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Review

Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms

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Abstract

The reduction of protochlorophyllide (Pchlide) is a key regulatory step in the biosynthesis of chlorophyll in phototrophic organisms. Two distinct enzymes catalyze this reduction; a light-dependent NADPH:protochlorophyllide oxidoreductase (POR) and light-independent Pchlide reductase (DPOR). Both enzymes are widely distributed among phototrophic organisms with the exception that only POR is found in angiosperms and only DPOR in anoxygenic photosynthetic bacteria. Consequently, angiosperms become etiolated in the absence of light, since the reduction of Pchlide in angiosperms is solely dependent on POR. In eukaryotic phototrophs, POR is a nuclear-encoded single polypeptide and post-translationally imported into plastids. POR possesses unique features, its light-dependent catalytic activity, accumulation in plastids of dark-grown angiosperms (etioplasts) via binding to its substrate, Pchlide, and cofactor, NADPH, resulting in the formation of prolamellar bodies (PLBs), and rapid degradation after catalysis under subsequent illumination. During the last decade, considerable progress has been made in the study of the gene organization, catalytic mechanism, membrane association, regulation of the gene expression, and physiological function of POR. In this review, we provide a brief overview of DPOR and then summarize the current state of knowledge on the biochemistry and molecular biology of POR mainly in angiosperms. The physiological and evolutional implications of POR are also discussed.

Abbreviations: Chl – chlorophyll; Chlide – chlorophyllide; DHFR – dihydrofolate reductase; DPOR – light-independent protochlorophyllide reductase; LHCB – light-harvesting chlorophyll *a*/*b*-binding protein complex of Photosystem II; LHPP – light-harvesting protochlorophyllide *a*/*b*-binding protein complex; Pchlide – protochlorophyllide; PLBs – prolamellar bodies; POR – light-dependent NADPH:protochlorophyllide oxidoreductase; SSU – small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; ZnPP*a* – zinc-protopheophorbide *a*; ZnPP*b* – zinc-protopheophorbide *b*

Introduction

Angiosperms require light for the biosynthesis of chlorophyll (Chl). Consequently, when germinated in the dark, they exhibit etiolated yellow leaves with a long hypocotyl, folded cotyledons and an apical hook. Ultrastructurally, plastid development is arrested at the stage of the etioplast. Such development in dark-grown etiolated plants is called skotomorphogenesis. Light-dependent NADPH:protochlorophyllide (Pchlide) oxidoreductase (POR, EC 1.3.1.33), which catalyzes the reduction of the double bond in ring D of the porphyrin molecule, is one of the key enzymes in Chl biosynthesis and chloroplast development (Griffiths 1991). It has been reported that POR exhibits several interesting features. First, its catalytic

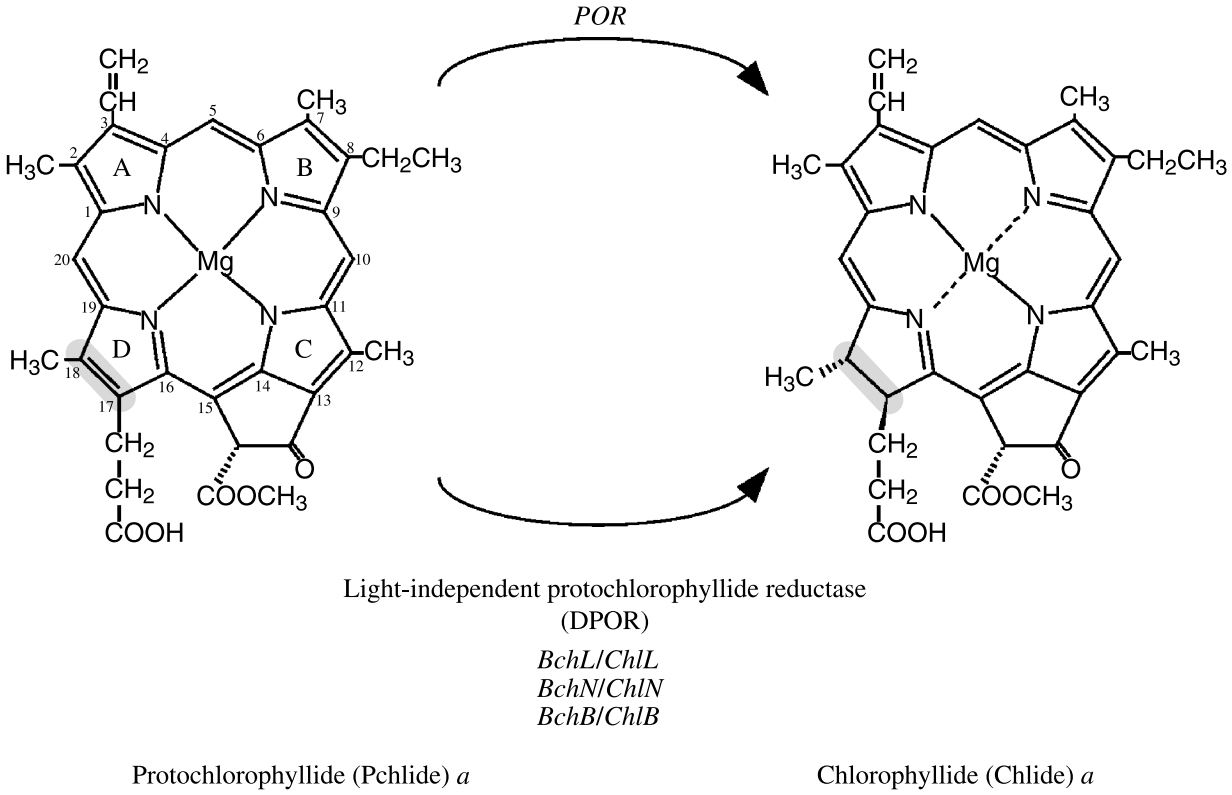

Figure 1. The reduction of Pchlide in the biosynthesis of Chl. The double bond of ring D of Pchlide (C17–C18, indicated by shade in Pchlide and Chlide) is reduced by two different enzymes: light-dependent enzyme POR and light-independent DPOR.

activity directly depends on light (Griffiths 1975, 1978; Apel et al. 1980). Second, in angiosperms, POR protein accumulates to high levels when seedlings are grown in the dark, whereas light-grown seedlings contain only trace levels of POR (Apel 1981; Batschauer and Apel 1984; Mosinger et al. 1985; Benli et al. 1991; Forreiter et al. 1991). The POR proteins that accumulates in the dark binds to the substrate, Pchlide, and cofactor, NADPH, to form aggregates, prolamellar bodies (PLBs), in etioplasts. PLBs have a highly regular, latticed structure, and extrude prothylakoids. Third, most of the POR is rapidly inactivated and subsequently degraded after catalysis (Hauser et al. 1984; Forreiter et al. 1991). Once the etiolated seedlings are illuminated, light-dependent reduction of Pchlide occurs, Chls are synthesized in parallel with the breakdown of PLBs, and chloroplasts are formed. Morphologically, greening and leaf expansion start and hypocotyl elongation stops. These phenomena are collectively called photomorphogenesis.

In contrast to angiosperms, most other photosynthetic organisms such as gymnosperms, algae, cyanobacteria and photosynthetic bacteria, have the ability to synthesize Chl in the dark as well as in the light, since they have another type of light-independent Pchlide reduction system (Figure 1). This light-independent protochlorophyllide reductase (DPOR), which also catalyses the reduction of the double bound in the Pchlide ring D, is similar functionally to POR, but differs completely in molecular structure, subunit composition, genomic coding and catalytic mechanism. The absence of DPOR in the Chl biosynthetic pathway makes the etiolated seedlings of angiosperms turn yellow when germinated in the dark.

POR has been characterized based on its light-dependent catalysis, the formation of PLBs in etioplasts, and unique fluorescence spectra that allow investigators to trace intermediates in the reaction. Moreover, recent molecular biological analysis has revealed multiple *POR* genes that differ in the pattern

of their expression. During the last decade, significant progress in our understanding of the regulation, function, and enzymatic mechanism of POR has been made. In this review, we have focused on recent advances in research into the regulation and functional significance of POR in angiosperms. Earlier studies on POR have been reviewed elsewhere (Griffiths 1991; Ryberg and Sundqvist 1991; Schulz and Senger 1993; Lebedev and Timko 1998; Aronsson et al. 2003b; Rüdiger 2003). The current status of research on DPOR has been reviewed in Timko (1998), Fujita (1996), Bauer et al. (1993), Armstrong (1998), and Fujita and Bauer (2003). Papers describing the overall or specific steps in the biosynthesis of Chl were reviewed by von Wettstein et al. (1995), Reinbothe and Reinbothe (1996), Porra (1997), Rüdiger (1997), Thomas (1997), Beale (1999) and Willows (2003). Here, we provide a brief overview of DPOR, and describe the molecular mechanism of the POR reaction, import and assembly in plastid membrane, regulation of expression and evolutionary aspects of POR.

Brief overview of the light-independent protochlorophyllide reductase

Many gymnosperms have the ability to synthesize Chl in the dark indicating the existence of a light-independent mechanism for Pchlide reduction. Although the dark-operative Pchlide reductase had also been presumed to provide the molecular basis for light-independent greening in lower plants, algae, and bacteria, features of the dark-enzyme had remained unknown until the last decade when significant advances were made. A major advance in the characterization of the dark-enzyme, DPOR, was made possible through the analysis of mutants of *Rhodobacter* species. *Rhodobacter capsulatus* and *R. sphaeroides*, both of which are facultative anoxygenic phototrophs, have been subjected to genetic screening to characterize mutants deficient in various steps in bacteriochlorophyll biosynthesis (Bauer et al. 1993). In studies of the pigment-deficient mutants, it was identified that three genetic loci, known as *bchL*, *bchB* and *bchN*, were required for light-independent Pchlide reduction in a photosynthetic gene cluster in both photosynthetic bacteria (Zsebo and Hearst 1984; Coomber et al. 1990; Yang and Bauer 1990; Burke et al. 1993a; Bollivar et al. 1994). One of the open reading frames (ORF) identified was previously found to encode a predicted protein homologous to a portion of the eubacterial NifH nitrogenase subunit (Hearst et al. 1985).

Independently, the ORF homologous to *bchL*, initially termed *frxC*, was identified in the chloroplast genome of liverwort (*Marchantia polymorpha*) (Ohyama et al. 1986) as a homologue of the Fe protein of nitrogenase that is distributed among a limited but diverse group of prokaryotes (Kohchi et al. 1988), but not in the chloroplast genome of tobacco (Shinozaki et al. 1986). The function of this ORF was revealed by targeted mutagenesis using a cyanobacterium, *Plectonema boryanum* (Fujita et al. 1992), and a green algae, *Chlamydomonas reinhardtii* (Suzuki and Bauer 1992). Both *frxC*-disrupted mutants showed a common phenotype with mutant cells able to synthesize Chl in the light but not in the dark. This phenotype clearly indicated that the protein encoded by *frxC* is involved in the light-independent Pchlide reduction and provided the surprising observation that DPOR has significant sequence similarity to the well-characterized nitrogenase enzyme that catalyzes the reduction of dinitrogen. The *frxC* gene was renamed *chlL* after the naming of *bchL* (Suzuki and Bauer 1992). In *C. reinhardtii*, the second DPOR gene *chlN* was identified in a chloroplast genome by complementation of a '*yellow-in-the-dark*' mutant (Choquet et al. 1992). The cyanobacterial *chlN* gene was found in a gene fragment just downstream of the *chlL* gene (Fujita et al. 1993). The third gene *chlB* was cloned based on homology to *bchB* and confirmed to be involved in the light-independent reduction of Pchlide by targeted mutagenesis (Li et al. 1993; Liu et al. 1993). The *chlB* gene was also cloned from *P. boryanum* (Fujita et al. 1996). Since *chlL*, *chlN*, and *chlB* homologs were identified in other oxygenic phototrophic organisms such as pine and ginkgo (Lindholm and Gustafsson 1991; Richard et al. 1994), it was strongly suggested that DPOR consists of three subunits similar to nitrogenase, and is an ancient enzyme that has been retained among a broad group of photosynthetic organisms from bacteria to gymnosperms, while angiosperms having appeared to have lost this enzyme (Burke et al. 1993b; Suzuki and Bauer 1995). Fujita and Bauer (2000) demonstrated this hypothesis by reconstitution of the activity of light-independent Pchlide reduction *in vitro* with recombinant *bchL*, *bchN*, and *bchB* proteins from *R. capsulatus*. The reconstituted DPOR enzyme showed nitrogenase-like features such as the requirement of ATP and thiol reductant for activity and extreme sensitivity to molecular oxygen. The requirements for ATP (energy)

and thiol reductant (reducing power) correspond to the requirements for light (and NADPH) and NADPH in the case of POR, respectively (see below). Considering the nitrogenase-like features of DPOR, the question arose as to how such an oxygen-sensitive enzyme operates in oxygenic phototrophs. In cyanobacteria, *Synechocystis* sp. PCC 6803, (Wu and Vermaas 1995) and *P. boryanum* (Fujita et al. 1998), *chlL*-disrupted (DPOR less) mutants have been constructed and characterized. DPOR contributes to Chl synthesis in cells growing in light, suggesting that DPOR is tolerant of oxygen to some extent or has a mechanism of protection from the oxygen evolved in oxygenic phototrophs. The extent of the contribution by DPOR decreases with increasing light intensity, suggesting inefficient catalysis when oxygen evolution is maximal (Fujita et al. 1998). Using the *chlL*-lacking mutant of *P. boryanum*, the distribution of preexisting Chl in both photosystems during dark adaptation was examined. It was suggested that the pre-existing Chl molecules in the periphery of Photosystem I could be released and re-distributed for Photosystem II biosynthesis during the dark 'etiolated' process (Kada et al. 2003).

Organization of the *POR* gene family

Gene identification

Until its discovery in cyanobacteria (Suzuki and Bauer 1995), POR had been thought to be distributed only among angiosperms and consequently to have evolved among eukaryotic phototrophs. Now, it is generally accepted that POR evolved from cyanobacteria and is a universal enzyme responsible for light-dependent Chl biosynthesis with a high degree of sequence similarity and ubiquitously distributed among most oxygenic photosynthetic organisms ranging from cyanobacteria to land plants (Fujita 1996; Armstrong 1998). The only organisms that appear to lack POR are the non-oxygen evolving photosynthetic bacteria that contain the most ancient photosystems. Thus, the evolution of POR seems to be closely related to the evolution of oxygenic photosynthesis (Reinbothe et al. 1996a).

Until now, it was considered that only a single POR-encoding gene is present in the cyanobacteria *Synechocystis* sp. PCC 6803 (Suzuki and Bauer 1995), *Phormidium laminosum* (Rowe and Griffiths 1995) and *P. boryanum* (Fujita et al. 1998), green alga *C. reinhardtii* (Li and Timko 1996), amoeboglagellate alga *Bigelowiella natans* (Archibald et al. 2003), and moss, liverwort (*Marchantia paleacea* var. *diptera*) (Takio et al. 1998). In fact, only one *POR* gene has been identified in cyanobacteria in which the complete genome sequence has been determined (Kaneko et al. 1996, 2001; Nakamura et al. 2002; Dufresne et al. 2003) (Table 1). In gymnosperms, PORs of loblolly pine (*Pinus taeda* L.) are encoded by a large multigene family, composed of two distinct subfamilies encoding *PORA* and *PORB* genes similar to those described in angiosperms (see below). The *PORA* subfamily has two members, and the *PORB* subfamily contains at least 11 potential members (Skinner and Timko 1998).

In angiosperms, initially, single cDNAs and/or genes were isolated 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* (Benli et al. 1991), and pea (Spano et al. 1992). In general, the expression of the *POR* gene showed a rapid and marked negative regulation after the illumination of etiolated seedlings, although such effects on the levels of *POR* mRNA varied depending upon plant species ranging from strict negative regulation to virtually no effect. The negative effects on the expression of *POR* are paradoxical, since plants require freshly synthesized Chl molecules throughout their lifetime to meet the demands of growth and pigment turnover. To address the puzzling absence of light-dependent POR during the period of the greatest demand for Chl biosynthesis, reports of multiple immunoreactive polypeptides led to the identification of two light-regulated isoforms, PORA and PORB, in *Arabidopsis* (Armstrong et al. 1995) and barley (Holtorf et al. 1995). In both plants, PORA and PORB are highly identical except in the N-terminal region that contains structural features such as chloroplast transit peptides, but their profiles of gene expression were strikingly different. Both *POR* mRNAs are formed in etiolated seedlings but only *PORB* mRNA continues to accumulate in light-grown plants, while *PORA* mRNA rapidly disappears after illumination. These results suggest that PORB operates throughout the greening process and in light-adapted mature plants, while PORA is active only in etiolated seedlings at the beginning of illumination. By analogy of the profiles of expression of *PORA* and *PORB* in both plants, it has been proposed that the existence of two differentially regulated *POR* genes may be a general phenomenon among angiosperms, and variations in the ratio of *PORA*- and *PORB*-type mRNAs in etiolated seedling could account for the observed species

Table 1. POR genes in various phototrophic organisms

Phylum	Class	Order/family	Species	Gene annotation[a]	Accession no.[b] (ORF ID)	Expression in dark-grown cells	Light-response during greening	Expression in mature cells	Reference/ comments
Cyanobacteria	Prochlorophytes	Prochlorococcaceae	*Prochlorococcus marinus* CCMP1375	por	AE017162 (Pro0543)				Dufresne et al. (2003)
			P. marinus MED4	por, pcr	BX572091 (PMM0542)				Prochlorococcus genome consortium
			P. marinus MIT9313	pcr	BX572098 (PMT1218)				Prochlorococcus genome consortium
	Cyanophyceae	Chroococcales	*Gloeobacter violaceus* PCC 7421	por	(glr2486)				CyanoBase[b]
			Synechocystis sp. PCC 6803	por	L37783 (slr0506)				Suzuki and Bauer (1995), CyanoBase[c]
			Synechococcus sp. PCC 7942	por	U30252 (SEA0022)				
			Synechococcus sp. WH8102	pcr, por	BX569693 (SYNW1726)				Synechococcus genome consortium
			Thermosynechococcus elongatus BP-1		AP005370 (tlr0575)				Nakamura et al. (2002), CyanoBase[c]
		Nostocales	*Anabaena* sp. PCC 7120	por	AP003587 (all1743)				Kaneko et al. (2001), CyanoBase[c]
		Oscillatoriales	*Plectonema boryanum*	por	AB005556				Fujita et al. (1998)
Chlorophyta	Chlorophyceae	Chlamydomonadales	*Chlamydomonas reinhardtii*	lpcr	U36752	High	Negative		Li and Timko (1996)
Chlorarachniophyta	Chlorarachniophyceae	Chlororarachiales	*Bigelowiella natans*		AY267660				Archibald et al. (2003)
Bryophyta	Hepatiocopsida	Marchantiales	*Marchantia paleacea* (liverwort)		AB007321	Undetectable	Positive	Yes	Takio et al. (1998)
Spermatophyta	Coniferopsida	Coniferales	*Pinus mugo* (mountain pine)	POR pPm1	S63824	High	Negative		Forreiter and Apel (1993)
				POR pPm2	S63825 (partial)	High	Constitutive		Forreiter and Apel (1993)
			P. taeda (loblolly pine)	porA	X66727	High	Positive		Spano et al. (1992)
				porB	AF027350 (partial)	High	Positive		Skinner and Timko (1998)
			P. strobes (white pine)	porA	AF027356 (partial)				Spano et al. (1992)
				lpcr/porB	AF027355 (partial)				Spano et al. (1992)
Anthophyta	Dicotyledonopsida	Capparales	*Arabidopsis thaliana*	PORA	U29699	High	Dramatically negative	Very low	Armstrong et al. (1995)
				PORB	U29785	High	Negative–constitutive	Yes	Benli et al. (1991)
				PORC	AB035746	Undetectable	Positive	Yes	Oosawa et al. (2000)

Table 1. Continued

Phylum	Class	Order/family	Species	Gene annotation[a]	Accession no.[b] (ORF ID)	Expression in dark-grown cells	Light-response during greening	Expression in mature cells	Reference/ comments
		Fabales	*Pisum sativum* (pea)	pcr	X63060	Medium	Constitutive		Spano et al. (1982)
			Vigna radiata	CipPor	AF279251	High	Constitutive	Yes	
		Solanales	*Nicotiana tabacum* (tobacco)	POR1	AB074570	High	Positive	Yes	Masuda et al. (2003)
				POR2	AB074571	Medium	Positive	Yes	Masuda et al. (2003)
			Lycopersicon esculentum (tomato)	POR1	AF243520 (partial)				
				POR2	AF243522 (partial)				
				POR3	AF243524 (partial)				
		Cucurbitales	*Cucumis sativus* (cucumber)	NPR	D50085	Low	Positive	Yes	Kuroda et al. (1995)
		Apiales	*Daucus carota* (carrot)	POR1	AF207691				
		Asterales	*Lactuca sativa* (lettuce)		AF162207				
		Caryophyllales	*Amaranthus tricolor*	POR1	AB050122 (partial)				Iwamoto et al. (2001)
				POR2	AB050124 (partial)				
	Monocotyledonopsida	Poales	*Triticum aestivium* (wheat)		X76532	High	Negative		Teakle and Griffiths (1993)
			Hordeum vulgare (barley)	PORA (PCR)	X15869	Very high	Dramatically negative	Undetectable	Schulz et al. (1989)
				PORB	X84738	High	Constitutive	Yes	Holtorf et al. (1995)
			Avena sativa (oat)		X17067	High	Negative		Darrah et al. (1990)
			Orysa sativa (rice)		AL606456				The rice full-length cDNA consortium
					AE017109				The rice full-length cDNA consortium
			Zea mays (corn)		AY103784				Maize mapping project
					AY106314				Maize mapping project

[a] Gene annotation is based on the annotation in the GenBank database or gene name appeared in the reference.
[b] (partial), only partial sequence is reported.
[c] Sequence was obtained from CyanoBase (www.kazusa.or.jp/cyano/).

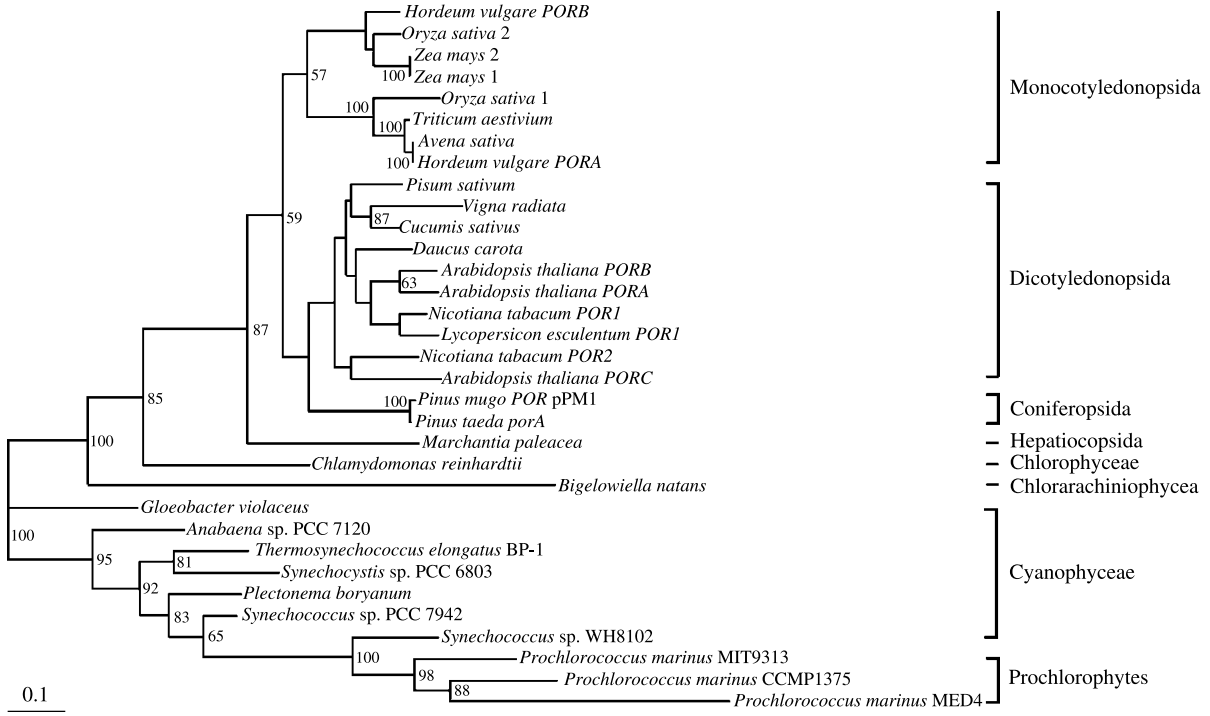

Figure 2. Phylogenetic relationship of the POR protein family. A phylogenetic tree was constructed by the maximum likelihood method using the program phyml (www.lirmm.fr/∼guindon/phyml.html) with a Dayhoff amino-acid substitution matrix. Numbers at the branch points represent the bootstrap values (>50%) of 100 replicate trees. Scale bars indicate the expected number of amino acid substitutions per site. The phylogeny derived using neighbor-joining analysis showed a similar topology at the basal node.

difference in the responses of the *POR* transcripts to light (Armstrong et al. 1995; Reinbothe et al. 1995a). Furthermore, it was suggested that PORA and PORB can not substitute for each other and have distinct functions.

The identification of a third POR isoform, PORC, from *Arabidopsis* (Oosawa et al. 2000), however, clearly ruled out the hypothesis that Chl biosynthesis and chloroplast biogenesis in angiosperms are generally controlled by two unique functions of POR isoforms. In etiolated seedlings, the *PORC* transcript was undetectable, but increased after illumination (Oosawa et al. 2000; Su et al. 2001). In addition, exceptions to the gene organization and light effects on the expression of the two *POR* genes have been reported in some plant species. In cucumber (*C. sativus* L.), levels of POR protein and mRNA increased in etiolated cotyledons when they were continuously illuminated (Kuroda et al. 1995; Yoshida et al. 1995) via transcriptional activation (Fusada et al. 2000). The light-induced expression of cucumber *POR* persisted in mature leaves of cucumber, the profile of which is well consistent with that of Chl accumulation (Kuroda et al. 2000). Extensive screening of the cucumber genome and cDNA libraries indicated that the cucumber *POR* is encoded by a single gene (Fusada et al. 2000). The presence of a single *POR* gene is also reported in pea, although two distinct polypeptides were detected using anti-POR antiserum (Spano et al. 1992; He et al. 1994; Sundqvist and Dahlin 1997). In tobacco, two POR isoforms have been isolated, the expression of which was not negatively regulated by light and persisted in matured green tissues. From the profiles of gene expression and similarity of deduced amino acid sequences, these isoforms were not possible to be categorized into the PORA- and PORB-type (Masuda et al. 2002). Phylogenetic analysis of the POR family showed that the proteins could basically be divided into the following groups: cyanobacteria, green algae, moss, monocotyledonous plants, gymnosperms, and dicotyledonous plants, with POR isoforms of the same plant closely related (Figure 2). Furthermore, no phylogenetic relationship was observed between the PORA and PORB isoforms of *Arabidopsis* and barley, indicating each isoform originated from an independent gene duplication event in individual plants

(Oosawa et al. 2000; Masuda et al. 2002). Table 1 summarizes the distribution of POR in various plant species found in databases, together with the expression profiles in etiolated and greening seedlings, and mature cells. As shown in this table and Figure 2, from cyanobacteria to higher plants, *POR* genes are singly or multiply encoded in the genome and phylogenetically divided into six groups, cyanobacteria, green algae, moss, monocotyledonous plants, gymnosperms, and dicotyledonous plants.

Structure and enzymology of POR

Structural characteristics of POR

The nuclear-encoded POR is translated in the cytoplasm as a higher molecular weight precursor and transported across the plastid envelope. Teakle and Griffiths (1993) showed that the wheat POR was translated as a 41-kDa precursor protein and imported *in vitro* into isolated wheat chloroplasts. The transit peptide was cleaved by a stromal-processing peptidase, resulting in an approximately 36-kDa mature protein. Comparisons of the amino acid sequences of POR with other sequences in the database have indicated that it is a short-chain alcohol dehydrogenase family (Baker 1994; Wilks and Timko 1995), which is a member of the 'RED' superfamily of enzymes (*r*eductases, *e*pimerases, *d*ehydrogenases). Complementation assays of Pchlide reduction mutants in the photosynthetic bacterium *R. sphaeroides*, revealed that highly conserved Tyr and Lys residues among short-chain alcohol dehydrogenase appeared to be essential for POR activity. It has been suggested that the conserved Tyr donates a proton to the C-18 position with the Lys residue thought to be important for lowering the pKa of Tyr, allowing deprotonation to occur (Wilks and Timko 1995). A secondary structure consisting of a central β-sheet built of seven β-strands and surrounded by nine α-helices was proposed by Dahlin et al. (1999) and Townley et al. (2001) as shown for the group of short-chain alcohol dehydrogenase (Birve et al. 1996). An unique feature of POR within the group is the presence of a large extra loop region between the 5th and 6th β-sheets. This hydrophobic loop-region was predicted to be involved in membrane anchoring (Birve et al. 1996) or protein complex formation (Reinbothe et al. 2003b) (see below). The model showed characteristics appropriate to a globular, soluble protein, the structure of which has a typical NAD(P)-binding Rossmann-fold providing a cofactor binding pocket. Charged-to-alanine scanning mutagenesis led to the identification of several domains within the enzyme that are required for catalytic activity and might be important in POR–Pchlide interaction. Mutations in predicted α-helical regions of the protein showed the least effect on enzyme activity, whereas mutations in predicted β-sheet regions of the protein showed a consistent adverse effect on enzyme function (Dahlin et al. 1999). Modeling of the ternary complex of POR, NADPH and Pchlide identified certain key residues involved in the binding of NADPH. The highly conserved Tyr and Lys catalytic motif is on the 6th α-helix (α-F), which forms a side of this cleft where the side chains of Tyr and Lys can interact with the substrate. Replacement of these residues by site-directed mutagenesis gave results consistent with this prediction, as did chemical modification of the generic types (Arg and Cys). A single buried Trp in the model was considered to be responsible for most of the fluorescence shown by the protein and this signal could be used to measure NADPH-binding affinity (Townley et al. 2001). The role of the conserved Tyr and Lys residues in the enzyme molecular organization and catalytic activity were investigated with highly purified recombinant pea POR by site-directed mutagenesis, and suggested that Tyr and Lys are responsible for coordinating the substrate and cofactor in the proper orientation in the enzyme. Their substitution does not prevent the enzyme from assessing the binding substrate and cofactor, but modifies the interaction as part of the POR–NADPH–Pchlide ternary complex and considerably suppresses the enzyme's active state (Lebedev et al. 2001). Meanwhile, site-directed mutagenized *Synechocystis* POR showed a significantly higher K_d value for NADPH binding to the K193R mutant than the wild-type enzyme, suggesting that the affinity for NADPH has also been reduced (Heyes and Hunter 2002). Structural domains important for the POR enzyme activity were also confirmed in pea POR heterologously expressed in the *R. capsulatus* complementation system (Lebedev and Timko 2002).

Thus, it can be concluded that POR is a member of the 'RED' subfamily that conserves Tyr and Lys residues among short chain alcohol dehydrogenase. These residues are essential for coordinating the substrate and NADPH in the enzyme.

Catalytic mechanism of POR

Considerable progress has been made in the identification of the POR structure/function relationship by analysis of the enzyme catalytic activity in solution.

Table 2. Kinetic parameters of POR

Source	Method	Km for Pchlide *a* (μm)	Kd for Pchlide *a* (μm)	Km for NADPH (μm)	Kd for NADPH (μm)	V_{max}	Reference
Hordeum vulgare (barley)	Isolated etioplast membrane	0.46		35			Griffiths (1978)
S. oliquus	Cell-free crude extract from the *yellow in the dark* mutant C-2A′	0.19		4.2		5.9 pmol s^{-1} (for NADPH) 6.5 pmol s^{-1} (for Pchlide)	Urbig et al. (1995)
Avena sativa (oat)	Purified from etioplasts	0.47				20.9 nmol min^{-1} mg^{-1}	Klement et al. (1999)
Pisum sativum (pea)	Overexpressed in *E. coli* as a fusion with maltose-binding protein	0.27		8.7		20.6 nmol min^{-1} mg^{-1}	Martin et al. (1997)
P. sativum (pea)	Overexpressed in *E. coli* as a fusion with maltose-binding protein-His6, followed by cleaving of these regions	0.18	0.030	11.0	21.8		Lebedev et al. (2001)
Synechocystis sp. PCC 6803	Overexpressed in *E. coli* as a His6-tagged protein	8.6		0.012	0.011	309 nmol min^{-1} mg^{-1} (in the presence of 3 μM NADPH) 270 nmol min^{-1} mg^{-1} (in the presence of 14.2 μm Pchlide)	Heyes et al. (2000)
			7.7				Heyes et al. (2002)
Synechocystis sp. PCC 6803	Overexpressed in *E. coli* as a fusion with maltose-binding protein			10	7.4		Townley et al. (2001)

Using either recombinant enzyme expressed in *Escherichia coli* and purified to apparent homology (Martin et al. 1997; Townley et al. 1998; Lebedev and Timko 1999; Heyes et al. 2000; Lebedev et al. 2001; Townley et al. 2001; Heyes and Hunter 2002; Heyes et al. 2002; Lebedev and Timko 2002; Heyes et al. 2003a, b) or pure protein isolated from etiolated plants (Klement et al. 1999) or *Scendesmus oliquus* (Urbig et al. 1995), a photoactive catalytic complex was reconstructed from the enzyme, substrate, and cofactor and detailed spectroscopic and kinetic analyses of the reaction were performed. Table 2 summarizes some parameters of PORs from various sources. Analysis of the dependency of the reaction on substrate and cofactor concentrations and light intensity has shown that one pigment and one NADPH molecule participate in the formation of the photoactive reaction complex and the reaction proceeds through a single-quantum mechanism (Griffiths et al. 1996; Lebedev and Timko 1999).

By using Triton X-100 solubilized PLBs from wheat etioplasts as a source, the substrate specificity of POR was determined. The isolated POR can photoreduce Pchlide *a* and zinc protopheophorbide *b* but not protochlorophylls *a* and *b* (Schoch et al. 1995) and Pchlide *a*' or any compound with substituents at C-13^2 different from Pchlide *a* (Helfrich et al. 1996). Chl c_1, the chemical structure of which is identical with Pchlide *a* except for a double bond in the side chain of C-17, is a competitive inhibitor for POR, tested with Pchlide *a* and zinc protopheophorbide *a* as substrates (Helfrich et al. 2003). The substrate specificity of pigment-free POR from etioplast of *A. sativa* seedlings was further determined. Among 13 analogues of Pchlide *a*, six analogues with different side chains at rings A or B are active substrates, while seven analogues with different side chains at ring D or E are not accepted as substrates by POR. The purified POR accepts Pchlide *a* and Pchlide *b* as substrates with almost identical high reaction rates and similar K_m values (Klement et al. 1999). The purified POR was used to reconstitute photoactive Pchlide *in vitro*. Together with the POR apoenzyme with NADPH and the zinc analog of Pchlide *a*, addition of plastid lipids and high concentrations of glycerol were required for formation of the photoactive long-wavelength complex (Klement et al. 2000).

The enzymatic steps in the photoreduction, involving Pchlide binding and an initial photochemical reaction, have been studied *in vitro* using low-temperature fluorescence and absorbance measurements. Using purified recombinant barley PORB protein fused to maltose-binding protein, Lebedev and Timko (1999) showed that the Pchlide reduction reaction consists of two steps, one photochemical and the other non-photochemical. The initial photochemical reaction follows a single quantum mechanism and leads to the formation of an unstable intermediate with a fluorescence maximum at 682 nm (F682) with a mixed pigment electronic structure and an electron paramagnetic resonance spectrum that suggests the presence of a free radical. The second step involves the spontaneous conversion of the unstable intermediate into Chlide as defined by its spectroscopic characteristics and migration on an HPLC column. Both steps of the reaction can be performed at subzero temperatures in frozen samples, suggesting that they do not include major changes in enzyme conformation or pigment rearrangement within the active site. Meanwhile, Heyes et al. (2000, 2002, 2003b) reported a series of works about the reaction mechanism using purified recombinant *Synechocystis* POR protein with a hexahistidine tag at the N-terminus. The authors reported that the first step in the reaction upon illumination is the formation of a non-fluorescent intermediate with a broad absorbance band at 696 nm (A696) that is suggested to represent an ion radical complex. The intermediate does not form when $NADP^+$ is included in place of NADPH, confirming that this initial photochemical step will only proceed if Pchlide is in a ternary complex with POR and NADPH. The temperature dependence of the rate of A696 formation has allowed the activation energy for the photochemical step to be 18.8 kJ mol^{-1} with 21% quantum efficiency. This step is followed by non-photochemical or 'dark' events prior to Chlide formation in which catalysis is assisted by thermally excited protein dynamics (Heyes et al. 2002). The first of these involves the conversion of the non-fluorescent A696 into a new intermediate that has an absorbance maximum at 681 nm and a fluorescence peak at 684 nm (A681, F684), which may correspond to the identified intermediate F682 (Lebedev and Timko 1999; Lebedev et al. 2001). The authors presumed this intermediate represented a POR-$NADP^+$–Chlide complex that can be formed directly from the non-fluorescent state. During the dark step, this species gradually shifts to yield the product, Chlide. The measurement of temperature dependence for each of these two processes showed that these steps could only occur close to or above the 'glass transition' temperature of proteins, suggesting that domain movements and/or reorganization of the protein

are required for these stages of the catalytic mechanism (Heyes et al. 2003b). Results from two groups are somewhat inconsistent in terms of the identification of the first intermediate, the determined cofactor dissociation constants, changes in the fluorescence yield of Pchlide by binding protein, and the role of two conserved Tyr and Lys residues in cofactor binding (Lebedev and Timko 1999; Heyes et al. 2000, 2002, 2003b; Lebedev et al. 2001; Heyes and Hunter 2002). The reason for these differences is not clear, but might reflect the origin of the protein, the presence of fusion peptides on the recombinant enzyme, the presence of detergent miscelles in the assay solution, or the level of photoconversion activity of obtained POR protein. In any case, however, the catalytic reaction by POR is basically composed of two reactions, one of which is a photochemical and a single quantum one, and the other is non-photochemical one to form POR-$NADP^+$–Chlide complex.

It had been proposed that hydride is transferred from the *pro-S* face of NADPH, *trans* to the C-17 position of Pchlide, together with the addition of H^+ to position C-18, forming Chlide. Recently, the ultrafast reaction dynamics in POR were analyzed in real-time after initiation of catalysis with a 50-fs laser pulse. Comparison of wild-type and a site-directed mutant of POR in which the conserved Tyr189 has been replaced with Phe indicated that hydride transfer from NADPH to Pchlide can still occur in the mutant, but cannot donate proton to Pchlide. It was shown that two different catalytic mechanisms, involving proton and hydride transfers proceeded with two different reaction rates: a concerted mechanism; where C17–C18 double bonds of Pchlide are broken in the transition state and the proton and hydride are simultaneously transferred in a single 3-ps step, and a sequential mechanism; involving a 3-ps proton transfer followed by a 'slow' 400-ps hydride transfer that produces Chlide. Together with the data of low-temperature spectroscopic measurements, the authors concluded that enzymatic reactions can involve molecular motions on a picosecond time scale (Heyes et al. 2003a).

Pchlide phototransformation

A number of spectroscopic forms of Pchlide and various reaction intermediates have been identified in etioplast membranes (Böddi and Franck 1997; Böddi et al. 1998, 2003; Schoefs et al. 2000a; Skribanek et al. 2000). At least three different spectral forms of Pchlide are recognized in intact tissues based on their fluorescence emission maximum (in nm): Pchlide F631, F644, and F655. The differences in the spectral properties of the pigments were interpreted as being due to the pigment structural arrangements, association of POR, and localization in PLBs and/or prothylakoids (Böddi et al. 1992, 1993). Fluorescence lifetime of Pchlide measured in plants showed that short- and long-wavelength Pchlide forms have fast ($0.3 \sim 0.8$ ns) and slow ($5.1 \sim 7.1$ ns) components with different proportions depending on plant species (Mysliwa-Kurdziel et al. 2003). Short-wavelength Pchlide forms are thought to be located in the prothylakoids, bound in a monomeric form to proteins other than POR (Böddi et al. 1998; Kis-Petik et al. 1999). The main photoactive form present in etiolated plants is Pchlide F655, which after illumination, is converted to Chlide and subsequently to Chl (F682) through the formation of several, long wavelength intermediates (Böddi and Franck 1997; Schoefs et al. 2000a). After flash illumination, photoactive Pchlide complex can be regenerated by reloading with non-photoactive Pchlide on a fast time-scale with concomitant release of Chlide (Franck et al. 1999; Schoefs et al. 2000b). The consecutive process causes a spectral blue shift, referred to as 'Shibata shift', which results in the appearance of a new form with emission maximum at 680 nm (Shibata 1957). The kinetics of this shift is shown to depend on environmental conditions, such as temperature (Eullaffroy et al. 1995; Böddi et al. 1997) and desiccation (Le Lay et al. 2001). In parallel, a species appears with emission maximum at 676 nm, which is presumes as a 'free' pigment, dissociated from POR (Le Lay et al. 2001), or Chl *a*, resulting from a rapid esterification process (Domanski and Rüdiger 2001). The molecular background of the Shibata shift is still not completely clear, but esterification of Chlide *a*, the disruption of the highly regular membrane structure of PLBs, disaggregation of POR complexes, and conformational changes of POR are proposed to be responsible for this blue shift (Smeller et al. 2003).

In most angiosperms, most of the POR and its substrate localizes specifically to PLBs in etiolated seedlings that are not detected in light-adapted plants. Some or all of the variability in the spectral composition of Pchlide *in vivo* may be a consequence of enzyme ternary complex formation and aggregation within the etioplast and PLBs. However, these processes, such as POR aggregation and PLB formation (Sundqvist and Dahlin 1997), can complicate

Pchlide photoreduction in etioplasts and, as a result, the catalytic mechanism of POR remains largely unresolved. In light-grown plants and the green alga, *C. reinhardtii*, only two Pchlide species, Pchlide F632 and Pchlide F644, were detected, both of which disappeared concomitantly with the appearance of Chlide F675 (Lebedev et al. 1985; Lebedev 1996). In greening barley leaves, however, Franck and Strzalka (1992) detected a photoactive Pchlide–protein complex with absorption and fluorescence maxima at 648 nm and 653 nm without any re-darkening, and suggested that Chl accumulation proceeded through continuous regeneration and phototransformation of the photoactive complex.

Consequently, among three fluorescent spectral forms of Pchlide in intact tissues, Pchlide F655 is the major photoactive form and is converted to Chlide and subsequently to Chl (F682) through several events such as the formation of several intermediates and disaggregation of POR.

Import into chloroplasts

As described before, the nuclear-encoded POR is translated in the cytoplasm as a higher molecular weight precursor and transported across the plastid envelope. Reinbothe et al. (1995b, c) suggested that the import of the precursor of barley PORA (pPORA) into chloroplasts was totally dependent on envelope-bound Pchlide, the substrate for the enzyme's catalytic activity. In barley etioplasts, pPORA was readily imported since the endogenous level of Pchlide met the demand for pPORA translocation. During the light-induced transformation of etioplasts into chloroplasts, the concentration of Pchlide dramatically declined, and chloroplasts rapidly lost the ability to import pPORA. The capacity to import pPORA was restored when the intraplastidic level of Pchlide was raised by incubation with 5-aminolevulinic acid, a common precursor of tetrapyrrole biosynthesis. The binding of pPORA to the envelope is mediated through the transit peptide of pPORA and receptor protein component of the outer envelope membrane in the ATP-dependent manner, and subsequently, the bound pPORA is vectorially translocated into plastid in the Pchlide-dependent manner (Reinbothe et al. 1995b). Exogenously applied Pchlide competitively released the envelope-bound pPORA into the cytosol (Reinbothe et al. 1996c). The transit peptide-dependent binding of pPORA to the envelope occurred even when pPORA formed substrate complexes, such as pPOR–Pchlide or pPOR–Pchlide–NADPH, but such binding was inhibited when chlorophyllide was bound to pPORA by direct addition or enzymatic production (Reinbothe et al. 1995b). The role of the transit peptide of pPORA was further analyzed by production of chimeric constructs, in which the transit sequences of pPORA and pPORB were exchanged and fused to either their cognate mature polypeptides or to a reporter protein of cytosolic dihydrofolate reductase (DHFR) from mouse. The transit peptide of pPORA conferred Pchlide-dependent import onto both the mature PORB and DHFR, while that of pPORB directed the mature PORA and DHFR into chloroplasts even without the endogenous Pchlide (Reinbothe et al. 1997), suggesting that the transit peptide of pPORA directly interacts with Pchlide in the plastid envelope for translocation. The formation of the pPORA–Pchlide complex may be effective for photoprotection by reducing the free form of Pchlide in the envelope of plastids, or for protection against proteolytic degradation (Reinbothe et al. 1996c), and together with transcriptional regulation, such a post-translational mechanism enables abundant PORA to exist in etioplasts (Reinbothe et al. 1995c). The Pchlide-dependent import of the transit peptide of barley pPORA and DHFR chimeric construct was also shown in chloroplasts of tobacco, *Arabidopsis thaliana*, and five other tested monocotyledonous and dicotyledonous plant species (Reinbothe et al. 2000). A competition assay with the precursors of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU) and ferredoxin and antibody-blocking experiment with anti-TOC86 and anti-TOC75 indicated that the import site is specific for pPORA (Reinbothe et al. 2000).

On the contrary, several reports indicated that Pchlide is not strictly required for the import of POR *in vitro* (Teakle and Griffiths 1993; Dahlin et al. 1995; Jarvis et al. 1998; Aronsson et al. 2000; Dahlin et al. 2000; Aronsson et al. 2001b). The import into chloroplasts in a homologous system from *Arabidopsis* (Jarvis et al. 1998; Jarvis and Soll 2001), pea (Dahlin et al. 1995; Aronsson et al. 2000, 2001b, 2003a) and wheat (Teakle and Griffiths 1993) was not dependent on Pchlide. The absence or presence of Pchlide did not significantly affect the capacity to import pPORA and pPORB in barley (Dahlin et al. 1995; Aronsson et al. 2000). A competition assay with pSSU and crosslinking analysis indicated that pPOR used the general import pathway into chloroplasts (Aronsson et al. 2000). Because of these discrepancies, instability

of the protein import receptor and the involvement of a high concentration of urea in the reaction mixtures have been considered (Aronsson et al. 2000). A major protein import receptor, TOC160, is extremely sensitive to proteolysis upon cell lysis, and degrades with a half-life of a few minutes if strict precautions are not taken (Botler et al. 1998). The degradation of TOC160 lowers the import efficiency of pSSU, although the degraded 86-kDa form still supports the import reaction. The import experiments of Reinbothe et al. (2000) were performed with partly denatured pPORA in the presence of 600–800 mM urea in addition to other salts and osmotic compounds, although other protocols for POR import exclude urea (Teakle and Griffiths 1993; Dahlin et al. 1995; Jarvis et al. 1998; Aronsson et al. 2000, 2001b; Dahlin et al. 2000). High concentrations of urea seem to influence chloroplast integrity and import competence. Aronsson et al. (2000, 2003b) proposed that differential functional integrity of the TOC complex might influence the requirement for import of pPOR into plastid *in vitro*. Thus, although the proposed mechanism of Pchlide *a*-dependent import of PORA precursor into plastids might be intriguing, it is generally untenable in view of many opposing arguments.

Assembly and disassembly of POR in plastids

Association of POR protein with the plastid membranes

In etiolated plants, POR accumulates in large aggregates as PLBs within the etioplasts. Some amounts of POR are detected in prothylakoid membranes. According to X-ray diffraction analysis, POR locates asymmetrically in PLBs (Williams et al. 1998). Analysis of the fluorescence and biochemical composition of different etioplast preparations showed that photoactive Pchlide–enzyme complexes with a fluorescence maximum at 655 nm were located mainly in PLBs (Ryberg and Sundqvist 1991) (see also before). A correlation between the formation of PLBs and appearance of Pchlide F655 was also observed *in vivo*, suggesting that the formation of the PLBs and photoactive Pchlide is due to POR aggregation (Lebedev et al. 1995; Younis et al. 1995; McEwen et al. 1996). Phospholylation of POR favors aggregation of POR proteins in etioplasts (Wiktorsson et al. 1996), but the exact role of the phospholylation is obscure (Kovacheva et al. 2000). Identification of the aggregation state of POR in PLBs using different cross-linkers showed that the most prominent product was a dimer of POR, but larger aggregates were also identified (Wiktorsson et al. 1993). Peptides of molecular mass 60 kDa, which might be a dimer of POR, were also immunodetected in *Phormidium* preparations (Rowe and Griffiths 1995). Dimerization of POR was identified *in vitro* with highly purified pea POR expressed as a fusion construct with maltose-binding protein (Martin et al. 1997).

In chloroplasts of light-adapted plants and green algae, POR and Pchlide were detected in stromal thylakoid and envelope membranes. Immunoprecipitation experiments revealed the presence of POR polypeptides in the envelope membrane fraction of mature spinach chloroplasts (Joyard et al. 1990). Pchlide and Chlide *a* were also detected in an envelope fraction from spinach chloroplasts. The presence of POR photoconversion activity was detected when the envelope membranes were illuminated in the presence of NADPH (Pineau et al. 1986, 1993). Fractionation assays followed by *in vitro* POR import into pea chloroplasts showed that the imported protein was targeted to the stromal side of thylakoid membranes as a peripheral protein (Dahlin et al. 1995). No radiolabeled proteins were detected in the stromal compartment or envelope membranes. In *Arabidopsis*, PORB and PORC proteins were immunodetected in both thylakoid and inner envelope membranes (Masuda and Block unpublished). Such a dual localization is observed in other tetrapyrrole biosynthetic enzymes such as ferrochelatase (Suzuki et al. 2002) and Mg-protoporphyrin IX methyltransferase (Block et al. 2002). It is possible that the distribution of POR protein in stromal thylakoid and inner envelope membrane is plant species-dependent.

Assembly reactions performed in chloroplast lysates showed that the maximum amount of radiolabeled protein was associated with thylakoid membranes with a thermolysin-resistant conformation when the assays were performed in the presence of hyrolyzable ATP and NADPH, but not in the presence of NADPH. Assembly assays performed with the mature form of POR showed that the transit peptide was not required for membrane assembly (Dahlin et al. 1995). To determine structural elements within POR potentially responsible for POR association to the membrane, an estimation of the secondary structure of isolated POR based on circular dichroism spectra was performed (Birve et al. 1996). The Rossmann-fold fingerprint motif identical to other NADPH-binding

enzyme was localized in the N-terminal region of POR. It is suggested that POR is anchored to the interfacial region of the membrane by either a β-sheet or an α-helical region containing tryptophan residues. Charge-to-alanine scanning mutagenesis was used on pea POR to substitute charged amino acids with uncharged alanine. Charges in the central region of POR or close to amino acids involved in NADPH or Pchlide binding were essential for the membrane association of POR in a thermolysin-resistant manner. Failure to be properly associated with the thylakoid membranes in a protease-resistant manner was only weakly correlated to a loss the of catalytic function (Dahlin et al. 1999; Aronsson et al. 2001b), showing that the POR–Pchlide–NADPH complex does not need to form before thylakoid association can occur *in vivo*. In general, the replacement of charged amino acids in the most distal parts of the N- and C-terminal regions of the mature protein did not significantly affect membrane association (Dahlin et al. 1999), but the membrane association of POR strongly depends on an intact C-terminus (Aronsson et al. 2001a). POR deletion mutants of pea in the C-terminus (Δ362–400, Δ338–400, Δ315–400 and Δ300–400) were attached to the thylakoid membrane prior to thermolysin treatment. However, except for the Δ396–400 mutant, no C-terminal deletion mutants resisted post-treatment with thermolysin after the thylakoid association reactions, suggesting that amino acids in the range 362–395 are vital for membrane association. The C-terminus may function as a membrane anchor for POR (Aronsson et al. 2001a). An explanation for the diverging results obtained could be that alanine substitutions generally do not affect the protein conformation, whereas a C-terminus deletion alters the conformation of POR and the anchoring ability (Aronsson et al. 2003b). After association, a part of the POR protein is still accessible to thermolysin as this protease produces a 2–4 kDa smaller degradation product (Dahlin et al. 1999; Aronsson et al. 2001a, b; Engdahl et al. 2001). Using *Synechocystis* POR, with a 6-His-tag added to the N-terminus, it was shown that the thermolysin-degraded part was at the C-terminus (Aronsson et al. 2001a). Based on these observations, it is proposed that the POR C-terminus is the most exposed part after the association to the membrane.

POR mutants, containing a Cys-to-Ser substitution (Cys119, Cys281 and Cys308), failed to associate with the thylakoid membranes (Aronsson et al. 2001a). Cys119 is necessary for the NADPH binding of the enzyme (Townley et al. 2001), whereas Cys281 is located within the predicted active site. It was predicted that Cys308 is not located within any of these two important sites (Dahlin et al. 1999). However, it is close to the predicted active site to exert an effect on Pchlide binding and/or reduction. Alternatively, it may very well be required for maintaining the three dimensional structure of the protein (Aronsson et al. 2001a).

Thus, it can be concluded that in etioplast, POR exists as aggregates (probably dimer) to form PLBs, which is composed of POR:Pchlide:NADPH (1:1:1). In chloroplast, POR is located in envelope and thylakoid membrane. Charged amino acids in the range 362–395 are vital for thylakoid membrane association.

Light-harvesting Pchlide a/b-*binding protein complex*

Reinbothe et al. (1999) performed *in vitro* reconstitution experiments with the two barley POR enzymes and synthetic zinc analogues of Pchlide *b* and Pchlide *a*, zinc-protopheophorbide *b* (ZnPP*b*) and zinc-protopheophorbide *a* (ZnPP*a*). These experiments led to the hypothesis of a novel light-harvesting Pchlide *a*/*b*-binding protein complex, named LHPP, and a new proposal for *in vivo* distinct functions of PORA and PORB. The LHPP complex is thought to consist of a 5:1 ratio of the dark-stable ternary complexes of PORA and PORB, which specifically bind to ZnPP*b* and ZnPP*a*, respectively, and are embedded into the lipid bilayers of the PLBs of etioplasts. Only the PORB-bound ZnPP*a* in the LHPP complex appears to be reduced immediately upon illumination, whereas the PORA-bound ZnPP*b* is proposed to function initially as a light-harvesting pigment. Energy transfer from ZnPP*b* to ZnPP*a* is speculated to provide a mechanism for photoprotection during the early stage of seedling greening. Functionally, LHPP has been hypothesized to play major roles in establishing the photosynthetic apparatus, in protecting against photooxidative damage during greening, and in determining etioplast inner membrane architecture (Reinbothe et al. 1999).

The existence of LHPP *in vivo* is controversial in several points, and a criticism has been made by Armstrong et al. (2000). First, according to the proposed LHPP model, Pchlide *b* would be predominant in dark-grown angiosperms rather than Pchlide *a*. Although Pchlide *b* was reported in several green plants (Shedbalkar et al. 1991), it was not detectable in extracts from etiolated seedlings of barley, and the

Pchlide *b* added to etioplast membrane was photoreduced to Chlide *b* by POR (Scheumann et al. 1999). Scheumann et al. (1999) also detected a substantial reduction of exogenously added ZnPP*b* to zinc-7[1]-hydroxy-protopheoporbide *a* in darkness, which is catalyzed by Chl *b* reductase (7-formyl reductase). The second question was the high degree of substrate specificity of barley PORA for ZnPP*b* and of barley PORB for ZnPP*a*. POR proteins solubilized from wheat PLBs (Schoch et al. 1995) and highly purified from oat etioplasts (Klement et al. 1999) did not show such substrate discrimination. In addition, both proteins were able to photoreduce ZnPP*b* or Pchlide *b* *in vitro*, which contradicts the presumed nonphotoactive complex of Pchlide *b*:NADPH:PORA in LHPP. Third, the proposed 5:1 stoichiometry of Pchlide *b*:NADPH:PORA to Pchlide *a*:NADPH:PORB in the LHPP was not consistent with the known population of photoactive and nonphotoactive Pchlide and that of POR isoforms in other plants. Furthermore, it is well documented that an approximately 70% of Pchlide can be phototransformed into Chlide *a* after 1 single msec flash (Kahn 1968; Henningsen et al. 1974; Ryberg and Sundqvist 1991), and this differs much from the figure that 1/5 of Pchlide is phototransformable in consequence of the LHPP model. Consequently, Armstrong et al. (2000) insisted that the following demonstrations are required for proving the existence of LHPP *in vivo*: (1) Etiolated angiosperms contain a large amount of Pchlide *b*; (2) About 85% of all the Pchlide in etioplasts is nonphotoactive; and (3) Enzymatically active complex of LHPP can be isolated from etioplast membranes.

To answer these questions, Reinbothe et al. (2003a–c) reported a series of papers. First, they reported that Pchlide *b* is abundant in barley etioplasts but metabolically unstable, since it is rapidly converted to Pchlide *a* by the activity of 7-formyl reductase, an enzyme involved in the Chl *b* to Chl *a* reaction cycle. During the pigment extraction procedure, plastid lysis and membrane solubilization collectively lead to the denaturation of PLBs, the release of PORA, and the conversion of most Pchlide *b* to Pchlide *a* via 7-hydroxy-Pchlide *a*. They proposed that 7-formyl reductase finely tunes the levels of Pchlide *b* and Pchlide *a*, and thereby may regulate the steady-state level of LHPP (Reinbothe et al. 2003c). Enzymes which may synthesize Pchlide *b* have not been identified, but Chlide *a* oxygenase (Tanaka et al. 1998; Oster et al. 2000) is the most likely candidate. Heterologous expression of the *Arabidopsis* Chlide *a* oxygenase in cyanobacteria leads to Pchlide *b* accumulation (Xu et al. 2001, 2002). However, recent re-examination of pigment extraction from etiolated barley failed to detect Pchlide *b* irrespective of the extraction protocol, and disputed the existence of LHPP complexes in etioplast of barley (Kollosov and Rebeiz 2003). Second, they reported the *in vitro* reconstitution of LHPP complexes from barley PORA and PORB, and chemically synthesized Pchlide *a*, Pchlide *b* and galacto- and sulfo-lipids. Pigment binding characteristics of PORA and PORB were specific to Pchlide *b* and Pchlide *a*, respectively. The reconstituted complex with a size of ∼480 kDa displayed the same characteristics as photoactive Pchlide. Upon illumination, only the PORB-bound Pchlide *a* was photoactive and it was converted to Chlide *a*, whereas Pchlide *b* bound to PORA remained photoinactive. They employed non-aqueous protein and pigment extraction and fractionation to isolate barley etioplasts, and isolated POR–pigment complexes with a similar size, and stoichiometric and photochemical properties to the reconstituted LHPP (Reinbothe et al. 2003a). Third, they reported that using deletion mutants of barley POR protein, the central region of POR polypeptide or so-called 'extra-loop', which distinguishes POR from the structurally related short-chain alcohol dehydrogenase, is dispensable for pigment binding but needed for the assembly of LHPP (Reinbothe et al. 2003b). This extra-loop region was previously predicted to be involved in the membrane-anchoring of POR protein (Birve et al. 1996).

The series of work by Reinbothe et al. (2003a–c) confirmed their proposed structure and properties of LHPP in barley (Reinbothe et al. 1999). However, so far, this model is limited to barley, and it is still difficult to generalize as the central structural determinant of the PLBs in etioplasts of angiosperms. The given 5:1 stoichiometry of PORA to PORB is not consistent with the levels of PORA and PORB mRNA in etiolated seedlings of *Arabidopsis* (Armstrong et al. 1995). Furthermore, the total quantities of either *PORA* or *PORB* mRNA in *Arabidopsis* can be specifically manipulated by their constitutive overexpression or knockout by DNA tagging (Sperling et al. 1997, 1998; Franck et al. 2000; Frick et al. 2003; Masuda et al. 2003). Such studies provide no evidence that alterations in the PORA to PORB ratio, independent of the total quantity of POR, dramatically influence the extent of PLB formation and the photoactive Pchlide-F655. Moreover, cucumber (Fusada et al. 2000) and pea (Spano et al. 1992; Sundqvist and

Dahlin 1997) provide an example of angiosperms that apparently contain only one *POR* gene. Therefore, the *in vivo* data from *Arabidopsis*, cucumber and pea are not consistent with specific functions for PORA and PORB within an LHPP complex of the type postulated for barley. It is possible that the LHPP complex is specific to monocotyledonous plants, since Reinbothe et al. (2003a) found a similar solubilization of PORA during the isolation of etioplast inner membranes from oat and wheat etioplasts. However, the predominance of non-photoactive Pchlide, which is one essential prediction of the LHPP model, is not fulfilled in these plants, since most of the Pchlide was phototransformed by flash illumination of wheat etioplast (Ryberg and Sundqvist 1988), etiolated wheat seedlings (Franck et al. 1999), and oat seedlings (Domanski and Rüdiger 2001). Thus, the distribution and functional significance of LHPP among angiosperms are necessary to be elucidated.

Proteolysis of POR protein

POR is abundant in etioplasts but inactivated after catalysis with concomitant rapid proteolytic degradation after illumination. Reinbothe et al. (1995d) showed that barley pPORA–Pchlide and pPORA–Chlide complexes synthesized from a full-length cDNA clone after *in vitro* transcription and translation differed markedly in sensitivity to proteolytic degradation. The pPORA–Pchlide complex was resistant to protease treatment independent of the presence or absence of NADPH. In contrast, the pPORA–Chlide complex was rapidly degraded. The naked pPORA without its substrates or products was less sensitive to proteolysis than the pPORA–Chlide complex, suggesting that both substrate binding and product formation during catalysis had caused differential changes in protein conformation (Reinbothe et al. 1995d). Such degradation was selective for PORA, and PORB remains operative in both illumination and green plants (Reinbothe et al. 1995a). The PORA-degrading protease is assumed to be a nuclear-encoded, energy-dependent, and plastid-localized protein in barley (Reinbothe et al. 1995d). Its activity is not detectable in etioplasts but is induced during illumination (Reinbothe et al. 1995a). However, Dahlin et al. (2000) examined post-import degradation of radiolabeled barley pPORA and pPORB on incubation with stroma-enriched fractions from etiolated and light-grown barley or wheat, but found no protease activity by any POR protein in any fraction. The reason for this discrepancy is currently unknown.

Regulation of gene expression by various factors

As described before, in angiosperms, the expression level of PORA mRNA is high in etiolated seedlings and rapidly reduced during greening, whereas the expression of PORB mRNA maintains similar levels in both etiolated and greening seedlings. In some cases, such as in *Arabidopsis*, PORC mRNA is not expressed in the etiolated seedlings but is induced on illumination. The expression of POR mRNAs is regulated by various factors as described below.

mRNA stability

The existence of a post-transcriptional mechanism that contributes to the light-induced decline of barley *PORA* mRNA steady-state levels is suggested by measuring the degradation kinetics of the *PORA* mRNA after inhibiting RNA synthesis with a transcription inhibitor, cordycepin. The *PORA* mRNA was inherently unstable, whereas the presence of cordycepin stabilized the *PORB* mRNA, suggesting that the mechanism of degradation of *PORB* mRNA is different from that of *PORA* mRNA. A plant-specific DST element in the 3′ untranslated region of transcripts was postulated that conferred *PORA* mRNA instability (Holtorf and Apel 1996a), although this motif is not commonly conserved in *POR* genes of other plants. Post-transcriptional regulation of *POR* mRNA is also proposed in liverwort. In the presence of cordycepin, not only incubation in the dark but also addition of DCMU or DBMIB in light stimulated the degradation of the *POR* transcript, suggesting that photosynthetic electron transport is involved in regulating the stability of the *POR* transcript (Eguchi et al. 2002).

Effect of light conditions

For the light-induced changes in the expression of the *POR* genes in angiosperms, the decline in *PORA* mRNA in barley had been shown to be mediated by phytochrome (Batschauer and Apel 1984; Mosinger et al. 1985; Holtorf et al. 1995). Unlike *PORA*, *PORB* in barley was not under the control of phytochrome (Holtorf et al. 1995). In *Arabidopsis*, involvement of phytochrome A in *POR* expression is suggested by experiment with far-red light-illuminated seedlings.

Etiolated seedlings grown under continuous far-red light are unable to green when subsequently transferred to white light. This process is dependent on phytochrome A, and known as far-red block of the greening response (Barnes et al. 1996). In the far-red light-illuminated seedlings of *Arabidopsis*, PORA and PORB transcripts were depleted, indicating expression of both genes is under the control of phytochrome A (Barnes et al. 1996). Similar results were also observed by Runge et al. (1996), but they reported that the effect of continuous far-red light was rather specific to PORA. In addition, constitutive photomorphogenic mutants of *Arabidopsis*, in which certain steps in phytochrome signaling are activated in the dark, lack *PORA* transcript (Lebedev et al. 1995). The involvement of phytochrome is also suggested in the positive light-dependent expression of *POR* in liverwort (Suzuki et al. 2001). In *Arabidopsis*, the *POR* gene expression is also controlled by light intensity. When seedlings were exposed to different light intensities, the amounts of *PORB* mRNA remained the same, while *PORA* and *PORC* mRNAs were modulated in an inverse way by these light intensities. *PORA* mRNA was still detectable in seedlings grown under low light intensities but disappeared at higher light intensities, while the mRNA concentration of *PORC* rose with increasing light intensities (Su et al. 2001). The maintenance of PORA protein under low light and the induction of PORC protein under high light was confirmed in greening seedlings of *Arabidopsis* (Masuda et al. 2003). In loblolly pine, transcripts arising from both gene subfamilies were shown to be present at high levels in the cotyledons of dark-grown seedlings, but the levels of both *PORA* and *PORB* mRNAs increased after exposure to illumination (Skinner and Timko 1999).

There are several reports that indicate POR gene expression is under the control of circadian and diurnal rhythms. In complete darkness, Pchlide accumulation and the capacity for Chl synthesis in leaves of 10–12-days-old etiolated *Phaseolus vulgaris* followed circadian oscillation (Argyroudi-Akoyunoglou and Prombona 1996). In greening barley kept under a diurnal 12 h light/12 h dark cycle, the *PORB* mRNA concentration showed a diurnal rhythm (Holtorf et al. 1995; Holtorf and Apel 1996b). Trace amounts of PORA protein and mRNA continue to reappear transiently at the end of the night period. This PORA might be involved in the synthesis and accumulation of Chl at the beginning of each day (Holtorf and Apel 1996a). In cucumber, *POR* gene expression was controlled by diurnal and circadian oscillations (Kuroda et al. 2000). In *Arabidopsis*, both *PORB* and *PORC* transcripts showed pronounced diurnal rhythmic fluctuations under a 16 h light/8 h dark cycle, but only the *PORB* transcript underwent a circadian oscillation when plants were exposed to continuous illumination (Armstrong et al. 1995; Su et al. 2001). Recent satellite macroarray analysis of tetrapyrrole biosynthetic pathways revealed that in *Arabidopsis*, *PORA* mRNA is also controlled by diurnal and circadian oscillations in mature leaves with an approximately two-magnitude lower level than that of *PORB* (Matsumoto and Masuda unpublished). In tobacco, transcripts of two *POR* isoforms showed diurnal fluctuations with a similar oscillation phase (Masuda et al. 2002). Diurnal and circadian regulation of *POR* gene expression seems to provide a way to adjust the synthesis of the POR polypeptide to varying needs of fully green plants for Chl during the day/night periods.

Thus, it can be concluded that depending on the *POR* genes of plant species, profiles of gene expression varied, such as up-regulation, down-regulation or constitutive on white light illumination in both etiolated and green seedlings. The light regulation is shown to be mediated by a phytochrome pathway. In some cases, *POR* gene expression is controlled under the control of endogenous rhythms.

Effect of phytohormones and developmental stage

The *POR* gene expression is regulated by phytohormones, particularly cytokinins, which are known to promote the greening of etiolated seedlings (Fletcher and McCullagh 1971). In the etiolated cotyledons of cucumber, a synthetic cytokinin, 6-aminobenzyladenine, remarkably increased the level of *POR* transcript (Kuroda et al. 1996). Nuclear transcription analysis and transient reporter assay of a fusion construct of the 5′-promoter region of cucumber *POR* and luciferase showed that the cytokinin-induced increase in the *POR* mRNA is mainly dependent on transcriptional activation, which accompanies *de novo* protein synthesis in the cytoplasm (Kuroda et al. 2001). Abscissic acid had an opposite effect on the *POR* expression (Kuroda et al. 1996). Such a cytokinin- and abscissic acid-dependent control of *POR* gene expression was also observed in etiolated lupine (*Lupinus luteus* L.) (Kusnetsov et al. 1998). The positive effects of cytokinin were also observed in the photomorphogenic *lip1* mutant of pea,

which lacks PLBs in plastids. Treatment with cytokinin, 2-isopentenyladenine restored the formation of PLBs and photoactive Pchlide in dark-grown seedlings, without an increase in the amount of Pchlide or POR (Seyedi et al. 2001a). Adversely, in *Arabidopsis*, treatment with cytokinin of etiolated seedling resulted in partial development of the thylakoid membrane with a concomitant loss of PLBs (Chory et al. 1994). Therefore, cytokinin-controlled regulation of *POR* may be different among plant species.

In *Arabidopsis*, *PORB* and *PORC* are expressed in all photosynthetic organs of mature, light-adapted plants, but not in roots (Armstrong et al. 1995; Oosawa et al. 2000). Such a photosynthetic organ-specific expression of *POR* is also observed in cucumber (Kuroda et al. 1995, 2000). For the developmental control of *POR* gene expression, studies on the effect of age on *POR* gene expression in *Arabidopsis* and barley showed that *PORA* is expressed nearly exclusively in young seedlings and *PORB* is expressed both in seedlings and in adult plants (Armstrong et al. 1995; Schunmann and Ougham 1996). In *Arabidopsis*, the expression of the *PORB* and *PORC* genes in mature green tissues is qualitatively similar (Oosawa et al. 2000; Su et al. 2001). In pea, the gene expression for *POR* was similarly controlled to that for aminolevulinic acid dehydratase and porphobilinogen diaminase regardless of whether the plants were grown in the dark or under white light. It is assumed that in pea, unidentified endogenous factors, but not light, are the primary regulatory factors controlling gene expression early in the development of the leaf (He et al. 1994). In cucumber, the expression of *POR* was developmentally controlled, the profile of which was closely related to that of Chl synthesis in adult plants (Kuroda et al. 2000).

Molecular genetic analyses of POR

Characterization of mutants that over-accumulate Pchlide

Genetic mutants in which the accumulation of Pchlide in darkness was affected were isolated from barley and *Arabidopsis*. In barley, four alleles of Pchlide overaccmulating *tigrina* mutants were identified that accumulate 2–10 times the wild-type amount of Pchlide in the dark (Nielsen 1974). Homozygous *tigrina-d* mutants are fully green, viable and fertile if grown in continuous weak light, but when they are grown under light/dark cycles, the seedlings show a green-white banded phenotype. In the *tigrina-d* mutants, only a normal level of POR is available, and when illuminated, the excess Pchlide causes photodynamic damage in plastids resulting in the formation of necrotic white domains. In *Arabidopsis*, Chl-deficient *xantha* mutants were isolated and classified into two groups, mutants that are blocked in various steps of the Chl biosynthetic pathway prior to POR, and mutants that accumulate Pchlide in the dark (Runge et al. 1995). More recently, Pchlide over-accumulating mutants of *Arabidopsis* were screened by fluorescence and designated *flu* (Meskauskiene et al. 2001). In the *flu* mutants, which resemble etiolated *tigrina* mutants of barely, the level of Pchlide but not that of free heme was higher than in wild-type seedlings. The FLU gene encoding a negative regulator of tetrapyrrole biosynthesis was identified as a nuclear-encoded plastid protein, which tightly associated with plastid membranes. Yeast two hybrid analysis indicated the physical interaction of FLU protein with glutamyl-tRNA reductase through the tetratricopeptide repeat domain of FLU (Meskauskiene and Apel 2002). Recently, it has been revealed that *TIGRINA d* gene of barley is an ortholog of the *FLU* gene of *A. thaliana* (Lee et al. 2003).

Characterization of organisms with altered expression of POR

In *C. reinhardtii*, *pc-1* mutants defective in Pchlide photoconversion *in vivo* have been isolated (Ford et al. 1981). Since *Chlamydomonas* cells possess DPOR, *pc-1* cells retain the capacity for light-independent Chl formation and synthesize about 52% of wild-type levels of Chl in the dark and about 36% in light. Map-based cloning of *pc-1* mutant demonstrated that the two-nucleotide deletion in *POR* gene causes a shift in the reading frame and results in a premature termination of translation (Li and Timko 1996). In cyanobacteria, *Synechocystis* sp. PCC 6803, (Wu and Vermaas 1995) and *P. boryanum* (Fujita et al. 1998), a POR-disrupted mutant has been constructed and characterized with comparison of ChlL-disrupted (DPOR less) mutants. Both POR and DPOR contribute to Chl synthesis in cells grown in the light, and the extent of the contribution by POR increases with light intensity; without it, the cells are unable to grow under strong light (Fujita et al. 1998). In *A. tricolor*, an ornament amaranth with red leaves, it is suggested that the repression of *POR* gene expression and resultant loss of Chl synthesis activity plays a role in red leaf formation (Iwamoto et al. 2001).

In vivo functions of each POR isoform have been explored mainly in *Arabidopsis* seedlings with altered endogenous expression of *PORA*. In the photomorphogenic mutant *cop1* (*det340*) of *Arabidopsis*, PORA is constitutively down-regulated, resulting in a lack of PORA and photoactive Pchlide-F655 in dark-grown seedlings. This mutant was very susceptible to photooxidative damage and accumulated Chl only at extremely low light intensities (Lebedev et al. 1995). The other PORA-depletion method employed was to grow wild-type seedlings of *Arabidopsis* under far-red light. As described before, it is well documented that etiolated seedlings grown under continuous far-red light are unable to green when subsequently transferred to white light. This process 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). From these studies, PORA has been proposed to play a special role in the formation of POR ternary complexes containing photoactive Pchlide-F655, PLB assembly, and protection against photo-oxidative damage caused by non-photoactive Pchlide (Reinbothe et al. 1996b). However, the photosensitive phenotype could be suppressed by expressing high levels of either the PORA or PORB isoform by the CaMV35S promoter in continuous far-red light-illuminated (Sperling et al. 1997) and *cop1* (Sperling et al. 1998) seedlings, and each POR isoform alone is sufficient for the accumulation of photoactive Pchlide F655 and the formation of the PLBs. Franck et al. (2000) examined in detail whether PORA and PORB play redundant roles in etioplast differentiation by manipulating the total POR content and the PORA-to-PORB ratio of *Arabidopsis* seedlings using antisense and overexpression approaches. Total POR content correlates closely with PLB formation, the amounts, spectroscopic properties, and photoreduction kinetics of photoactive Pchlide, and the ratio of photoactive to non-photoactive Pchlide. The authors concluded that the typical characteristics of etioplasts are closely related to POR content, but not obviously to the specific presence of PORA or PORB. Thus, rather than the intrinsic nature of PORA protein itself, it is the high-level expression of PORA in etiolated seedlings that plays a key role in promoting resistance to photooxidative damage. PORA and PORB appeared to be qualitatively interchangeable with respect to their functions in etioplast formation and photoprotection.

Similar to *Arabidopsis*, a pea mutant in the *COP1* locus, termed *lip1*, lacked PLBs and photoactive Pchlide-F655 in etioplasts, but the levels of POR were the same as in the wild-type. Treatment with cytokinin, 2-isopentenyladenine restored the formation of PLBs, but was not accompanied by an increase in the amount of Pchlide or POR (Seyedi et al. 2001a). In dark-grown *lip1* mutant, Pchlide-containing plastids were distributed in stem and root, which lacked both the photoactive Pchlide-F655 and the regular PLBs, but had a higher content of POR. No harmful photodestruction occurred in *lip1* mutant as the seedlings were observed to rapidly green in the light (Seyedi et al. 2001b). Thus, the formation of PLBs in pea is not directly correlated with the levels of endogenous POR and Pchlide, and it is possible that an unknown factor which is modifiable by cytokinin may be of importance for the regulation of PLB formation.

Although the depletion of endogenous POR by continuous far-red light illumination or *cop1* mutation is a useful tool to investigate the *in vivo* function of POR, the depletion potentially involves pleiotropic effects on chloroplast biogenesis, since continuous far-red light illumination or *cop1* mutation affects the central light signaling pathway of angiosperms. In fact, in addition to the repression of PORA, the levels of PORB transcript are also affected in far-red illuminated or *cop1* seedlings (Lebedev et al. 1995; Barnes et al. 1996; Runge et al. 1996). Furthermore, the expression of *Lhcb* and *HEMA1*, which encode light-harvesting chlorophyll *a*/*b*-binding protein complex and early tetrapyrrole synthesis enzyme glutamyl-tRNA reductase, respectively, under white light was repressed by preillumination under far-red light (McCormac and Terry 2002). The physiological function of specific POR isoforms *in vivo* has been recently elucidated by isolation and characterization of knockout mutants of *Arabidopsis* (Frick et al. 2003; Masuda et al. 2003). Mutants of *PORB* and *PORC*, in which the corresponding genes are interrupted by a derivative of the maize Dissociation (*Ds*) transposable element (Frick et al. 2003) or *Agrobacterium*-mediated random T-DNA insertion (Masuda et al. 2003), lack the respective POR transcript and specific POR isoforms. Single *por* mutants display no obvious phenotypes at the whole plant or chloroplast ultrastructural levels, except that *porB* mutants have less extensive etioplast inner membranes. Frick et al. (2003) produced a *porB porC* double mutant, which displayed a seedling-lethal *xantha* phenotype at the cotyledon stage, contained only a small amount of Chl *a*, and possessed chloroplasts with mostly unstacked thylakoid membranes, and they proposed that PORB and PORC play redundant roles in maintaining light-

dependent Chl biosynthesis in green plants, and are together essential for growth and development. Meanwhile, Masuda et al. (2003) focused on the greening process of *por* mutants, and showed that 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 authors proposed 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. In addition, it was found that 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.

Putative regulatory roles of POR in intracellular signaling and metabolism

The developmental and metabolic status of plastids affects the expression of nuclear genes that encode plastid proteins, and distinct plastid-to-nucleus signaling pathways have been hypothesized on the basis of physiological, genetic, and molecular studies. Plastid signals are also important for efficient metabolism and proper leaf development (Rodermel 2001; Surpin et al. 2002). Collective evidence suggests that the tetrapyrrole intermediate is one of the plastid-derived signals. Susek and Chory (1992) devised a genetic strategy for isolating mutants that do not repress *Lhcb* transcription completely in *Arabidopsis* seedlings, in which chloroplast development is prevented due to photooxidative damage by norflurazon treatment. Using this approach, recessive mutants were isolated in five nuclear genes, in which the normal coordinated expression of nuclear and chloroplast genomes is disrupted. This group of mutants is referred to as *gun* for *genomes uncoupled* (Susek et al. 1993; Mochizuki et al. 2001). GUN5 appeared to encode the ChlH subunit of Mg-chelatase (Mochizuki et al. 2001) and GUN4 binds to the ChlH subunit (Larkin et al. 2003), indicating that the tetrapyrrole intermediate (Mg-)protoporphyrin IX acts as a signaling molecule in one of the signaling pathways between the chloroplast and nucleus. Recently, Strand et al. (2003) showed that the accumulation of Mg-protoporphyrin IX is both necessary and sufficient to regulate the expression of many nuclear genes encoding chloroplastic proteins associated with photosynthesis, supporting the hypothesis that this molecule functions as plastid-derived signaling factor.

Interestingly, transgenic *Arabidopsis* lines overexpressing *PORA* or *PORB* restored the loss of nuclear gene expression due to the norflurazon treatment, indicating that the transgenic lines phenotypically resembles *gun* mutants. The expression of a nuclear gene, *HEMA1*, encoding glutamyl-tRNA reductase is inhibited by the norflurazon treatment. In transgenic *Arabidopsis* lines, overexpression of *PORA* or *PORB* restored the loss of *HEMA1* gene expression caused by the norflurazon treatment. In addition, the overexpression of POR also restored the continuous far-red induced loss of expression of *HEMA1* (McCormac and Terry 2002). It is not yet known whether the POR overexpression results in a reduced level of plastid damage or has a direct effect on plastid signaling. The regulatory role of POR on cell metabolism is also suggested in heterologously expressed pea POR in *R. capsulatus*. Amino acid substitutions in various POR domains affected the endogenous level of Mg-protoporphyrin IX accumulation, indicating the possible role of POR fragments as a putative regulatory factor (Lebedev and Timko 2002).

Physiological and evolutional implication of Pchlide reduction systems – concluding speculation

Chl biosynthesis in angiosperms is generally accepted to be light- and POR-dependent. Nevertheless, there are persistent reports of *de novo* Chl biosynthesis in various dark-adapted angiosperms, and, in a few cases, even in etiolated seedlings (Adamson et al. 1997). In this regard, so far, there is no evidence that angiosperms contain a DPOR enzyme that would permit them to reduce Pchlide in the dark (Armstrong 1998). Angiosperms, therefore, seem to be a lineage that has simply lost the DPOR genes. Fundamental questions are why phototrophs acquired POR in addition to DPOR and why angiosperms maintained POR but lost DPOR during evolution. The identification of POR in cyanobacteria indicates that the POR system evolved before the emergence of eukaryotic photosynthesis, and the acquisition of POR is not directly related to the endosymbiotic evolution of the chloroplast or mul-

ticellularity. Rather, it is likely that POR evolved to fulfill a fundamentally cell-autonomous role (Suzuki and Bauer 1995), and that its appearance reflects the transformation from anoxygenic to oxygenic photosynthesis (Reinbothe et al. 1996a). Considering the nitrogenase-like features as described above, it is possible that the activity of DPOR is repressed under oxygenic photosynthesis. It has been reported that DPOR is operative even under light in POR-deficient cells in cyanobacteria (Wu and Vermaas 1995; Fujita et al. 1998) and green algae (Ford et al. 1981), and thus it is likely that an unidentified oxygen protective mechanism has developed in these organisms. However, the DPOR activity was not high enough to compensate for the loss of POR, especially under high light intensities where oxygen evolution is maximal (Fujita et al. 1998). This inefficiency of DPOR under maximal oxygen-evolving conditions may be a selective pressure that drove the evolution of a new type of Pchlide reduction system, POR.

The origin of POR protein has not been identified yet, but it is highly likely that the ancestral protein evolved from a short-chain alcohol dehydrogenase belonging to the RED family, which is among the most widespread enzyme family in living organisms, playing a key role in many metabolic and biosynthetic pathways. Recently, complete genome sequences of several cyanobacteria have been reported (Kaneko et al. 1996, 2001; Nakamura et al. 2002; Dufresne et al. 2003). Interestingly, in all subspecies of *P. marinus* and *Synechococcus* sp. WH8102, the *POR* gene was located just downstream of *chlN-chlB-chlL* gene clusters in the genome, although such a gene order is not conserved, except for the *chlN-chlL* operon in other cyanobacterial genomes and chloroplast genome in algae (Fujita and Bauer 2003). It is possible that the tight linkage of *chlN-chlB-chlL* and *POR* genes has some significance to the regulation of their gene expression. Alternatively, the ancient POR protein may have physically interacted with DPOR, since the coupling of gene order with functional and physical interactions at the encoded protein level has been observed in the genomes of a number of nonphotosynthetic bacterial and archaeal species (Dandekar et al. 1998). Thus, it can be speculated that ancient POR functioned as an electron donor to DPOR to reduce Pchlide in an NADPH-dependent manner in the early evolutionary step of oxygenic photosynthesis. This ancient POR might acquire Pchlide-binding domain by adapting the extra-loop region. In this connection, it is of interest that most of the DPOR genes in eukaryotic phototrophs other than angiosperms still remain in the plastid genome, whereas the newcomers have been preferentially transferred to the nuclear genome. One of the reasons for this might be related with the evolution of oxygenic photosynthesis because the oxygen insensitivity and simple subunit composition of POR might allow an ancient host cell to more easily and independently control the biogenesis of an ancient symbiont through the expression and import of POR rather than DPOR.

Present data indicate that there is no distinct evolutional order in the emergence of PORA and PORB. For example, first, phylogenetic analysis clearly indicated that *Arabidopsis* PORA and PORB were generated by a recent gene duplication event (Oosawa et al. 2000). In addition, two POR genes identified from tobacco were not possible to classify into PORA- and PORB-types from their sequences and expression profiles (Masuda et al. 2002). Second, in gymnosperms, two gene families named PORA and PORB have been identified, but their terminology is not based on sequence similarity but the resemblance of gene expression profiles (Skinner and Timko 1998). Furthermore, the POR of gymnosperms is not ancestral, but rather closely related to angiosperm POR (Oosawa et al. 2000). Third, a single POR gene is present in cucumber (Fusada et al. 2000) and pea (Sundqvist and Dahlin 1997). Interestingly, these organisms showed unique profiles of gene expression for *POR* that persisted in mature tissues and not negatively regulated by light. Thus, it is likely that the evolution of POR in angiosperms proceeded in two ways: one is a gene duplication event, which may occur independently in individual plant families or genera, like in *Arabidopsis*, barley and tobacco, and also in gymnosperms. The resultant gene redundancy may allow individual modification of gene expression dependent on the requirement of POR proteins during evolution, causing differential expression of POR isoforms in these organisms. It is possible that in some plants belonging to Gramineae like barley, the produced POR isoforms have differentiated to possess distinct physiological functions to form the LHPP complex. The other way is the preservation of a single *POR* gene by modifying transcriptional control, like in cucumber and pea. In any case, however, angiosperms seem to retain a high level of POR expression in dark-grown leaves or cotyledons to form PLBs in etioplasts.

Why do angiosperms accumulate POR protein as PLBs in etioplasts even after losing DPOR. It is generally accepted that the formation of PLBs correlates

with the photoprotection of etiolated plants at the beginning of illumination (Lebedev et al. 1995; Barnes et al. 1996; Reinbothe et al. 1996a; Runge et al. 1996). In this case, PLBs should possess a specific mechanism for quenching excited pigments. However, if this is the only reason, angiosperms do not necessarily need to accumulate photosensitizing Pchlide in the dark even with the potential risk of suffering photooxidative damage under subsequent illumination. In addition, quantitatively transformed Chlide has similar photosensitizing activity. The other explanation may be that the close location of POR in PLBs allows energy migration among their pigments and thus increases the efficiency of light harvesting at low light intensities similar to the antenna of photosynthetic machinery (Ignatov and Litvin 1981). In this sense, PLBs function as a pool of Pchlide that is immediately transformable to Chlide upon illumination (Ryberg and Sundqvist 1991), although the plants cannot use the newly formed Chlide until the PLBs are disintegrated. Recent study of *PORB* knockout mutant of *Arabidopsis* supports this hypothesis since the *porB* mutant accumulated a reduced level of Chl under low light conditions, while comparable Chl accumulation to the wild type was observed under medium and high light conditions (Masuda et al. 2003).

The next question is why they chose this step for accumulating Chl intermediates in the dark. Except for the Chl-binding proteins, such as the core proteins of Photosystems I and II and light-harvesting Chl *a*/*b*-binding proteins, POR is the only protein that has been shown to form stable complexes with Chl precursors (Ryberg and Sundqvist 1991). There is only limited information available on protein complexes of earlier Chl precursors than Pchlide that one of subunit of Mg-chelatase, ChlH, binds to protoporphyrin IX (Willows and Beale 1998), and GUN4 protein, which is involved in plastid-to-nucleus signal transduction, binds to Mg-protoporphyrin IX and protoporphyrin IX (Larkin et al. 2003) (also see before). In *Arabidopsis*, the expression of ChlH was almost negligible in etiolated seedlings (Matsumoto and Masuda unpublished), and the endogenous levels of ChlH mRNA and protein are tightly regulated in mature leaves (Gibson et al. 1996; Nakayama et al. 1998). In *Arabidopsis*, GUN4 protein is accumulated as a pool in the stroma of chloroplasts in mature leaves (Larkin et al. 2003), but low levels of Mg-protoporphyrin IX and protoporphyrin IX were detected in angiosperms in the dark (Popperl et al. 1998; Papenbrock et al. 1999). Moreover, several disadvantages have been considered if protoporphyrin IX and Mg-protoporphyrin IX accumulate as Chl precursors in the dark. First, they are known as potential photosensitizers, and to metabolite them, more enzymatic steps are necessary to convert protoporphyrin IX to Chl than POR, which might have more risk to suffer photooxidative damage in angiosperms. Furthermore, a substantial delay of Chl synthesis may occur after illumination. Second, Mg-protoporphyrin IX and its methyl ester are thought to be plastid-derived signals that repress the expression of nuclear-encoded photosynthetic genes, such as *Lhcb*. Although light-dependent trafficking of these compounds is proposed (Kropat et al. 2000), the accumulation of such compounds in the dark may interfere with the expression of photosynthetic genes under subsequent illumination. The expression of Chl-binding proteins is regulated in accord with the supply of Chl. Many Chl-binding proteins are known to be very unstable in the absence of Chl, and Chl is required for the translation of certain plastid-encoded Chl-binding proteins. Thus, phototrophic organisms need to synthesize Chl, synchronized with the synthesis of Chl-binding proteins. The highly concentrated POR protein, together with Pchlide and NADPH, in etioplasts ensures not only that Chl is synthesized for photosynthesis as quickly as possible upon illumination but also, at the same time, Pchlide, a highly toxic compound, is converted into harmless state bound Chl. Furthermore, angiosperms do not need to produce Chl and Chl-binding proteins to generate photosystems that are useless in darkness. Such a reasonable and efficient POR-dependent mechanism of chloroplast development may give a greater opportunity to survive.

Future perspectives

As summarized in this review, more than one hundred articles about the Pchlide reduction system of phototrophs have been published during the last decade, and our knowledge on POR has dramatically increased in terms of enzyme catalysis, transport, membrane binding, localization, function, and gene expression. Enzymatic and structural analyses of POR proteins clarified the very efficient light-dependent catalytic mechanism of Pchlide photoconversion and suggested key amino acid residues involved in the cofactor binding, catalysis and membrane association. However, the catalytic mechanism of POR is still somewhat inconclusive and the mechanism of the membrane binding of POR has not been fully clarified. Although the

stereo structure of POR has been predicted by homology modeling based on other short-chain alcohol dehydrogenases, the crystallization of POR protein has not been successful which prevents the determination of the precise stereo structure of POR. Future studies must address such subjects to provide knowledge on the molecular nature of POR protein. Characteristics of POR reported in barley, such as the substrate-dependent import of POR precursor protein into plastids, the LHPP complex formation in etioplasts, and the selective proteolytic degradation are intriguing but still open for discussion as to whether these characteristics are specific depending on species or not. Such a distinct transport system, function and stoichiometry of POR isoforms in barley are apparently not applicable to some of the other angiosperms, such as cucumber, pea and *Arabidopsis*. Studies are necessary to demonstrate whether aggregated POR complexes in etioplasts of such plants are different from the LHPP complex of barley, and whether they contain large amounts of Pchlide *b*. Presumed 7-formyl reductase needs to be identified as responsible for the rapid turnover of Pchlide *b in vivo*. In any case, however, almost all angiosperms accumulate POR proteins to form PLBs in etioplasts of dark-grown seedlings, and we really do not know why PLBs are necessary for efficient chloroplast development. Further detailed studies are necessary to clarify whether PLBs function for photoprotection or capturing light energy or both, and such studies will give further insight into the physiological function of this unique and very important enzyme.

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