Identification and light-induced expression of a novel gene of NADPH-protochlorophyllide oxidoreductase isoform in *Arabidopsis thaliana*[1]

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Abstract In *Arabidopsis thaliana*, we identified a novel gene of a NADPH-protochlorophyllide oxidoreductase (POR) isoform, which catalyzes the light-dependent protochlorophyllide *a* reduction in the chlorophyll (Chl) biosynthetic pathway. The deduced amino acid sequence of the novel POR isoform (PORC) showed significant identities (~75%) with the previously isolated two POR isoforms of *A. thaliana*. Contrasting with these POR isoforms, the *PORC* transcript increased in etiolated seedlings by illumination, and was dominantly expressed in immature and mature tissues. The present results demonstrated that Chl biosynthesis and chloroplast biogenesis in *A. thaliana* are controlled by three POR isoforms, which are differentially controlled by light and development.

Key words: Chlorophyll biosynthesis; Chloroplast biogenesis; Greening; NADPH-protochlorophyllide oxidoreductase; *Arabidopsis thaliana*

1. Introduction

The light-dependent NADPH-protochlorophyllide oxidoreductase (POR) catalyzes the photoreduction of protochlorophyllide (Pchlide) *a* to chlorophyllide *a* in plastids [1]. POR protein is present in high levels as ternary complex with Pchlide and NADPH forming prolamellar body in etioplasts of dark-grown seedlings of angiosperms [2]. Two distinct isoforms of POR, PORA and PORB, which were differentially regulated by light, were isolated from barley [3] and *Arabidopsis thaliana* [4]. In both plants, PORA is only transiently active in etiolated seedlings at the beginning of illumination, whereas PORB is active in green leaves, indicating that the synthesis of chlorophyll (Chl) might be controlled by the differential action of the two distinct isoforms of POR in angiosperms [3,4]. Reinbothe et al. [5] suggested that in addition to the Chl synthesis, PORA mainly functions for photoprotection during the transitory stage from dark to light growth, and housekeeping PORB may function for Chl biosynthesis. Furthermore, it is recently hypothesized that PORA and PORB might have unique functions at the onset of greening. By in vitro reconstitution experiment, it was suggested that in barley, PORA–Pchlide *b* complex functions for light-harvesting to transfer light energy to Pchlide *a* for photoreduction by PORB [6]. However, this model is being disputed [7] since Pchlide *b* was undetectable in barley etioplasts [8].

Previously, we cloned *POR* cDNA from cucumber, and showed that it was encoded by a single gene and its expression was light-inducible, the pattern of which was well consistent with Chl synthesis both in cotyledons [9] and fully green leaves [10]. Since the profile of expression of cucumber *POR* was strikingly different from those of barely and *A. thaliana*, the regulation of POR-dependent Chl biosynthesis is considered to be different among angiosperms. In this study, however, we isolated a novel *POR* isoform gene from *A. thaliana*, whose expression was induced on illumination. The data presented here imply the reconsideration of the concept that Chl biosynthesis and chloroplast biogenesis in angiosperms are generally controlled by two unique functions of POR isoforms.

2. Materials and methods

For the amplification of a *PORC* cDNA fragment, total RNA was extracted from 4 week old light-grown *A. thaliana* (ecotype Columbia) seedlings [11]. First strand cDNA was synthesized from 1 μg of total RNA using an oligo dT primer and RNA PCR kit (AMV) Ver. 2.1 (Takara). With the first strand cDNA as template, we amplified by PCR with a pair of primers, 5′-CTCTCCAAGCTGCCTATTC-TCTTC-3′ and 5′-ATCTTCCTTGGACATTCCAACAGATCTC-3′, designed based on the sequence on chromosome 1 deposited in the database. With LA *Taq* polymerase (Takara), PCR amplification was carried out as follows: 95°C for 1 min, followed by 30 cycles of 98°C for 0.5 min, 55°C for 1 min and 72°C for 1 min. The resulting PCR products were cloned and sequenced. Subsequently, with the obtained cDNA fragment as a probe, we screened the *A. thaliana* cDNA library constructed from whole rosette leaves grown in continuous light for 4–5 weeks. Hybridization was carried out at 65°C for 16 h, and the blot was washed twice with 0.2×SSC, 0.1% sodium dodecyl sulfate for 15 min at 65°C.

For genomic Southern blot analysis, genomic DNA was prepared from *A. thaliana* seedlings [11]. 10 μg of genomic DNA was digested with restriction enzymes, separated on agarose gel, and transferred onto a nylon membrane. We analyzed hybridization by using PCR-amplified and ^{32}P-labeled probes specific for *PORC* as described above. For RNA gel blot analysis, 5 μg of total RNA prepared from illuminated *A. thaliana* was electrophoresed on a 1.2% agar-

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number AB035746.

Abbreviations: Chl, chlorophyll; Pchlide, protochlorophyllide; LHC, light-harvesting chlorophyll protein; POR, NADPH-protochlorophyllide oxidoreductase

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ose/formaldehyde gel and blotted onto a nylon membrane. In addition to the *PORC* gene specific probe, *PORA* and *PORB* gene specific probes were prepared [4], and used for hybridization.

3. Results and discussion

Database search of the *A. thaliana* genome with the *A. thaliana PORB* nucleotide sequence showed existence of two *POR* homologs. One of them was identical to the genomic sequence of *PORB*, whereas the other, which was contained in BAC F21B7 clone of chromosome 1 (accession no. AC002560), was apparently different from that of *PORA*, but the predicted gene product showed high homology with POR protein sequences. To determine whether this *POR* homolog is actually transcribed in *A. thaliana*, we amplified the cDNA fragments originating from its transcripts by RT-PCR. The DNA fragment of 416 bp, which was identical to the predicted coding sequence of the *POR* homolog, was obtained. This fragment was used to screen a cDNA library of *A. thaliana*, and subsequently, one positive clone was isolated out of 30 000 plaques. The sequence of obtained cDNA clones containing a full length coding sequence was identical to the predicted one of the *POR* homolog.

This cDNA clone encoded a protein of 402 amino acids with a calculated molecular mass of 43.9 kDa and 74.4% and 75.8% amino acid identity to *A. thaliana* PORA and PORB, respectively (Fig. 1). This sequence identity was unexpectedly low, because POR proteins of higher plants are highly evolutionary conserved with sequence identities more than 74%. In fact, PORA and PORB share 87% amino acid identity [4]. Most of the sequence divergence between POR proteins occurs in the N-terminal putative chloroplast transit peptides. The sequence alignment indicated that the putative mature region of the POR isoform sequence was highly conserved with other POR proteins (82.8% and 83.2% amino acid identity to *A. thaliana* PORA and PORB, respectively), in-

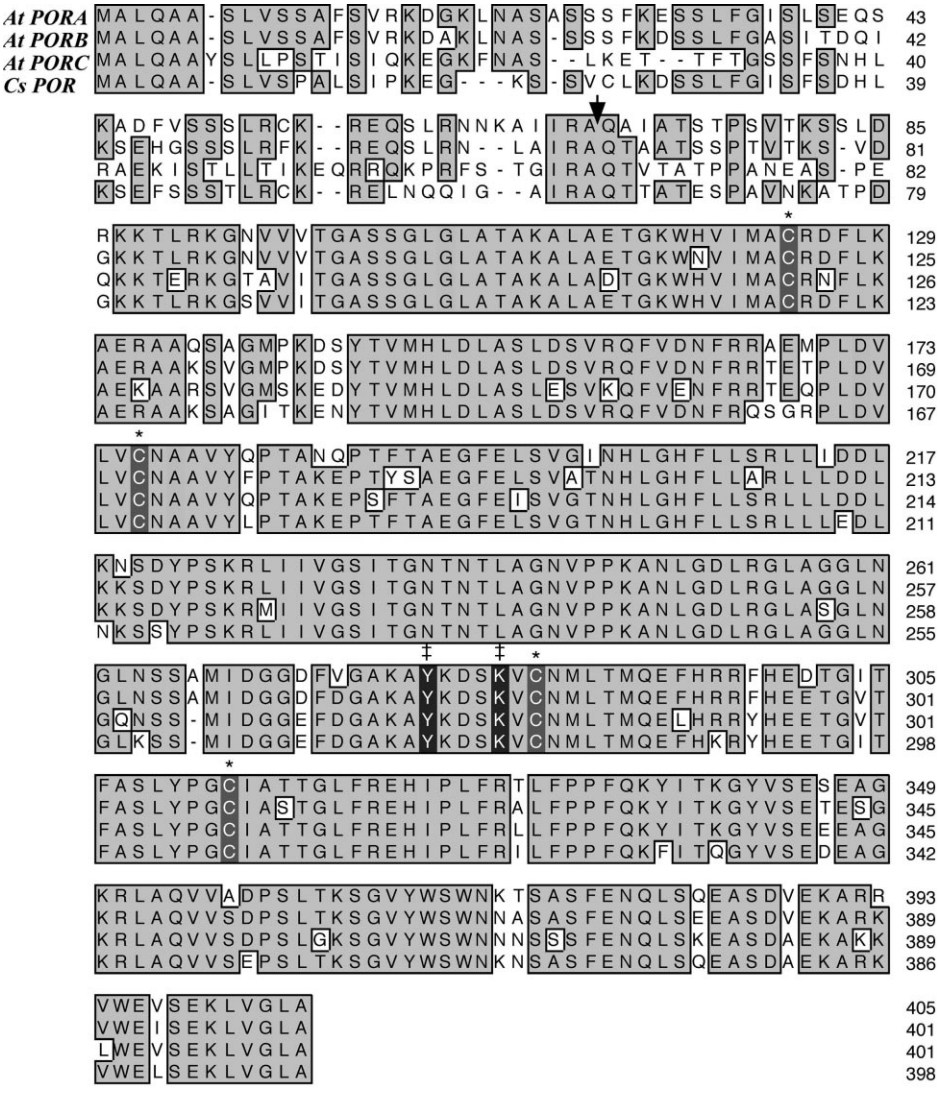

Fig. 1. Multiple alignment of the deduced amino acid sequence of *A. thaliana PORC* with those of *A. thaliana PORA* and *PORB* [4] and cucumber *POR* [9]. The amino acids identical among more than three PORs are shaded in gray. The putative N-terminal cleavage sites for the plastid transit peptides are indicated by the arrow. Cys residues located within the mature POR polypeptides are highlighted and indicated by (*). Essential amino acid residues for POR catalytic activity, such as Tyr-276 and Lys-280 of PORC [11], are highlighted and indicated by (‡).

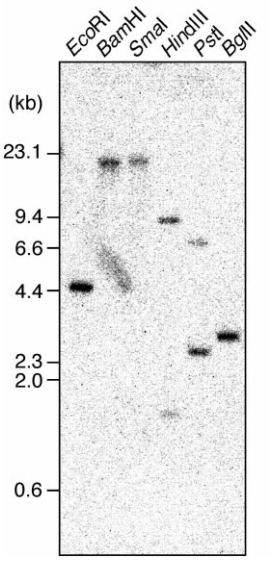

Fig. 2. Genomic Southern hybridization of the *A. thaliana PORC* gene. Genomic DNA from *A. thaliana* was cut with *Eco*RI, *Bam*HI, *Sma*I, *Hin*dIII, *Pst*I or *Bgl*II. The genomic Southern blot was hybridized with the *PORC* PCR product as a probe. DNA size markers are indicated in the left margin. Two bands in the lanes of *Hin*dIII and *Pst*I were caused by internal sites of the *PORC* probe. ←

cluding four Cys residues which may correlate with substrate or cofactor binding [4], and several amino acid residues which were essential for POR catalytic activity [12]. We therefore concluded that the sequence found on chromosome 1 is actually functioning as a gene for a novel POR isoform, and designated here the gene as *PORC*. Genomic Southern hybridizations with the radiolabeled *PORC* fragment showed a single band, indicating that this gene is encoded by a single gene in *A. thaliana* (Fig. 2). No cross hybridization with *PORA* or *PORB* was observed, probably because the *PORC* probe comprising the N-terminal region of the open reading frame (ORF) possessed low homology with *PORA* and *PORB* (less than 63% in nucleotide sequence), resulting in specific hybridization with the *PORC* gene.

We clarified the genomic organization of the gene for

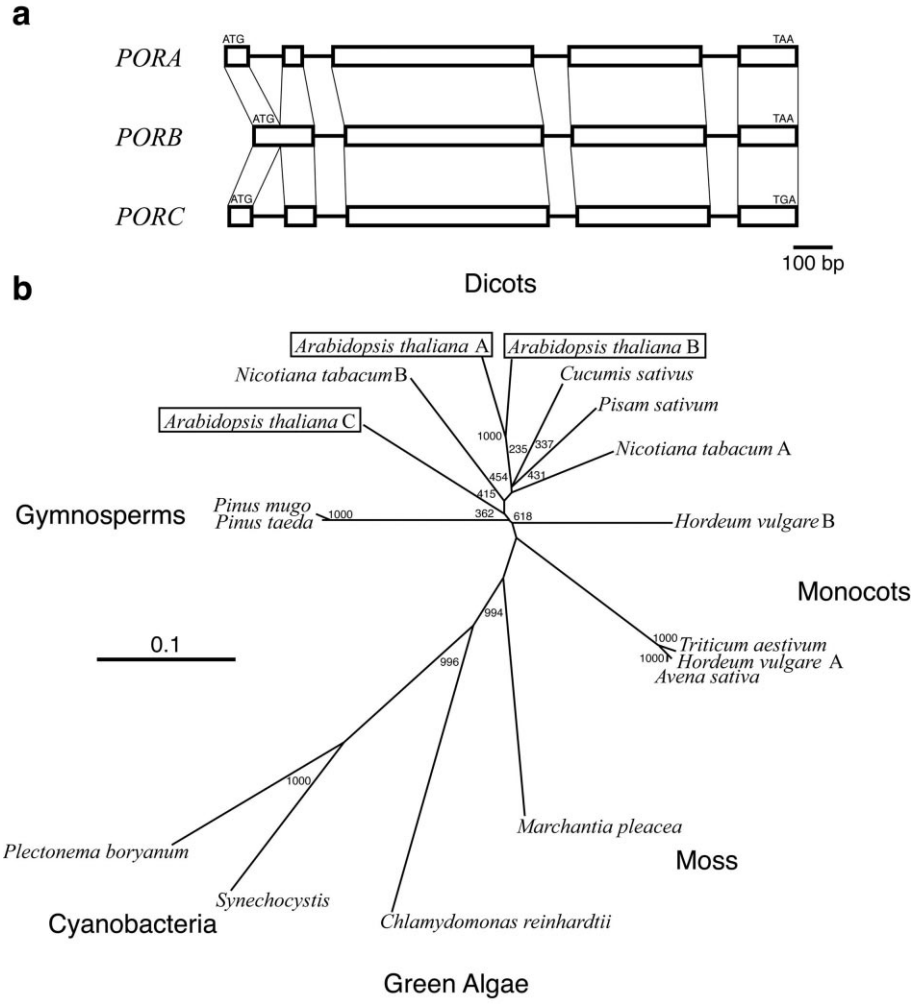

Fig. 3. a: Comparison of the gene structures of *PORA*, *PORB* and *PORC* of *A. thaliana*. Open boxes show the regions encoding the POR ORFs. Initiation and termination codons are indicated at the top. The scale bar represents 100 bp. b: Phylogenetic relationship of the POR protein family. The unrooted tree was constructed by the neighbor-joining method [12] with ClustalX and TREEVIEW software. Numbers at the branch points represent the bootstrap values of 1000 replicate trees. The scale bar represents 0.1 mutation/site.

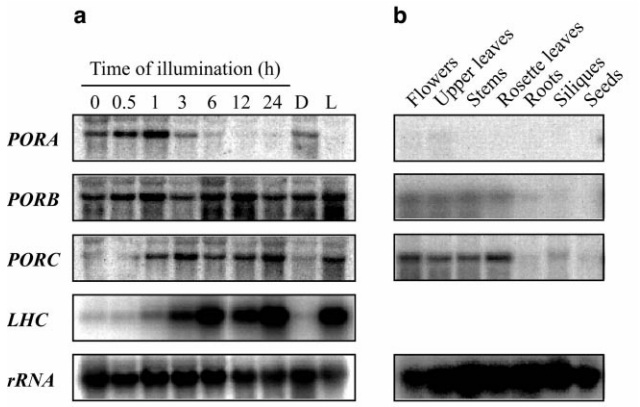

Fig. 4. a: The effects of light on the levels of *POR* transcripts during greening. Northern blots are shown for the genes indicated at the left. Four-day-old *A. thaliana* etiolated seedlings were illuminated for the time indicated (0–24 h). Lanes D and L show dark and continuous light for 24 h as controls, respectively. *LHC* mRNA served as a positive control for the inductive effect of the light treatment. b: Organ specific expression of the *POR* transcripts in continuously illuminated adult plants. Total RNA was isolated from each tissues of 5 weeks grown *A. thaliana* seedlings. Organs of the plant from which RNA was isolated are indicated at the top.

PORC by aligning its cDNA sequence with the genomic sequence. Since the nucleotide sequence of the *PORA* gene was not deposited in the database, the *PORA* genomic sequence was also determined. In Fig. 3a, the genomic organization of the *PORC* gene is aligned with those of *PORA* and *PORB* genes. The sequence encoding *PORC* was found to be separated into five exons, as was found in the *PORA* gene. The ORFs of both *PORA* and *PORC* were interrupted by introns at exactly the same sites. In *PORB*, the first intron was removed, resulting in the separation into four exons of coding sequence. Other interrupted sites were identical to those of *PORA* and *PORC*. To examine the evolutional relationship of the POR protein family, we constructed a phylogenetic tree by the neighbor-joining method [13] (Fig. 3b). POR proteins were roughly divided into four groups, i.e. monocots, dicots, gymnosperms, and algae and cyanobacteria. Although *A. thaliana* PORC protein together with PORA and PORB was grouped in the dicot group, PORC was branched earliest, while PORA and PORB were closest relatives. Therefore, it is likely that in *A. thaliana*, *PORC* is ancestral, and the *PORB* gene may be recently duplicated from *PORA* with concomitant removal of the first intron of *PORA*.

Northern blot analysis in *A. thaliana* etiolated seedlings showed that the expression of *PORC* transcript was not detectable in darkness, but upon illumination, it gradually increased and reached maximum at 6 h (Fig. 4a). In contrast, as reported previously [4], the level of *PORA* transcript rapidly decreased by onset of illumination, and the level of *PORB* remained constant during illumination. When compared with *PORB*, the light inductive profile of *PORC* transcript showed good agreement with that of light-harvesting Chl protein (LHC). Furthermore, when compared with the level of *PORB*, the level of the *PORC* transcript was much higher in mature tissues, such as rosette leaves and stems, as well as immature seedlings (Fig. 4b). The *PORC* transcripts was not detectable in non-photosynthetic tissues, such as roots and seeds. Since *PORC* was not expressed in etiolated seedling in darkness, PORC may function for Chl biosynthesis together with PORB, rather than for photoprotection or light-harvesting during greening like PORA [5,6]. Very recently, we isolated a T-DNA inserted *A. thaliana* mutant of *PORB*, and found that this mutant did not show any pale green phenotype, indicating that PORC alone could maintain the Chl biosynthesis in PORB-depleted mature tissues (Oosawa et al., unpublished data). Since *PORC* was dominantly expressed among POR isoforms in mature tissues, it is most likely that in *A. thaliana* PORC mainly or redundantly functions for Chl biosynthesis in all developmental stages.

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