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Identification of NADPH:Protochlorophyllide Oxidoreductases A and B: A Branched Pathway for Light-Dependent Chlorophyll Biosynthesis in *Arabidopsis thaliana*[1]

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Illumination releases the arrest in chlorophyll (Chl) biosynthesis in etiolated angiosperm seedlings through the enzymatic photoreduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide), the first light-dependent step in chloroplast biogenesis. NADPH: Pchlide oxidoreductase (POR, EC 1.3.1.33), a nuclear-encoded plastid-localized enzyme, mediates this unique photoreduction. Paradoxically, light also triggers a drastic decrease in the amounts of POR activity and protein before the Chl accumulation rate reaches its maximum during greening. While investigating this seeming contradiction, we identified two distinct *Arabidopsis thaliana* genes encoding POR, in contrast to previous reports of only one gene in angiosperms. The genes, designated *PorA* and *PorB*, by analogy to the principal members of the phytochrome photoreceptor gene family, display dramatically different patterns of light and developmental regulation. *PorA* mRNA disappears within the first 4 h of greening, whereas *PorB* mRNA persists even after 16 h of illumination, mirroring the behavior of two distinct POR protein species. Experiments designed to help define the functions of POR A and POR B demonstrate exclusive expression of *PorA* in young seedlings and of *PorB* both in seedlings and in adult plants. Accordingly, we propose the existence of a branched light-dependent Chl biosynthesis pathway in which POR A performs a specialized function restricted to the initial stages of greening and POR B maintains Chl levels throughout angiosperm development.

Chls form one of the most abundant classes of natural pigments and play an essential role in radiant energy absorption during photosynthesis in bacteria, algae, and higher plants. In biological systems Chls occur as noncovalently bound components of pigment-protein complexes, represented by the chloroplast-localized light-harvesting antennas and photosynthetic reaction centers of PSI and PSII in plants (reviewed by Thornber et al., 1991).

Two genetically and biochemically distinct pathways have been described for the biosynthesis of Chl, depending on whether the enzymatic reduction of Pchlide to Chlide requires light as an essential cofactor (reviewed by Bauer et al., 1993; Schulz and Senger, 1993). Light-dependent Chl biosynthesis occurs in all oxygenic photosynthetic organisms, including cyanobacteria, algae, gymnosperms, and angiosperms. With the exception of angiosperms, this pathway coexists with a separate light-independent Chl biosynthetic pathway.

Because etiolated angiosperm seedlings rely strictly on the light-dependent Chl biosynthesis pathway, they accumulate Pchlide in the dark. The light-activated reduction of Pchlide to Chlide and the simultaneous photoconversion of the Pr form of phytochrome to the Pfr form trigger plant photomorphogenesis (reviewed by Chory, 1991; Thompson and White, 1991). This developmental program includes changes in gene expression, formation of chloroplasts, cotyledon expansion, leaf development, and inhibition of stem elongation. The light-dependent POR catalyzes the photoreduction of Pchlide in plastids (Griffiths, 1978; Apel et al., 1980) using Pchlide itself as the photoreceptor (Koski et al., 1951). The action spectrum for Pchlide reduction also closely matches that of chloroplast development, indicating a tight coupling between light-dependent Chl biosynthesis and the assembly of a functional photosynthetic membrane (Virgin et al., 1963). POR is encoded in the nucleus, translated as a precursor protein in the cytosol, and ultimately transported into plastids (Apel, 1981). Single angiosperm cDNAs and/or genes encoding POR (also referred to as LPCR or PCR in some studies) have been isolated and characterized from monocotyledonous plants, such as barley (Schulz et al., 1989), oat (Darrah et al., 1990), and wheat (Teakle and Griffiths, 1993), and dicotyledonous plants, such as *Arabidopsis thaliana* (Benli et al., 1991) and pea (Spano et al., 1992a). The barley POR, expressed in *Escherichia coli* as a β-galactosidase fusion protein, converts Pchlide to Chlide in the predicted light- and NADPH-dependent manner (Schulz et al., 1989).

Paradoxically, although plants require freshly synthesized Chl molecules throughout their lifetimes to meet the demands of growth and pigment turnover (Brown et al., 1991), light exerts a rapid and dramatic negative regulation on POR-mediated light-dependent Pchlide reduction at the levels of enzyme activity and protein accumulation

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Abbreviations: LHC, light-harvesting complex; PHY A, phytochrome A; PHY B, phytochrome B; POR, NADPH:Pchlide oxidoreductase; POR A, NADPH:Pchlide oxidoreductase A; POR B, NADPH:Pchlide oxidoreductase B.

(Mapleston and Griffiths, 1980; Santel and Apel, 1981; Ikeuchi and Murakami, 1982; Meyer et al., 1983; Forreiter et al., 1990). During the greening of etiolated monocotyledonous seedlings substantial amounts of Chl first begin to accumulate after more than 2 h, although POR activity plummets to almost undetectable levels within the first 15 min in the light (Mapleston and Griffiths, 1980; Santel and Apel, 1981). The disappearance of POR activity and protein following Pchlide photoreduction results from the proteolysis of the enzyme in the absence of its substrate (Kay and Griffiths, 1983; Häuser et al., 1984).

Light also strongly negatively regulates *Por* mRNA levels during the greening of most monocotyledonous plants (Apel, 1981; Meyer et al., 1983; Batschauer and Apel, 1984; Häuser et al., 1987; Kay et al., 1989; Darrah et al., 1990). Dicotyledonous species, however, display little negative light regulation at the level of mRNA (Forreiter et al., 1990; Kittsteiner et al., 1990; Benli et al., 1991; Spano et al., 1992a; He et al., 1994). The light-induced disappearance of barley *Por* mRNA results both from a phytochrome-mediated reduction in transcription initiation (Apel, 1981; Mösinger et al., 1985) and from posttranscriptional effects (Mösinger et al., 1988).

Negative light regulation of the strictly light-dependent POR at the levels of enzyme activity, protein accumulation, and mRNA accumulation would seem to be inconsistent with maintenance of Chl biosynthesis after extended periods of illumination and in fully greened plants. Several hypotheses have been advanced during the past 15 years to explain this striking contradiction.

One proposal suggests that angiosperms activate a pathway for light-independent Chl biosynthesis in a developmentally controlled fashion only after dark adaptation of previously illuminated plants (Adamson et al., 1985; Walmsley and Adamson, 1989). Anoxygenic photosynthetic bacteria, cyanobacteria, algae, and gymnosperms, in contrast, do not require any preillumination for dark Chl biosynthesis (Bauer et al., 1993; Schulz and Senger, 1993). Angiosperms also lack the known genes required for light-independent Chl biosynthesis in other organisms (Shinozaki et al., 1986; Hiratsuka et al., 1989; Lidholm and Gustafsson, 1991; Suzuki and Bauer, 1992), leaving the proposed pathway genetically undefined.

Another model suggests that a small amount of the light-dependent POR enzyme characterized from etiolated seedlings can accumulate in illuminated monocotyledonous plants (Griffiths, 1978; Mapleston and Griffiths, 1980; Griffiths et al., 1985). The residual POR activity, measured against the high endogenous Chl background, has been proposed to provide enough new Chl to support plant growth and to replace photooxidized pigments.

A further possibility to reconcile the unusual regulation of angiosperm POR with the requirement for Chl biosynthesis throughout plant development would be the existence of distinct and differentially regulated *Por* genes. Evidence demonstrating the presence of more than one *Por* gene in angiosperms has been lacking, however. Several puzzling observations have nonetheless been made at the protein level. Multiple polypeptide species recognized by anti-POR antibodies have been described in several angiosperms but have usually been attributed to unspecific cross-reaction or to modification or processing of a single POR protein (Oliver and Griffiths, 1981; Ikeuchi and Murakami, 1982; Dehesh et al., 1986; Forreiter et al., 1990; Spano et al., 1992a). In addition, several studies have described the persistence of POR-immunoreactive polypeptides after prolonged periods of illumination (Meyer et al., 1983; Schrubar et al., 1990; Spano et al., 1992a; He et al., 1994).

To address the puzzling absence of the light-dependent POR during the period of greatest demand for Chl biosynthesis in angiosperms, we have studied the greening process in *A. thaliana*. While investigating the origin of two differentially light-regulated POR immunoreactive polypeptides we discovered a novel *Por* cDNA and identified two genes, *PorA* and *PorB*, subject to differential regulation by light and developmental state. Our expression data suggest distinct functions for POR A and POR B and identify Pchilde reduction as a branch point during light-dependent Chl biosynthesis in angiosperms.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia, obtained from Dr. C. Somerville (Carnegie Institute of Washington, Stanford, CA), was used for all experiments. Seeds were surface sterilized and distributed on Petri plates containing Murashige-Skoog agar. After an overnight treatment at 4°C, plated seeds were exposed to 1 h of white light with an intensity of 180 μE m^{-2} s^{-1} supplied by growth chambers (Weiss Umwelttechnik, Lindenstruth, Germany) fitted with Osram L 36W/21–1 Lumilux cool-white lamps. Light intensities were measured with an LI-185B quantum meter coupled to an LI-190SB quantum sensor (Li-Cor, Inc., Lincoln, NE). Standard growth conditions, except as noted below, were 22°C and 120 μE m^{-2} s^{-1} for experiments requiring subsequent illumination. The end of the 180 μE m^{-2} s^{-1} light treatment was defined as time 0 for seedlings grown in continuous darkness or continuous light, and for redarkened samples. After the light treatment the Petri plates were, depending on the experiment, placed in darkness, continuously illuminated, or exposed to a 16-h day, 22°C/8-h night, 18°C cycles for 4 weeks. In the experiments in which etiolated seedlings were illuminated after 4 d, time 0 corresponded to removal of the plates from darkness. In one experiment seeds or seedlings were dark adapted after varying durations of white light illumination. Adult plants dissected for the isolation of RNA from individual organs were grown for 8 to 9 weeks on Murashige-Skoog medium supplemented with 1.0% (w/v) Suc under continuous illumination. Plant material was harvested in liquid nitrogen at the times indicated for each experiment and stored at −80°C until analysis. Dark-grown samples were collected using separate Petri plates for each time under a dim green safelight that does not permit photoconversion of Pchlide to Chlide.

Pigment Extraction and Analysis

Chl accumulation rates were determined under the conditions described above for following the greening of etiolated seedlings. Pigments were extracted by grinding three seedlings in 1 mL of acetone supplemented with ammonia to a final concentration of 0.0083% (v/v). Three parallel samples were extracted for each time. After a brief centrifugation to pellet cell debris, Chl fluorescence at 666 nm was measured in acetone extracts with a Perkin-Elmer Cetus LS 50 luminescence spectrometer using an excitation wavelength of 440 nm. Chl concentrations were calculated as nanograms per microgram of total protein using a specific extinction coefficient of 92.45 for Chl in pure acetone after conversion of the measured fluorescence to absorption units (Lichtenthaler, 1987).

Nucleic Acid Isolation and Analysis, PCR, and Plasmid Construction

Routine nucleic acid manipulations were performed according to standard protocols (Sambrook et al., 1989). Total DNA from adult *A. thaliana* plants was isolated by slightly modifying the extraction method of Doyle and Doyle (1990) and quantitated by UV absorption with a Beckman DU-70 spectrophotometer. Samples of DNA (2.5 μg) were digested with *Bam*HI, *Eco*RI, *Pst*I, or *Xba*I restriction endonucleases, separated electrophoretically on 0.7% (w/v) native agarose gels, denatured, and blotted onto Schleicher & Schuell nitrocellulose membranes with 20× SSC solution (Sambrook et al., 1989).

Total RNA from *A. thaliana* seeds, seedlings, or plants was isolated using a small-scale method adapted from Ausubel et al. (1987). RNA concentrations were measured by UV absorption, and the quantitations were verified by examination of the rRNA bands by ethidium bromide staining after native agarose gel electrophoresis. From each RNA sample equal amounts (5 μg) were denatured and electrophoretically separated on denaturing formaldehyde-agarose gels (Ausubel et al., 1987). Separated RNAs were blotted and immobilized on NEN DuPont GeneScreen membranes according to the manufacturer's instructions.

Por sequences encoded in the *A. thaliana* genome were analyzed by PCR using 25 ng of total DNA as the template. *Por* complementary oligonucleotide primers, AtPor1.1 and AtPor1.2, were produced with an Applied Biosystems model 392 DNA/RNA synthesizer. These primers bracket the protein-coding region of the *A. thaliana PorB* cDNA clone denoted AR, previously isolated from ecotype Antwerpen 2 (Benli, 1990; Benli et al., 1991). The sequences of these oligonucleotides are Atpor1.1, 5′-GG GAATTC**AC-CATGGCCCTTCAAGCTGCTTC**-3′, and Atpor1.2, 5′-GG-GTCGAC**TATTAGGCCAAGCCCACGAG**-3′. Nucleotides shown in bold type correspond to the sequence of clone AR. The 5′ and 3′ primers contain engineered *Eco*RI and *Sal*I restriction sites (single underline), respectively. Codons representing the translation start in Atpor1.1 and the complement of the translation stop in Atpor1.2 are indicated (double underline). *A. thaliana Por* cDNAs were obtained by reverse transcriptase-PCR as follows. AMV reverse transcriptase was added to oligo(dT_{10}) and 2 μg of total DNase-treated RNA, isolated from 4-d-old etiolated seedlings, to produce a single-stranded cDNA population derived from poly(A)$^+$ RNA. These cDNAs were then amplified by PCR using the AtPor1.1 and AtPor1.2 primers. PCR amplifications using total *A. thaliana* genomic or poly(T)$^+$ cDNA as the template were performed with a Perkin-Elmer Cetus DNA thermal cycler as follows: 94°C, 30 s; 48°C, 30 s; 72°C, 30 s; 30 cycles total with a 2-min ramp to 72°C for the first three cycles. The resulting reverse transcriptase-PCR cDNA products were directionally cloned into the Stratagene pBS$^+$ vector using the engineered restriction sites. Both DNA strands of vector inserts were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a Pharmacia LKB T7 DNA polymerase sequencing kit. Examination of 10 independent clones revealed two distinct *Por* sequences, one of which corresponded to the previously described *PorB* cDNA (Benli, 1990; Benli et al., 1991). The novel sequence was designated *PorA*.The absence of PCR errors in the *Por* cDNAs was confirmed by analysis of *A. thaliana* genomic sequences (G.A. Armstrong, unpublished data) and by comparison with the known *PorB* cDNA sequence. Plasmids containing the *PorA* and *PorB* cDNAs were designated pPORA1.0 and pPORB1.0, respectively.

Nucleic Acid Hybridizations and Probe Preparation

A. thaliana genomic DNA Southern blots were hybridized overnight (Sambrook et al., 1989) with hexamer-primed 5′-[α-^{32}P]dATP-oligolabeled (Feinberg and Vogelstein, 1983) gel-purified inserts prepared from pPORA1.0 or pPORB1.0. After hybridization, blots were washed with 2× SSC, 0.1% (v/v) SDS for 10 min at 63°C, followed by 1× SSC, 0.1% (v/v) SDS for 20 min at 63°C, and exposed to Kodak X-OMAT AR film at −80°C in the presence of an intensifying screen to obtain autoradiograms. The Southern blots were subsequently rewashed at a higher stringency (0.1× SSC, 0.1% (v/v) SDS for 20 min at 63°C) to reduce *PorA*/*PorB* cross-hybridization (data not shown).

Gene-specific northern blot hybridization probes for *PorA* and *PorB* mRNAs were obtained by PCR using standard conditions (94°C, 30 s; 55°C, 30 s; 72°C, 30 s; 30 cycles) with the cloned *Por* cDNAs as templates. The 150-bp *PorA* and the 141-bp *PorB* PCR products, amplified from the corresponding portions of pPORA1.0 and pPORB1.0, represent the most highly divergent regions of the respective cDNAs (72% sequence identity). These probes discriminated completely between *PorA* and *PorB* in test hybridizations under the wash stringencies described below (data not shown). The sequences of the PCR primers are AtPor2.1, 5′-GGGAATTC**GCCTCCATTACCGACCA**-3′, and AtPor2.2, 5′-GGGTCGAC**GCCGTCCACGGATTTTG**-3′ (*PorB*); AtPor3.1, 5′-GGGAATTC**ATTTCACTTTCGGA-GCA**-3′, and AtPor3.2, 5′-GGGTCGAC**GCGGTCTAAG-GAAGATT**-3′ (*PorA*). *Eco*RI and *Sal*I restriction sites (underline) were engineered into the 5′ and 3′ primers, respectively. Nucleotides shown in boldface derive from the *Por* cDNA portions of clones pPORA1.0 or pPORB1.0.

A portion of the *A. thaliana Lhcb1*2* (previously *cab3*) sequence cloned in pAB180 (Leutwiler et al., 1986), kindly provided by Dr. E. Tobin (University of California, Los Angeles), was amplified by PCR using standard conditions. *Lhcb1*2* encodes a type I LHC B polypeptide of PSII. The sequences of the PCR primers used are AtLhcb1.1, 5′-ATCCACAGCAGGTGGGCTATG-3′, and AtLhcb1.2, 5′-GAACAAAGTTGGTTGCGAAGGC-3′. The sequence of the resulting 493-bp *Lhcb1*2* PCR product is nearly identical (91–97%) with the corresponding portions of four other *Lhcb1* genes described thus far in *A. thaliana* (Leutwiler et al., 1986; McGrath et al., 1992). Under the conditions used in this study the radioactively labeled *Lhcb1*2* probe would be expected to yield an overall picture of the expression of the *Lhcb1* gene family.

To obtain a control mRNA hybridization probe, reverse transcriptase-PCR was performed using standard conditions with specific oligonucleotide primers derived from the sequence of the *A. thaliana AAc1* actin gene (Nairn et al., 1988). The sequences of the PCR primers are AtAc1.1, 5′-TGGGATGACATGGAGAAGAT-3′, and AtAc1.2, 5′-ATACCAATCATAGATGGCTGG-3′. The resulting 572-bp PCR product was ligated to *Eco*RI linker adaptors and cloned into *Eco*RI-digested pUC18 plasmid vector, generating pAC. The identity of the cloned insert was verified by dideoxynucleotide sequencing as described above.

PorA-specific, *PorB*-specific, and *Lhcb1* PCR products and the *AAc1* insert from pAC were gel purified. Northern blot hybridization probes were prepared by oligolabeling (Feinberg and Vogelstein, 1983), with the modification that single primers (AtPor3.2, *PorA*; AtPor2.2, *PorB*; AtLhcb1.2, *Lhcb1*; AtAc1.2, *AAc1*) replaced the random hexamers to generate strand-specific radioactive antisense probes. Hybridizations were performed according to protocols supplied with the GeneScreen membranes. *Lhcb1*-probed blots were subsequently washed with 1× SSC, 0.1% (v/v) SDS for 10 min at 25°C, 1× SSC, 0.1% (v/v) SDS for 20 min at 63°C and 0.1× SSC, 0.1% (v/v) SDS for 20 min at 63°C before exposure to x-ray film for varying durations, as described above. For northern blots hybridized with other probes the last wash step was omitted. Quantitations of northern blots were performed by exposing filters to a PhosphorImager screen and analyzing the collected signals with a Molecular Dynamics (Sunnyvale, CA) imaging device and associated software.

Protein Isolation and Analysis and Computer-Based Sequence Analyses

Total *A. thaliana* protein was isolated by grinding samples under liquid nitrogen and transferring the powder to a 2-mL Eppendorf centrifuge tube containing M1 buffer (250 mM Suc, 25 mM KCl, 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 0.5% (v/v) β-mercaptoethanol, 100 mM EDTA, 0.5% (v/v) Triton X-100). Samples were sonicated with a sonifier (Branson Ultrasonics, Danbury, CT) twice for 30 s on ice and centrifuged for 5 min at 5,000 rpm in a model 5415 C Eppendorf centrifuge. The supernatant was recentrifuged for 5 min at 14,000 rpm, and proteins were subsequently precipitated for 2 h on ice by addition of one-third volume of 20% (v/v) TCA. After the sample was centrifuged for an additional 5 min at 10,000 rpm, the protein pellet was washed twice with acetone, washed twice with 96% (v/v) ethanol, dried, resuspended in protein gel-loading buffer, and stored at −20°C.

Protein concentrations were determined by the method of Esen (1978). Denaturing SDS-polyacrylamide gradient gels (10–20%, v/v) were prepared and loaded with equal amounts (15 μg), as previously confirmed by Coomassie blue staining, of heat-denatured total protein from each sample. After electrophoretic separation the proteins were electroblotted onto nitrocellulose membranes. Western blots were incubated either with a polyclonal antiserum raised against a β-galactosidase:POR fusion protein expressed in *E. coli* (Schulz et al., 1989) or with an LHC B-specific polyclonal antibody (Apel, 1979), followed by goat anti-rabbit IgG and rabbit anti-goat alkaline phosphatase conjugate (Sigma). Protein bands were visualized by detection of alkaline phosphatase activity using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a precipitating substrate (Sigma).

Sequence alignments, comparisons, and data base searches were performed using programs from the Genetics Computer Group software package (version 7.3, June 1993) available for VAX computers (Devereux et al., 1984).

RESULTS

Differential Light Regulation of Two POR Polypeptides during Greening of *A. thaliana*

When initiating our study of the greening process in *A. thaliana* we decided to reexamine the accumulation of the POR protein, whose levels had previously been reported to be negatively light regulated (Benli et al., 1991). Seedlings that had been etiolated for 4 d were shifted to white light for varying amounts of time, and samples were collected for analysis of protein and Chl contents. We prepared western blots using equal amounts of total protein from each sample (see "Materials and Methods"), extracted using an alternative procedure to that previously described (Benli et al., 1991). After the blots were incubated with a polyclonal antiserum we observed POR protein bands I and II, with apparent molecular masses of 37 and 36 kD, respectively, in samples from etiolated seedlings (Fig. 1). The POR-I protein species disappeared within 4 h of illumination, whereas the POR-II polypeptide persisted through 16 h, albeit at reduced levels at later times. For comparison we prepared a replicate western blot for immunoreaction with a polyclonal antiserum that recognizes the LHC B polypeptides, the dominant LHC proteins of PSII (Thornber et al., 1991). The light-induced accumulation of LHC B polypeptides, with apparent molecular masses of 29 and 28 kD, first became detectable at 4 h and thereafter increased dramatically (Fig. 1).

We next examined the Chl accumulation rate for comparison with the protein measurements. During the first 2 h of illumination approximately 0.09 ng Chl μg^{-1} total protein h^{-1} accumulated, indicative of the initial lag phase in the greening of etiolated angiosperm seedlings. Between

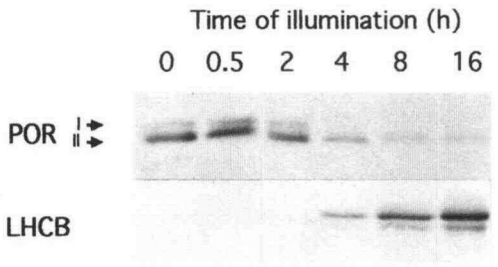

Figure 1. Light differentially regulates the levels of two POR polypeptides during greening of *A. thaliana* seedlings. Western blots were performed using antisera raised against the proteins indicated at the left. Two POR polypeptides are indicated (I and II). Time 0 represents a sample collected at the end of etiolated growth (4 d), and other times indicate the subsequent duration of seedling exposure to light. LHC B polypeptides serve as a positive control for the inductive effect of the light treatment during greening.

2 and 16 h of illumination the linear rate of Chl accumulation increased to 0.64 ng Chl μg^{-1} total protein h^{-1}. This increase coincided with the first appearance of LHC B polypeptides at 4 h (Fig. 1).

Two Genes Encode Products Homologous to Light-Dependent POR in *A. thaliana*

The identification of two *A. thaliana* POR polypeptides differentially regulated by light led us to consider the possibility that POR might be encoded by a multigene family. To evaluate the number of expressed *Por* genes present in *A. thaliana*, we applied several different techniques, including PCR with *A. thaliana* genomic DNA or poly(T)$^+$ cDNA as templates, and low-stringency hybridizations with genomic DNA. PCR was selected as an experimental approach because of the 75% or greater amino acid sequence identity between POR proteins described thus far from angiosperms (Schulz et al., 1989; Darrah et al., 1990; Benli et al., 1991; Spano et al., 1992a; Teakle and Griffiths, 1993) and gymnosperms (Spano et al., 1992b; Forreiter and Apel, 1993).

Using the nucleotide sequence of a previously isolated *A. thaliana Por* cDNA (Benli, 1990; Benli et al., 1991) we designed two nondegenerate, antiparallel oligonucleotide PCR primers to amplify the entire protein-coding region. When total *A. thaliana* DNA was used as the template this pair of primers yielded two PCR products of approximately 1450 and 1550 bp, each of which contained several unique restriction sites (data not shown). Both of these products exceeded the 1203 bp predicted for the coding region of the *Por* cDNA, suggesting the presence of an intron(s).

To confirm these results we examined the expression of *Por*-related mRNAs by reverse transcription of total RNA prepared from etiolated seedlings, coupled with amplification of the resulting single-stranded cDNAs using the above-mentioned PCR primers. This approach led to the synthesis of a single reverse transcriptase-PCR product of approximately 1200 bp (data not shown), consistent with the predicted size of the *Por* coding region. Cloning of this PCR product and restriction endonuclease screening of individual recombinant plasmids revealed two classes of inserts, however.

Nucleotide sequencing of plasmids containing both types of insert identified the first class as cDNAs of 1203 bp in length that were 100% identical with the *Por* cDNA, designated here *PorB*, previously isolated from *A. thaliana* ecotype Antwerpen 2 (Benli, 1990). The second class of inserts, however, were found to encode a novel *Por* cDNA of 1215 bp, designated here *PorA*. The *PorA* and *PorB* cDNAs exhibit 83% nucleotide sequence identity. No additional *Por* sequences could be detected in the *A. thaliana* genome by PCR or in cDNA populations by reverse transcriptase-PCR when degenerate primers were used.

To confirm that *PorA* and *PorB* represent the entire *Por* gene family in *A. thaliana* we hybridized replicate Southern blots prepared from total DNA with radioactively labeled *PorA* or *PorB* cDNAs. A relatively simple banding pattern was observed with each probe under low-stringency wash conditions (Fig. 2). Cross-hybridizing fragments could be assigned to either *PorA* (*Pst*I, 2.2 kb; *Xba*I, 12.5 kb; *Eco*RI, 10.5 kb) or *PorB* (*Pst*I, 8.0 kb; *Xba*I, 8.5 kb; *Eco*RI, 5.2 kb; *Bam*HI, >20 kb) by increasing the wash stringency. Based on these results and the known distribution of restriction sites within the *Por* cDNAs and corresponding genomic sequences (G.A. Armstrong, unpublished data), we conclude that *PorA* and *PorB* represent the only *Por* genes present in *A. thaliana*. In retrospect, an earlier study indicated the existence of only one *Por* gene because the experimental conditions selected allowed hybridization only to *PorB* genomic fragments (Benli et al., 1991).

Evolutionary Conservation of Higher Plant POR Proteins

The *A. thaliana Por* cDNAs encode predicted precursor POR A and POR B polypeptides of 405 and 401 amino acids, respectively, that are 88% identical (Fig. 3). POR A and POR B are equally well conserved with each of the other higher plant POR proteins that have thus far been

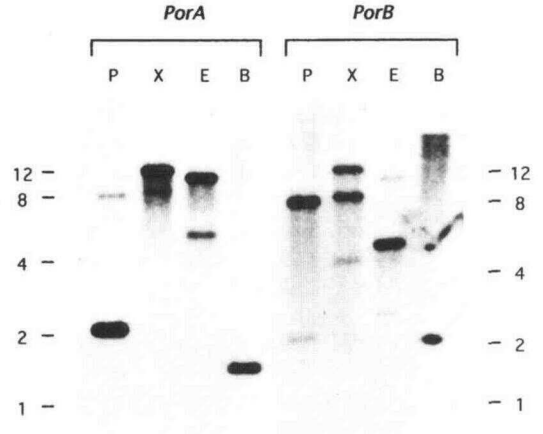

Figure 2. Two distinct genes encode POR polypeptides in *A. thaliana*. Genomic Southern blots of total DNA were hybridized with the probes indicated at the top. Restriction endonuclease digests were: P, *Pst*I; X, *Xba*I; E, *Eco*RI; B, *Bam*HI. DNA size markers in kb are indicated in the left and right margins.

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AtPOR A     MALQAASLVSSAFSVRKDGKLNASASSSFKESSLFGI-SLSEQSKADFVSSSLRCKREQSLRNNKAIIRA QAIATSTPSVTK-----SSL    84
            ****************** ***** ***** *****  *   * *    ***** ********    *** *  *** * ***      *
AtPOR B     MALQAASLVSSAFSVRKDAKLNAS-SSSFKDSSLFGA-SITDQIKSEHGSSSLRFKREQSLRN--LAIRA QTAATSSPTVTK------SV    80
PsPOR       MALQTASMLPASFSIPKEGKIGA----SLKDSTLFGVSSLSDSLKGDFTSSALRCKRE--LRQKVGAVRA ETAAPATPAVNK-----SSS    79
PmPOR 1     MGTLLQTHIGSVAFALQKEGGHSASA----KDSAFLGV-SLVEHGKKEFSFPVIRAKKVTSRNTNVPRIRA QTVAA--PVETK-----DAP    79
HvPOR       MALQ---LLPSTLSVPKKGS-SMGAVAVKDTAAFLGVSSKA--------------------KKASLAVRT QVATAPSPVTTSPGSTASSP    66
Consensus   MALQ        FS  K G   A      K S   G  S     K         R K           RA Q  A   P  TK      S

                  +++   + +++    +        +     ++      + +       +     ++   + + +   +  +      +++++
AtPOR A     DRKKTLRKGNVVVTGASSGLGLATAKALAETGKWHVIMA C RDFLKAERAAQSAGMPKDSYTVMHLDLASLDSVRQFVDNFRRAEMPLDVLV C  176
            * ********************************** **** * ********** * ***************************** * ****** *
AtPOR B     DGKKTLRKGNVVVTGASSGLGLATAKALAETGKWNVIMA C RDFLKAERAAKSVGMPKDSYTVMHLDLASLDSVRQFVDNFRRTETPLDVLV C  172
PsPOR       EGKKTLRKGNVVITGASSGLGLATAKALAESGKWHVIMA C RDYLKAARAAKSAGLAKENYTIMHLDLASLDSVRQFVDNFRRSEMPLDVLI N  171
PmPOR 1     ASKKTDRKGNVIITGASSGLGLATAKALGESGKWHIIMA C RDFLKAERMARSVGIPKENYSVMHLDLASLESVRQFADNFRRSGRPLDVLV C  171
HvPOR       SGKKTLRQGVVVITGASSGLGLAAAKALAETGKWHVVMA C RDFLKASKAAKAAGMADGSYTVMHLDLASLDSVRQFVDAFRRAEMPLDVLV C  158
Consensus     KKTLRKGNVV TGASSGLGLATAKALAE GKWHVIMA C RDFLKA RAA S G  K  YTVMHLDLASLDSVRQFVDNFRR E PLDVLV C

            ++      +    +     +     + +       +    +
AtPOR A     NAAVYQPTANQPTFTAEGFELSVGINHLGHFLLSRLLIDDLKNSDYPSKRLIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLNGLNSSAM    268
            ***** ***  **  ********  ******** *** **** ************************************************
AtPOR B     NAAVYFPTAKEPTYSAEGFELSVATNHLGHFLLARLLLDDLKKSDYPSKRLIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLNGLNSSAM    264
PsPOR       NAAVYFPTAKEPSFTADGFEISVGTNHLGHFLLSRLLLEDLKKSDYPSKRLIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLTGLNSSAM    263
PmPOR 1     NAAIYLPTAKLPTYTAEGFELSVGTNHLGHFLLSRLLLEDLKTSDFNSKRVIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLNGVNISPM    263
HvPOR       NAAIYRPTARTPTFTADGHEMSVGVNHLGHFLLARLLMEDLQKSDYPSRRMVIVGSITGNSNTLAGNVPPKASLGDLRGLAGGLSGASGSAM    220
Consensus   NAA Y PTA  PT TA GFE SVG NHLGHFLL RLL  DLK SDYPSKR IIVGSITGNTNTLAGNVPPKANLGDLRGLAGGL G N SAM

AtPOR A     IDGGD-FVGAKAYKDSKV C NMLTMQEFHRRFHEDTGITFASLYPG C IATTGLFREHIPLFRTLFPPFQKYITKGYVSESEAGKRLAQVVADP  359
            ***** * ********** * ************** ** ******** * ** ************ **************** * ******** **
AtPOR B     IDGGD-FDGAKAYKDSKV C NMLTMQEFHRRFHEETGVTFASLYPG C IASTGLFREHIPLFRALFPPFQKYITKGYVSETESGKRLAQVVSDP  355
PsPOR       IDGGD-FDGAKAYKDSKV C NMLTMQEFHRRYHEETGITFASLYPG C IATTGLFREHIPLFRTLFPPFQKYITKGYVSEEESGKRLAQVVSDP  354
PmPOR 1     IDGGE-FDGAKAYKDSKV C NMLTMQEFHRRYHEETGITFASLYPG C IATTGLFREHIPLFRLLFPPFQKYITKGFVSEEEAGKRLAQVVSNP  354
HvPOR       IDGGESFDGAKAYKDSKV C NMLTMQEFHRRYHEETGITFSSLYPG C IATTGLFREHIPLFRTLFPPFQKFVTKGFVSEAESGKRLAQVVAEP  342
Consensus   IDGG  FDGAKAYKDSKV C NMLTMQEFHRR HEETGITFASLYPG C IATTGLFREHIPLFR LFPPFQKYITKG VSE E GKRLAQVV  P

AtPOR A     SLTKSGVYWSWNKTSASFENQLSQEASDVEKARRVWEVSEKLVGLA   405
            ************ ********* ********* *** ********
AtPOR B     SLTKSGVYWSWNNASASFENQLSEEASDVEKARKVWEISEKLVGLA   401
PsPOR       SLTKSGVYWSWNNASASFENQLSQEASDAEKARKVWEVSEKLVGLA   400
PmPOR 1     SLTKSGVYWSWNNNSGSFENQLSEEASDPEKAKKLWEISEKLVGLA   400
HvPOR       VLTKSGVYWSWNKDSASFENQLSQEASDPEKARKVWELSEKLVGLA   388
Consensus   SLTKSGVYWSWN  SASFENQLS EASD EKARKVWE SEKLVGLA
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Figure 3. Structural features of higher plant POR polypeptides. Representative sequences from dicotyledonous (*A. thaliana*, pea) and monocotyledonous (barley) angiosperms and from a gymnosperm (pine) have been aligned: AtPOR A, *A. thaliana* POR A (this work); AtPOR B, *A. thaliana* POR B (Benli et al., 1991; this work); PsPOR, pea (*Pisum sativum*; Spano et al., 1992a); PmPOR 1, mountain pine (*Pinus mugo*; Forreiter and Apel, 1993); HvPOR, barley (*Hordeum vulgare*; Schulz et al., 1989). POR sequences from wheat (Teakle et al., 1993) and oat (Darrah et al., 1990) are about 95% identical with the barley HvPOR, whereas a loblolly pine POR polypeptide (Spano et al., 1992b) is 99% identical with the mountain pine PmPOR 1. Residue numbers for the POR polypeptides are given on the right side. Putative cleavage sites for the plastid transit peptides are indicated by a triangle. Hyphens represent gaps inserted to optimize the sequence alignments, asterisks highlight residues conserved between AtPOR A and AtPOR B, and plus signs indicate residues identical with those found in the NADPH-dependent human carbonyl reductase (Wermuth et al., 1988). Cys residues located within the mature POR polypeptides are indicated. The consensus sequence represents those residues conserved in at least four of the five higher plant POR proteins, including underlined residues that form a putative $\beta\alpha\beta$-ADP-binding fold involved in NADPH binding.

described, with sequence identities ranging between 74 and 82%. Most of the sequence divergence between POR proteins occurs in the N-terminal regions that contain structural features consistent with their roles as putative chloroplast transit peptides (von Heijne et al., 1989). Despite this sequence divergence, the first four amino acids of the *A. thaliana* POR A and POR B transit peptides (MALQ) are also conserved in the barley, pea, and wheat POR sequences (Schulz et al., 1989; Spano et al., 1992a; Teakle and Griffiths, 1993).

Both *A. thaliana* POR A and POR B contain a tetrapeptide, IRAQ (residues 67–70 in POR A), which may constitute the processing site for the cleavage of the plastid transit peptides (Fig. 3). When the *A. thaliana* POR sequences are compared with the pea POR sequence, Gln^{70} of *A. thaliana* POR A aligns with the N-terminal residue determined for the mature pea POR protein (Spano et al., 1992a). POR proteins from several species of pine also retain the IRAQ tetrapeptide (Spano et al., 1992b; Forreiter and Apel, 1993). Processing of chloroplast transit peptides occurs at or near IRAQ motifs in the case of the precursors for flavin adenine dinucleotide-containing Fd-$NADP^+$ oxidoreductases (Jakowitsch et al., 1993) and the NADPH-dependent 3-ketoacyl-acyl carrier protein oxidoreductase involved in fatty acid biosynthesis (Klein at al., 1992). Whether the IRAQ motif reflects purely structural requirements of the precursors of NADPH-dependent oxidoreductases or indicates a conserved interaction of this class of enzymes with the plastid protein transport and processing machinery remains to be investigated. If processing were to occur at the IRAQ motifs in the *A. thaliana* POR A and POR B precursors, the calculated sizes of the 90% identical mature polypeptides would be approximately 36 kD, composed of 336 and 335 amino acids, respectively.

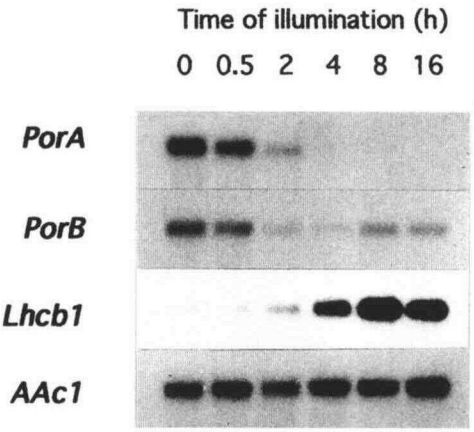

Figure 4. Light differentially regulates the levels of *PorA* and *PorB* mRNAs during greening. Northern blots are shown for the genes indicated at the left. *Lhcb1* mRNA, which serves as a positive control for the inductive effect of the light treatment, derives from a family of at least five highly related nuclear genes that encode the cytosolic precursors of type I LHC B polypeptides of PSII (Leutwiler et al., 1986; McGrath et al., 1992). *AAc1* mRNA, which encodes actin (Nairn et al., 1988), provides a constitutive control. Other experimental details are given in the legend to Figure 1.

The respective N termini of the mature POR A and POR B proteins contain putative $\beta\alpha\beta$-ADP-binding folds (Fig. 3) likely to be involved in the interaction with NADPH (reviewed by Baker, 1994). A similar observation was originally made from a partial deduced sequence for oat POR (Darrah et al., 1990). Data base searches revealed conservation of the *A. thaliana* POR ADP-binding folds and other short stretches of amino acids from residues 90 to 220 with several NADPH-dependent oxidoreductases that use nonporphyrin substrates (Fig. 3; data not shown), such as the human carbonyl reductase (Wermuth et al., 1988).

The mature *A. thaliana* POR A and POR B proteins share four conserved Cys residues with other higher plant POR polypeptides (Fig. 3). *A. thaliana* POR A also contains a fifth Cys residue within the transit peptide (Cys^{54}) that is conserved in the pea POR (Spano et al., 1992a). Chemical modification studies have implicated one or more of the four Cys residues conserved among mature plant POR proteins in substrate or cofactor binding (Oliver and Griffiths, 1981; Dehesh et al., 1986). Cys^{124} and Cys^{176} of *A. thaliana* POR A are embedded within conserved stretches of amino acids common to other NADPH-dependent oxidoreductases, such as human carbonyl reductase (Fig. 3), and therefore may be involved in NADPH binding.

PorA mRNA Disappears, whereas *PorB* mRNA Persists during Light-Induced Greening

With the knowledge that light differentially regulates two *A. thaliana* POR polypeptides and that two genes encode POR, we turned our attention to the regulation of *PorA* and *PorB* during greening. Seedlings that had been etiolated for 4 d were shifted to white light for varying amounts of time and samples were collected for analysis of RNA. Hybridization of northern blots containing equal amounts of total RNA (see "Materials and Methods") with *PorA* or *PorB* gene-specific probes revealed transcripts of approximately 1400 bp in both cases (Fig. 4), in agreement with the size of the previously isolated *PorB* cDNA from *A. thaliana* (Benli et al., 1991). Given that the *Por* gene-specific probes are of similar size and were labeled to similar specific activities, we conclude that both *Por* transcripts accumulate to approximately equivalent levels in etiolated seedlings. Light differentially regulated the levels of *PorA* and *PorB* mRNAs during photomorphogenesis, however. The amount of *PorA* mRNA decreased rapidly and became undetectable after 4 h of illumination. *PorB* mRNA initially decreased in abundance in parallel to *PorA* from 0 to 4 h but reaccumulated from 4 to 16 h, coinciding with the 7-fold increase in *Lhcb1* mRNA relative to the *AAc1* control mRNA during the same period.

The parallel negative light regulation of the steady-state levels of *PorA* mRNA (Fig. 4) and the POR-I polypeptide (Fig. 1) suggest that this protein species may correspond to the mature POR A. At the 4-, 8-, and 16-h points, subsequent to the lag phase in Chl accumulation, only the POR-II polypeptide could be detected. Since *PorB* but not *PorA* mRNA was present at these times, the POR-II protein may represent the mature POR B. The anti-POR antibody used in this study indeed recognizes bacterially expressed POR A and POR B with approximately equal efficiencies (B. van Cleve, personal communication). We note, however, that other possible explanations for the presence of the POR-I and POR-II polypeptides exist.

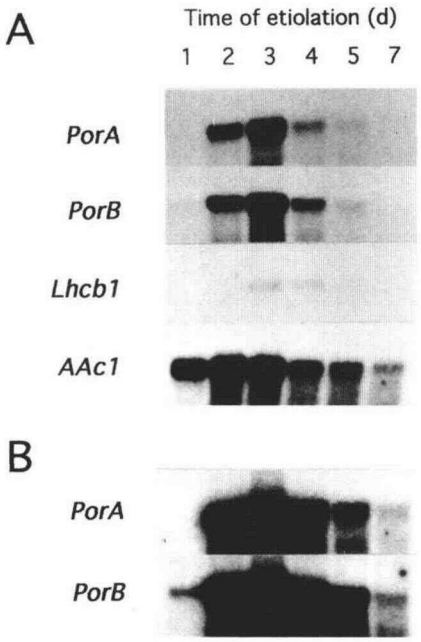

Figure 5. Regulation of *Por* mRNA levels during skotomorphogenic development of seedlings. Northern blots are shown for the genes indicated at the left. The duration of etiolated growth after plating of seeds is given at the top. B, Longer exposures of the same *Por* autoradiograms presented in A. Note the absence of *PorA* and the presence of *PorB* mRNA after 1 d of etiolation.

Young Seedlings Express PorA and PorB, whereas Older Plants Express only PorB

To obtain further insight into the functions of POR A and POR B we next performed a series of experiments to characterize the expression patterns of the *Por* genes throughout plant development. The effects of light and developmental state on *Por* gene regulation were studied by plating seeds and allowing them to germinate in the dark or in continuous light. Seeds or seedlings were collected at various times and their RNAs were analyzed in northern blots. Under all growth conditions described in this work seedlings germinated after approximately 2 d.

The ratio between *PorA* and *PorB* mRNAs remained constant at each time during skotomorphogenic growth from 2 to 7 d, although the *Por* mRNA concentrations changed substantially relative to total RNA (Fig. 5A). Levels of both *Por* mRNAs at first increased dramatically, reaching maxima after 3 d of darkness, before decreasing to almost undetectable levels by 7 d. The sharp decline after 3 d of skotomorphogenic growth may reflect selective degradation relative to *AAc1* mRNA. A striking deviation from the parallel regulation of the *Por* mRNAs occurred after 1 d, prior to seed germination, when *PorB* mRNA had accumulated in seeds but *PorA* mRNA could not be detected (Fig. 5B). The amount of *PorB* mRNA increased 25-fold between 1 d and its maximum at 3 d, normalized to the levels of *AAc1* mRNA. As has been previously described in *A. thaliana* (Brusslan and Tobin, 1992), *Lhcb1* mRNA accumulated to a small extent after 3 to 4 d of darkness, slightly later than maximal *Por* mRNA accumulation (Fig. 5A).

When *A. thaliana* seeds were plated and maintained in continuous light, *PorA* mRNA accumulated exclusively during germination, at 2 d (Fig. 6). In contrast, although the level of *PorB* mRNA also reached a maximum at 2 d, detectable amounts were present both prior and subsequent to germination. As a consequence of the strong negative regulation of *PorA* mRNA amount by light (Fig. 4) and assuming equal hybridization efficiencies for the radioactive probes, the steady-state level of *PorB* mRNA exceeded that of *PorA* by a factor of 12 after 2 d of continuous illumination (Fig. 6). *Lhcb1* mRNA could not be detected at 1 d but reached a constant level, about 10-fold higher than the etiolated control, when normalized to the levels of *AAc1* mRNA, starting at 2 d of illumination (Fig. 6A).

In an additional experiment we studied the expression of *Por* mRNAs during dark adaptation. Seedlings were maintained in continuous light for 1 to 6 d before being shifted to darkness for 2 d. Comparison of mRNA levels in continuously illuminated seedlings (Fig. 6) and in dark-adapted seedlings of the same total age (Fig. 7) revealed different patterns of regulation by light and developmental state for *PorA*, *PorB*, and *Lhcb1*. *PorB* and *Lhcb1* mRNAs were present throughout the entire illumination:redarkening time course. *Lhcb1* mRNA was always less abundant after dark adaptation, underlining the requirement of light for maintenance of this mRNA. *PorB* mRNA accumulated to higher levels in 1 d:2 d and 2 d:2 d dark-adapted seedlings versus 3- and 4-d-old illuminated seedlings, but this pattern was reversed in 4 d:2 d dark-adapted seedlings. *PorA* mRNA reaccumulated in 1 d:2 d, 2 d:2 d, and 3 d:2 d dark-adapted seedlings (Fig. 7B), contrasting with the complete absence of this mRNA in continuously illuminated seedlings of comparable age (Fig. 6B). Seedlings older than 3 d:2 d lost the ability to reaccumulate *PorA* mRNA upon dark adaptation, however (Fig. 7B). Neither *Por* mRNA ever reaccumulated to the levels present in etiolated control seedlings (Fig. 7).

The results obtained above suggested that the plant developmental program temporally restricts *PorA* expression to a small window coinciding with germination and greening of seedlings. To determine the expression patterns of *PorB* and *PorA* later in plant development, several experiments were performed. In one case *A. thaliana* seedlings were grown for 4 weeks under LD conditions (16-h day/8-h night cycles). At the end of this time samples were harvested at 4-h intervals either in the light or in the dark during a 24-h diurnal cycle.

PorA mRNA could not be detected under LD conditions, although hybridization with an *AAc1* probe confirmed that RNA was present on the northern blot (Fig. 8). *PorB* mRNA was readily detectable, however, and exhibited a clear diurnal oscillation with a midday maximum, 8 h after the onset of illumination. No significant reaccumulation was observed during the 8-h dark period, suggesting that the amount of *PorB* mRNA remains constant during a normal night. The maximal amount of *PorB* mRNA exceeded that present at its minimum by a factor of about 5, normalized

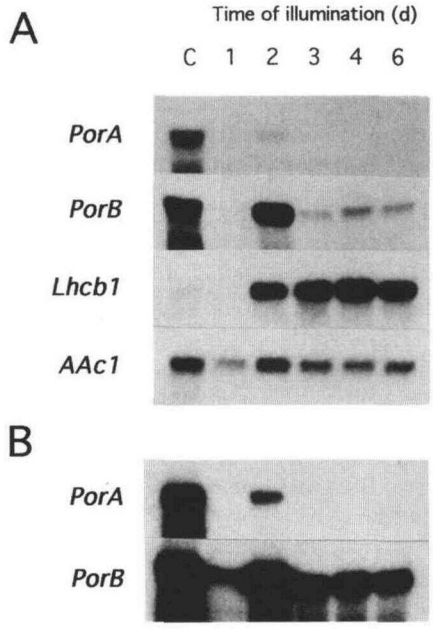

Figure 6. Regulation of *Por* mRNA levels during photomorphogenic development of seedlings. Northern blots are shown for the genes indicated at the left. The duration of growth in the light after plating of seeds is given at the top. C indicates a control lane containing RNA from etiolated seedlings. B, Longer exposures of the same *Por* autoradiograms presented in A. Note the absence of *PorA* and the presence of *PorB* mRNA after 1 d of illumination and the appearance of *PorA* mRNA exclusively at 2 d.

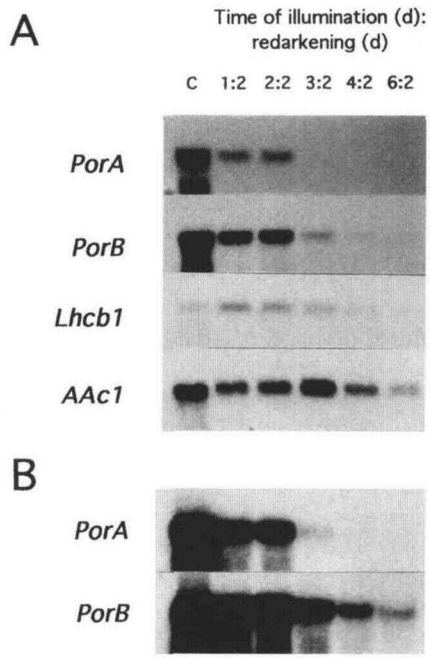

Figure 7. Regulation of *Por* mRNA levels after dark adaptation of continuously illuminated seedlings of various ages. Northern blots are shown for the genes indicated at the left. The duration of growth in the light followed by a constant redarkening period of 2 d is given at the top. C indicates a control lane containing RNA from etiolated seedlings. Differences with respect to Figure 6 for seedlings of the same age indicate whether dark adaptation results in net mRNA reaccumulation (i.e. *PorA*) or degradation (i.e. *Lhcb1*). B, Longer exposures of the same *Por* autoradiograms presented in A. Note the absence of *PorA* mRNA after a total of 6 or 8 d of growth, and the presence of *PorB* mRNA under the same conditions.

to the levels of *AAc1* mRNA. Similar expression patterns were obtained using SD growth conditions (8-h day/16-h night cycles; data not shown).

The organ specificity of *Por* mRNA expression was examined by dissecting adult plants grown under continuous illumination for 8 to 9 weeks. Northern blots prepared with total RNA revealed that *PorA* mRNA could not be detected in any adult plant part. In contrast, *PorB* was expressed strongly in all photosynthetic organs but only weakly in roots relative to the *AAc1* control (Fig. 9). The pea *Por* mRNA displays similar organ-specific expression patterns in illuminated seedlings (Spano et al., 1992a). Dormant *A. thaliana* seeds lacked detectable mRNA for *PorA*, *PorB*, and *AAc1*. Comparison with Figures 5 and 6 indicates that *PorB* and *AAc1* mRNAs increase to measurable levels before germination after seeds have imbibed for 1 d.

DISCUSSION

Evidence for Distinct Functions of POR A and POR B in a Branched Light-Dependent Chl Biosynthesis Pathway

In this study we analyzed the POR-mediated light-dependent reduction of Pchlide as a critical regulatory point in the biosynthesis of Chl and chloroplast biogenesis in angiosperms. Using *A. thaliana* we have demonstrated that,

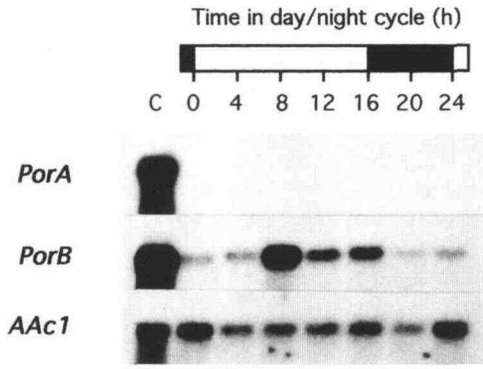

Figure 8. Diurnal regulation of *PorB* mRNA during LD growth. Northern blots are shown for the genes indicated at the left. The time of sample collection with respect to the day/night cycle (white and black boxes, respectively) is given at the top. C indicates a control lane containing RNA from 4-d-old etiolated seedlings.

in contrast to the results of several previous studies of angiosperms (Schulz et al., 1989; Darrah et al., 1990; Spano et al., 1992a; Teakle and Griffiths, 1993), two distinct and differentially regulated genes encode POR. The negative light regulation of *A. thaliana PorA* mRNA and the putative POR A protein (Figs. 1 and 4) corresponds to that originally reported for POR in etiolated monocotyledonous seedlings (Mapleston and Griffiths, 1980; Apel, 1981; Santel and Apel, 1981; Batschauer and Apel, 1984). These earlier findings inspired several proposals to reconcile the apparently paradoxical negative light regulation of POR enzyme activity and protein with the maintenance of Chl biosynthesis at all stages of plant development (Griffiths, 1978; Mapleston and Griffiths, 1980; Adamson et al., 1985; Griffiths et al., 1985; Walmsley and Adamson, 1989).

The identification of *A. thaliana PorB* offers a novel solution to this paradox. Fully greened *A. thaliana* seedlings and adult plants do not express *PorA* but rather *PorB* throughout the day/night cycle and in all photosynthetic organs, respectively (Figs. 8 and 9). These data support the idea

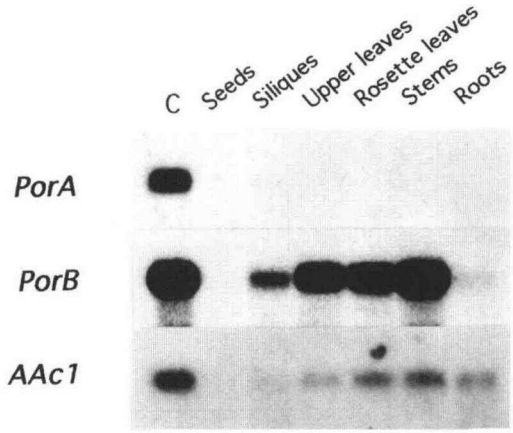

Figure 9. Organ-specific expression of *PorB* mRNA in continuously illuminated adult plants. Northern blots are shown for the genes indicated at the left. The portion of the plant from which RNA was isolated is given at the top. C indicates a control lane containing RNA from 4-d-old etiolated seedlings.

that the residual POR activity that sustains Chl biosynthesis in green plants (Griffiths, 1978; Mapleston and Griffiths, 1980; Griffiths et al., 1985) derives solely from POR B. We therefore propose that POR B assumes responsibility for directing light-dependent Chl biosynthesis during most of plant development, particularly subsequent to greening.

The question arises of whether the closely related POR A and POR B enzymes perform distinct or redundant biological functions. At no point during greening and early seedling development is *A. thaliana PorA* expressed without the concomitant expression of *PorB* (Figs. 1 and 4–7). Indirect evidence strongly suggests that *A. thaliana* POR A and POR B are both light-dependent enzymes. For example, exposure of *A. thaliana* to far-red light eliminates *PorA* but not *PorB* expression, implying that the remaining light-dependent POR activity results from POR B (S. Runge and G.A. Armstrong, unpublished data). Recent data also indicate that both the barley POR A and POR B enzymes convert Pchlide to Chlide in a strictly light- and NADPH-dependent manner in vitro (Schulz et al., 1989; Holtorf et al., 1995). One POR enzyme should therefore, in principle, suffice to provide all of a seedling's early needs for Chl biosynthesis. On the other hand, the impairment of the greening process in *A. thaliana* by far-red light pretreatment provides a compelling argument that POR B alone is not sufficient for normal greening and suggests a crucial biological function for POR A (S. Runge and G.A. Armstrong, unpublished data).

What might be the distinct function of POR A in light-dependent Chl biosynthesis? The expression of *PorA* is both transient and highly negatively regulated by light, allowing mRNA accumulation only close to the time of germination (Figs. 1 and 4–7). *PorA* mRNA could not be detected in adult plants in an experiment using the exquisitely sensitive technique of reverse transcriptase-PCR (data not shown). These data reinforce the conclusion that POR A acts exclusively during the initial stages of greening and the assembly of functional photosynthetic membranes in developing chloroplasts.

According to our working hypothesis, POR A and POR B define two functionally distinct branches of the light-dependent Chl biosynthesis pathway. Whether the POR A and POR B branches merge again subsequent to Pchlide photoreduction or remain completely independent, leading to the incorporation of Chl into distinct pigment-binding polypeptides, remains to be addressed. An examination of the in vivo functions of *A. thaliana* POR A and POR B throughout the angiosperm life cycle should prove revealing.

Significance of Two Differentially Regulated *Por* Genes in Angiosperms

The existence of two differentially regulated *Por* genes may be a general phenomenon among angiosperms rather than a special feature of *A. thaliana*. In barley a second expressed *Por* gene has very recently been identified (Holtorf et al., 1995). By analogy to *A. thaliana*, the originally described barley cDNA encoding the negatively light-regulated POR from etiolated seedlings (Schulz et al., 1989) has been designated *PorA*, and the constitutively expressed *Por* gene has been denoted *PorB* (Holtorf et al., 1995). Evidence also consistent with the possible existence of undiscovered *Por* genes in other angiosperms comes from previous reports of multiple or relatively light-stable POR-immunoreactive polypeptides (Oliver and Griffiths, 1981; Ikeuchi and Murakami, 1982; Meyer et al., 1983; Forreiter et al., 1990; Schrubar et al., 1990; Spano et al., 1992a; He et al., 1994).

The presence of the differentially regulated *PorA* and *PorB* genes in both *A. thaliana* and barley could also offer an explanation for observations that the influence of light on *Por* mRNA levels ranges from a drastic negative regulation to virtually no effect depending on the plant species (Apel, 1981; Meyer et al., 1983; Batschauer and Apel, 1984; Häuser et al., 1987; Kay et al., 1989; Darrah et al., 1990; Forreiter et al., 1990; Kittsteiner et al., 1990; Benli et al., 1991; Spano et al., 1992a; He et al., 1994). *A. thaliana* expresses *PorA* and *PorB* at almost equal levels in etiolated seedlings (Figs. 4 and 5). Upon illumination *PorA* mRNA disappears completely within 4 h, whereas the *PorB* mRNA initially dips below its etiolated levels before recovering by 16 h (Fig. 4). These two overlapping expression patterns create a situation in which total *A. thaliana Por* mRNA exhibits a modest decrease upon illumination, exactly the effect previously reported when a hybridization probe that did not distinguish between *PorA* and *PorB* was used (Benli et al., 1991). Thus, by analogy to *A. thaliana*, variations in the ratio of *PorA* to *PorB* mRNAs in etiolated seedlings of other angiosperms could account for the observed species-specific differences in the responses of total *Por* mRNA to light. Such a situation occurs in barley, in which *PorA* expression dominates in etiolated seedlings (Holtorf et al., 1995).

Parallels between Regulation of *PorA* and *PorB* and the Phytochrome *PhyA* and *PhyB* Genes

Striking similarities exist between the regulation and expression of the *A. thaliana Por* genes and the angiosperm *Phy* genes that encode the two principal members of the phytochrome photoreceptor family, PHY A and PHY B (reviewed by Quail, 1994). In *A. thaliana*, levels of *PorA* mRNA and the POR-I protein decrease rapidly and in parallel in response to light (Figs. 1 and 4). If one assumes that illumination of etiolated seedlings immediately blocks all further transcription of *PorA*, the maximum half-life of this mRNA must be less than 2 h. *PorA* thus belongs to a small group of negatively light-regulated higher plant genes, including monocotyledonous plant *PhyA*, that encode rapidly degraded transcripts (Thompson and White, 1991; Quail, 1994). Reminiscent of *PhyB* mRNA and PHY B, the amounts of *PorB* mRNA and the POR-II protein remain readily detectable after 16 h of illumination. Also, *A. thaliana PorB* but not *PorA* is expressed in green plant tissues (Figs. 8 and 9).

These parallels between the regulation of the *Por* and *Phy* genes and, possibly, the gene products are intriguing for at least two reasons. First, the POR-mediated photoreduction of Pchlide to Chlide and the photoconversion of phytochrome from the Pr to the Pfr form occur simultaneously

during the illumination of etiolated seedlings, and together activate the interconnected processes of plant photomorphogenesis and chloroplast biogenesis (Chory, 1991; Thompson and White, 1991). Second, phytochrome has been shown to negatively regulate transcription of total *Por* mRNA in barley (Apel, 1981; Mösinger et al., 1985). With the genetic tools that are currently available the influence of the various phytochromes on the expression of the *Por* genes should be amenable to study in *A. thaliana*.

Superimposed Programs of Light and Developmental Regulation Control *PorA* and *PorB* Expression

A. thaliana exhibits developmental regulation of *PorA* and *PorB* mRNAs during growth in continuous darkness, continuous illumination, and in dark-adapted seedlings (Figs. 5–7). The regulation of the two *Por* mRNAs is tightly coordinated during etiolated growth from 2 to 7 d but quantitatively diverges during continuous illumination from 2 to 6 d because of the differential effects of light. The expression of *PorA* and *PorB* peaks in concert whether growth takes place skotomorphogenically (at 3 d) or photomorphogenically (at 2 d). Limited developmental regulation of the total amount of *Por* mRNA observed in etiolated barley and dark-adapted oat seedlings (Häuser et al., 1987) qualitatively agrees with the much more dramatic effects seen with the *PorA* and *PorB* mRNAs in *A. thaliana* (Figs. 5 and 7). mRNA from the pea *Por* gene has also recently been shown to accumulate in a developmentally controlled fashion, with light playing a secondary role in regulation of mRNA levels (He et al., 1994). In *A. thaliana*, in contrast, we find strong developmental regulation of both *PorA* and *PorB* superimposed on the parallel negative regulation by light of *PorA*.

Dormant seeds contain neither *PorA* nor *PorB* mRNAs (Fig. 9). After 1 d in the dark or the light, however, imbibed seeds express *PorB* but not *PorA* mRNA (Figs. 5 and 6). *PorA* expression first appears between 1 and 2 d under either dark or light growth conditions, coinciding in each case exactly with seed germination. Why seed imbibition activates *PorB* and *PorA* expression sequentially is under study. Interestingly, PHY B and PHY A, which perform distinct and nonoverlapping functions during *A. thaliana* seed germination, also accumulate sequentially (Shinomura et al., 1994).

We observed a strong midday *PorB* mRNA maximum under LD conditions in *A. thaliana* (Fig. 8). Studies of oat and barley seedlings have shown pronounced diurnal regulation of the levels of total *Por* mRNA (Darrah et al., 1990), POR protein, and enzymatic activity (Griffiths et al., 1985) during or at the end of dark periods of various durations. In agreement with our findings for *PorB* mRNA (Fig. 8), however, young monocotyledonous seedlings typically reaccumulated only small amounts of *Por* mRNA and POR protein during night periods of physiological length (Häuser et al., 1987). Also consistent with our observations for young *A. thaliana* seedlings dark adapted for 2 d (Fig. 7), the amount of oat *Por* mRNA increased dramatically during an extended dark period (Häuser et al., 1987; Darrah et al., 1990).

Coordinated Expression of *PorB* and the *Lhcb1* Genes

Newly synthesized Chl partitions between the PSI and PSII core complexes and their respective LHC antenna polypeptides. The LHC B polypeptide family of PSII binds an estimated 40 to 45% of the total Chl in the chloroplast (Thornber et al., 1991). Although the requirements for Chl and photosynthetic membrane component synthesis differ during greening and in adult plants, expression of *PorB* and *Lhcb1* mRNAs occurs concomitantly in both cases. *PorB* and *Lhcb1* mRNAs accumulate in parallel to Chl during the later stages of the transition from etiolated growth to the light (Fig. 4). Similarly, during continuous illumination *PorB* mRNA displays a maximum at the time of seed germination coordinated with the appearance of high steady-state levels of *Lhcb1* mRNA (Fig. 6).

The biological function, if any, of the pronounced midday maximum in the amount of *A. thaliana PorB* mRNA is not known (Fig. 8). The overall level of mRNA from the *A. thaliana Lhcb1* genes also undergoes a weak diurnal oscillation, and the *Lhcb1*1* and *Lhcb1*2* mRNAs, in fact, cycle strongly with a 25-fold increase at the midday maximum (Millar and Kay, 1991). On the other hand, experiments performed in barley suggest that the coordinated synthesis of LHC B polypeptides and Chl may not be controlled at the level of Pchlide reduction (Beator and Kloppstech, 1993). Whether coordinated diurnal oscillations of *A. thaliana PorB* and *Lhcb1* mRNAs help to fulfill a biological function in co-regulating the supply of Chl and Chl-binding sites remains to be investigated.

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