different size were observed. We then examined whether the *PORC* gene is normally transcribed even in the presence of the T-DNA insertion in these two mutants by RT-PCR (Fig. 2B). Several primers specific to the *PORC* gene were designed to amplify regions containing T-DNA inserts or introns and exons of *PORC* (Fig. 1B). When RT-PCR was performed with primers F3 and R1 to amplify the third exon of the *PORC* transcript, the PCR product was readily detected in both *porC-1* and *porC-2* mutants as well as in wild types, indicating that the *PORC* gene is transcribed in these mutants (Fig. 2B). In *porC-1*, however, primers F1 and R1, which flanked both ends of the T-DNA of *porC-1*, failed to amplify, and primers F2 and R1, which were designed to include the first and second exons of *PORC*, produced a higher molecular weight band than that of the wild type. These results suggest that in *porC-1*, the *PORC* gene is transcribed by a probable read through from the T-DNA insert, but aberrant splicing of the *PORC* transcript may occur. Similarly, in *porC-2*, aberrant splicing of the *PORC* transcript is likely to have occurred as products with different sizes were produced by RT-PCR with primers F3 and R1. Furthermore, no band was detected with primers F4 and R2 (Fig. 1B). These results suggest that in *porC-1* and *porC-2* mutants, the *PORC* gene is transcribed by a probable read through from the T-DNA and endogenous promoter, respectively, but splicing of the transcript to produce vital *PORC* mRNA may be affected in both lines.

To evaluate the effects of such gene disruption or aberrant splicing of mRNA, we then analyzed the level of POR proteins in total proteins extracted from mature leaves by Western blotting. When the blot was probed with polyclonal anti-wheat POR antibody (provided by H. Aronsson), two bands were detected in wild types, Col (Fig. 2C) and Ws (data not shown). In *porB*, only the upper band with an apparent molecular mass of ~37 kDa was detectable, while in *porC-1* and *porC-2*, only the lower band with an apparent molecular mass of ~35 kDa was detectable (Fig. 2C). Since *PORA* expression is almost negligible at this stage (Fig. 2A), it is likely that the upper and lower bands represent PORC and PORB proteins, respectively. To further confirm the specific level of PORC protein, we then produced anti-PORC specific antibody by the immunization of oligo-peptides specific to the N-terminal PORC amino acid sequence. In wild type and *porB*, this antibody clearly cross-reacted with a single band, but this band was absent in *porC-1* and *porC-2*. These results clearly demonstrated that in *porB*, null expression of the *PORB* gene caused a complete absence of PORB protein, and in *porC-1* and *porC-2*, reduced expression and aberrant splicing of the *PORC* transcript caused the null translation of PORC protein. Furthermore, our data obtained using knockout mutants of *porB* and *porC* revealed that the PORB and PORC proteins are distinguishable by Western blot analysis with anti-wheat POR antibody in mature leaves. The upper band corresponds to PORC and the lower band to PORB.

por mutants are photosynthetically competent under continuous white light

Phenotypes of homozygous mutants of *porB*, *porC-1* and *porC-2* grown under continuous white light were visibly normal (data not shown). As there was no visible difference between *porC-1* and *porC-2*, we chose *porC-1*, which has a single insertion of the T-DNA tag in the *PORC* gene, for further analysis.

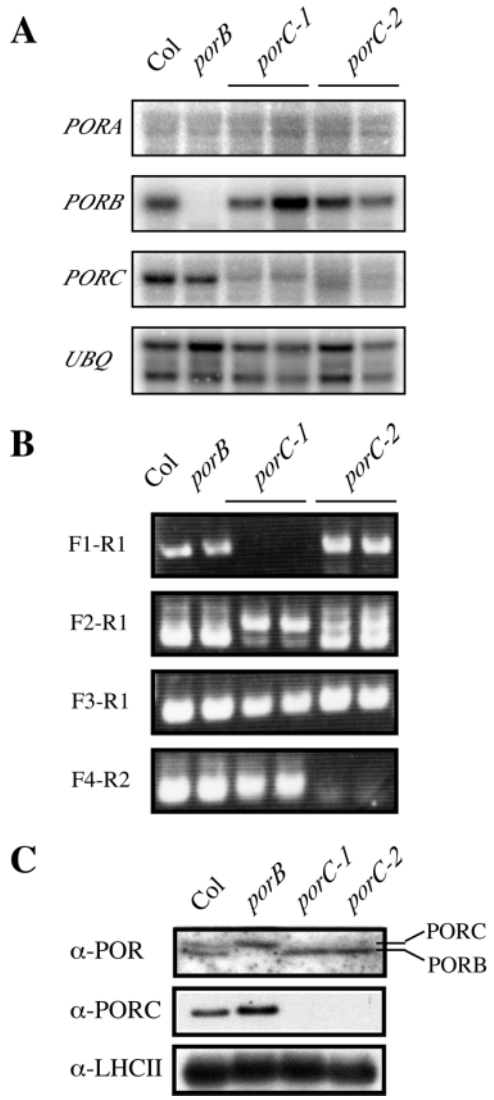

Fig. 2 Depletion of PORB or PORC isoform in respective *por* mutants. (A) The levels of *POR* transcripts in mature rosette leaves of wild types and homozygous *porB*, *porC-1* and *porC-2* mutants. Total RNA was isolated from mature leaves of 4-week-old seedlings. For analysis of *porC-1* and *porC-2*, total RNA was isolated from two independent lines of each mutant. RNA gel blots were prepared using 4 μg of total RNA extracted from mature leaves of (1) Col, (2) *porB*, (3, 4) *porC-1* and (5,6) *porC-2*. Obtained blots were probed with [α-^{32}P]dCTP-labeled *PORA*, *PORB*, *PORC* and *UBQ* gene-specific probes. (B) RT-PCR analysis of *porC* mutants. RT-PCR was carried out with 1 μg of total RNA extracted from etiolated seedlings of (1) Col, (2) *porB*, (3, 4) *porC-1* and (5, 6) *porC-2*. Combinations of primers used for RT-PCR (Fig. 1B) are indicated on the left. (C) Western blot analysis of POR proteins. Western blots were prepared using 10 μg of total protein extracted from mature leaves of (1) Col, (2) *porB*, (3) *porC-1* and (4) *porC-2*. Obtained blots were probed with polyclonal anti-wheat POR antibody (upper), anti-PORC antibody (middle) or anti-light-harvesting complex (LHC-II). POR isoforms corresponding to the upper and lower bands in the Western blot are indicated on the right.

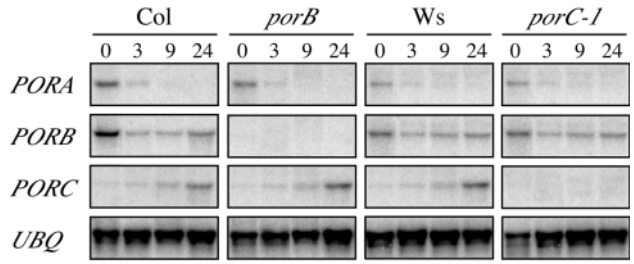

Fig. 3 Effect of *PORB* or *PORC* knockout on the expression of other *POR* isoforms. The 3-day-old dark-grown etiolated seedlings were sampled after 0, 3, 9 and 24 h of illumination, and total RNA was isolated. RNA gel blots were prepared using 4 μg of total RNA extracted from mature leaves of Col, *porB*, Ws and *porC-1*. Obtained blots were probed with [α-^{32}P]dCTP-labeled *PORA*, *PORB*, *PORC* and *UBQ* gene-specific probes.

We first characterized the photosynthetic parameters of these mutants (Table 1). No obvious difference in total Chl content, Chl *a*/*b* ratio and total carotenoid content was observed between Col and *porB*, and Ws and *porC-1*. We also measured the efficiency of two photosystems of these mutants using a pulse-amplitude-modulation (PAM) apparatus, but they exhibited comparable PSI and PSII activities to those of wild types. These results suggest that PORB and PORC proteins are redundant, functioning to maintain Chl biosynthesis and chloroplast development in developed plants under normal growth conditions.

Etioplast differentiation in por mutants

It is well characterized that PORA and PORB proteins accumulate and form PLBs as a ternary complex with Pchlide and NADPH in etioplasts of dark-grown seedlings in *Arabidopsis*. To determine the effect of PORB or PORC deficiency on etioplast differentiation, we then characterized etiolated seedlings of obtained *por* mutants. We first analyzed the effect of specific *POR* gene knockout on the expression of other *POR* genes. Non-illuminated and 3, 9 and 24 h illuminated 3-day-old etiolated seedlings were sampled, and total RNA was isolated and analyzed by Northern blotting with *POR* gene-specific probes. At these developmental stages, Ws cotyledons were apparently smaller than Col cotyledons. In Ws, the levels of *POR* gene expression and POR protein accumulation were lower than in Col, although such a difference became less pronounced during greening. Thus, we separately compared Col and *porB*, and Ws and *porC-1*. Although a specific depletion in *PORB* mRNA in *porB* and *PORC* mRNA in *porC-1* was detected, no obvious changes in the levels of *POR* mRNA were observed in these mutants when compared with those in the respective wild type (Fig. 3). Thus, it is confirmed that the specific knockout of *POR* genes did not affect the expression of *POR* genes in these mutants.

Then, we carried out Western blot analysis with total protein extracted from etiolated seedlings of *por* mutants. Western blot analysis of Col and *porB* seedlings with anti-wheat POR

Table 1 Photosynthetic parameters of wild types, and *porB* and *porC-1* mutants of *A. thaliana*

Parameters	Col	*porB*	Ws	*porC-1*
Total Chl (μg (mg FW)$^{-1}$)	2.44±0.30	2.77±0.25	2.40±0.20	2.35±0.16
Chl *a*/*b* (mol : mol)	2.54±0.19	2.98±0.09	2.74±0.11	2.88±0.02
Total carotenoid (μg (mg FW)$^{-1}$)	0.43±0.06	0.53±0.06	0.44±0.05	0.45±0.03
F_v/F_m	0.77±0.01	0.78±0.01	0.78±0.01	0.78±0.01
ΦPSII	0.67±0.00	0.67±0.00	0.65±0.02	0.64±0.02
ΦPSI	0.81±0.07	0.84±0.04	0.81±0.05	0.82±0.04

Data are means from five independent measurements. Values shown are means ± SD.

antibody detected a single band, although the level of this band was severely decreased in *porB* seedlings (Fig. 4A). This result confirms that the anti-wheat POR antibody, which recognizes PORA and PORB proteins in wild-type etioplasts, cross-reacted with only PORA protein accumulated in *porB* etioplasts. In Ws and *porC-1*, a similar level of POR protein was detected by Western blot analysis (Fig. 4A). Anti-PORC antibodies failed to detect PORC in all etiolated seedlings, indicating that PORC is not accumulated in etioplasts and not responsible for PLB formation.

We then quantified total and photoactive Pchlide amounts based on fluorescence measurements of pigment concentrations after acetone extraction of non-flashed and flashed seedlings. As shown in Fig. 4B, in the *porB* mutant, a slightly smaller amount of total Pchlide was accumulated. The level of photoactive Pchlide in *porB* seedlings was less but significant, and ~15% of that in Col (Fig. 4B). These results suggest that a PORB deficiency in *porB* severely affects the amount of total POR protein and photoactive Pchlide in etiolated seedlings, but less so total Pchlide content. Furthermore, since *porB* still possessed photoconversion activity for a certain level of Pchlide, it is demonstrated that in *Arabidopsis* PORA protein itself possesses activity for the photoconversion of Pchlide. On the contrary, the levels of total and photoactive Pchlide in *porC-1* were comparable to those in Ws (Fig. 4B). Thus, the knockout of the *PORC* gene did not affect the etioplast differentiation due to the absence of *PORC* gene expression in etiolated seedlings.

Since the level of PORA and PORB proteins in etioplasts may correlate with the assembly of PLBs, we then analyzed the etioplast morphology in *porB* seedlings using transmission electron microscopy. Observations of ultrathin sections of etiolated cotyledons showed that in the *porB* mutant the shape and size of etioplasts was similar, but the PLBs were much smaller than, in Col (Fig. 5A, B arrowheads). The lens-shaped plastids of *porB* contained unstacked prothylakoids. When seedlings were illuminated for 7 h under white light, the PLBs disappeared in Col and *porB*, and only (pro)thylakoids remained (Fig. 5C, D).

Light intensity-dependent greening of etiolated seedlings of porB mutant

It has been postulated that the accumulation of PLBs in etioplasts is important for photoprotection, via binding with

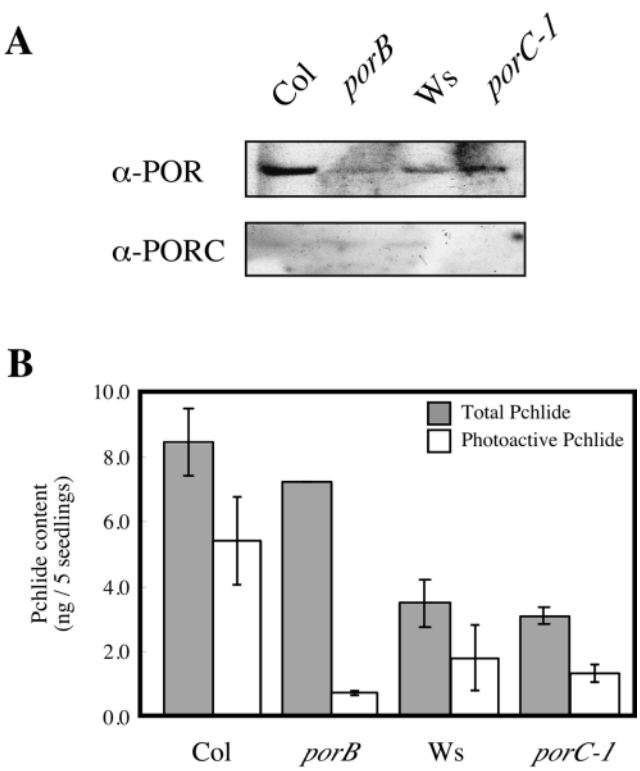

Fig. 4 Specific depletion of POR proteins and photoactive Pchlide population in etiolated wild-type, *porB* and *porC-1* seedlings. (A) Western blot analysis of POR proteins. Western blots were prepared using 10 μg of total protein extracted from etiolated seedlings of wild types (Col and Ws), *porB* and *porC-1*. Obtained blots were probed with polyclonal anti-wheat POR antibody (upper) or anti-PORC antibody (lower). (B) Quantitative determination of total Pchlide and photoactive-Pchlide in etiolated seedlings. Fluorescence intensities of total pigments extracted with 80% acetone from non-illuminated and flash-illuminated cotyledons of seedlings were determined with five replications. Total Pchlide was calculated from the fluorescence emission band at 634 nm of non-illuminated extract. Photoactive Pchlide was determined based on the emission at 672 nm of flash-illuminated extract that results from its quantitative photoenzymatic reaction to chlorophyllide. Pigment contents are given on a per-5-seedlings basis. Error bars represent SD.

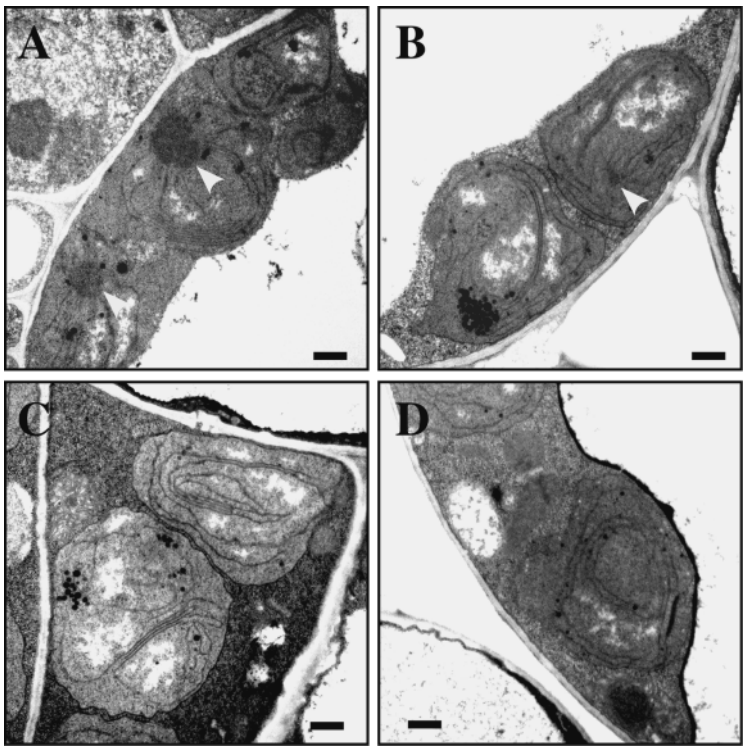

Fig. 5 Plastid ultrastructure in cotyledons of Col and *porB* seedlings. (A, B) Plastid ultrastructure of Col and *porB* seedlings, respectively, grown in the dark for 4 d. (C, D) Plastid ultrastructure of Col and porB seedlings, respectively, illuminated for 7 h. Bar = 0.5 μm.

Pchlide, the free form of which will cause photooxidative damage under illumination (Reinbothe et al. 1996). Considering the reduced levels of POR proteins (Fig. 4A), and the increased accumulation of non-photoactive Pchlide in *porB* seedlings (Fig. 4B), we expected *porB* etiolated seedlings to be sensitive to photooxidation. To test this possibility, etiolated seedlings of *porB* mutants were exposed to lights of different intensities and chloroplast development was evaluated by measuring Chl. Three different light conditions (high light (HL), 1,000 μmol m^{-2} s^{-1}; medium light (ML), 50 μmol m^{-2} s^{-1}; low light (LL), 0.1 μmol m^{-2} s^{-1}) were examined. Surprisingly, as shown in Fig. 6, under the HL condition, 3-day-old *porB* seedlings showed a comparable Chl accumulation to that of Col up to 72 h (Fig. 6A). Moreover, the Chl accumulation in *porB* was slightly less extensive under ML (Fig. 6B), and this difference was more prominent under the LL conditions (Fig. 6C). These results suggest that in *porB*, the remaining PORA protein in etioplasts can photoconvert Pchlide under conditions of excess light with the same efficiency as that of Col, but when the light intensity is not sufficient, the efficiency of photoconversion by PORA was less than that of Col, which contains both PORA and PORB proteins in etioplasts. Thus, it is suggested that the size of PLBs in etioplasts composed of PORA and PORB proteins is important for efficiently capturing light energy for photoconversion of Pchlide, rather than photoprotection against excess exposure to light.

Induction of PORC protein under HL during greening contributes to photoprotection

For photoprotection from the excess light, it is possible that a particular POR gene(s) is induced by HL, which makes it possible to synthesize Chl even under excess light. To test this possibility, we then determined the levels of POR proteins under different light intensities by Western blot analysis. Total protein was extracted from 72 h-illuminated seedlings under different light intensities. In Col, two bands were detected under the ML conditions when the Western blot was probed with anti-wheat antibody (Fig. 7A). In *porB*, only the upper band was detectable under the same conditions (Fig. 7A). Under LL conditions, the level of the lower band was higher than that for the ML and HL conditions. Given the findings that the level of the lower band in LL conditions was much higher than that in ML or HL conditions (Fig. 7A) and a reduced but significant level of the lower band was also detectable in *porB* seedlings (Fig. 7A), it is most likely that the high level of the lower band is mainly caused by PORA protein. The level of the upper band, PORC, was prominent under HL conditions and was faint under LL conditions. Such light-dependent changes in PORC protein were confirmed with anti-PORC antibody (Fig. 7A).

The opposite effects on the levels of POR transcripts under HL irradiation were confirmed with the Affimetryx microarray system. As shown in Table 2, when 10-day-old seedlings were exposed to HL irradiation for 3 h, the level of *PORC* transcripts increased 2-fold, while the levels of *PORA* and *PORB* transcripts decreased. Thus, it is demonstrated that

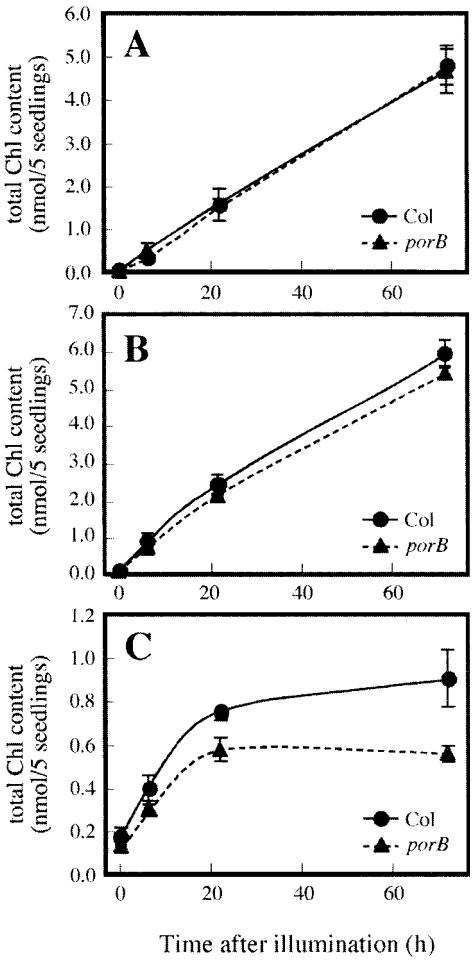

Fig. 6 Effects of light intensities on the accumulation of Chl in Col and *porB* mutant at the onset of greening. (A) High light (HL), 1,000 μmol m^{-2} s^{-1}, (B) medium light (ML), 50 μmol m^{-2} s^{-1} and (C) low light (LL), 0.1 μmol m^{-2} s^{-1} conditions. The 3-day-old dark-grown etiolated seedlings were illuminated under different light conditions. Five seedlings were sampled after 0, 9, 24 and 72 h of illumination, and extracted with 80% acetone. Chl concentration was determined by measuring fluorescence intensity as described by Armstrong et al. (1995) with five replications. Chl contents (nmol) are given on a per-5-seedlings basis. Error bars represent SD.

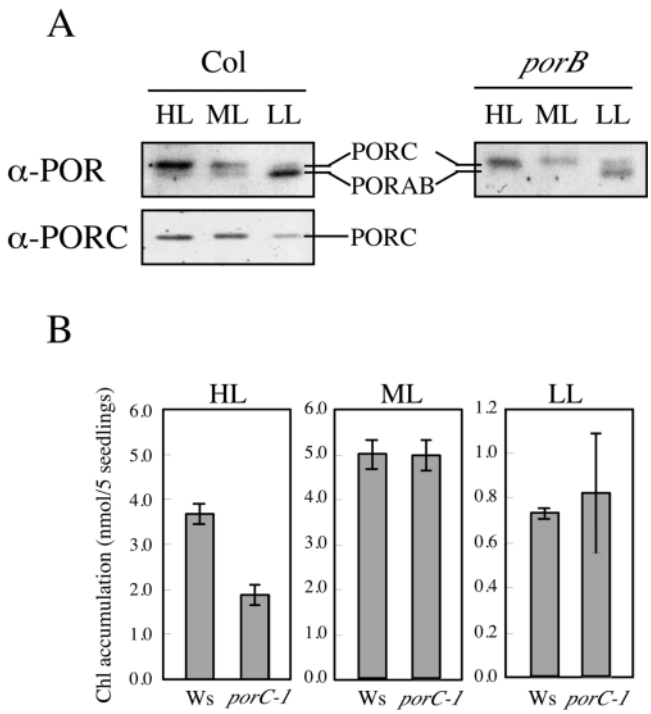

Fig. 7 Induction of PORC under HL conditions during greening contributes to photoprotection. (A) Western blot analysis of POR proteins in greening seedlings under different light intensities. Western blots were prepared using 10 μg of total protein extracted from 72 h greening seedlings of Col under different light intensities: (HL) 1,000 μmol m^{-2} s^{-1}, (ML) 50 μmol m^{-2} s^{-1} and (LL) 0.1 μmol m^{-2} s^{-1} conditions. Obtained blots were probed with polyclonal anti-wheat POR antibody (upper) or anti-PORC antibody (lower). POR isoforms corresponding to the upper and lower bands in the Western blot are indicated on the right. (B) Chl accumulation in Ws and *porC-1* mutant under different light intensities. The 3-day-old dark-grown etiolated seedlings of Ws and *porC-1* were illuminated under different light conditions: (HL) 1,000 μmol m^{-2} s^{-1}, (ML) 50 μmol m^{-2} s^{-1} and (LL) 0.1 μmol m^{-2} s^{-1}. Five seedlings were sampled after 72 h of illumination, and extracted with 80% acetone. The Chl concentration was determined by measuring fluorescence intensity as described by Armstrong et al. (1995) with five replications. Chl contents (nmol) are given on a per-5-seedlings basis. Error bars represent SD.

the expression of *PORC* is induced by HL, whereas the expression of *PORA* is maintained under dim light. These results are basically consistent with Su et al. (2001) in that the level of *PORC* transcript was increased by HL intensities during greening and that of *PORA* was maintained under LL, while it was undetectable under other lighting conditions.

Then, we examined whether PORC is really responsible for photoprotection under HL conditions. As described previously, Ws and *porC-1* etiolated seedlings were exposed to three different light intensities, and the level of Chl accumulation after 72 h was determined. As shown in Fig. 7B, the level of Chl in *porC-1* under HL conditions was about 50% of that in Ws, while the levels under other lighting conditions were comparable to those in Ws. Therefore, it is most likely that in *Arabidopsis*, the light-induced accumulation of PORC protein is light-intensity dependent, and such an induction of PORC protein contributes to photoprotection against excessive light.

porB but not porC is highly sensitive in the far-red block of the greening response

As described previously, it has been proposed that POR proteins accumulated in etioplasts have an important role in photoprotection against photo-oxidative damage. This hypothesis has been mainly based on studies using *Arabidopsis* seedlings, which were severely depleted of endogenous POR by the illumination of continuous far-red (FR) light (Barnes et al. 1996, Runge et al. 1996, Sperling et al. 1997) or by using *cop1*

Table 2 Microarray analysis of *POR* transcripts under high light irradiation

	500 μmol m^{-2} s^{-1} 1 h	500 μmol m^{-2} s^{-1} 3 h	1,200 mmol m^{-2} s^{-1} 3 h
PORA	0.59	0.32	0.21
PORB	0.57	0.2	0.23
PORC	1.36	1.29	2.11

Total RNA isolated from 10-day-old *Arabidopsis* seedlings exposed for 1–3 h at 500 or 1,200 μmol m^{-2} s^{-1} was analyzed with an Affymetrix *Arabidopsis* GeneChip covering 22,500 genes. The values are the ratio of hybridization signal of the HL-treated samples to the untreated light-irradiated samples (control).

constitutive photomorphogenic mutants (Sperling et al. 1998). It is well documented that etiolated seedlings grown under continuous FR are unable to green when subsequently transferred to white light. This process, known as the FR block of the greening response, involves depletion of PORA, partial depletion of PORB and the concomitant loss of PLBs and photoactive Pchlide, resulting in photo-oxidative damage caused by non-photoactive Pchlide acting as a photosensitizer (Barnes et al. 1996, Runge et al. 1996, Sperling et al. 1997). Here, higher levels of non-photoactive Pchlide and poor PLBs were also observed in etiolated *porB* seedlings, nevertheless, the seedlings were able to green even under the subsequent HL conditions. Thus, to compare the effect of continuous FR-dependent depletion of POR proteins and specific loss of PORB or PORC on photo-oxidative damage, etiolated seedlings of wild types and *por* mutants were illuminated on continuous FR, and the subsequent FR block of the greening response was evaluated.

Fig. 8A shows that 2 d of continuous FR irradiation (30 μmol m^{-2} s^{-1}) of etiolated wild-type seedlings fully inhibited their ability to green under subsequent illumination (ML conditions), whereas dark-grown seedlings at the same developmental stage could green normally (data not shown). The

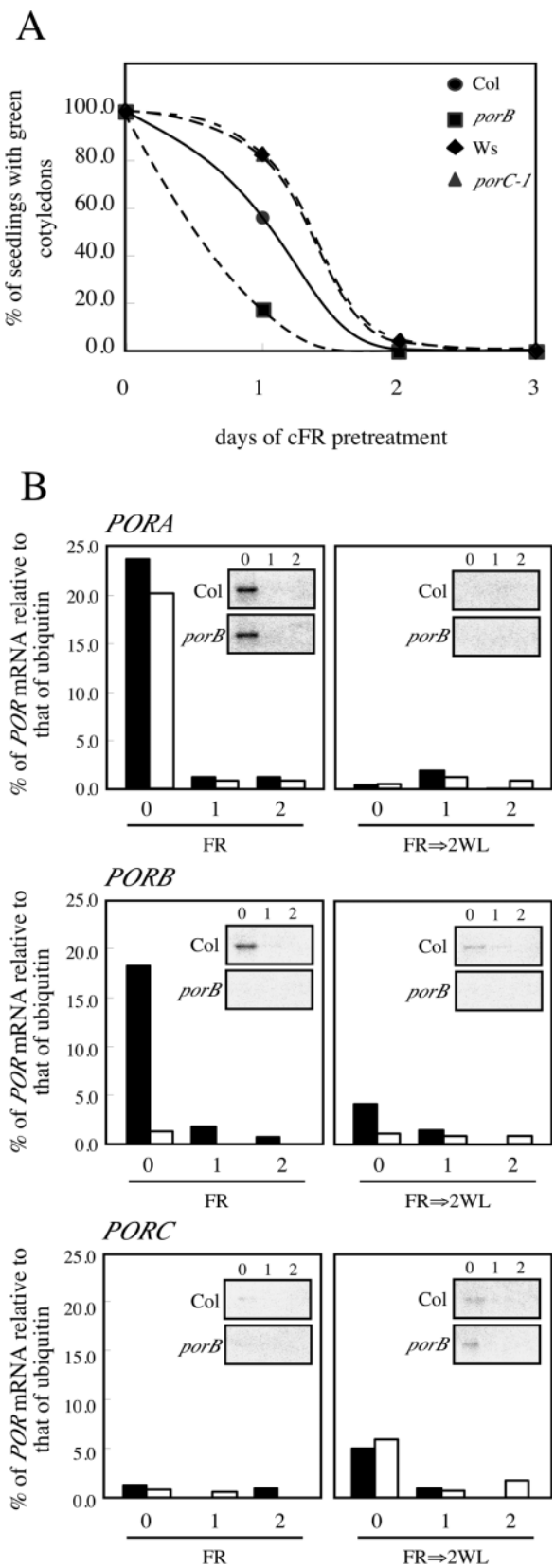

Fig. 8 *porB* is highly sensitive in the far-red block of the greening response. (A) Greening of cotyledons under white light (50 μmol m^{-2} s^{-1}) after growth in continuous FR. Seedlings of Col, *porB*, Ws and *porC-1* were germinated in the dark for 2 d and were subsequently grown for a further period (as indicated) under continuous FR. Seedlings were then transferred to a white light source for 2 d. Greening is shown as the percentage of seedlings with visibly green cotyledons. (B) Quantification of Northern blot analysis of *POR* isoforms in etiolated seedlings of Col (black box) and *porB* (white box) after pre-illumination of continuous FR (each left panel) and following white light illumination (each right panel). Seedlings of Col and *porB* were germinated in the dark for 2 d and then transferred under continuous FR. After 0, 1, and 2 d of continuous FR illumination seedlings were subsequently transferred to a white light source for 2 d and sampled (as indicated). Total RNA was isolated from these seedlings, and RNA gel blots using 4 μg of total RNA were probed with [α-^{32}P]dCTP-labeled *PORA*, *PORB*, *PORC* and *UBQ* gene-specific probes. Radioactivity of each band was quantified, and normalized to that of *UBQ*. Values are presented as percent of *POR* mRNA relative to that of *UBQ*. Northern blots are shown in inset of each panel.

sensitivity to continuous FR of Ws was slightly less than that of Col at this stage. When compared to Col, *porB* seedlings showed a higher sensitivity to continuous FR. After 1 d of FR treatment, less than 20% of etiolated *porB* seedlings were able to green, compared to 60% of Col seedlings, under subsequent illumination (Fig. 8A). No difference in sensitivity to continuous FR was observed between Ws and *porC-1*.

We then analyzed the effects of continuous FR illumination on the subsequent white-light-induced changes in the expression of each *POR* gene. First, 2-day-old etiolated seedlings of Col and *porB* were illuminated with continuous FR for 0–2 d and then with 2 d of white light. Under our experimental conditions in Col, 1 d of FR illumination severely repressed the levels of both *PORA* and *PORB* transcripts, and also repressed the subsequent light-induced expression of *PORC* (Fig. 8B). In *porB*, profiles of FR-dependent repression of *PORA* and *PORC* were similar to those of Col, indicating that the absence of PORB did not affect FR-dependent repression of these PORs. Thus, it is likely that the higher sensitivity to continuous FR in *porB* was simply caused by the decrease in total *POR* transcripts due to the specific loss of *PORB*.

Discussion

This paper describes the identification and characterization of *Arabidopsis* mutants with T-DNA insertions in the *PORB* and *PORC* genes. We have identified null mutations that resulted in the absence of PORB and PORC in the respective mutants and demonstrated that the knockout of *PORB* or *PORC* did not affect the remaining *POR* gene expression (Fig. 3). Thus, these mutants are ideal tools for investigations of the specific function of PORB and PORC proteins.

PORB and PORC are functionally equivalent and redundant in mature plants grown under normal growth conditions

As shown in Table 1, homozygous *porB*, *porC-1* and *porC-2* mutants were fertile and photosynthetically competent under continuous white light. Furthermore, we also examined the effect of the HL illumination on the levels of Chl and PSII activity in mature seedlings with a PAM apparatus, but no difference was observed between wild types, and *porB* and *porC-1* (data not shown). Therefore, it is likely that PORB and PORC are functionally equivalent and redundant, acting to maintain Chl turnover in mature leaves. However, there are a few examples showing PORB and PORC are differentially controlled in mature leaves, for instance, circadian control of the *PORB* but not *PORC* gene (Su et al. 2001). Another example is that on HL illumination the level of *PORC* mRNA is constant, whereas that of *PORB* mRNA decreased significantly (data not shown). In addition, we cannot exclude the possibility that the two POR enzymes are required to maintain Chl biosynthesis under particular conditions, such as in response to environmental stress. Actually, we have observed a reduction in both *PORB* and *PORC* transcripts following dehydration and 250 mM NaCl treatment in mature leaves (Masuda et al. unpublished result). The effects of these stresses and other untested conditions on *por* mutants will be further elucidated.

Roles of POR proteins in etioplast differentiation and photo-oxidative protection in Arabidopsis

Recent studies have hypothesized that PORA specifically functions to protect against photooxidative damage by the binding of free Pchlide, structural organization of PLB, and assembly of photoactive Pchlide (Reinbothe et al. 1996), while PORB persists with Chl biosynthesis in light-adapted plants. More recently, Reinbothe et al. (1999) proposed the LHPP model based on in vitro reconstitution experiments with barley PORA and PORB.

In this study, using *porB* mutant, we demonstrated a reduction in photoactive-Pchlide (Fig. 4B) and the amount of PLBs (Fig. 5) in etioplasts caused by the specific depletion of PORB. These results clearly demonstrated that PORB, as well as PORA, is also responsible for normal PLB assembly and the formation of a photoactive Pchlide in etioplasts. It seems that, at least in *Arabidopsis*, the absolute level of photoactive Pchlide in the etioplast does not necessarily parallel the greening activity and photosynthetic activities, because for example, the levels of photoactive Pchlide in *porB*, Ws and *porC-1* are similar to each other but quite low compared with Col (Fig. 4), nevertheless, these strains similarly green and show photosynthetic activity to Col. Furthermore, since *porB* seedlings still possessed low but significant photoconversion activity for Pchlide and were able to green under subsequent illumination, it is apparent that PORA protein itself has photoconversion activity in *Arabidopsis*, which apparently contradicts the LHPP model in barley (Reinbothe et al. 1999). Our results are basically consistent with recent observations that PORA and PORB are qualitatively interchangeable with respect to their functions in etioplast formation (Sperling et al. 1997, Sperling et al. 1998, Franck et al. 2000). Thus, PORA and PORB equally form a complex to stabilize PLBs and photoactive Pchlide in *Arabidopsis*, unlike LHPP. In this sense, the LHPP model is rather specific, and is not a general mechanism for POR-dependent chloroplast development in angiosperms, especially in dicot plants.

The low levels of photoactive Pchlide and poor PLBs in etiolated *porB* seedlings (Fig. 4, 5) were somewhat similar to those in continuous FR illuminated or *cop1* etiolated cotyledons, which were severely depleted of endogenous POR resulting in the accumulation of non-photoactive Pchlide (Barnes et al. 1996, Sperling et al. 1997, Sperling et al. 1998). Therefore, we firstly expected that *porB* seedlings would be sensitive to photo-oxidative damage under subsequent illumination. However, even under HL and ML conditions, *porB* seedlings showed a comparable greening ability to Col (Fig. 6). In addition, we discovered that PORC, which is induced by HL, contributed to adaptation to a HL environment. The light intensity-dependent increase in PORC protein (Fig. 7) is apparently caused by the rapid induction of *PORC* expression by HL

(Table 2). As the level of Chl accumulation in *porC-1* under HL conditions was about 50% of that of Ws, it is likely that PORC is not essential but is supplementary to PORA and PORB proteins for the turnover of Pchlide under excess light. Here, we demonstrated that PORC protein was negligible in etioplasts (Fig. 4A) and the expression of PORC is totally light dependent (Fig. 3, Oosawa et al. 2000). In this sense, the strategy for protection against photo-oxidative damage of *Arabidopsis* is not preparatory by forming PLBs in etioplasts, but is adaptive by induction of PORC under HL intensities.

To compare the effects of continuous FR-induced depletion of POR and specific loss of PORB in *porB*, we further examined the FR block of the greening response in etiolated seedlings. Continuous FR actually blocks greening by depleting endogenous POR (Fig. 8A). This FR block of the greening response is dependent on phytochrome A activity (Barnes et al. 1996). Northern blot analysis with *POR*-specific probes showed that continuous FR depleted *PORA* and *PORB* transcripts of dark germinated seedlings, and also depleted light-induced expression of *PORC* (Fig. 8B). Interestingly, *porB* seedlings showed higher sensitivity to FR than Col. It is likely that this difference of sensitivity is due to the absence of PORB in the mutant, and the full FR block of the greening response occurs when endogenous *POR* expression is completely depleted. Therefore, it is likely that the FR block of the greening response simply depends on the total amount of *POR* transcripts. It should be noted that there was no difference of sensitivity to the FR block of the greening response between Ws and *porC-1*, because there is no PORC protein in etiolated seedlings and at the early stage of greening, while photo-oxidative damage may occur immediately after white light illumination.

The occurrence of photo-oxidative damage is well known in several transgenic lines depleted of tetrapyrrole biosynthetic enzymes (Mock and Grimm 1997, Papenbrock et al. 2001). In this sense, the FR block of the greening response by the depletion of endogenous POR is a well-characterized photo-oxidative damage caused by tetrapyrrole intermediates for example, free Pchlide, and may not be directly related to the photoprotective function of POR. In addition, we would emphasize that continuous FR treatment or *COP1* mutation had pleiotropic effects on tetrapyrrole biosynthesis and chloroplast development, since they affect the central light-signaling pathway during photomorphogenesis. In fact, the continuous FR treatment also reduced the expression of the *Lhcb* gene and *HEMA1* gene encoding glutamyl-tRNA reductase, which catalyzes the earlier step of tetrapyrrole biosynthesis (McCormac and Terry 2002). Therefore, it is possible that such pleiotropic effects caused a more severe block of greening than specific depletion of PORB in *porB* mutants.

PLB functions to capture light energy for photoconversion of Pchlide

If the formation of PLBs in etioplasts is not for photoprotection, the question is why POR proteins accumulate in darkness, and what is the function of PLBs. An answer to this question may come from the reduced level of Chl accumulation in *porB* under LL conditions (Fig. 6). Even under dim light, etiolated seedlings develop chloroplasts to some extent to perform photosynthesis. It is interesting that under such conditions, the expression of *PORA* was maintained (Su et al. 2001) and a certain amount of PORA protein was still detectable in seedlings (Fig. 7A), while the expression of PORB remained constant under ML. In *porB* seedlings, PORA protein was still detectable under LL light conditions, but the level was lower than that in Col (Fig. 7A). Thus, it is reasonable to consider that PORA expression is maintained under dim light for capturing light energy for photoconversion rather than for photoprotection. It has been suggested that the function of PLBs as a pool of Pchlide that is immediately transformable to Chlide upon illumination (Ryberg and Sundqvist 1991). Therefore, we would conclude that in *Arabidopsis*, PLBs, which are mainly composed of PORA and PORB proteins, are important for the efficient capture of light energy for the photoconversion of Pchlide under various light conditions.

During chloroplast development, Chl begins to accumulate rapidly in etiolated seedlings after illumination with an initial lag phase, and soon the rate of Chl accumulation reaches a maximum. We are now speculating on the strategy for PLB-dependent chloroplast development in angiosperms as follows: plants prepare for chloroplast development during skotomorphogenesis by converting plastids into etioplasts, which massively accumulate POR proteins and Pchlide as PLBs, and when illuminated, PLBs efficiently capture light energy under various light conditions to photoconvert Pchlide for chloroplast development to perform photosynthesis. Such an efficient development of chloroplasts may give greater opportunity to survive and overcome other germinating plants. We have now identified putative mutants of *PORA* in *Arabidopsis*. A detailed analysis of each mutant of the *POR* isoforms and crossing of mutants to generate double mutants will give further insight into the function of POR isoforms in the future.

Materials and Methods

Plant materials and growth conditions

Seed stocks of *Arabidopsis thaliana* (L.) Heynh from Col and Ws ecotype were used for all experiments. Surface-sterilized seeds were plated on an 0.8% (w/v) agar medium containing Murashige and Skoog salts (Murashige and Skoog 1962) supplemented with 1% (w/v) sucrose. Plates were placed at 4°C in darkness for 2 d prior to receiving 6 h white light irradiation to synchronize germination. Then, plates were exposed to white fluorescent light (50 μmol m^{-2} s^{-1}) or darkness. For obtaining mature plants, they were grown at 22°C in an environmental growth chamber under continuous illumination. For the experiment of different light intensities, seeds were allowed to germinate in darkness at 22°C for 3 d before white light illumination. For HL irradiation, a projector light (Cabin, Tokyo, Japan) was passed through two layers of flat water bottles to avoid the effects of heat and UV. For LL conditions, seedlings were placed in a box shielded by a black net to control the light intensity. Light intensity was measured with an LI-

250 light meter (LI-COR, Lincoln, NE, U.S.A.) equipped with a quantum probe LI-190SA (LI-COR). For irradiation of continuous FR, the light-emitting diodes with a peak at 730 nm (Sanyo, Japan) were used for irradiation. The light was passed through a cut-off filter to remove <700 nm. The final fluence rate of FR was ~30 μmol m^{-2} s^{-1}. In this experiment, seeds were plated on plates in the absence of sucrose.

T-DNA insertion-line identification

A primary polymerase chain reaction (PCR) screen was performed on pooled chromosomal DNA from ~20,000 individual lines of a collection of *Arabidopsis* T-DNA insertion mutants (constructed by Kazusa DNA Research Institute, Kisarazu, Japan). Since open reading frames of *PORA* and *PORB* possess identical sequences at the 5′-end (44 bp) including the initiation codon and the 3′-end (24 bp) including termination codon, oligonucleotide primers were designed from these sequences for primary screening to broaden the possibility of obtaining either *porA* or *porB* mutants (5′-atggcccttcaagctgcttctttgg-3′ and 5′-ttaggccaagcccacgagcttc-3′ for 5′- and 3′-ends, respectively). PCR amplification was carried out as follows: 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min. After the primary screen, one positive line was detected and subjected to a secondary screen with gene-specific primers for *PORA* or *PORB*. Sequences of 5′- and 3′-ends of *PORA*-specific primers are 5′-ggagcaaagcaaagctgactttgtc-3′ and 5′-gacatcgctagcttcttgagacag-3′**,** respectively, and of 5′- and 3′-ends of *PORB*-specific primers are 5′-cgaccaaatcaaatccgaacatgga-3′ and 5′-cgtcacttgcttcttctgataactgg-3′, respectively. Likewise, to screen mutants of the *PORC* gene, gene-specific primers for *PORC* were designed (F2; 5′-CTCTCCAAGCTGCCTATTCTCTTC-3′ and R2; 5′-CCACAGTTTCTTTGCCTTCTCCG-3′), and screened by PCR. The resulting PCR products were separated on 1% agarose gels. After migration, gels were immersed in 0.4 M NaOH. DNA was transferred onto nylon membrane (Amersham Bioscience, Piscataway, NJ, U.S.A.). Blots were hybridized with radiolabelled *POR* probes and washed at high stringency.

Genomic Southern and analysis of POR transcripts

Genomic DNA was isolated from *Arabidopsis* seedlings according to Murray and Thompson (1980). Genomic DNA (2 μg) was digested with restriction enzymes, separated on agarose gels, transferred onto nylon membrane, and hybridized in 0.25 M sodium phosphate, 7% SDS, and 1 mM EDTA at 65°C. Total RNA was prepared with RNeasy Mini Kit (Qiagen, Chatsworth, CA, U.S.A.) according to the manufacturers. For Northern blot analysis, 4 μg of total RNA was electrophoresed on 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane. Hybridizations with [α-^{32}P]dCTP-labeled *PORA*, *PORB*, *PORC* and ubiquitin (*UBQ*) gene-specific probes were performed as described in Oosawa et al. (2000). Hybridization was analyzed with the Bio-image Analyzer BAS 2000. For reverse transcriptase (RT)-PCR analysis of *porC* mutants, in addition to the primers used for screening (F2 and R2), several primers specific to the *PORC* gene were designed to amplify particular regions of *PORC*. Sequences of primers are F1; 5′-AGCCTGTGTTTCCCATTGTTGTGAG-3′, F3; 5′-GAAGACAGAAACCGCGATTTTCCAC-3′, F4; 5′-GAGGAAGCTGGCAAAAGACTAGC-3′, and R1; 5′-ATCTTCCTTGGACATTCCAACAGATCTC-3′. RT-PCR was carried out with RNA PCR Kit (AMV) Ver. 2.1 (Takara, Otsu, Japan) with 1 μg of total RNA isolated from 4-week-old mature leaves according to the manufacturers. PCR amplification was carried out as follows: 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min. For microarray analysis, 10-day-old *Arabidopsis* seedlings were exposed for 1–3 h at 500 or 1,200 μmol m^{-2} s^{-1} and harvested (Kimura et al. 2001). RNA was prepared using the RNeasy Kit (QIAGEN). Double strand cDNA was synthesized with a SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, U.S.A.), and labeled with a BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY, U.S.A.). Probe hybridization to Affymetrix *Arabidopsis* oligoarrays covering 22,500 genes was carried out at the University of Pennsylvania School of Medicine according to the manufacturer's suggestions (Affymetrix, Santa Clara, CA, U.S.A.).

Determination of photosynthetic parameters

Seedlings were harvested and homogenized in 80% acetone, followed by centrifugation to remove debris. In mature leaves, Chl was determined by measuring spectrophotometrically the absorbance at 710 nm, 663 nm and 645 nm according to Arnon (1949) with five replications. To determine the Chl accumulation in greening seedlings, Chl fluorescence of 80% acetone extracts at 666 nm was measured with a fluorescence spectrophotometer (model 850, Hitachi Instruments, Tokyo Japan) using an excitation wavelength of 440 nm (Armstrong et al. 1995). To evaluate the FR block of greening, greening is shown as the percentage of seedlings with visibly green cotyledons (McCormac and Terry 2002), since the response of the block of greening varied among seedlings and this assessment was reported to be linearly correlated to total Chl content (Moran 1982). To determine photosynthetic activity, a Mini-PAM (Heinz Walz GmbH, Effeltrich, Germany) and PAM 101/103 fluorometer equipped with an ED 800 T (Heinz Walz) were used to determine the efficiency of PSII and PSI, respectively. The calculation of each activity was carried out according to Meurer et al. (1996).

Analysis of POR proteins

Total protein was extracted from frozen samples and 10 μg was subjected to SDS-PAGE on a gel containing 12.5% polyacrylamide for separation (Laemmli 1970), and then electro-transferred onto nitrocellulose membranes. Obtained blots were probed with polyclonal anti-wheat POR antibodies, which recognize all three POR isoforms of *Arabidopsis*. To detect PORC protein, we produced polyclonal anti-oligopeptide antibodies against the N-terminal PORC-specific amino acid sequence (QTVTATPPANFASPEQKKTERKG). The obtained antibody was cross-reacted with a single band at around 37 kDa (see Results). Both antibodies were used at a dilution of 1 : 1,000, and immunodetection was performed using the ECL Western blotting kit as described by the manufacturer (Amersham Bioscience). Determination of the protein concentration was carried out by the method of Bensadoun and Weinstein (1976) with BSA as a standard.

Measurement of total and photoactive Pchlide concentrations

From five etiolated seedlings, pigments were extracted with 80% (v/v) acetone with five replications. Calibration was performed by the method of Anderson and Boardman (1964). The total Pchlide content in dark-grown cotyledons was calculated from the fluorescence emission intensity at 634 nm. The concentration of photoactive Pchlide was obtained from the pigment extracts of flash-treated cotyledons by measuring the chlorophyllide fluorescence intensity at 672 nm, after subtraction of the Pchlide fluorescence intensity at that wavelength. Flashes were repeated for 100 ms at room temperature, until the kinetics of conversion of photoactive Pchlide reached a plateau.

Transmission electron microscopy

Cotyledons were excised and fixed in 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde. The samples were dehydrated serially in ethanol and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan) according to standard procedures. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then observed with a JEM 1200EX transmission electron microscope.

Note added in proof

After this manuscript was submitted, a related study appeared in *Plant J.* (2003) 35: 141–153.

Acknowledgments

We thank Henrik Aronsson (Leicester University, Leicester, U.K.) and Christer A. Sundqvist (Göteborg University, Sweden) for kindly providing polyclonal antibody against wheat POR. This research was financially supported by the Grant of the 21st Century COE Program, Ministry of Education, Culture, Sports, Science and Technology.

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(Received June 16, 2003; Accepted August 4, 2003)