GENETIC MANIPULATION OF NADPH: PROTOCHLOROPHYLLIDE OXIDOREDUCTASE CONTENT IN *ARABIDOPSIS* REVEALS ESSENTIAL ROLES IN PROLAMELLAR BODY FORMATION AND PLANT DEVELOPMENT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree Doctor of Philosophy in the Graduate

School of The Ohio State University

By

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The Ohio State University
2008

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ABSTRACT

Chlorophyll synthesis in angiosperms requires light because, in contrast to other photosynthetic organisms, they rely exclusively on a light-dependent mechanism to reduce protochlorophyllide during chlorophyll biosynthesis. NADPH:protochlorophyllide oxidoreductase (POR) catalyses a light-dependent reduction of protochlorophyllide to chlorophyllide, which is subsequently transformed to chlorophyll. In etioplasts during skotomorphogenesis, POR forms a photolabile aggregate of NADPH-POR-Pchlide localized to the prolamellar bodies. In Arabidopsis there is a three-member gene family encoding structurally related but differentially regulated POR enzymes denoted PORA, PORB and PORC. PORA and PORB accumulate during skotomorphogenesis. PORB and PORC accumulate during seedling development and throughout the life of the plant, during which they are responsible for bulk chlorophyll synthesis. Here I describe the detailed molecular–genetic dissection of the functions of the different POR isoforms. While single porB-1 or porC-1 null mutants display no distinct light-grown phenotypes, the porB-1 porC-1 double mutant displays a severe xantha (highly chlorophyll-deficient) phenotype. In response to illumination, chlorophyll production, thylakoid stacking and photomorphogenesis are restored in the PORA-overexpressing porB-1 porC-1 transgenic lines. Therefore, the porB-1 porC-1
double mutant is functionally rescued by ectopically expressed PORA, which suffices in the absence of either PORB or PORC to direct bulk chlorophyll synthesis and normal plant development. Using reverse genetic approaches, our lab identified a \textit{porA-1} null mutant which I have characterized here; additionally I have characterized \textit{PORA RNAi} knockdown lines. The \textit{porA-1} and \textit{PORA RNAi} lines display photoautotrophic growth blocks which are partially rescued on sucrose-supplemented media. \textit{porA-1} mutant seedlings display defects in etioplast development with reductions in prolamellar body accumulation and photoactive Pchlide conversion. Further analysis of the \textit{porB-1 porC-1} double null mutant reveals independent PORA catalytic activity in the cauline leaves of low-light grown plants. The \textit{porA-1 porB-1} and \textit{porA-1 porC-1} double mutants were used to investigate the contributions of each POR individual isoform to Chl biosynthesis and growth in green plants. The \textit{porA-1 porB-1} and \textit{porA-1 porC-1} mutants have photoautotrophic growth defects which resemble that of the \textit{porA-1} single mutant. In addition, etioplast development in the \textit{porA-1 porB-1} double mutant is defective, with no detected prolamellar body formation or photoactive protochlorophyllide conversion.
Dedicated to Courtney and my family
ACKNOWLEDGMENTS

I wish to thank my adviser, Greg Armstrong for his efforts, support and patience in correcting my stylistic and scientific errors.

I thank Mary Mason for her diligence and precision in transgenic line screening, seedling propagation, and for biologically and statistically challenging discussions.

I am grateful to Daniel Lima for his focused efforts in establishing pigment quantification methodology and then applying these techniques to a variety of plant lines.

I thank Rosario Barbieri for her intellectually stimulating discussions, encouragement, advice, support and understanding.

I thank Rebecca Lamb for her support and editing contributions, and Patrice Hamel and Randy Scholl for their advice.

I am indebted to Courtney my first line of defense against the grammar police.

I also wish to thank Kathy Wolken at The Ohio State University Campus Microscopy and Imaging Facility for assistance with the electron microscopy, Dick Sayre for providing access to the fluorescence spectrophotometer, and the AGRIKOLA consortium, The Nottingham Arabidopsis Stock Centre, UK, and The Arabidopsis Biological Resource Center for the generous donation of seed lines.

This research was financially supported by The Ohio State University and The National Science Foundation (IOB#0450114).
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CHAPTER 1
INTRODUCTION

1.1. Summary

The production of chlorophylls (Chl) is indispensable in photosynthetic organisms. Chl is essential for the plant photochemistry of photosynthesis during which solar energy is harvested and transferred from the light-harvesting antenna systems to the photosynthetic reaction center. In the reaction center Chl undergoes charge separation, which initiates photosynthetic electron transport. Excess porphyrin accumulation is potentially a lethal photosensitizer, therefore the study of Chl biosynthesis and its regulation has been a central area of plant biology research.

Chl synthesis in angiosperms requires light because, in contrast to other photosynthetic organisms, they rely exclusively on a light-dependent mechanism to reduce protochlorophyllide during chlorophyll biosynthesis. NADPH:protochlorophyllide oxidoreductase (POR) catalyses a light-dependent reduction of protochlorophyllide \( a \) to chlorophyllide \( a \), which is subsequently transformed to chlorophyll. In the etioplast during skotomorphogenesis in angiosperms POR forms a photolabile aggregate of NADPH-POR-Pchlide ternary complexes localized to the prolamellar bodies.

Within Arabidopsis there is a structurally related but differentially regulated three member POR gene family denoted \( \text{PORA} \), \( \text{PORB} \) and \( \text{PORC} \). The PORA and PORB
proteins accumulate during skotomorphogenesis. Upon illumination PORA and PORB are negatively light-regulated. PORA has a very low level of expression that persists even in continuously illuminated plants in cauline leaves and in response to low light at the shoot apex. In contrast, PORB and PORC are not only expressed during seedling development but also throughout the later life of the plant, during which they are responsible for bulk chlorophyll synthesis. The Arabidopsis porB-1 porC-1 double null mutant displays a severe xantha (highly chlorophyll-deficient) phenotype.

Analysis of the porB-1 porC-1 double null mutant demonstrated smaller prolamellar bodies in etioplasts and decreased thylakoid stacking in chloroplasts. Introduction of an ectopic PORA overexpression construct into the etiolated porB porC double mutant suffices to restore prolamellar body formation. In response to illumination, light-dependent chlorophyll production, thylakoid stacking and photomorphogenesis are also restored in PORA-overexpressing porB-1 porC-1 seedlings and adult plants. An Arabidopsis porB porC double mutant can therefore be functionally rescued by the addition of ectopically expressed PORA, which suffices in the absence of either PORB or PORC to direct bulk chlorophyll synthesis and normal plant development.

We have tested the hypothesis that PORA has critical significance in skotomorphogenesis by assisting in etioplast development. Furthermore, we evaluated the proposition that PORA is required for normal photomorphogenic development. Using reverse genetic approaches, we identified a porA-1 null mutant that contained the maize Dissociation transposable element in the PORA gene; additionally we characterized PORA RNAi lines. The porA-1 and PORA RNAi lines display
photoautotrophic growth blocks which can be partially rescued on sucrose-supplemented media. Elimination of PORA during skotomorphogenesis results in reductions in prolamellar body accumulation and photoactive Pchlide conversion. The porA-1 mutant characterization establishes the requirement for PORA in normal etioplast development. Analysis of the porB-1 porC-1 double null mutant reveals independent PORA catalytic activity in the cauline leaves of low light-grown seedlings.

The porA-1 porB-1 and porA-1 porC-1 double null mutants of Arabidopsis were used to investigate the contributions of each POR isoform to Chl biosynthesis and growth in green plants. Photoperiodically-grown porB-1 and porC-1 single mutants resemble the wild type, whereas the porA-1, porA-1 porB-1 and porA-1 porC-1 mutants are light-green and seedling growth is arrested at the cotyledon stage. When supplied with sucrose, these growth arrested mutants develop as light-green dwarves. Etioplast development in the porA-1 porB-1 double mutant is defective with no detected prolamellar body formation or photoactive protochlorophyllide conversion, whereas the porA-1 porC-1 double mutant etioplasts resemble those of the porA-1 mutant. The elimination of the other POR family members establishes the individual activities of the PORB and PORC isoforms, in addition to suggesting PORA-specific functions in photomorphogenesis and plant development.
1.2. Chlorophyll biosynthesis through Mg-protoporphyrin IX

The regulation, genetics and biochemistry of Chl biosynthesis have been recently reviewed (Tanaka and Tanaka, 2007; Masuda, 2008). In oxygenic photosynthetic organisms the biosynthesis of tetrapyrroles begins with the formation of delta-aminolevulinic acid. This C₅ compound is the first committed precursor of Mg-tetrapyrroles, such as the Chls, and Fe-tetrapyrroles, such as heme. Delta-aminolevulinic acid participates in a series of condensation reactions, accompanied by decarboxylation and oxidation steps that culminate in the formation of protoporphyrinogen IX, a nonconjugated macrocyclic tetrapyrrole. Protoporphyrinogen IX is oxidized to protoporphyrin IX, a conjugated tetrapyrrole, by protoporphyrinogen oxidase. In plants, inhibition of this enzyme with diphenylether herbicides causes tetrapyrrole over-accumulation leading to a photodynamic effect in which the Chl precursor acts as a photosensitizer (Lermontova and Grimm, 2000). In humans, a common version of photodynamic therapy relies on the similar overproduction of endogenous protoporphyrin IX or the introduction of synthetic porphyrins into tumor cells, followed by laser irradiation of the cancerous tissue (McCaughan, 1999). Protoporphyrin IX serves as the common precursor for Chl and heme biosynthesis in photosynthetic organisms, and is the substrate for the enzymatic insertion of either Mg or Fe mediated by Mg-chelatase or ferrochelatase, respectively. The initial product of the Chl branch of the pathway is Mg-protoporphyrin IX. Regulation of this portion of the tetrapyrrole biosynthetic pathway is exerted primarily at the levels of delta-aminolevulinic acid formation and Mg chelation (Beale, 1999; Meskauskiene et al., 2001; Papenbrock and Grimm, 2001).
1.3. Two distinct protochlorophyllide reduction mechanisms

Divinyl-Pchlide $a$ is synthesized by a set of reactions including the methylation of Mg-protoporphyrin IX and the formation of an isocyclic ring. To reduce Pchlide $a$ to Chlide $a$ two biochemically and genetically distinct mechanisms have evolved (Schoefs, 1999). The more ancient, light-independent reaction is performed by a multisubunit enzyme known as DPOR (dark-active Pchlide oxidoreductase) that is present in anoxygenic photosynthetic bacteria and in most photosynthetic eukaryotes other than angiosperms (Fujita, 1996; Armstrong, 1998). This enzyme consists of three subunits that are plastid-encoded in eukaryotes and that are structurally related to the subunits of eubacterial nitrogenase. The oxygen-sensitive activity of DPOR has been demonstrated \textit{in vitro} (Fujita and Bauer, 2000).

The POR-mediated Pchlide $a$ reduction pathway was first characterized in detail using etioplast inner membranes of angiosperms (Griffiths, 1978). This strictly light-dependent activity is present in all oxygenic photosynthetic organisms examined thus far, but is not found in anoxygenic photosynthetic bacteria (Rüdiger, 1997; Beale, 1999). POR catalyzes the only light-dependent reaction in Chl biosynthesis and is hence essential to angiosperms, which lack DPOR and do not green in the dark.
1.4. Biochemical and structural properties of light-dependent POR

The biochemical properties and regulation of POR have been recently summarized (Heyes and Hunter, 2005). Experimentally, the catalytic properties and substrate specificity of POR have been studied using both partially solubilized and native etioplast inner membrane preparations (Schoch et al., 1995; Chahdi et al., 1998; Scheumann et al., 1999), a purified and reconstituted oat POR preparation (Klement et al., 1999), and bacterially-expressed POR fusion and native polypeptides (Martin et al., 1997; Townley et al., 1998; Lebedev and Timko, 1999; Heyes et al., 2000; Lebedev et al., 2001). These studies indicate that POR can be active as a monomer or dimer in vitro.

One particularly remarkable result is the in vivo complementation of Rhodobacter capsulatus DPOR mutants by almost undetectable amounts of the pea POR enzyme (Wilks and Timko, 1995). As Rhodobacter does not have an endogenous POR, this suggests that plant POR is quite insensitive to the exact nature of the enzymes with which it interacts during substrate and product transfer.

POR proteins themselves are structurally conserved throughout evolution, with sequence identities of about 54 and 65 % between the higher plant, and cyanobacterial and algal enzymes, respectively (Suzuki and Bauer, 1995; Li and Timko, 1996). Among higher plant POR enzymes, sequence identities of >70 % for the precursor polypeptides and >80 % for the mature proteins are typical. PORs are also structurally related to an NADPH-dependent family of short-chain alcohol dehydrogenases. Site-directed mutagenesis performed on the basis of predictions made from this structural relationship has helped to identify critical amino acid residues required for the enzyme activity,
cofactor binding and plastid membrane association of pea POR (Wilks and Timko, 1995; Dahlin et al., 1999; Aronsson et al., 2001; Engdahl et al., 2001).

1.5. Intraorganellar localization and membrane association of POR ternary complexes

Early studies demonstrated that POR is encoded in the nucleus, synthesized in the cytosol as a precursor polypeptide, and imported into plastids with concomitant processing to remove an N-terminal transit sequence (Apel, 1981). The resulting mature polypeptide of about 37 kDa is targeted to etioplast inner membranes. POR is a basic, peripheral membrane protein lacking obvious transmembrane helices (Grevby et al., 1989; Benli et al., 1991; Spano et al., 1992a).

In angiosperm seedlings germinated in the dark, POR is the most abundant protein component of the paracrystalline PLB inner membrane of etioplasts (Dehesh and Ryberg, 1985; Ryberg and Dehesh, 1986). Dark-stable Pchlide a:NADPH:POR ternary complexes occur in an aggregated state in the PLB. These complexes are poised such that absorption of a single photon by the pigment ($\lambda_{\text{max}} = 650$ nm) leads to the immediate, NADPH-dependent trans-reduction of the C$_{17}$,C$_{18}$ double bond in ring D of the macrocycle (Figure 1.1). This reaction occurs at temperatures as low as -70°C, and in response to a 150 femtosecond light pulse, underscoring its biochemical novelty (Griffiths et al., 1996). The Pchlide a bound to the POR active site in aggregated ternary complexes displays an in situ low temperature (77 K) fluorescence emission maximum at about 655 nm in intact etiolated cotyledons and is referred to as "photoactive" Pchlide a-F655 (Böddi et al., 1989). Photoactive Pchlide a present in a less highly aggregated state typically fluoresces at slightly shorter wavelengths (Chahdi et al., 1998).
“Nonphotoactive” Pchlide a-F632, ie. the pigment fraction that is not bound to the POR active site, is a mixture of unesterified Pchlide and esterified protochlorophyll (Pchl), which is not a POR substrate (Schoch et al., 1995; Chahdi et al., 1998; Klement et al., 1999). Because nonphotoactive Pchlide is a dangerous sensitizer for photooxidative damage (Lebedev et al., 1995), negative feedback control normally prevents its overaccumulation in etiolated angiosperms (Meskauskiene et al., 2001).

The light-dependent reduction of photoactive Pchlide a by POR yields Chlide a, which is subsequently esterified and further modified to produce Chls a and b (Beale, 1999). This process occurs in conjunction with the disintegration and reorganization of the PLB into thylakoids (Figure 1.1) to produce a functional photosynthetic membrane (Sundqvist and Dahlin, 1997). During greening, POR activity and polypeptide levels decrease dramatically in angiosperms (Forreiter et al., 1991), due to the action of a membrane-localized protease that degrades POR in the absence of its substrate, Pchlide, and cofactor, NADPH (Häuser et al., 1984). Greening seedlings therefore typically contain a low steady-state amount of POR that is distributed between the thylakoid and plastid envelope membranes (Forreiter et al., 1991; Barthélemy et al., 2000).

1.6. Distribution of bicontinuous cubic phase membranes

The PLB is perhaps the best-characterized and most widespread example of a bicontinuous cubic membrane found in nature (Bruce, 1998). However, similar types of membranes have been reported in the context of the mitochondrial inner membrane and the smooth endoplasmic reticulum (Landh, 1995). Such a membrane forms in giant amoebae mitochondria in response to the absence of food, which is intriguingly
associated with oxidative stress (Deng and Mieczkowski, 1998, Almsherqi et al., 2006). However, the functions of these non-plant cubic membranes have not been determined. Although the primary biological context of the PLB is clearly within etiolated angiosperms, the distribution of this membrane in plants is not strictly limited to etioplasts. A few gymnosperms that green poorly in the dark, presumably due to a low level of DPOR activity (Armstrong, 1998), contain etiochloroplasts in which PLBs and Chl-containing thylakoids coexist (Selstam and Widell, 1986; Mariani et al., 1990). The chloroplasts of young angiosperm seedlings that have been dark- or low-light-adapted also often contain small PLBs (Henningsen et al., 1993; Sundqvist and Dahlin, 1997).

1.7. Formation of the PLB

The determinants of PLB formation are not well understood. Lipid composition almost certainly facilitates or allows the formation of PLBs, which contain a high content of monogalactosyldiacylglyceride that would tend to form tubular arrays (Sundqvist and Dahlin, 1997; Bruce, 1998). All three components of the NADPH:Pchlide:POR ternary complex also seem to be necessary for PLB formation (Henningsen et al., 1993; Runge et al., 1995; Sperling et al., 1997, 1998; Engdahl et al., 2001) and indeed there is a close correlation between POR content and PLB extent (Franck et al., 2000; Frick et al., 2003; Masuda et al., 2003). Because POR is a peripheral membrane protein it may act to stabilize the curvature of the lipids. Nevertheless, two examples exist which suggest that the presence of POR, presumably in ternary complexes, is not sufficient for membrane formation. The first example is provided by the lip1 photomorphogenic mutant of pea (Seyyedi et al., 1999), and the second by a newly described carotenoid isomerase crtISO.
mutant of Arabidopsis (Park et al., 2002). In the latter case, it has been argued that carotenoids, specifically all-trans-xanthophylls, are required for PLB formation. Finally, it is unknown whether minor protein components in addition to POR are required for PLB formation.

A major question regarding the PLB has been whether it performs a distinct function. One hypothesis has been that this membrane structure confers a competitive advantage to angiosperms by accelerating greening at the onset of photomorphogenesis (Sundqvist and Dahlin, 1997; Park et al., 2002). Certainly the compact, regular structure of the PLB provides the means to store a large amount of lipids, carotenoids and Chl precursors, in the form of Pchlide, within a relatively small volume in anticipation of light-induced Pchlide reduction and chloroplast differentiation (Figure 1.1). By analogy, higher plant chromoplasts accumulate a structural protein called fibrillin, which forms a supramolecular lipoprotein structure together with carotenoids, galactolipids and phospholipids (Deruere et al., 1994). Although the geometry of this storage structure is not related to the PLB, and fibrillin is not an enzyme, fibril organization is reminiscent of the participation of POR ternary complexes in PLB formation. The counterpoint to a defined function for the PLB argues that this membrane is simply a passive product of its individual components and exists only in organisms with no or little DPOR activity (i.e. angiosperms and a few gymnosperms) that would deplete Pchlide content in the dark.

1.8. The Arabidopsis POR gene complement

Further complicating models of the formation and function of the PLB, two or more differentially regulated POR genes have been identified in angiosperms including
Arabidopsis (Armstrong et al., 1995; Oosawa et al., 2000), barley (Holtorf et al., 1995), tobacco (Masuda et al., 2002) and the ornamental species *Amaranthus tricolor* (Iwamoto et al., 2001). Species of pine, a gymnosperm, also contain fairly extensive *POR* gene families (Spano et al., 1992b; Forreiter et al., 1993; Skinner and Timko, 1998). On the other hand, the best evidence available from studies of pea (Spano et al., 1992a; Seyyedi et al., 1999) and cucumber (Kuroda et al., 1995; Fusada et al., 2000) suggests that these angiosperms contain single *POR* genes, as do lower photosynthetic organisms, such as the bryophyte *Marchantia paleacea* (Takio et al., 1998), the green alga *Chlamydomonas reinhardtii* (Li and Timko, 1996) and the cyanobacteria *Synechocystis* sp. PCC 6803 (Suzuki and Bauer, 1995), *Phormidium laminosum* (Rowe and Griffiths, 1995) and *Plectonema boryanum* (Fujita et al., 1998).

Arabidopsis is currently the only higher plant for which the entire *POR* gene complement is known with certainty. Furthermore, this species provides a superior molecular-genetic system in which to explore questions related to the biology of light-dependent Pchlide reduction and the roles of the *POR* enzymes in plastid membrane formation. The Arabidopsis genome encodes three structurally related but differentially regulated *POR* proteins, denoted PORA, PORB and PORC, on chromosomes 5, 4 and 1, respectively (Benli et al., 1991; Armstrong et al., 1995; Oosawa et al., 2000). Their enzymatic activities have been qualitatively demonstrated *in vivo* in *POR*-overexpressing transgenic plants (Sperling et al., 1997, 1998, 1999; Franck et al., 2000) and/or *in vitro* using bacterially-expressed *POR* fusion proteins (Su et al., 2001). The Arabidopsis *POR* genes are regulated in a remarkably complex fashion in response to the light environment and plant developmental state. The *PORA* and *PORB* genes, but not *PORC* are highly
expressed early in seedling development, particularly in etiolated seedlings (Armstrong et al., 1995; Oosawa et al., 2000; Su et al., 2001). In contrast, vigorous expression of PORB and PORC, but not PORA, is observed in older seedlings and adult plants. During these stages of development the abundance of the PORB transcript is subject to a circadian regulation whereas the PORC transcript amount is diurnally regulated (Su et al., 2001). Furthermore, light perceived by phytochrome photoreceptors negatively regulates PORA such that its expression rapidly becomes undetectable at the onset of greening (Armstrong et al., 1995; Barnes et al., 1996; Runge et al., 1996). PORC, however, is positively regulated by light in a fluence rate-dependent manner (Su et al., 2001), and upon illumination during deetiolation (Oosawa et al., 2000). These patterns of expression are highly suggestive of evolutionary adaptations to optimize plant growth under specific conditions and at discrete developmental stages.

1.9. Functions of angiosperm PORA and PORB

The functional significance of the multiple, differentially expressed POR enzymes in angiosperms are unknown. Ecotopic overexpression and antisense approaches to study the functions of Arabidopsis PORA and PORB (Sperling et al., 1997, 1998, 1999; Franck et al., 2000). Some of the overexpression studies have been performed using Chl-deficient seedlings depleted of these endogenous POR proteins. This can be achieved by growing wildtype seedlings in continuous far-red light with a $\lambda_{\text{max}}$ of 740 nm (Runge et al., 1996; Frick et al., 1999), or as the consequence of a pleiotropic mutation, such as cop1, which leads to constitutive photomorphogenesis in the dark (Deng et al., 1991; Lebedev et al., 1995). In either case, not only are endogenous PORA and PORB
depleted, but the cotyledon plastids have small or no PLBs, little photoactive Pchlide-F655, and unusually large amounts of photosensitizing nonphotoactive Pchlide-F632. In particular, we have focused on the roles of the POR proteins in the protection of seedlings against Pchlide-sensitized photooxidative damage (Runge et al., 1996; Sperling et al., 1997), and in mediating etioplast development as assayed by PLB membrane formation and the appearance of photoactive Pchlide (Sperling et al., 1998; Franck et al., 2000).

The total POR content in various ecotopic overexpressing and antisense transgenic plants correlates well with the extent of the PLB and the amount of Pchlide-F655. One intriguing result obtained with PORA- and PORB-overexpressing seedlings grown in far-red light is that they, unlike the wildtype, can use this light quality for photoactive Pchlide reduction to the extent that photosystems I and II can be detected by their characteristic Chl fluorescence emission maxima in \textit{in situ} 77 K fluorescence measurements of intact cotyledons (Sperling et al., 1999). Thus, modulation of POR levels seems to allow Arabidopsis seedlings to grow in unusual light qualities that mimic the dim far-red-rich light available below a forest canopy. In summary, the above studies have demonstrated that either PORA or PORB can offer substantial protection against nonphotoactive Pchlide-mediated photooxidative damage, and can serve as an endogenous organizer for the assembly of the PLB membrane and photoactive Pchlide-F655. The caveat is that these studies have been conducted with POR-depleted systems rather than with \textit{por} null mutants.

In barley, a number of reports have detailed a novel mechanism involving the Pchlide-dependent plastid import of the cytosolic precursor of PORA (prePORA), in contrast to the constitutive import of prePORB (Reinbothe et al., 1995, 1996). Various
functions have been ascribed to this phenomenon. The Pchlide requirement has been identified as a binding of the pigment substrate to the transit peptide of prePORA (Reinbothe et al., 1997). Subsequent experiments have, however, failed to reproduce a Pchlide requirement for pPORA plastid uptake (Aronsson et al., 2000; Dahlin et al., 2000), casting doubt on the original observations. It has also been suggested that the in vitro pigment-binding and aggregation properties of barley PORA and PORB and, by extension, their in vivo functions with respect to deetiolation, light-harvesting and protection against photooxidative damage are unique (Reinbothe et al., 1999). At the heart of this proposal is the contention that PORA and PORB in etiolated barley seedlings form a novel light-harvesting Pchlide $a/b$-binding supercomplex, termed LHPP, containing Pchlide $b$:NADPH:PORA and Pchlide $a$:NADPH:PORB ternary complexes in a 5:1 ratio. LHPP is considered in this model to be the pigment-protein complex that defines photoactive Pchlide in the PLB. Key aspects of this proposal are not correct, however (Armstrong et al., 2000), including the hypothesized presence of Pchlide $b$ in etiolated angiosperms (Scheumann et al., 1999; Franck et al., 2000). Nevertheless, the specific prediction that both PORA and PORB must be present to assemble photoactive Pchlide-F655 within the PLB cannot be completely discounted. Finally, it has been discussed that angiosperm PORA and PORB may contribute to separate Chl biosynthetic pathways involving spectroscopically distinct sets of Pchlide and Chlide intermediates in greening seedlings and in fully green plants, respectively (Lebedev et al., 1995; Lebedev and Timko, 1998, 1999).

Until recently, a true in vivo functional analysis of the contribution of individual angiosperm POR enzymes to plant development and plastid membrane differentiation has
not been possible in the absence of a complete set of \textit{por} mutants. \textit{por} mutants have been described in unicellular oxygenic photosynthetic organisms that contain single \textit{POR} genes, such as species of cyanobacteria (Fujita et al., 1998; He et al., 1998) and \textit{Chlamydomonas reinhardtii} (Li and Timko, 1996). However, in contrast to angiosperms, these organisms can also reduce Pchlide to Chlide using DPOR (Fujita, 1996; Armstrong, 1998).

This introduction was written to explain surrounding literature that is not necessarily included in the introduction to each chapter. Each chapter investigates specific hypotheses using molecular and biochemical techniques to further elucidate the consequences of the \textit{POR} gene family. This thesis describes the recent progress in the analysis of the angiosperm \textit{Arabidopsis thaliana} \textit{POR}. I have characterized the \textit{porA-1}, \textit{porB-1} and \textit{porC-1} mutants and their respective double mutants in the following three chapters. Ectopic expression as well as RNAi suppression has been used to alter PORA gene accumulation. Topics pertinent to each chapter are introduced in context in more detail to the addressed subjects of each chapter.
Figure 1.1 The process of light activated greening in Arabidopsis (top). Stacked thylakoids form in the place of the prolamellar body in the etioplast (middle). The chemical reaction catalyzed by POR (bottom).
CHAPTER 2

ARABIDOPSIS PROTOCHLOROPHYLLIDE OXIDOREDUCTASE A (PORA)
RESTORES BULK CHLOROPHYLL SYNTHESIS AND NORMAL DEVELOPMENT
TO A PORB PORC DOUBLE MUTANT

ABSTRACT

In angiosperms the strictly light-dependent reduction of protochlorophyllide to chlorophyllide is catalyzed by NADPH:protochlorophyllide oxidoreductase (POR). The Arabidopsis thaliana genome encodes three structurally related but differentially regulated POR genes, PORA, PORB and PORC. PORA is expressed primarily early in development—during etiolation, germination and greening. In contrast, PORB and PORC are not only expressed during seedling development but also throughout the later life of the plant, during which they are responsible for bulk chlorophyll synthesis. The Arabidopsis porB-1 porC-1 mutant displays a severe xantha (highly chlorophyll-deficient) phenotype characterized by smaller prolamellar bodies in etioplasts and decreased thylakoid stacking in chloroplasts. Here we have demonstrated the ability of an ectopic PORA overexpression construct to restore prolamellar body formation in the
*porB* *porC* double mutant background. In response to illumination, light-dependent chlorophyll production, thylakoid stacking and photomorphogenesis are also restored in *PORA*-overexpressing *porB-1 porC-1* seedlings and adult plants. An Arabidopsis *porB* *porC* double mutant can therefore be functionally rescued by the addition of ectopically expressed *PORA*, which suffices in the absence of either *PORB* or *PORC* to direct bulk chlorophyll synthesis and normal plant development.

### 2.1. INTRODUCTION

Within angiosperms, the strictly light-dependent reduction of protochlorophyllide *a* (Pchlide) to chlorophyllide *a* (Chlide) is performed by the nuclear-encoded, plastid-targeted, NADPH:protochlorophyllide oxidoreducase (POR, EC 1.3.1.33) (Griffiths, 1978). Dark-stable, ternary complexes of Pchlide:NADPH:POR catalyze the only light-dependent step in chlorophyll (Chl) biosynthesis, a *trans* addition of hydrogen across the C17-C18 double bond of the D-ring of Pchlide to create Chlide *a*, which is subsequently esterified and further modified to yield Chls *a* and *b* (Lebedev and Timko, 1998). Because of the light requirement of POR for catalysis, etiolated and in some cases dark-adapted angiosperm seedlings possess plastids known as etioplasts with a membrane ultrastructure typified by a paracrystalline matrix composed primarily of POR, lipids, carotenoids and Chl precursors, known as the prolamellar body (PLB) (Rosinski, 1972; Selstam and Sandelius, 1984). Pchlide bound by POR at the active site is termed ‘photoactive’ because it is photoconverted instantaneously to Chlide in the presence of light (Heyes and Hunter, 2005). Pchlide not bound at the POR active site is termed
'nonphotoactive' and has been implicated as a photosensitizer for the formation of singlet oxygen that can cause photooxidative damage (Reinbothe et al., 1996; Sperling et al., 1997; Sperling et al., 1998). Extensive feedback control of tetrapyrrole accumulation to limit the amount of nonphotoactive Pchlide has been demonstrated (Meskauskiene et al., 2001). One central feature of angiosperm photomorphogenesis is that illuminated seedlings begin to green, due to the visible accumulation of Chl synthesized as a downstream result of the activity of the photoactive Pchlide:NADPH:POR ternary complex. In parallel, the cotyledon plastids transition from being etioplasts with PLBs and unstacked (pro)thylakoids to photosynthetically-competent chloroplasts with mature thylakoids in which POR functions in bulk Chl biosynthesis (Solymosi et al., 2007).

Gymnosperms, nonvascular plants, algae and cyanobacteria, almost all do not require light to green, there are generally two distinct enzymes, light-independent POR (DPOR) and light-dependent POR, available for Chl biosynthesis. In contrast, angiosperms have only light-dependent POR. The loss of plastid-encoded DPOR in angiosperms, coupled with the development in many cases of small POR gene families suggests probable adaptive advantages of specific POR regulation. Phylogeny analysis indicates that gene duplication events likely led to the formation of POR gene families encoding closely related proteins (Masuda and Takamiya, 2004). Tobacco (*Nicotiana tabacum* L.) and barley (*Hordeum vulgare* L.), for example, there are two POR gene family members characterized (Holtorf et al., 1995; Masuda et al., 2002). In a few cases, such as pea (*Pisum sativum* L.) and cucumber (*Cucumis sativus* L.), only single POR genes have been reported (Spano et al., 1992; Fusada et al., 2000). A variety of *in vitro* and *in vivo* studies have been performed with the above-mentioned species, as well as
with wheat (*Triticum aestivum*), *Amaranthus tricolor*, maize (*Zea mays*), cabbage (*Brassica oleracea* cv. *capitata*) and Arabidopsis (*Arabidopsis thaliana*) to understand the function, plastid import, and effects on PLB formation and etioplast differentiation of POR (Teakle and Griffiths, 1993; Iwamoto et al., 2001; Selstam et al., 2002; Solymosi et al., 2004).

There are three structurally related Arabidopsis genes that encode light-dependent POR have been characterized in the genome. *PORA* is strongly down regulated by light, *PORB* is moderately negatively regulated by light, and *PORC* is positively regulated by light (Armstrong et al., 1995; Su et al., 2001). Although *PORA* is highly expressed in etiolated seedlings, *PORA* mRNA and PORA protein are very difficult to detect after only a few hours of illumination (i.e. by the time that greening becomes evident). In contrast, *PORB* mRNA and PORB protein are readily detectable both in etiolated seedlings, and also in light-grown seedlings and plants, with *PORB* mRNA accumulation following a circadian pattern. The expression of *PORC* is initiated upon illumination and thereafter increases to a level related to the photon fluence used for plant growth. The pronounced expression of one POR isoform in etiolated barley seedlings has been taken as evidence for the existence of a barley PORA ortholog (Holtorf et al., 1995). Tobacco, pea and barley also each express a POR isoform regulated in a comparable fashion to Arabidopsis PORB (Spano et al., 1992; Holtorf et al., 1995; Masuda et al., 2002). Further, tobacco has a POR isoform with an expression profile analogous to that of the Arabidopsis PORC isoform (Masuda et al., 2002).

PORA-dependent Chl production was observed in cotyledons of *porB-1 porC-1* double mutant seedlings germinated in the light (Frick et al., 2003). These greening
cotyledons were indistinguishable from those of the wild type for 3-4 d but then gradually faded to a pale yellow as the highly Chl-deficient xantha double mutant phenotype was revealed. This study, which utilized a reverse genetic approach to identify the porB-1 and porC-1 null mutants, also found that under normal laboratory light growth conditions the single mutants exhibited no obvious phenotypes, indicating that PORB and PORC are functionally redundant with respect to bulk Chl biosynthesis.

In addition to the result of null insertion mutations in PORB and PORC, POR levels can be manipulated in planta by a variety of techniques including far-red light treatment of seedlings, generation of transgenic plants expressing ectopic POR expression or antisense constructs, and by the mutation of plastid import machinery and photomorphogenic pathway genes. For example, seedlings grown for several days under continuous far-red light contained little Chl and did not green even when transferred thereafter to white light (WL), due to a phytochrome A-mediated block of photomorphogenesis (Runge et al., 1996). These seedlings were depleted of PORA and partially depleted of PORB, causing a reduction in PLB size. Similarly, no PORA and reduced amounts of PORB were detected in the constitutive photomorphogenic mutant cop1-18, in which normally phytochrome-dependent processes occur even in the absence of light (Sperling et al., 1998). When germinated in the dark this mutant displayed a light-grown seedling morphology with a plastid ultrastructure that resembled a pseudochloroplast with no PLB and photoactive Pchlide. Photoactive Pchlide and PLBs were restored, however, when PORA or PORB was expressed ectopically either in the cop1-18 mutant background (Sperling et al., 1998), or in far-red light-grown wild type plants (Sperling et al., 1997). Although the resulting partial rescue of the greening
defects in POR-depleted 
*cop1-18* mutants and far-red light-treated seedlings suggests a possible functional redundancy of the PORA and PORB isoforms, the use of ectopic expression constructs complicates this conclusion. When antisense and overexpression approaches were used to manipulate PORA and/or PORB protein amounts in etiolated wild type seedlings, it was found that total POR content correlated with the spectroscopic properties of photoactive Pchlide, the ratio of photoactive to nonphotoactive pigment, the photoreduction kinetics of Pchlide, and the formation and amount of the PLB (Franck et al., 2000). Again, these results strongly suggest but do not conclusively demonstrate the functional redundancy of PORA and PORB in etiolated seedlings.

With regard to isoform-specific mechanisms for POR plastid import from the cytosol, much uncertainty remains in the literature. It has been reported that there is an organ-specific, Pchlide-dependent import of the cytosolic precursor of PORA but not of PORB in Arabidopsis cotyledons, whereas both are imported independent of the pigment substrate in true leaves (Kim and Apel, 2004). Recently, there have been conflicting reports pertaining to PORA protein plastid import and the role of outer envelope solute channel protein Oep16. In an Oep16 mutant Philippar et al. (2007) demonstrated normal PORA import and etioplast ultrastructure, whereas Pollmann et al. (2007) reported a seedling conditional lethal phenotype, as well as a PORA import defect. There is also a lack of clarity in the literature from *in vitro* and *in vivo* research regarding the role of Toc33, a putative member of the outer plastid envelope translocon, in the import of PORA and PORB into plastids. Some data suggest that Toc33 is required for the import of PORB but not PORA in cotyledons, whereas in true leaves contrasting data support a
model in which Toc33 is required for PORA but not PORB import in the dark (Kim et al., 2005; Reinbothe et al., 2005).

The Arabidopsis PORs therefore define a complex story of a protein family whose three members (1) perform a photoregulated enzymatic reaction followed by plastid ultrastructural transformation, (2) display differential substrate-dependent and organ-specific plastid import requirements and (3) exhibit differential light regulation of gene expression. The *porB-1 porC-1* double mutant demonstrates clearly *in vivo* how the negatively light-regulated expression of the PORA promoter causes a pleiotropic phenotype in the absence of PORB and PORC, due to the very low level of expression of PORA in light-grown plants. Here we further elucidate the *porB-1 porC-1* double mutant phenotype by analyzing etioplast ultrastructure in plastids containing only PORA, as well as by examining PORA-mediated greening ability under high light conditions, thereby defining the endogenous activity of PORA expressed from its native promoter. Using an ectopic expression construct we also investigated the capacity of PORA to direct bulk Chl synthesis in the *porB-1 porC-1* double mutant background. Our results demonstrate that Arabidopsis seedlings and adult plants are capable of using PORA as the sole means of producing sufficient Chl to support normal plant growth and development.

### 2.2. RESULTS

A transgene encoding the *PORA* cDNA under the control of the ubiquitously active cauliflower mosaic virus (CaMV) 35S promoter was introduced into the *porB-1 porC-1* double mutant though a genetic cross to establish *in vivo* whether ectopic *PORA*
expression can replace the functions of \textit{PORB} and \textit{PORC}. Previous work had identified doubly homozygous \textit{porB-1 porC-1} mutants among the progeny of a doubly heterozygous population (Frick et al., 2003). The \textit{PORA} transgene described above had also been previously introduced into ecotype C24 wild type plants, creating strong ectopic \textit{PORA} overexpressor (PAO) transgenic lines (Sperling et al., 1997). A cross was performed between homozygous line PAO-3 and a line homozygous for the \textit{porC-1} and heterozygous for the \textit{porB-1} mutations, followed by a PCR screen of the F1 generation for the presence of all three insertions. This step was essential due to the segregating \textit{porB-1} allele. Triply positive lines were allowed to self-pollinate. F2 progeny were screened for lines homozygous for either \textit{porB-1} or \textit{porC-1}, as well as positive for the presence of the respective second mutation, and the presence of the \textit{PORA} cDNA. F3 lines were screened for the presence of the \textit{PORA} cDNA in all progeny. PCR using gene-specific \textit{PORA} primers created a 247 base pair (bp) genomic PCR fragment that spanned an 82 bp intron, versus a 165 bp fragment when the PAO-3 cDNA fragment was present (Figure 2.1A). Not all \textit{porB-1 porC-1} lines carrying the homozygous PAO-3 insertion showed a fully rescued phenotype; some lines had bleaching in sectors of the cotyledons, as well as in stems and leaves. Lines 41 and 31 displayed no visible sectoring, and were therefore chosen for further analysis. From these lines the PAO-3 cDNA PCR fragment was amplified in all progeny, \textit{Ds (Dissociation)} element: genomic border fragments were amplified for both the \textit{porB-1} and \textit{porC-1} insertions in all progeny, and no wild type PCR amplification products were obtained with primers flanking the respective \textit{Ds} insertion sites (Figure 2.1A).
Phenotypically rescued porB-1 porC-1 double mutants overexpress the PORA protein

PORA mRNA was not detected in northern blot analyses of light-grown wild type Arabidopsis seedlings whereas the PORB and PORC mRNAs were relatively abundant (Armstrong et al., 1995; Su et al., 2001). In contrast, northern blot analysis of the porB-1 and porC-1 single homozygous mutants showed no mRNA for the respective mutated genes in 7 d-old light-grown seedlings (Frick et al., 2003). Furthermore, as reproduced in Figure 2.1B, each of the single mutants was deficient in one of the two distinct POR-immunoreactive species detected in total protein extracts of light-grown plants that had been reacted with a polyclonal anti-Arabidopsis POR antiserum which recognizes all three POR enzymes. The porB-1 mutant was deficient in the POR isoform with a molecular mass of about 37 kDa, whereas the porC-1 mutant lacked an isoform with an apparent molecular mass of 38 kDa. These data suggested the identity of PORB and PORC to be the 37- and 38-kDa polypeptides, respectively (Frick et al., 2003). The presence of both isoforms in the wild types, as well as their absence in the porB-1 porC-1 double mutant (Figure 2.1B), also supports this conclusion. The lack of any detectable POR isoforms in the porB-1 porC-1 double mutant further agrees with the lack of detectable expression of PORA mRNA in light-grown seedlings (Armstrong et al., 1995). The presence of a higher amount of POR in the PAO-3 line compared to the two wild types is due to the ectopic accumulation of PORA, in addition to the endogenous levels of PORB and PORC (Sperling et al., 1998). Because the PORB and PORC proteins are not produced in the PORA-rescued porB-1 porC-1 double mutant lines 41 and 31, only the
PORA isoform with an apparent molecular weight of 37 kDa remains (Figure 2.1B). These data support the conclusion that PORB and PORC are not produced in the *porB-1 porC-1* double null mutant, as expected, and that PORA is functioning independently to support bulk Chl synthesis in the rescued double mutant lines.

**PORA-rescued porB-1 porC-1 lines have a standard growth profile**

A functional PORA protein expressed throughout seedling and plant development should be able to support growth of a *porB-1 porC-1* double mutant plant that resembles the wild type if PORA can function in place of PORB and PORC in bulk Chl biosynthesis. PAO lines in a wild type background do not show any obvious morphological differences relative to the wild type when grown under normal photoperiodic conditions, so PORA overexpression *per se* would not be expected to cause pleiotropic side effects (Sperling et al., 1997). The expression profile of PORA shows a high level of transcription in emerging cotyledons of light-grown gerninating seedlings at 2 d, which apparently yields adequate PORA for Chl biosynthesis during early greening (Armstrong, et al., 1995). PORA expression diminishes by 3 d, coinciding with the visible onset of gradual Chl turnover that ultimately reveals the yellow (*xantha*) phenotype of the *porB-1 porC-1* mutant (Frick et al., 2003). In Figure 2.2A, the first true leaves of the PORA-rescued *porB-1 porC-1* double mutants sown on MS (Murashige and Skoog) agar are visible. Whereas, the *porB-1 porC-1* double mutant is unable to grow photoautotrophically and hence does not develop past the cotyledon stage (Frick et al., 2003). The PORA-rescued *porB-1 porC-1* mutant matured similarly to both the C24 and Landsberg erecta (*Ler*)
wild types from the young seedling stage through the rosette stage (Figure 2.2B-D). At this point, a delay in the flowering time was observed, causing an extension of vegetative growth in the rescued lines (Figure 2.2E). Delays in flowering have been previously reported in the progeny of ecotype crosses related to the segregation of FRI and FLC alleles. It was shown that a polymorphism exists between C24, a Columbia-derived ecotype, and Ler ecotypes in the FRI locus (Sanda and Amasino, 1995). Therefore, in the PORA-rescued porB-1 porC-1 lines the delay in flowering time is likely due to the interaction of FRI-late and FLC-late loci in the crossed plants. From these data, we conclude that ectopically expressed PORA can support plant development that resembles that of the wild type in the porB-1 porC-1 double mutant background.

**Bulk Chl accumulation is quantitatively restored to the porB-1 porC-1 mutant by ectopically expressed PORA protein**

To determine whether PORA can function quantitatively as the sole Pchlide-reducing enzyme used for bulk Chl synthesis in green plants, we examined whether Chl accumulation in PORA-rescued porB-1 porC-1 double mutants is similar to that of wild type. We quantified the restoration of Chl in the rescued double mutants by extracting Chls a and b from adult rosette leaves. Extracts were measured by fluorescence emission spectroscopy and the amounts of Chls a and b were determined (Figure 2.3). Rosette leaves of the PORA-rescued porB-1 porC-1 double mutants accumulated approximately wild type levels of Chls a and b relative to the C24 control and marginally lower levels relative to the Ler control. The calculated Chl a/b ratios were 3.11 and 3.35 for rosette
leaves from rescued lines 31 and 41, respectively, 3.32 and 3.14 for *Ler* and C24, respectively, and 3.04 for line PAO-3. These ratios suggest that the photosynthetic pigment-protein complexes present in the PORA-rescued *porB-1 porC-1* rosette leaves may be similar to those of the wild type grown in the same light environment. Therefore, under normal growth conditions the rescued *porB-1 porC-1* double mutants display PORA-dependent Chl accumulation that is similar to that seen in the wild type when all three PORs are expressed. This underscores the apparent redundancy of enzymatic activities between PORA, which is normally expressed primarily in etiolated and greening cotyledons, and PORB and PORC, which are expressed in cotyledons but also in both older seedlings and plants, and are normally responsible for most post-cotyledon Chl synthesis.

*Photoperiodically-grown PORA-rescued porB-1 porC-1 seedlings display normal chloroplast membrane ultrastructure*

Restoration of extensive thylakoid stacking to the *porB-1 porC-1* double mutant by PORA overexpression would be consistent with the production of a normal complement of photosynthetically active Chl. Ultrathin sections of photoperiodically-grown cotyledons were analyzed by transmission electron microscopy (Figure 2.4). A detailed observation of chloroplast inner membrane architectures revealed the wild type and PORA-rescued *porB-1 porC-1* seedlings to be similar, whereas the membrane organization of the *porB-1 porC-1* double mutant plastids differed substantially. The wild type, PAO-3 and PORA-rescued *porB-1 porC-1* double mutant chloroplasts
contained numerous grana stacks (Figure 2.4A, D, E, F, G) in addition to unstacked thylakoids and starch granules. In contrast, the double mutant chloroplasts contained primarily unstacked thylakoids, as well as occasional double membrane stacks (Figure 2.4B). A small but significant population of porB-1 porC-1 plastids contained irregularly organized unstacked thylakoids along with rare membrane stacks (Figure 2.4C). The comparison of porB-1 porC-1 double mutant plastids to PORA-rescued porB-1 porC-1 chloroplasts clearly demonstrates the normal chloroplast membrane architecture of the rescued lines.

**PORA overexpression allows normal greening of previously etiolated porB-1 porC-1 seedlings**

The ability of ectopically available PORA to suffice for greening as the sole POR present was demonstrated by a rescue of the porB-1 porC-1 double mutant greening deficiency seen at high light fluences. Chl accumulation was quantified in etiolated seedlings that had been shifted for 24 h to medium light (50 µE m⁻² s⁻¹) or high light (400 µE m⁻² s⁻¹). This approach was used to establish the greening efficiency of the PORA-rescued porB-1 porC-1 double mutant compared to the por single mutants, line PAO-3, the two wild types and, in high light, the porB-1 porC-1 double mutant (Figure 2.5). Arabidopsis seedlings were etiolated for 5 d on MS plates and then exposed to the respective light fluence for 24 h, followed by collection, acetone extraction and total Chl quantification by fluorescence emission spectroscopy. At WL fluences of either 50 or 400 µE m⁻² s⁻¹ the PORA-rescued porB-1 porC-1 double mutants synthesized equivalent
amounts of Chl to the wild type seedlings (Figure 2.5A, B). The seed set of the doubly homozygous *porB-1 porC-1* mutant was not sufficient to perform this measurement. Furthermore, in 50 µE m$^{-2}$ s$^{-1}$ of WL it was not possible to phenotypically distinguish segregating *porB-1 porC-1* double mutant seedlings among a heterozygous population after only 24 h (data not shown). However, *porB-1 porC-1* double mutants obtained from a segregating population during growth at 400 µE m$^{-2}$ s$^{-1}$ could be phenotypically selected for pigment analysis as bleached *xantha* seedlings. No such bleached seedlings were observed in the controls. These *xantha* seedlings produced 21-fold less Chl than did the two wild types, underscoring the severe greening deficiency evident in high light due to the absence of PORB and PORC. Ectopically expressed PORA can therefore function to prevent the early greening deficiency in *porB-1 porC-1* double mutant cotyledons, thereby demonstrating both the normal importance of PORB and PORC in the wild type, and establishing the enzymatic potential of PORA to assume their functions.

**Specific defects in porB-1 and porC-1 mutant Chl accumulation at very low and very high light fluences are compensated for by ectopic PORA expression**

Under very high light and very low light conditions there are reductions in Chl accumulation in *porC* and *porB* mutants respectively, relative to the wild type. In Masuda et al. (2003), a unique role of PORB activity in very low light was demonstrated. Using a *porB* mutant allele in the Columbia ecotypic background they found that after 3 d of etiolation followed by 3 d of 0.1 µE m$^{-2}$ s$^{-1}$ WL, Chl accumulation in mutant seedlings was reduced 2-fold relative to the wild type. Here, these growth conditions were
reproduced and the harvested seedlings of various genotypes subjected to Chl quantification using fluorescence emission spectroscopy (Figure 2.5C). The porB-1 mutant in the Ler ecotypic background confirmed the Masuda et al. (2003) result with a more than 2-fold decrease in Chl accumulation versus its respective wild type. Unfortunately, making this measurement with the porB-1 porC-1 double mutant was technically impossible for the same reasons noted for the experiment shown in Figure 2.5A. The Chl accumulation of the porC-1 mutant and the PORA-rescued porB-1 porC-1 double mutant lines was similar to or slightly higher than that of the Ler wild type (Figure 2.5C). Therefore, under these very low light conditions PORC does not seem to play a major role in Chl production. This agrees with the observation that PORC expression is induced by high light conditions and is almost undetectable in etiolated seedlings and in low light (Su et al., 2001). PAO-3, a PORA overexpressor line, produced slightly more Chl than C24, its respective wild type. Overall these data demonstrate that ectopic production of the PORA protein in very low light conditions increases the capacity for the synthesis of Chl, and that PORA can replace PORB in this respect.

Masuda et al. (2003) also demonstrated that the PORC protein was produced in 3 d-old etiolated seedlings that were subsequently exposed for an additional 3 d to 1000 μE m⁻² s⁻¹ of WL and that under these conditions seedlings carrying a porC mutant allele in the Wassilewskija ecotypic background accumulated 2-fold less Chl than the wild type. To confirm this experiment in the porC-1 mutant, 3 d-old etiolated seedlings were transferred into 1000 μE m⁻² s⁻¹ light for 3 d, and thereafter collected, acetone-extracted and their Chl contents quantified using fluorescence emission spectroscopy. This very
high light fluence did not hinder Chl accumulation in the porB-1 single mutant compared to the Ler wild type, whereas Chl accumulation in the porC-1 mutant was modestly reduced (Figure 2.5D), in qualitative agreement with Masuda et al. (2003). The PORA-rescued porB-1 porC-1 double mutant lines, however, as well as PAO-3, all produced as much Chl as the C24 wild type. Therefore, both the very high light and the very low light experiments verified specific conditions under which PORC and PORB, respectively, individually play crucial roles in Chl production. These roles can, however, be fulfilled by PORA alone in PORA-rescued porB-1 porC-1 double mutant seedlings.

**PORA protein can independently direct the formation of PLBs**

The current hypothesis suggests a close correlation between the levels of photoactive Pchlide, the total amount of POR protein, and the extent of the PLB. It was therefore necessary to test whether the reduction of PLB volume found in porB mutants (Frick et al., 2003; Masuda et al., 2003) would also be observed in the porB-1 porC-1 double mutant, and furthermore if the PLB would be restored in PORA-rescued porB-1 porC-1 double mutants. Ultrastructural analysis of cotyledon etioplasts of 5 d-old etiolated seedlings was performed by transmission electron microscopy using the porB-1 porC-1 mutants and the PORA-rescued porB-1 porC-1 lines. Typical etioplasts from porB-1 porC-1 double mutants contained PLBs that were somewhat smaller than those seen in Ler wild type etioplasts of the same age (Figure 2.6A, B). The PORA-rescued porB-1 porC-1 double mutant lines each contained etioplasts with characteristically larger PLBs (Figure 2.6C, D) than both those of the porB-1 single mutant (Frick et al., 2003) and the
porB-1 porC-1 double mutant (Figure 2.6B). As expected, the most extensive PLBs were observed in line PAO-3 (Figure 2.6E), which contains ectopically expressed PORA, as well as endogenously expressed PORA and PORB (Sperling et al., 1998). PORA-rescued porB-1 porC-1 lines, as well as porB-1 porC-1 plants, only contain PORA protein. Therefore, our experiments demonstrate that PORA can function independently of the other PORs to create and maintain a PLB.

**PORA can independently function to produce wild type levels of photoactive Pchlide**

POR-mediated Pchlide photoreduction is strictly light-dependent; in etiolated seedlings POR accumulates as a dark-stable photoreactive ternary complex with bound NADPH and Pchlide. Pchlide not bound to POR, however, can act readily as a photosensitizer (Matringe et al., 1989; Mock et al., 1998). Photoconversion of Pchlide to Chlide in porB null mutants under continuous very low light conditions, as shown here (Figure 2.5C) and elsewhere (Masuda et al., 2003), would be expected to be PORA-limited because PORC is not present under these conditions. Using fluorescence emission spectroscopy to measure extracted Pchlide and Chlide from etiolated seedlings before and after flash illumination treatment we calculated the amounts of photoactive, nonphotoactive and total Pchlide pigment present. The amounts of pre-flash total Pchlide and photoactive Pchlide, respectively, were similar in Ler, porC-1 and the PORA-rescued porB-1 porC-1 lines, whereas porB-1 had modestly reduced total Pchlide and 4-fold less photoactive Pchlide than Ler wild type (Figure 2.7). Expressing this in a different way, if the ratio of pre-flash photoactive to total Pchlide in Ler is taken as 100%, then the porB-1 single
mutant ratio of photoactive to total Pchlide pre-flash was reduced to only 42%. The highest amounts of total and photoactive Pchlide among all of the genotypes were measured in line PAO-3, as expected (Sperling et al., 1998). The level of Pchlide photoconversion in the PORA-rescued porB-1 porC-1 lines resembles wild type, clearly demonstrating that the amount of PORA is limiting for Chlide formation in etiolated porB-1 seedlings. Furthermore, that PORA is sufficient in the absence of PORB and PORC to produce photoactive Pchlide.

2.3. DISCUSSION

In the angiosperms studied to date, one to three light-dependent POR gene family members have been identified per species, but the significance of the corresponding protein families has not been definitively established. The Arabidopsis POR gene family encodes three homologous, structurally similar protein members, denoted PORA, PORB and PORC, which are synthesized as cytosolic precursors and are processed upon plastid import. Pairwise amino acid sequence alignment comparisons between the predicted mature Arabidopsis POR proteins show that they share 84-89% identity. Despite this high degree of sequence conservation, many studies have investigated the potential for functional equivalence of the mature POR isoforms because of their differential expression patterns and uncertainties surrounding their exact roles in plant development (Sperling et al., 1998; Masuda et al., 2003; Frick et al., 2003; Franck et al., 2000). Pairwise amino acid sequence alignments for the Arabidopsis POR plastid transit peptides show that PORA and PORB share 75% identity with one another, whereas these
sequences only share ~40% identity with the PORC transit peptide, suggesting there may be several different pathways for import. POR isoform-specific plastid import has indeed recently been a topic of much interest (Kim and Apel 2004; Philippar et al., 2007; Pollmann et al., 2007; Kim et al., 2005; Reinbothe et al., 2005).

In light-grown angiosperms, POR functions as an essential component in the pathway for bulk Chl biosynthesis, whereas in etiolated seedlings it is required for the existence of the PLB and then upon illumination for the photoconversion of Pchlide to Chlide in Chl synthesis during the early stages of greening (Sperling et al., 1998). The lack of obvious visible phenotypes in single mutants deficient in either PORB or PORC demonstrates the functional redundancy of these isozymes in greening seedlings and green plants under normal light intensities and growth conditions. However, etiolated seedlings lacking PORB do have smaller PLBs, as well as decreased photoconversion of substrate at the onset of greening, indicating that the in vivo levels of PORA are limiting for the production of the PLB and Chlide (Frick et al., 2003; Masuda et al., 2003). Chl accumulation in the porB-1 porC-1 double mutant presents a clear in vivo demonstration of the unmasked catalytic function of PORA (Frick et al., 2003). The porB-1 porC-1 pleiotropic xantha phenotype is most likely due to severely reduced Chl accumulation, and hence impaired photosynthetic metabolism, and possibly also to photosensitization due to unbound Pchlide. A Pchlide-mediated photodynamic effect is suggested by the fact that the double mutant phenotype is exacerbated at higher light intensities.
Ectopic PORA expression is functionally equivalent to endogenous PORB and PORC expression in green plants

PORA is normally expressed preferentially in etiolated seedlings and briefly during greening, suggesting a specialized PORA function in response to these conditions and developmental stages (Armstrong et al., 1995). On the other hand, all three POR family members share a high degree of amino acid sequence identity, indicating a probable overlap of at least some functions. The result of ectopic expression of PORA in the porB-1 porC-1 double mutant background demonstrates conclusively its potential in vivo catalytic functionality in bulk Chl synthesis.

The wild type Chl production measured in porB and porC single mutants suggests that the corresponding POR proteins do not limit Chl accumulation under normal growth conditions (Masuda et al., 2003). Growth of the porB-1 porC-1 double mutant under different light intensities affects the degree of photobleaching (Figure 2.5), highlighting the ability of endogenous PORA to delay Chl loss from cotyledons maintained at a normal light intensity for 3-4 d (Frick et al., 2003). In contrast, for double mutant seedlings grown in high light the loss of Chl synthesized during early greening is more pronounced. Ectopically expressed PORA in a porB-1 porC-1 mutant background can sustain wild type development, including bulk Chl synthesis, demonstrating the capability of PORA to assume the functions of all three PORs that contribute to the whole plant phenotype (Figure 2.2). The porB-1 mutant produces less Chl than the wild type under very low light conditions (Figure 2.5), indicating a specific adaptation function for the
PORB protein that neither PORA nor PORC normally fulfill. However, when PORA is ectopically expressed in the *porB-1 porC-1* double mutant it supplies the additional Pchlide-reducing capacity under very low light conditions needed for wild type Chl production. Similarly, reduced Chl production under very high light conditions in the *porC-1* mutant is also restored by the ectopic expression of PORA in the double mutant. These data demonstrate the *in vivo* catalytic function of PORA over a wide range of light fluences, including extreme environmental conditions. By analogy, previous work has shown that ectopically expressed PORA allows far-red light-grown PAO seedlings to synthesize much larger amounts of Chl that do PORA- and PORB-depleted wild type seedlings under the same conditions (Runge et al., 1996; Sperling et al., 1997). Ectopically expressed PORA can thus maintain Chl synthesis and development, and likely provide photoprotection by limiting unbound substrate accumulation, under not only normal but also extreme light conditions when PORB and PORC are absent due to mutations in the respective genes.

With regard to parameters that indicate the presence of a functional photosynthetic membrane, a wild type Chl *a/b* ratio was quantified in the PORA-rescued *porB-1 porC-1* plants (Figure 2.3). The chlorotic double mutant cotyledons normally have a highly skewed Chl *a/b* ratio, due to the extremely low endogenous PORA content (Frick et al. 2003). The wild type ratio can therefore be restored by ectopic PORA expression. Independent of PORB and PORC, PORA increases the availability of Chl *b* in the *porB-1 porC-1* double mutant for binding by the light-harvesting Chl *a/b*-binding photosynthetic antenna complexes (Melkozernov et al., 2006), presumably by boosting overall Chl biosynthesis capacity. The wild type thylakoid stacking observed in the
PORA-rescued \textit{porB-1 porC-1} mutants (Figure 2.4) underscores the apparent assembly of a normal photosynthetic membrane. Taken together with the apparently wild type visible phenotype and growth of the rescued plants, these data collectively demonstrate that neither PORB nor PORC are functionally required either alone or in a heteromeric complex for \textit{in vivo} bulk Chl biosynthesis or for functionally robust photosynthetic membrane assembly, provided that a sufficient amount of PORA is available.

\textit{Ectopically expressed PORA protein compensates for the absence of PORB and PORC to restore normal etioplast membrane ultrastructure and photoactive Pchlide formation}

The partial PLB deficiency seen in \textit{porB-1 porC-1} double mutant etiolated seedlings (Figure 2.6) is similar to the \textit{porB-1} mutant phenotype, and is also consistent with the normal PLB observed in the \textit{porC-1} mutant (Frick et al., 2003), because \textit{PORC} expression is extremely weak under these conditions. Therefore, \textit{porB-1 porC-1} etioplasts contain abnormally small PLBs primarily, if not exclusively, due to the absence of PORB. The PORA-rescued \textit{porB-1 porC-1} double mutant, however, has etioplasts containing only PORA with PLBs that display the normal paracrystalline matrix in size as well as shape, a typical prothylakoid structure, and a cotyledon cell morphology that also shows no obvious alterations. In the \textit{porB} mutant used by Masuda et al. (2003), a reduction in the amount of photoactive Pchlide paralleled a decrease in PLB volume. This correlation would also be expected for the \textit{porB-1 porC-1} double mutant, but making the photoactive Pchlide measurement was technically impossible due to the poor seed set of the mutant versus the number of doubly homozygous etiolated seedlings.
complementary porB-1 porC-1 double mutant rescued by ectopically expressed PORA, in which normal Pchlide photoconversion ability was demonstrated (Figure 2.7) in conjunction with a restoration of the PLB (Figure 2.6). Interestingly, we also found a reduction in total pre-flash Pchlide in the porB-1 mutant, which could be symptomatic of a possible feedback regulation of Pchlide accumulation by the nonphotoactive pigment pool in that genotypic background. Published miniarray data have identified PORC expression as being positively light-correlated, but have also indicated a relatively low yet detectable expression in 3 d-old etiolated seedlings (Matsumoto et al., 2004). The similarity between the PLBs in dark-grown seedlings of the porB-1 porC-1 double mutant and the porB-1 mutant agrees with earlier protein and mRNA data, in which PORC expression was not detected in western blots and northern blots, respectively (Su et al., 2001; Masuda et al., 2003). The smaller PLB previously observed in the porB-1 mutant (Frick et al., 2003) demonstrated that PORA, the only detectably expressed POR in porB-1 etioplasts, could most likely independently produce a PLB. However, the possibility of an undetectable amount of PORC participating in this process could not be completely excluded. Therefore, the identification of a PLB, albeit smaller than in the wild type, in the porB-1 porC-1 double mutant establishes the fundamental ability of PORA to direct the formation of a morphologically normal PLB independent of either PORB or PORC. Taken together, these data are consistent with the extent of the PLB and the amount of photoactive Pchlide correlating with the total amount of PORA and PORB in an additive manner, as has been proposed (Franck et al., 2000). Furthermore, both PORA and PORB
specifically function to protect against photosensitization during early greening by the binding of free nonphotoactive Pchlide, consequently creating photoactive Pchlide in addition to promoting PLB formation.

The consequences of ectopic PORA expression in the absence of other PORs highlights the ability of plants to use PORA as the sole means of producing Chl

The tight integration of overall plant physiology and development with POR gene regulation emphasizes its central role in controlling the activity of a key biosynthetic enzyme required for photosynthesis. The robust Chl biosynthetic activity of ectopically expressed Arabidopsis PORA in the \textit{porB-1 porC-1} double mutant background is not consistent with the proposed LHPP model (Reinbothe et al., 1999), which has proven remarkably persistent in the literature despite serious doubts regarding its validity (Armstrong et al., 2000; Masuda and Takamiya, 2004). Basically, the LHPP model suggests the formation of a specific PORA:PORB protein complex, based on \textit{in vitro} reconstitution assays using the barley PORs and protein gel blot analysis of Arabidopsis extracts, as a prerequisite for deetiolation and greening in plants (Reinbothe et al., 1999; Pollmann et al., 2007). The clear demonstration of PORA-dependent functional bulk Chl synthesis allowing normal growth in the absence of the PORB and PORC isoforms indicates that an \textit{in vivo} heteromeric POR complex involving PORA, if it occurs at all, is not essential for plant growth and development. Similarly, the previously reported isolation of \textit{porB} and \textit{porC} single null mutants that display no phenotypes except under
extreme growth conditions largely rules out the possibility of essential heteromeric POR complexes involving either PORB or PORC (Frick et al., 2003; Masuda et al., 2003).

The tissue specificity, environmental response to light, and developmental timing of native PORA gene expression contrast with the broad ectopic PORA-mediated rescue of PORB and PORC deficiency described here. This suggests a constitutive ability of plastids to import and process the cytosolic precursor of PORA, and to concentrate PORA and Pchlide at the proper intraorganellar site to synthesize Chlide for Chl synthesis, even in situations in which PORA protein is not normally present. Collectively, the experiments described here and elsewhere indicate that the apparent roles of the POR gene family members in vivo in Arabidopsis are chiefly due to their specific expression patterns, presumably to optimize growth, and that PORB and PORC, to the extent that it has been tested, can be functionally replaced by PORA. Whether PORA, in addition, serves any unique roles in plant growth and development remains to be determined.

It has clearly been established that low POR protein content in light-grown seedlings can lead to photosensitization and impaired growth, although negative consequences of excess POR accumulation have not been identified. The differential expression patterns of the POR gene family members act to coordinate the availability of multiple isozymes in response to physiological signals including light, thereby maintaining photosynthesis and preventing photosensitization.
2.4. MATERIALS AND METHODS

Arabidopsis Ecotypes and Lines Used, Plant Growth Conditions, Sample Collection and Genotyping

Ecotypes Landsberg erecta stock number CS20 and C24 stock number CS906 Arabidopsis seeds were obtained from the Arabidopsis Biological Resource Center at The Ohio State University. A detailed description of the plasmid vector used for the ectopic overexpression of PORA in Arabidopsis ecotype Columbia derivative C24 plants and the generation of the homozygous PORA-overexpressing transgenic line studied here have been presented elsewhere (Sperling et al., 1997). The porB-1 and porC-1 single mutants, and the porB-1 porC-1 double mutant, all with an Arabidopsis ecotype Landsberg erecta background, have been previously described (Frick et al., 2003). For seed propagation, photoperiodically-grown putative porB-1 porC-1 double mutants were maintained on MS agar supplemented with 2% sucrose at a fluence rate of 12 µE m$^{-2}$ s$^{-1}$. A segregating porB-1 heterozygous and porC-1 homozygous line created in the process of screening for a doubly homozygous porB-1 porC-1 line was used for crosses with line PAO-3, a homozygous PORA-overexpressing transformant. In these crosses, the double mutant was the pollen donor and the PAO-3 plant the pollen acceptor. To isolate phenotypically rescued porB-1 porC-1 plants that ectopically express PORA, seedlings of subsequent generations of the above crosses were initially germinated by sowing seeds on MS agar plates. Thereafter, 10- to 21-d-old seedlings grown at 50 µE m$^{-2}$ s$^{-1}$ were screened for the
desired genotypes by PCR. PAO-3 homozygous lines were first isolated using primers for the PORA cDNA, 1.9 (formerly Atpor3.1; Armstrong et al., 1995) and 2.0 (formerly Atpor3.2; Armstrong et al., 1995), which also amplify an intron-containing portion of the endogenous PORA gene. These lines were further screened for plants that possessed both the porB-1 and porC-1 Ds insertions (Frick et al., 2003). A wild type PORB genomic fragment amplifies using primers 2.7 and 6.0, whereas the porB-1 Ds insertion:PORB genomic border fragment amplifies using primers 2.7 and DS3. Similarly, a wild type PORC fragment amplifies using primers 5.1 and 5.6, whereas the porC-1 Ds insertion:PORC genomic border fragment amplifies using primers DS3 and 5.6.

For seed propagation and phenotypic analysis, photoperiodically-grown plants were used. 21-d-old seedlings were transplanted from MS agar to soil and grown to maturity at a fluence rate of 120 µE m\(^{-2}\) s\(^{-1}\). Photoperiodically-grown plants were maintained in long-day conditions (16 h days at 22°C / 8 h nights at 20°C). Continuous WL conditions at 22°C were provided by a combination of TLD 36W/84 (4100 K) cool-white fluorescent lamps (Philips), F17T8/TL741 17 watt (4100 K) cool-white fluorescent lamps (Philips) and ES27 27 watt (2700 K) compact fluorescent bulbs (TCP). Seedlings were germinated by sowing surface-sterilized seeds on MS agar plates. To help synchronize germination, plates were placed in the dark at 4°C for at least 24 h to allow seed imbibition and thereafter exposed at 22°C to 1-2 h WL at a fluence rate of 160 µE m\(^{-2}\) s\(^{-1}\). The illuminated imbibed seeds were either returned to darkness at room temperature to produce etiolated seedlings of various ages, or were moved to continuous or photoperiodic WL of various fluence rates to produce light-grown seedlings. Different fluence rates were obtained using combinations of neutral density filters (Rosco). High
light-treated seedlings were maintained at 25-27°C and a WL fluence rate of 1000-1100 µE m⁻² s⁻¹, which was attained using a 1000 watt metal halide bulb (Hortilux). All fluence rates were measured with an LI-250 light meter (LI-COR).

Plant material was harvested and either frozen in liquid nitrogen and stored at -80°C until its use for DNA and protein isolation or was processed immediately for electron microscopy and spectroscopic measurements. Light-grown plant material was harvested in the laboratory at an ambient fluence rate similar to that at which plants had been grown immediately prior to harvest. Mutant xantha seedlings from segregating porB-1 porC-1 populations were selected visually for pigment quantitation. All other wild type, mutant and transgenic lines used were homozygous. In flash illumination experiments, a 1 ms light pulse was supplied to each plate of etiolated seedlings using a 550FD photographic camera flash (Vivitar) to ensure full conversion of all photoactive Pchlide to Chlide. Etiolated seedlings were harvested before and after flash treatment under a green safe light (Kodak 7B filter). Images of seedlings and plants were collected using a Zeiss Stemi 2000-C stereomicroscope fitted with a Sony DSC-S75 digital camera, or with the camera alone.

Protein Analyses and Transmission Electron Microscopy

Whole 14-d-old seedlings grown in long-day conditions at a fluence rate of 80 µE m⁻² s⁻¹ and harvested 5 h into the day phase were used for protein analysis. Cotyledons from both 5-d-old etiolated seedlings harvested under a green safe light and 7-d-old seedlings grown in long day conditions at 80 µE m⁻² s⁻¹ and harvested 2 h into the day phase, were
collected for plastid ultrastructure analysis using transmission electron microscopy. Total protein extracts were prepared by grinding seedlings under liquid nitrogen and combining one volume of the frozen powder with one volume of homogenization buffer (0.0625 M Tris-HCl, pH 6.8; 1% (v/v) SDS; 10% (v/v) glycerol; 0.5% (v/v) β-mercaptoethanol). Samples were incubated at 95°C and centrifuged at 17,000 g for 10 min. Protein content was assayed using the RC DC Protein Assay, a modification of the method of Lowry et al. (1951) (Bio-Rad Laboratories). Samples containing total protein extracts were separated electrophoretically under denaturing conditions by SDS-PAGE on a 10% polyacrylamide gel and electroblotted onto an Immun-Blot PVDF membrane (Bio-Rad Laboratories). Western blot analysis of the electroblotted antigens was performed using a 1:500 dilution of a polyclonal anti-Arabidopsis POR antiserum (Sperling et al., 1997), combined with an Immun-Star™ Goat Anti-Rabbit IgG Detection Kit (Bio-Rad Laboratories). An identical protein gel was stained with Coomassie Brilliant Blue R250 to confirm that equal loadings had been used for the immunoblot.

Cotyledons collected for plastid ultrastructure analysis were vacuum infiltrated at 10 psi for 16 h in a fixative composed of 0.1 M sodium phosphate buffer, pH 7.0, 3% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in the dark at room temperature. Fixed tissues were each washed five times for 5 min in 0.1 M sodium phosphate buffer, pH 7.0 and post-fixed for 1 h in 1 M sodium phosphate buffer, pH 7.0 containing 1% (v/v) osmium tetroxide. Post-fixed tissues were washed 3 times for 5 min in 0.1 M sodium phosphate buffer, pH 7.0, four times in distilled water, stained for 2 h in 1% (v/v) uranyl acetate and washed three times in distilled water. A gradual dehydration with ethanol over 1 h was followed by two 10 min washes in propylene oxide. Over 12 h
propylene oxide was gradually replaced with Spurr resin (Ted Pella, Inc.) until the
cotyledons were embedded in 100% (v/v) resin. After polymerization, samples were
sectioned and mounted on Formvar-coated copper grids, then stained with 2% (v/v)
uranyl acetate and Reynolds lead citrate. The plastid inner-membrane architectures were
viewed on a Technai G2 Spirit transmission electron microscope from at least three
independent seedlings of each genotype.

*Quantitative Pigment Determinations by Fluorescence Spectroscopy*

Room temperature fluorescence emission analyses of the Pchlide, Chlide, Chl *a* and Chl *b* present in pigment extracts of etiolated and light-treated etiolated seedlings, as well as leaves of older light-grown plants were performed. Total photosynthetic pigments were extracted from 10 pooled cotyledons or one leaf per sample in 3 ml of 80% (v/v) acetone supplemented with 0.1 N ammonium hydroxide at 4°C. Measurements were repeated at least 5 times using independent pigment extracts. Fluorescence emission spectra of the pigment extracts were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian) with both the excitation and emission slit widths set at 5 nm. The pigment extracts were excited at 433 nm, which preferentially causes Chl *a* fluorescence emission at 670 nm, and at 455 nm, which preferentially causes Chl *b* fluorescence emission at 655 nm, and spectra were recorded. Chl *a* and *b* amounts were determined from their respective emission bands using simultaneous equations derived from measurements of authentic standards (Fluka Chemica) excited at 433 and 455 nm, taking into account the published extinction coefficients of the pure pigments in 80% acetone. Correction factors
were applied by determining the amount of fluorescence overlap at different excitation wavelengths (Inskeep and Bloom, 1985). Pchlide was extracted from 5-d-old etiolated ecotype Col (stock number CS60000) seedlings according to Carey and Rebeiz (1985). Calibration was performed by adjusting the extinction coefficient of Chl a to reflect the lower molecular weights of Pchlide and Chlide. The Pchlide and Chlide amounts were determined by excitation of the pigment extracts at 433 nm and measurement of the resulting emission bands at 634 and 672, respectively. Data were analyzed in Minitab version 14.1 using one-way ANOVAs with a 95% confidence interval around the mean.

2.5. REFERENCES


Figure 2.1. **Isolation of PORA ectopic overexpressing seedlings in the porB-1 porC-1 double null mutant background.**

(A) PCR identification of doubly homozygous porB-1 porC-1 mutants rescued by ectopic expression of PORA. Genotypes of genomic DNA templates are given in the upper line. Primers sets used are as follows: PORA, 1.9/2.0; PORB, 2.7/6.0; porB insertion, 2.7/DS3; PORC, 5.1/5.6; porC insertion, DS3/5.6. All PCR fragments represent genomic sequences, with the exception of the lower PORA doublet band, which corresponds to the cDNA carried in transgenic line PAO-3.

(B) PORA protein is detectable in the porB-1 porC-1 mutants rescued by ectopic expression of PORA. A gel blot was prepared using 45 µg of total protein extracted from seedlings grown in photoperiodic conditions and reacted with a polyclonal anti-Arabidopsis POR antiserum that recognizes with all 3 Arabidopsis PORs. Lines 31 and 41 are genotype porB-1 porC-1 PAO-3. Ler and C24 are the wild type controls. The molecular mass of the nearest molecular weight marker is indicated in kilodaltons. A duplicate Coomassie-stained gel highlighting the amounts of the abundant Rubisco L-subunit in these extracts is included as a loading reference.
Figure 2.2. **Ecotypic PORA expression rescues the severe chlorophyll-deficient phenotype of porB-1 porC-1 double mutants.**

Representative growth phenotypes of PORA-rescued porB-1 porC-1 mutant lines 41 and 31. Genotypes from left to right and top to bottom are as follows: C24, Ler, PAO-3, line 41 and line 31. Panels A,B,C,D: bar signifies 0.5 cm. (A) 14 d after germination, seedlings grown in long-day photoperiodic conditions at a light fluence rate of 120 μE m⁻² s⁻¹, (B) 21 d after germination after which seedlings were transferred from MS agar to soil, (C) 31 d after germination, (D) 40 d after germination, (E) 58 d after germination, bar represents 2.0 cm.
Figure 2.3. **Rosette leaves of the PORA-rescued porB-1 porC-1 double mutant lines 31 and 41 accumulate wild type levels of chlorophyll.**

The amounts of chlorophylls $a$ and $b$ were determined in acetone-extracted total pigments from seedlings grown for 45 d after germination under long-day conditions at a fluence rate of 120 µE m$^{-2}$s$^{-1}$. *Ler* and C24 are the wild type controls. Pigments were extracted from five rosette leaves individually for each data point, with error bars indicating 95% confidence intervals.
Figure 2.4. Chloroplast membrane ultrastructure in wild type seedlings, doubly homozygous porB-1 porC-1 mutants and doubly homozygous porB-1 porC-1 mutants ectopically expressing PORA. Ultrathin sections of plastids from 7 d-old seedlings grown under photoperiodic conditions at a fluence rate of 80 µE m⁻² s⁻¹ were examined by transmission electron microscopy. Genotypes (A) Ler, (B) porB-1 porC-1, (C) porB-1 porC-1, (D) porB-1 porC-1 PAO-3 line 41, (E) porB-1 porC-1 PAO-3 line 31, (F) PAO-3 and (G) C24. Bars = 0.5 µm.
Figure 2.5. Chlorophyll accumulation in the porB-1 porC-1 double mutant is restored by ectopic PORA expression.

Chlorophyll was extracted from 5-d-old etiolated seedlings subsequently exposed to 24 h of WL at (A) 50 µE m$^{-2}$ s$^{-1}$ or (B) 400 µE m$^{-2}$ s$^{-1}$ to test the effect of ectopic PORA expression during greening. In addition, chlorophyll was extracted from 3-d-old etiolated seedlings thereafter exposed to 72 h of WL at (C) 0.1 or (D) 1000 µE m$^{-2}$ s$^{-1}$ to test the effect of ectopic PORA expression during extreme high and low light growth conditions. Lines 31 and 41 are genotype porB-1 porC-1 PAO-3. Ler and C24 are the wild type controls. Each data point represents at least seven replicates of ten seedlings with error bars indicating 95% confidence intervals. Chlorophyll content could not be determined for the porB-1 porC-1 double mutant grown at fluence rates of 50 and 0.1 µE m$^{-2}$ s$^{-1}$ because of the difficulty in identifying double mutant seedlings among a segregating population under these conditions.
Figure 2.6. Etioplast membrane ultrastructure in wild type, doubly homozygous porB-1 porC-1 mutants and doubly homozygous porB-1 porC-1 mutants ectopically expressing PORA. Ultrathin sections of etioplasts from 5-d-old etiolated seedlings grown in complete darkness were examined by transmission electron microscopy. Genotypes: (A) Ler, (B) porB-1 porC-1, (C) porB-1 porC-1 PAO-3 line 41, (D) porB-1 porC-1 PAO-3 line 31, (E) PAO-3 and (F) C24. Bars = 0.5 µm.
Figure 2.7. **Total and photoactive protochlorophyllide pigment forms deficient in the etiolated and flash-illuminated porB-1 mutant are restored by ectopic by PORA expression.** Pigments were extracted from 5-d-old etiolated seedlings before (dark bars) and after flash treatment (dark bars minus light bars). Photoactive Pchlide was calculated as the difference between the pre- and post-flash measurements. Lines 31 and 41 are genotype porB-1 porC-1 PAO-3. *Ler* and C24 are the wild type controls. Each data point represents at least ten replicates of ten seedlings with error bars indicating 95% confidence intervals.
CHAPTER 3

ARABIDOPSIS LIGHT-DEPENDENT PROTOCHLOROPHYLIDE OXIDOREDUCTASE A (PORA) IS ESSENTIAL FOR NORMAL PLANT GROWTH AND DEVELOPMENT

ABSTRACT

During skotomorphogenesis in angiosperms, NADPH:protochlorophyllide oxidoreductase (POR) forms an aggregate of photolabile NADPH-POR-protochlorophyllide (Pchlide) ternary complexes localized to the prolamellar bodies within etioplasts. During photomorphogenesis, POR catalyses the light-dependent reduction of Pchlide $a$ to chlorophyllide $a$, which is subsequently converted to chlorophyll. In Arabidopsis there are three structurally related $POR$ genes, denoted $PORA$, $PORB$ and $PORC$. The PORA and PORB proteins accumulate during skotomorphogenesis. During illumination, PORA is only transiently expressed, whereas PORB and PORC persist and are responsible for bulk chlorophyll synthesis throughout plant development. Here we have tested whether PORA is important to skotomorphogenesis by assisting in etioplast development, and that it is also required for
normal photomorphogenic development. Using reverse genetic approaches, we have identified the *porA-1* null mutant, which contains an insertion of the maize Dissociation transposable element in the *PORA* gene. Additionally, we have characterized *PORA* RNAi lines. The *porA-1* and *PORA* RNAi lines display severe photoautotrophic growth defects, which can be partially rescued on sucrose-supplemented growth media. Elimination of PORA during skotomorphogenesis results in reductions in the volume and frequency of prolamellar bodies, and in photoactive Pchlide conversion. The *porA-1* mutant characterization thus establishes a quantitative requirement for PORA in etioplast development by demonstrating significant membrane ultrastructural and biochemical defects, in addition to suggesting PORA-specific functions in photomorphogenesis and plant development.

3.1. INTRODUCTION

NADPH:protochlorophyllide (Pchlide) oxidoreductase (POR, EC 1.3.1.33) has defined functions in light dependent chlorophyll (Chl) biosynthesis and, in angiosperms, in prolamellar body (PLB) formation. During angiosperm skotomorphogenesis, POR proteins accumulate in a ternary complex of POR-NADPH-Pchlide in the etioplast. This photoactive ternary complex is the major protein component of the PLB (reviewed by Schoefs and Franck, 2003). Other than POR and other minor proteins, PLBs are composed of membrane lipids, carotenoids and Chl precursors (in particular Pchlide). The PLB displays a regular, honeycombed, lattice-like structure, with (pro)thylakoids protruding into the etioplast stroma (Rosinski and Rosen, 1972; Selstam and Sandelius,
Upon absorption of a photon by the bound pigment substrate, POR catalyzes a reduction across the C17-C18 double bond of the D-ring of Pchlide $a$ to yield chlorophyllide $a$ (Chlide) (reviewed by Heyes and Hunter, 2005). Chlide $a$ is then esterified and further modified to form chlorophylls $a$ and $b$. During this process of light-induced greening the PLBs in etioplasts disintegrate and are gradually replaced by newly formed thylakoids as the plastids differentiate into chloroplasts. In light-grown angiosperms POR is associated with chloroplast thylakoid membranes, where it accumulates at much lower levels than in etiolated seedlings (Forreiter et al., 1991).

There are Arabidopsis three POR isoforms (PORA, PORB and PORC) with distinct developmental and light-regulated expression patterns, whose biological roles have sparked vigorous investigation (reviewed by Masuda and Takamiya, 2004). The $PORA$ mRNA and PORA protein are present at relatively high levels in etiolated seedlings, and their abundances are negatively regulated upon illumination, with the transcript no longer detectable by northern blotting within several hours of light exposure (Armstrong et al., 1995; Holtorf et al., 1995). In contrast, $PORB$ mRNA and PORB protein are readily detectable both in etiolated seedlings, and, at lower levels, in light-grown plant tissues, with $PORB$ mRNA accumulation following a circadian regulation pattern. Whereas the amount of $PORC$ mRNA in etiolated seedlings is very low, the $PORC$ gene is positively regulated by light and the transcript becomes detectable within several hours of seedling illumination in a fluence rate-dependent manner (Oosawa et al., 2000; Su et al., 2001; Masuda et al., 2003).
In contrast to angiosperms, gymnosperms and non-vascular plants also possess a second Pchlide-reducing pathway that relies on light-independent POR, a three subunit (ChIL, ChIN and ChIB) enzyme that is structurally unrelated to light-dependent POR. The loss of plastid-encoded light-independent POR in angiosperms, together with the evolution of small POR gene families in many species, indicate a probable adaptive advantage of specific POR regulation (Masuda and Takamiya, 2004). For example, in tobacco (Nicotiana tabacum L.) and barley (Hordeum vulgare L.) there are two POR gene family members characterized (Holtorf et al., 1995; Masuda et al., 2002). The POR gene regulation for barley is reminiscent of Arabidopsis PORA and PORB gene regulation. A few angiosperms studied to date apparently lack small POR gene families. A single constitutively expressed POR gene, regulated most similarly to PORB, has been reported in pea (Pisum sativum L.) (Spano et al., 1992). Within cucumber (Cucumis sativus L.) there is also a single POR gene, which is positively regulated by light by analogy to PORC (Fusada et al., 2000). In addition to these observations of gene regulation, a range of plant model systems have been used for in vitro and in vivo studies of POR using molecular-genetic, and biochemical approaches. Plants studied include the above-mentioned species, as well as wheat (Triticum aestivum), Amaranthus tricolor, maize (Zea mays) and cabbage (Brassica oleracea cv. capitata) (Teakle and Griffiths, 1993; Iwamoto et al., 2001; Eguchi et al., 2002; Selstam et al., 2002; Solymosi et al., 2004).

One function of PORA (and PORB) is to bind Pchlide in dark-grown seedlings. When Pchlide is not bound to the active site of POR, it is considered to be non-
photoactive Pchlide, because it is not immediately available to make Chlide. Non-
photoactive Pchlide that accumulates can act as a lethal sensitizer for the production of
singlet oxygen, which can lead to photooxidation and potentially even seedling death
upon illumination (op den Camp et al., 2003). Plants thus go to great efforts to prevent
the accumulation of unbound Chl biosynthetic intermediates that could serve as
photosensitizers. Major tetrapyrrole biosynthesis control points relevant for Chl
production include the feedback regulation of 5-aminolaevulinic acid (ALA) formation,
and the control of Mg$^{2+}$ ion insertion into the porphyrin macrocycle of protoporphyrin IX
(Moulin and Smith, 2005). In addition, the accumulation of excess Pchlide is prevented
by negative feedback control in etiolated angiosperms (Meskauskiene et al., 2001).

Defects in angiosperm seedling photomorphogenesis have been reported to
eliminate PORA and reduce PORB accumulation. An example comes from seedlings
grown for several days under continuous far-red light. Such seedlings appear
morphologically similar to white light-grown seedlings but contain little Chl because this
light quality is not used efficiently for POR catalysis, and do not green when
subsequently transferred to white light. This effect is attributable to a phytochrome A-
mediated block of photomorphogenesis which acts by decreasing $\text{POR}$ (in particular
$\text{PORA}$) gene expression in continuous far-red light, thus causing POR depletion and a
reduction in PLB size (Runge et al., 1996). Simultaneously, the accumulation of non-
photoactive Pchlide is increased because ALA formation is deregulated by continuous
far-red light. The second example comes from the constitutive photomorphogenic mutant
$\text{cop1-18}$, in which normally phytochrome-dependent processes occur even in the absence

65
of light (Sperling et al., 1998). When the cop1-18 mutant is germinated in the dark it displays a light-grown morphology. Further, the cop1-18 mutant displays a plastid ultrastructure that resembles a pseudochloroplast with unstacked prothylakoid membranes, no PLB and virtually no photoactive Pchlide but a large excess of non-photoactive Pchlide. When PORA or PORB was ectopically expressed either in the cop1-18 mutant background (Sperling et al., 1998), or in far-red light-grown wild type plants (Sperling et al., 1997), photoactive Pchlide and PLBs were restored, and the amount of non-photoactive Pchlide was decreased. Ectopic expression further resulted in a partial rescue of the greening defects in these POR-depleted seedlings, thus suggesting functional redundancy of the PORA and PORB isoforms.

Recent studies have examined the plastid import of the nuclear-encoded cytosolic precursors of the mature POR isoforms. Arabidopsis cotyledons demonstrate a Pchlide-requirement for the import of the cytosolic precursor of PORA but not that of PORB, whereas both are imported independent of the pigment substrate in true leaves (Kim and Apel, 2004). Conflicting import studies on the cytosolic precursor of PORA have raised questions regarding the role of plastid outer envelope solute channel protein Oep16. Using the Atoep16-1 mutant Philippar et al. (2007) demonstrated normal PORA import and etioplast ultrastructure. In contrast, Pollmann et al. (2007) reported a PORA import defect in the same mutant, in addition to a seedling-conditional, Pchlide accumulation-related lethal phenotype. Additional POR plastid import data have revealed complexities regarding the role of a putative member of the plastid outer envelope translocon Toc33. 

In vivo data suggest that Toc33 is required for the import of PORB but not PORA in
Arabidopsis cotyledons, whereas in vitro data indicate that in true leaves Toc33 is required for PORA but not PORB import in the dark (Kim et al., 2005; Reinbothe et al., 2005).

Targeted manipulation of isoform accumulation has revealed individual contributions of the Arabidopsis PORA and PORB proteins to plant development. The results of the introduction of antisense and ectopic overexpression constructs that manipulated PORA or PORB protein accumulation in vivo in transgenic Arabidopsis seedlings and plants have been reported (Runge et al., 1996; Sperling et al., 1997, 1998; Franck et al., 2000). It was demonstrated in etiolated seedlings of different genotypes that the total POR content (ie. PORA plus PORB, because endogenous PORC is not expressed under these conditions) is proportional to the photoactive Pchlide content, and correlates well with the ratio of photoactive to nonphotoactive pigment and the accumulation of the PLB (Franck et al., 2000). More recently, reverse genetic approaches have allowed the isolation of Arabidopsis por null mutants (Frick et al., 2003; Masuda et al., 2003). No obvious phenotypes were found under normal laboratory light growth conditions for the porB-1 and porC-1 single null mutants, suggesting that in general and particularly beyond the cotyledon stage PORB and PORC perform functionally redundant bulk Chl biosynthesis (Frick et al., 2003). This point is underscored by the severe xantha (ie. near achlorophyllous) phenotype of a porB-1 porC-1 double mutant. A clear demonstration of temporally-restricted and organ-specific PORA-dependent Chl production is, however, seen in light-germinated porB-1 porC-1 double mutant cotyledons, which are indistinguishable from those of the wild type for
about 3-4 days. Thereafter the Chl gradually disappears, presumably due to degradation, to reveal the pale yellow *xantha* double mutant phenotype (Frick et al., 2003). To assess the ability of POR to direct bulk Chl biosynthesis independent of PORB and PORC, a *POR* cDNA was ectopically expressed in the *porB-1 porC-1* double mutant background, thereby rescuing the double mutant impaired growth and Chl-deficient phenotypes. This demonstrates the clear ability of ectopically expressed POR to function as the only POR required for bulk Chl synthesis.

Thus, the currently available data indicate that POR functions in skotomorphogenesis as well as photomorphogenesis. During skotomorphogenesis, POR contributes to the formation of the prolamellar body, and binds the potent photosensitizer Pchlide, thus preparing the etioplast to minimize photooxidative damage during subsequent illumination (Franck et al., 2000). In photomorphogenesis, the activity of POR in Chl synthesis during the first few days suggests a role during early greening in cotyledons (Frick et al., 2003). However, the studies performed thus far do not indicate whether any of these (or other as yet unknown) functions of POR are essential.

To help address this question, we describe here the isolation and phenotypic characterization of the Arabidopsis *porA-1* null mutant, the only *porA* mutant documented thus far. Our goal has been to establish whether angiosperms require the function of POR for normal growth and development.
3.2. RESULTS

*Isolation of an Arabidopsis mutant containing a Ds element insertion in the PORA gene*

A collection of mutagenized Arabidopsis lines carrying insertions of a modified maize *Dissociation (Ds)* transposable element at random chromosomal locations has been described (Parinov et al., 1999). Preliminary genomic flanking sequences for a number of the *Ds* mutations were determined and assembled into a database of putative insertion sites (http://www.plantcell.org/cgi/content/full/11/12/2263/DC1). Transgenic seed family SGT4757 was predicted on the basis of a flanking sequence to segregate for a *Ds* insertion within the *PORA* gene (http://www.arabidopsis.org/servlets/TairObject?type=polyallele&id=500165528). A number of plants derived from this seed family were propagated on Murashige-Skoog (MS) agar at 80 µE m⁻² s⁻¹ under photoperiodic conditions and examined for visual phenotypes. In this preliminary screen, it was noted that approximately ¼ of the seedlings were light-green (ie. *chlorina*) in appearance, and did not grow beyond the cotyledon stage. Based on this result, those plants with a wild type appearance were propagated further on MS agar, whereas the *chlorina* seedlings were transferred to MS agar.
supplemented with sucrose, which allowed them to resume growth although they remained dwarfed (see below for details of the growth conditions and phenotype).

Both populations of plants were subsequently screened by PCR for the presence and orientation of the Ds insertion provisionally predicted to lie within intron 4 of PORTA (Figure 3.1A). To determine the exact Ds insertion site, we attempted to generate Ds::PORTA genomic border fragments by PCR to be used as templates for cycle sequencing with nested primers. Whereas the PORTA sequence flanking the 5’ end of the transposon could be amplified, we were not able to obtain a similar PCR product from the 3´ end of the transposon, despite the use of a number of different primers pairs. The same observation was also made in the preliminary sequencing of this Ds insertion site (V. Sundaresan, personal communication). However, using a PORTA-specific primer complementary to the sequence immediately downstream of the predicted Ds insertion site, primer 7.3 (Figure 3.1A), we were able to demonstrate that the 3’ flanking region immediately after the predicted location of the transposon insertion could be amplified by PCR (data not shown). These results indicated that no detectable deletions or rearrangements of the PORTA sequence had occurred downstream of the insertion site, and suggested that the failure to amplify the Ds::PORTA genomic border fragment at the 3´ end of the transposon was most likely due to a rearrangement or deletion within the Ds sequence itself. Nucleotide sequencing of the 5’ Ds::PORTA genomic border revealed the porA-1 insertion to lie 1 bp downstream of the originally proposed location (http://www.plantcell.org/cgi/content/full/11/12/2263/DC1), between nucleotides 25431 and 25432 (gi:3128134) (Figure 3.1B). In the absence of additional evidence, we assume
that this represents an exact insertion without a duplication of the genomic sequence bordering the 3´ end of the transposon. The corrected insertion site of the \textit{porA-1 Ds} element therefore lies 82 bp into intron 4, relative to the 5´ end of the gene.

Those seedlings containing the mutation due to the \textit{Ds} element (hereafter denoted \textit{porA-1}) were then screened for the presence of homozygous versus heterozygous \textit{Ds} insertions. As shown in Figure 3.1C, it was possible to identify homozygous \textit{porA-1} mutants based on the presence of the \textit{Ds} element within the \textit{PORA} gene and the inability to amplify a wild type genomic fragment using PCR primers flanking the \textit{Ds} insertion site. In heterozygotes, the latter PCR product was also present. Although interruption of a genomic sequence with the roughly 6.5 kb \textit{Ds} element (Sundaresan et al., 1995; Parinov et al., 1999), might, in principle, have caused the synthesis of a much larger PCR product with flanking primers, no such products were observed using our standard PCR methods. The heterozygous \textit{porA-1} mutants displayed no unusual phenotypes and could be grown to maturity on soil, whereas the homozygous mutants corresponded in every case to the light-green dwarves that were only viable on MS agar supplemented with sucrose.

After this initial round of phenotyping and genotyping, heterozygous \textit{porA-1} mutants were backcrossed once to the Landsberg \textit{erecta} wild type as an essential step to eliminate possible linked-insertion mutations that could lead to non-PORA-specific phenotypes. Indeed, one such spurious phenotype was detected fortuitously in the original SGT4757 seed stock; non-backcrossed families containing the \textit{porA-1} mutation in a heterozygous state also segregated in an unlinked fashion for seedlings with long and short hypocotyls when grown under continuous far-red light, a growth condition used in
the laboratory to cause POR depletion (Runge et al., 1996; Sperling et al., 1997). After backcrossing, we screened for \( F_2 \) \emph{porA-1} heterozygous families that had only short hypocotyls under far-red light, which is the wild type phenotype under this condition (Parks et al., 2003), and used those families for all further experiments.

Using the backcrossed \emph{porA-1} mutant, the originally observed stunted \emph{chlorina} phenotype was confirmed and characterized in detail. In contrast to the wild type, the \emph{porA-1} homozygous mutant arrested growth at the cotyledon stage when grown on MS agar (Figure 3.2A). These \emph{porA-1} seedlings resumed visible growth only when transferred to MS agar supplemented with 2% sucrose (Figure 3.2B). The \emph{porA-1} mutant eventually produced limited amounts of viable seed when propagated on this growth medium in moderate light (Figure 3.2C and D).

The \emph{porB-1 porC-1} double mutant is propagateable beyond the cotyledon stage as a slowly growing dwarf plant with a severely reduced seed set when maintained under low light conditions on sucrose-supplemented growth media (Figure 3.2E and F). A low level of greening was observed in the \emph{porB-1 porC-1} double mutant young rosette leaves when maintained under low light conditions (Figure 3.2E). Therefore, PORA dependent greening is observed in cauline leaves of \emph{porB-1 porC-1} double mutant plants when maintained in low light conditions on sucrose supplemented media.

The backcrossed heterozygous \emph{porA-1} seedlings were germinated on kanamycin-containing, sucrose-supplemented MS agar to estimate the number of independent \emph{Ds} insertions present. 7-d-old seedlings were observed to segregate in a 3:1 ratio (i.e. 279 kanamycin-resistant:89 kanamycin-sensitive). Co-segregation between kanamycin
resistance and the presence by PCR of a homozygous or heterozygous *porA-1 Ds* insertion was always observed. F₂ backcrossed *porA-1* seedlings were also germinated on MS media to determine the segregation ratio for the stunted *chlorina* seedling phenotype of the homozygous *porA-1* mutants. In this analysis of 14-d-old seedlings a 2.37:1 segregation ratio was observed (i.e. 316 wild type growth:133 stunted growth). Co-segregation between the stunted *chlorina* phenotype and the presence by PCR of the *porA-1* homozygous *Ds* insertion was always observed. Both of these genetic results therefore are consistent with the presence of a single locus *Ds* insertion in the homozygous *porA-1* mutant.

The etiolated *porA-1* null mutant lacks POR A transcript and a specific POR isoform

In the analysis of the *porA-1* mutant it was critical to demonstrate that the *Ds* insertion actually alters *POR A* gene expression, hence providing a potential basis for understanding the observed phenotype. Because * POR A* is expressed primarily in young seedlings and particularly in the absence of light (Armstrong et al., 1995), *POR A* transcript and POR protein levels were determined in etiolated rather than light-grown wild type and homozygous *por* mutant seedlings, as shown in Figure 3.3. *POR B* transcript levels were also monitored because this gene encodes the other highly expressed POR protein in etiolated seedlings. The *POR C* transcript level in etiolated wild type seedlings has been observed to be extremely low to undetectable (Oosawa et al., 2000; Su et al., 2001), as was the case in this set of experiments (data not shown). The
homozygous porB-1 and porC-1 mutants were included as controls because they have been previously demonstrated to lack the corresponding transcripts and specific polypeptide species in 7-d-old photoperiodically-grown seedlings (Frick et al., 2003).

Northern blot analysis revealed the etiolated porA-1 mutant to be completely deficient in PORA transcript (Figure 3.3A). This suggests degradation of any PORA transcript produced from the PORA gene upstream of the location of the Ds mutation in intron 4. No obvious cross-regulation of POR transcript amounts was seen in the etiolated por null mutants. In particular, the PORB transcript amount was not affected in the porA-1 mutant, demonstrating that this mutant retains a significant ability for POR enzyme synthesis.

In the western blot analysis, the etiolated homozygous porA-1 and porB-1 mutants were found to be deficient in distinct POR-immunoreactive species when challenged with an anti-Arabidopsis POR antiserum that recognizes all three isoforms (Figure 3.3B). Whereas the etiolated wildtype contained POR isoforms with apparent molecular masses of 37 and 36 kDa, the less abundant 37 kDa form was missing in the porA-1 mutant. In contrast, the porB-1 mutant lacked the more abundant POR isoform with a molecular mass of 36 kDa. The remaining POR-immunoreactive signal in this mutant appeared diffuse and was spread between 37 and 36.5 kDa. No obvious difference was seen in the patterns of POR-immunoreactive species between the porC-1 mutant and the wildtype. Based on these data, it therefore seems likely that PORA corresponds to the diffuse 37 – 36.5 kDa immunoreactive band observed in etiolated porB-1 seedlings, whereas the more abundant 36 kDa polypeptide represents PORB. These assignments are in agreement with
earlier studies of the wildtype in which correlations were drawn between the absence of $PORA$ mRNA and the 37 kDa POR-immunoreactive species (Armstrong et al., 1995; Runge et al., 1996). Although we cannot entirely exclude the possibility that a portion of the diffuse immunoreactive band represents PORC, whose absence in the $porC-1$ sample might have been obscured by the other immunoreactive signals, we emphasize that $PORC$ transcripts were not detected by northern blotting in this set of experiments or in other studies of etiolated seedlings.

**Light-grown porA-1 mutants display severe growth defects beyond the cotyledon stage**

As discussed earlier, homozygous $porA-1$ null mutants display an arrested growth phenotype when germinated on MS agar medium in the light. When germinated on or transferred to sucrose-supplemented media, however, they developed beyond the cotyledon stage and continued to grow as chlorina dwarf seedlings to maturity (Figure 3.2C and D). Growth conditions were optimized for sterile propagation of reproductively viable $porA-1$ mutants (see Materials and Methods). Under these optimized growth conditions the $porA-1$ mutant bolted and flowered at a similar time as the wild type grown on sucrose-supplemented media, although the morphologies of the mutant and wild type seedlings were very different. Not only did older $porA-1$ mutant plants appear stunted, but they also had a bushy appearance, apparently due to decreased apical dominance. It is noteworthy that when growth-arrested $porA-1$ seedlings were maintained on MS agar for several weeks at a moderate light intensity cotyledon-size
primary leaves eventually developed at the shoot apex (data not shown), demonstrating that these seedlings do not always die in the absence of sucrose but that their growth has almost ceased. Similarly, when the porA-1 mutant was germinated and grown under optimized conditions on sucrose-supplemented media for several weeks and thereafter transferred to soil as a stunted plant with a relatively well developed root system little or no further growth occurred (data not shown). Under no growth condition tested was the mutant phenotype completely rescued.

**Reduced PORA expression in PORA RNAi lines induces growth and greening defects**

In order to obtain a second independent porA mutant line, we surveyed database listings of existing Arabidopsis mutant populations that contained either random insertional events (T-DNA or transposon) or targeted RNAi constructs that might affect the expression of PORA. Among these populations, the AGRIKOLA consortium utilized the CATMA collection of Arabidopsis gene-specific tags to generate binary hairpin RNAi vectors (Hilson et al., 2004). These vectors were used to create a collection of gene-specific silenced transgenic Arabidopsis lines. The AGRIKOLA consortium database identified CATMA code CATMA5a50110 as a collection of RNAi lines targeted to the PORA gene. In these lines CATMA primers 5a50110.5’ and 5a50110.3’ had been used to create a 172 bp Columbia ecotype PORA fragment in a hairpin construct under the control of the cauliflower mosaic virus 35S promoter (Figure 3.1; Supplemental Figure 3.10). 12 CATMA5a50110 lines were received as a generous gift from the AGRIKOLA
consortium. These T$_2$ generation lines were plated on sucrose-supplemented MS agar and displayed no obvious phenotypes on this medium. Basta-resistant lines were selected from these seed families for propagation as soil-grown plants. T$_3$ generation seedlings germinated on MS agar formed two populations, one whose growth ceased at the cotyledon stage and another that resembled the wild type. After 7 days, the stunted seedlings were transferred to sucrose-supplemented MS agar after which they resumed normal growth, and after 14 days these seedlings were transferred to soil. The resulting T$_4$ generation was screened for basta resistance. When all progeny displayed resistance the line was considered to be homozygous for the RNAi transgene.

Homozygous PORA RNAi seedlings from CATMA5a50110 6470 and 6475, denoted lines 70 and 75, respectively, displayed abnormal growth phenotypes after transfer to soil and were therefore selected for further analysis. Quantitative Reverse Transcriptase PCR (qRT-PCR) was used to evaluate the effect of the PORA RNAi construct on each of the POR genes in these lines. The amount of the PORA transcript in etiolated seedlings was reduced 10.8-fold in line 70 and 102-fold in line 75 (Figure 3.4). There was also a much smaller effect on the PORB transcript levels in lines 70 and 75, with expression reductions of 1.6 and 4.4-fold, respectively. The transcript level of PORC in etiolated seedlings was shown to be very low in the wild type, as well as in lines 70 and 75 containing the RNAi construct, and did not differ significantly between these genotypes.

The pronounced growth defects observed in the porA-I mutant are similar to the growth deficiencies observed in lines 70 and 75. To evaluate the extent of the growth
abnormalities caused by the *PORA* RNAi construct, lines 70 and 75 were sown on MS agar, and MS agar supplemented with sucrose. Seedling development was monitored over time on each growth medium, as shown in Figure 3.5. Growth similar to wild type was observed in lines 70 and 75 on sucrose-supplemented medium, whereas there was an arrest of growth similar to that of the *porA-1* mutant in 40-60% of the *PORA* RNAi seedlings observed in the T$_3$-T$_5$ generations (Figure 3.5A). When these stunted seedlings were transferred to sucrose-supplemented medium normal growth ensued. As the *porA-1* null mutant shows a *chlorina* phenotype, the Chl content of the *PORA* RNAi lines was evaluated to determine whether alterations in POR catalytic capacity caused by the reduction of *PORA* expression would be reflected in the final products of the Chl biosynthetic pathway. To evaluate Chl content in the *PORA* RNAi lines it was necessary to harvest 9-d-old light-grown seedlings with emerging primary leaves so that stunted and not stunted seedlings could be clearly distinguished from one another. For both seedling classes fresh weights were measured and pigments were extracted as a prelude to spectrofluorimetric determination of their total Chl contents. The stunted RNAi seedlings of both lines accumulated several-fold less Chl than did the wild type (Figure 3.5B). This reduction was also measured when *porA-1* Chl levels were compared to those of the wild type. In contrast, the *PORA* RNAi seedlings that were not stunted accumulated Chl at approximately wild type levels. A additional growth difference observed between the wild type and the stunted *PORA* RNAi plants was reduced apical dominance in the inflorescences, resulting in a dwarf stature at maturity (Figure 3.5C). As described above, this effect was also evident in the *porA-1* mutant (Figure 3.2D). There were slight
differences between the adult plant phenotypes of line 70 versus line 75. Compared to Columbia wild type, line 70 produced several stunted inflorescences with cauline leaves that cup the floral organs, whereas line 75 produced a smaller rosette and only a few undersized inflorescences.

*Etioplast-localized Pchlide photoconversion and prolamellar body ultrastructure are altered in the porA-1 mutant.*

Total Pchlide and photoactive Pchlide concentrations were quantified by room temperature fluorescence measurements of pigment extracts prepared from flash-illuminated and non-illuminated etiolated seedlings. The total Pchlide concentrations in wild type, *porA-1* and *porC-1* seedlings were similar, whereas the *porB-1* mutant accumulated about 33% less total Pchlide (Figure 3.6). Photoactive Pchlide formation was also similar in the wild type and the *porC-1* mutant. In contrast, there were reductions in the amounts of photoactive Pchlide in the *porA-1* and *porB-1* mutants of roughly 50% and 66%, respectively, compared to the wild type. When photoactive Pchlide was compared as a percentage of total Pchlide for each genotype, standardized to the wild type ratio, the reduction in the amounts of photoactive Pchlide was revealed to be 54% and 42%, respectively, in the *porA-1* and *porB-1* mutants. These results clearly demonstrate that both PORA and PORB are required for wild type levels of photoactive Pchlide formation, although each can form ternary complexes which are catalytically active independent of the other *in vivo*. The effect of the *porC-1* mutation was negligible
on both total Pchlide accumulation and photoactive Pchlide formation, suggesting no measurable accumulation of PORC in etioplasts. This agrees with the fact that PORC expression is almost undetectable in etiolated seedlings (Figure 3.4; Oosawa et al., 2000; Su et al., 2001).

As POR proteins are known to be localized to the prolamellar body in the etioplasts of dark-grown seedlings, we examined the membrane ultrastructure of porA-1 etioplasts to reveal the role of PORA in the formation of this suborganellar structure. Ultrathin sections of cotyledons of the porA-1 mutant and wild type were viewed by transmission electron microscopy. The plastid inner membrane architectures were examined from multiple ultrathin sections of at least three independent seedlings. Representative membrane ultrastructures are seen in Figure 3.7. The wild type etioplasts contained extensive single or multiple prolamellar bodies with unstacked prothylakoids extending between them and throughout the stroma (Figure 3.7A). In the porA-1 mutant there was a reduction in the extent of the typical prolamellar body, although there were still many prothylakoids present (Figure 3.7B). The size ranges of the etioplasts observed in the wild type and the porA-1 mutant were similar (data not shown).

**Light-dependent greening in the absence of PORA**

The observed reductions in photoactive Pchlide formation in the etiolated porA-1 and porB-1 mutants (Figure 3.6) suggested reduced capacities for light-dependent Chl biosynthesis during early greening. Furthermore, the decreased ratios of POR-bound,
photoactive Pchlide to nonphotoactive (ie. possibly free or unbound) Pchlide in these mutants suggested that they might display impaired greening due to the deleterious effects of nonphotoactive Pchlide acting as a potent photosensitizer (op den Camp et al., 2003). By quantifying the early greening characteristics of the por null mutants we evaluated possible Chl accumulation differences triggered by the reductions in the levels of photoactive Pchlide formation and nonphotoactive Pchlide binding. Within Figure 3.8, the contributions of each POR to the greening of Arabidopsis cotyledons were evaluated by quantitative measurements of Chl accumulation in 5-d-old etiolated porA-1, porB-1, and porC-1 mutant seedlings as well as the wild type, during a subsequent 48 h period of illumination in either moderate light (50 μE m⁻² s⁻¹) or high light (400 μE m⁻² s⁻¹). In all genotypes tested, Chl accumulation was much faster in moderate light, presumably due to decreased photooxidation under this condition as well as an increased need for Chl for the synthesis of more extensive light-harvesting antenna complexes. There was no statistically significant in Chl accumulation differences between genotypes that were measured during the first 48 h of greening in moderate light. However, the trends in this experiment indicated somewhat slower greening of the porA-1 and porB-1 mutants than the wild type (Figure 3.8A and B). These trends were accentuated in high light (Figure 3.8D and E). For example, Chl accumulation in the porA-1 mutant was statistically lower than in the wild type at the 36 and 48 h time points. The data suggest that the porA-1 and porB-1 mutants tend to accumulate less Chl than the wild type after the first 12 h in moderate light, with the divergence between the mutant and wild type curves more accentuated in the high light experiment to the point that the differences in pigment
accumulation become statistically significant at later times. In moderate light conditions, the \textit{porC-1} mutant Chl accumulation curve mirrored that of the wild type (Figure 3.8C). Although in high light, Chl accumulation in the mutant was slightly attenuated starting at 24 h although these differences versus the wild type were not statistically significant (Figure 3.8F).

To demonstrate the influence of varying light intensities and sucrose supplementation on the \textit{porA-1} mutant phenotype, seedlings were grown under various conditions and their appearances were documented. The three continuous light treatments used were low light (5 \( \mu \text{E m}^{-2} \text{s}^{-1} \)), moderate light (50 \( \mu \text{E m}^{-2} \text{s}^{-1} \)) and high light (450 \( \mu \text{E m}^{-2} \text{s}^{-1} \)). The reduced growth of the \textit{porA-1} mutants compared to the wild type was most obvious on unsupplemented medium in moderate light (Figure 3.9A and C). In high light both genotypes displayed reduced greening, relative to moderate light, and in low light both genotypes exhibited reduced cotyledon expansion, as well as hypocotyl elongation that was particularly prominent in the wild type. The growth of the \textit{porA-1} mutant on sucrose-supplemented medium at any of the light intensities was reduced compared to the wild type but the growth impairment was not as dramatic as it was on unsupplemented medium (Figure 3.9B and D). Relative to the wild type, growth of the \textit{porA-1} mutants on sucrose-supplemented medium in low and moderate light resulted in seedlings with smaller cotyledons and leaves. The high light-treated wild type and \textit{porA-1} seedlings initially greened in a similar fashion (Figure 3.9B) although the Chl gradually disappeared from the \textit{porA-1} mutant (Figure 3.9D). High light treatment in the presence
of sucrose also caused red pigment formation that is most likely due to anthocyanins in seedlings of both genotypes (Solfanelli et al., 2006; Steyn et al., 2002).

By assessing Chl production during greening at extreme light intensities the differential in vivo roles of each of the POR proteins were investigated. Chl was quantified by room temperature fluorescence emission measurements of pigment extracts. To evaluate por mutant Chl production at very high light intensities, 3-d-old etiolated seedlings were transferred directly into continuous illumination of 1000 μE m\(^{-2}\) s\(^{-1}\) for 3 days at a growth temperature of 25-27\(^{\circ}\)C. The most dramatic reduction in Chl accumulation was observed in the porA-1 mutant, with roughly 25% of the wild type Chl level (Figure 3.9E). The porC-1 mutant accumulated 66% of the wild type Chl level, whereas the porB-1 mutant and the wild type produced similar levels of Chl. The reductions in Chl accumulation indicate the photoprotective importance of the PORA and PORC gene products, as well as their contributions to overall Chl biosynthesis capacity. To evaluate Chl production in very low light, 3-d-old etiolated seedlings were transferred from the dark into continuous illumination of 0.1 μE m\(^{-2}\) s\(^{-1}\). In the por mutants, the loss of PORA and PORB expression led to reductions in Chl accumulation to roughly 60% and 35% of the wild type levels, respectively (Figure 3.9F). The porC-1 mutation, in contrast, did not have an effect on Chl accumulation in very low light. The specific conditions under which PORA, PORB and PORC individually play crucial roles in Chl production are thus emphasized when their expression is abolished in the corresponding por null mutants. The resulting picture indicates that the POR gene family members in Arabidopsis are catalytically redundant, but because of the specific expression patterns of
each family member, the gene products perform both overlapping and unique Chl biosynthesis activities during plant development.

3.3. DISCUSSION

We have paper demonstrated that PORA is required for growth during all stages of the plant life cycle. Etiolated seedlings of the porA-1 mutant have severe defects in etioplast development. Etiolated cotyledons develop with greening similar to wild type when exposed to light for the first several hours. After several days, cotyledon growth subsides thus indicating the transition to photoautotrophic never occurs. These observations were accomplished by creating reductions in PORA accumulation due to a Ds insertion in the PORA gene and by a PORA targeted binary hairpin construct resulting in the silencing of the PORA mRNA. The porA-1 mutation resulted in the absence of PORA mRNA and protein accumulation that does not affect PORB or PORC expression (Figure 3.2). Consequently, we have prepared a system to investigate the phenotype of an independent Arabidopsis PORA knock out. The PORA RNAi silencing was created with gene specific primers to target a divergent sequence between PORA and PORB, there was partial silencing of the PORB mRNA (Figure 3.5). Each of these independent reverse genetics approaches were used to confirm phenotypical and biochemical abnormalities observed due to the reduction of PORA expression. The null porB and porC null mutants did not demonstrate any phenotypical growth defects under normal growth conditions (Frick et al., 2003; Masuda et al., 2003). An antisence expression approach reduced
POR A and POR B expression with no phenotypical abnormalities (Franck et al., 2000). The photoautotrophic growth defect observed in PORA depleted seedlings demonstrates a strict requirement for PORA accumulation for normal seedling growth. Heterotrophic growth of the porA-1 mutant on sucrose supplemented media along with the abnormal plant growth of PORA depleted PORA RNAi lines suggest PORA is required for normal plant growth. Consequently, the requirement for PORA makes it unique within the Arabidopsis POR family.

The loss of PORA causes developmental defects which are partially rescued by sucrose supplementation.

Based on expression analysis and the Chl accumulation in the porB-1 porC-1 double mutant PORA was predicted to function in etiolated and early in greening seedlings (Frick et al., 2003). The arrest of growth in the porA-1 seedling on unsupplemented media suggests it supplies a required activity for the transition from the use of embryonic reserves to photoautotrophic growth. porA-1 seedlings maintained in the dark displayed etiolated growth similar to wild type. Within etiolated or light germinated porA-1 mutant seedlings photomorphogenesis commenced in a similar manner to wild type in continuous or photoperiodic light conditions. However, after several days of growth, a reduced growth rate is observed compared to wild type. In plastid import mutant Atoep16-1, proposed to eliminate the import of PORA, Pollmann et al., (2007) demonstrated a Pchlide dependent cell death phenotype in cotyledons of etiolated
seedling after light treatment. The *porA-1* null mutant phenotype is less severe but does display abnormal arrested growth under similar conditions. The *Atoep16-1* mutant most likely disrupts import of other plastid resident proteins as well as POR A, perhaps explaining its more extreme phenotype.

Supplementation with sucrose enabled the *porA-1* mutant to partially overcome the photoautotrophic arrested growth arrest. When germinated on supplemented media the *porA-1* mutant grows heterotrophically at reduced rate. The sucrose dependent growth phenotype is also observed in portion of the *PORA* RNAi seedlings; the fact that only some RNAi seedlings are stunted is due to variable penetrance and expressivity associated with RNAi linked phenotypes (Wang et al., 2005). The *porB-1* *porC-1* double mutant’s growth on unsupplemented media declined as the seedlings *xantha* phenotype emerged (Frick et al., 2003). Growth defects observed in the *porB-1* *porC-1* double mutant on sucrose supplemented media included light fluency dependent extreme chlorotic growth under low light conditions (Figure 3.2E and F). Combined, these results show a reduction in POR activity can be partially subverted by sucrose supplementation suggesting either a metabolic carbon deficit or systemic regulation block that reduces overall growth. In Osuna et al., (2007), they quantified gene expression in seedlings that were carbon-deprived then maintained on sucrose supplemented media. Induced genes were involved in carbohydrate synthesis, glycolysis, respiration, amino acid and nucleotide, DNA, RNA and protein synthesis whereas those genes involved in amino acid and lipid catabolism, photosynthesis and chloroplast protein synthesis were repressed. It is possible that these adaptations in combination with sucrose as a carbon source are
required for the partial rescue as well as responsible for the phenotypic differences observed. Light and carbon signaling pathways have complex interactions at the gene expression level (Thum et al., 2004). This systems approach to connect light and carbon signaling pathways has not yet integrated the metabolic effects of PORA, but the partial rescue of the porA-1 mutant by sucrose suggests possible interactions. 

The reduced growth rate throughout porA-1 mutant development leads to the observed dwarf mutant phenotype. The requirement for the missing cryptic light expression of PORA is observed in the porA-1 mutant when seedlings are maintained on sucrose supplementation. A reduction in PORA leads to a declined growth rate on soil observed in the adult PORA RNAi plants. Reproductive defects were observed in both the porA-1 mutant and the PORA RNAi lines resulting in reduced silique formation and viable seed yield. The porA-1 mutant can be heterotrophically propagated on sterile sucrose supplemented media for several months with gradual production of viable seed. There were some differences between the two independent PORA RNAi lines, with Line 70 displaying a bushy dwarf inflorescence, whereas in Line 75 overall growth was reduced producing a smaller plant than wild type, but with normal apical dominance. The severity of these phenotypes correlates with the relative reductions in PORA transcript. Further, the porA-1 mutant displays the most extreme phenotype. Both the knockout and knockdown plants suggest PORA supplies an essential factor for normal inflorescence development.
Etioplast development in the porA-1 null mutant is abnormal.

POR binds Pchlide to prevent photooxidative damage in etiolated tissues. Furthermore, a photoactive complex of POR-Pchlide-NADPH is proposed to aggregate into the prolamellar body. Expression studies have implicated PORA and PORB as the principal dark expressed Arabidopsis POR isoforms (Armstrong et al., 1995; Holtorf et al., 1995). Studies have shown the photoactivated conversion of Pchlide is reduced when the levels of PORB are depleted. This accompanies a reduction in the observed extent of PLB (Frick et al., 2003; Masuda et al., 2003; Franck et al., 2000; Runge et al., 1996).

Interestingly, the total Pchlide accumulation in the porB-1 mutant was reduced whereas in porA-1 and porC-1 mutants total Pchlide accumulations were similar to wild type. This suggests a mechanism that adjusts the amounts of total Pchlide when PORB is depleted. However, the ratio of photoactive Pchlide to the total Pchlide accumulated in each mutant reveals that PORA and PORB are each responsible for photoconverting about half of the wild type levels.

In our study using the complete set of POR mutants we have been able to account for the portions of photoactive Pchlide accumulation from each POR isoform and thereby showed that PORB is responsible for a larger portion of photoactive Pchlide than PORA, with PORC not contributing significantly. In addition, the ability of PORB to photoconvert Pchlide in the porA-1 mutant suggests the independent catalytic activity of PORB. While there is a slight possibility that PORC could be partially responsible for the photoactive Pchlide accumulation in the porA-1 and porB-1 mutants, expression data
indicates PORC is at very low levels in etiolated seedlings (Oosawa et al., 2000; Su et al., 2001). Moreover, the photoconversion activity observed independently in our porA-1 and porB-1 mutants, in addition to a porB mutant from Masuda et al., (2003), collectively reject a strict requirement for the light-harvesting NADPH:POR:Pchlide (LHPP) complex model. The LHPP model proposes the formation of a specific PORA:PORB protein complex based on in vitro reconstitution assays using the barley PORs and protein gel blot analysis in Arabidopsis (Reinbothe et al., 1999; Pollmann et al., 2007). The porA-1 mutant phenotype demonstrates that there is a an unknown requirement that only PORA fulfills; if this activity helps maintain a POR heteromeric complex, then PORA expression is a strict requirement whereas PORB is not required.

The low levels of photoconverted Pchlide correlate with an observed reduction in PLB accumulation in the porA-1 mutant. The remaining PLB observed in the porA-1 mutant contains only the PORB isoform, indicating PORB is capable of independent aggregation and Pchlide photoconversion. This result mirrors those found in porB mutants correlating with a reduction in PLB and Pchlide photoconversion (Frick et al., 2003; Masuda et al., 2003). These combined results suggest PORA and PORB can act independently in PLB formation and therefore each account for a significant portion of wild type PLB. PORA and PORB act jointly in etioplast development bringing about PLB formation where they accumulate in a photoactivated complex. This corroborates previous hypotheses that PORA and PORB are functionally interchangeable in developing etioplasts (Sperling et al., 1997, 1998; Franck et al., 2000; Frick et al., 2003; Masuda et al., 2003).
The deficiency in POR leads to light fluency dependent Chl accumulation defects

A reduction in the total Pchlide accumulation in the porB-1 mutant accompanies a reduction in photoactive Pchlide whereas the photoactive Pchlide reduction in the porA-1 mutant is present despite wild type levels of total Pchlide. This additional accumulation of the potent photosensitizer Pchilde in the porA-1 mutant implies that these mutants have possible sensitivity to photooxidation.

The similarity in greening in all three mutants during the first 12 hours indicates the depletion of a single isoform is not enough to cause significant early greening defects. This redundancy between each of the isoforms at moderate as well as high light during initial greening indicates POR is not limiting during early greening. After 24 hrs the requirement of PORA and possibly PORB is suggested by reduced Chl accumulation in the respective mutants. All POR mutant Chl deficits were intensified at higher light fluencies. The reduced Chl accumulation in the porA-1 mutant could be due to the reduced growth or a reduction in PORA specific catalytic activity still observed in the porB-1 porC-1 double mutant (Frick et al., 2003).

Quantification of reduced Chl accumulation in the PORA RNAi lines was complicated by partial penetrance of the stunted growth phenotype on unsupplemented media. When Chl was quantified in the stunted seedlings, the accumulated Chl amounts correlated with the reduction in PORA mRNA in etiolated seedlings and these levels were similar to those of the porA-1 mutant. This supports the specificity of the phenotypical
Supplementation with sucrose fully rescued PORA RNAi as well as partially rescued the porA-1 mutant’s early photoautotrophic growth defects.

Specific roles of PORB and PORC under extreme light conditions are suggested by the single mutant phenotypes; in extremely low light porB mutants accumulated less Chl than the wild type, whereas a porC mutant had reduced Chl accumulation in very high light conditions (Masuda et al., 2003). Our data confirmed those measurements in the porB-1 and porC-1 mutants, with the depletion of PORB at low light leading to a 2.6 fold reduction in Chl and a slight Chl reduction observed in the porC-1 mutant at very high light. PORA gene expression is required for normal Chl accumulation at very low light and more evidently at very high light, suggesting a light fluency dependent PORA requirement such as that seen with the other PORs. The PORA requirement increases over time to result in chlorotic seedlings with reduced photoautotrophic growth in the porA-1 mutant. The stunted growth of the porA-1 mutant is possibly a contributing pleiotropic factor to the reduction of Chl synthesis. Overall, very high light fluencies resulted in an increase in total Chl accumulation compared to the very low light. This suggests a model with partially overlapping functions at extreme light conditions. Where each Arabidopsis POR isoform has specialized expression optimizations, for instance PORA is required in a light fluency dependent manner, PORB is optimized for low light conditions and PORC is specifically needed at high light conditions.
The porA-1 mutant phenotypes imply PORA has a physiological role throughout plant development. Data published by Oosawa et al. (2000) indicated the presence of trace amounts of PORA mRNA in a northern blot containing total RNA from cauline leaves. However, the authors did not comment on this fact. A RNA mini-array study of 35 genes involved in the Chl biosynthetic pathway noted persistent low level PORA expression in 3-week-old light-grown plants with true leaves (Matsumoto et al., 2004). In this case, no functional significance was attached to this observation.

Low light-induced PORA is suggested by the visible Chl biosynthesis revealed in young rosette leaves of the porB-1 porC-1 double mutant under these conditions (Figure 3.2E). Substantial PORA-dependent Chl accumulation was observed in cauline leaves of the porB-1 porC-1 double mutant, which was successfully propagated for the first time beyond the seedling stage using modified growth conditions (Figure 3.2E and F). These data indicate a direct role for PORA in adult plants in cauline leaf Chl biosynthesis. For cauline leaves, the PORA Chl synthesis activity reported here underscores the greater sensitivity of Chl production versus earlier northern blot analyses in which the steady-state accumulation of PORA mRNA was found to be at or below the limit of detection (Armstrong et al., 1995; Oosawa et al., 2000).

Collectively these data indicate PORA activity is required for normal adult plant Chl synthesis. Furthermore, while PORB and PORC have redundant bulk Chl biosynthetic activities (Frick et al., 2003), PORA supplies a unique post-cotyledon
function. Greening in the \textit{porB-1 porC-1} double mutant cauline leaves due to PORA activity is clear evidence that supports light grown function for PORA activity.

Photoautotrophic growth blocks in the \textit{porA-1} and \textit{porB-1 porC-1} mutants can both be partially rescued with heterotrophic growth on sucrose supplemented media in moderate or low light. Thus, suggesting defects related to photosynthetic growth due to POR depletion.

3.4. MATERIALS AND METHODS

\textit{Generation of Transgenic Plants, Plant Growth Conditions and Sample Collection}

Landsberg \textit{erecta} stock number CS20 and Columbia stock number CS60000 Arabidopsis seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. \textit{porB-1} and \textit{porC-1} alleles described in Frick et al., (2003). Seeds of transgenic line SGT4757-5-3 were obtained from the Nottingham Arabidopsis Stock Centre, UK. Homozygous mutants derived from seed family SGT4757-5-3, denoted as \textit{porA-1}, were back-crossed once with their ecotypic background Landsberg \textit{erecta}. The mutant lines were used as the F\textsubscript{0} pollen donors and the wild type as the F\textsubscript{0} pollen acceptor to allow selection with kanamycin for the \textit{Ds} element in the F\textsubscript{1} generation. The genotypes of the F\textsubscript{2} progeny were determined by PCR and by the kanamycin-resistance segregation ratios of F\textsubscript{3} seedlings. Analysis of the F\textsubscript{2} kanamycin-resistance segregation ratios confirmed the presence of single-locus \textit{Ds} insertion in the back-crossed \textit{porA-1} line. For propagation, the photoperiodically grown homozygous \textit{porA-1} mutant was
maintained on MS agar supplemented with 2% sucrose at a fluence rate of 50 µE m⁻² s⁻¹. Seeds of lines CATMA5a50110 6469-6480 were a kind gift of Magdalena Weingartner (The AGRIKOLA consortium, Max-Planck-Institute for Molecular Plant Physiology, Potsdam, Germany). Seeds of 12 F₂ CATMA5a50110 69-80 lines were bulked for analysis. The presence of the AGRIKOLA RNAi construct in F₃ progeny were determined by the basta-resistance segregation ratios of F₃ seedlings. Fully basta-resistant lines were considered homozygous and F₄ and F₅ progeny were used for analyses.

Photoperiodically-grown plants were maintained in long day conditions (16 h day at 22°C / 8-h night at 20°C). Continuous white light (WL) conditions at 22°C were provided by a combination of TLD 36W/84 (4100 K) cool-white fluorescent lamps, F17T8/TL741 17 watt (4100 K) cool-white fluorescent lamps (Philips) and ES27 27 watt (2700 K) compact fluorescent bulbs (TCP). Seedlings were germinated by sowing surface-sterilized seeds on MS agar plates. To help synchronize germination, sowed seeds were placed in the dark at 4°C for a least 24 h to allow seed imbibition and thereafter exposed at 22°C to 1-2 h WL at a fluence rate of 160 µE m⁻² s⁻¹. The illuminated imbibed seeds were returned to darkness at room temperature to produce etiolated seedlings of various ages. Imbibed seeds were moved to continuous WL or photoperiodic WL of various fluence rates to produce light-grown seedlings. Different fluence rates were obtained using combinations of neutral density filters (Rosco). High light with a fluence rate of 1000 µE m⁻² s⁻¹ was attained using a 1000 watt metal halide bulb (Hortilux). All fluence rates were measured with a Li250 light meter (Li-Cor).
Plant material was harvested and either frozen in liquid nitrogen and stored at -80°C until its use for RNA, DNA and protein analysis or processed immediately for electron microscopy and spectroscopic measurements.

In flash illumination experiments, one flash per plate of seedlings was given using a 550FD (Vivitar) camera flash attachment to ensure full conversion of all photoactive Pchlide to Chlide. Etiolated seedlings were harvested using a green safe light (Kodak 7B filter). Images of seedlings and plants were collected using a Sony DsC-S75 digital camera.

**DNA isolation, PCR and Realtime quantitative PCR**

To determine the genotypes of individual plants grown on MS agar, total DNA was extracted and analyzed using an REDExtract-N-Amp™ Plant PCR kit (Sigma) with appropriate oligonucleotide primers to test for the presence of the respective *Ds* element and the intact wild type POR genomic sequence. Primer sequences are included in Supplementary Figure 3.1 and Frick et al., (2003). The *porA-1* insertion, *Ds::genomic border* fragments were amplified with primer pairs 4.0/Ds5, intact genomic sequences were amplified using 4.0/5.8. PCR reactions to identify *Ds* insertions assay for intact genomic sequences, and generate *Ds::genomic border* fragments were performed as follows: 1 cycle of 5 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 55°C, 2 min at 72°C, and one cycle of 7 min at 72°C.
RNA from 5-d-old etiolated seedlings were extracted essentially as previously described by Armstrong et al., (1995). DNase treatment was performed using DNase I on the RNeasy mini spin columns as described by manufacturer (Qiagen Inc.). RNA from Arabidopsis seedlings was reverse-transcribed to first-strand cDNA using Omniscript Reverse Transcriptase (Qiagen Inc) as described by the manufacturer. Two reactions were performed on each mRNA sample, either in the presence or absence of reverse transcriptase. Each reaction contained 2 µg of total RNA in a 20-µl total volume. The thermal cycling parameters for the reactions were 60 min at 37°C. The oligonucleotide primers were designed using Beacon Designer 4.0 to yield target products ranging 100-200 bp (Supplementary Figure 3.10).

For qRT-PCR amplification the BioRad iQ SYBR Green Supermix with all the primers at final concentrations of 0.2 µM was used. Triplicate reactions of three independent biological replicates were performed. The PCR conditions were optimized on the iCycler (Bio-Rad). The cycling conditions were: 3 min at 95°C; 40 cycles: 30 s at 95°C, 30 s at 58°C, and 45 s at 72°C. The amplicons were verified by sequencing the qRT-PCR products from wild type samples. We chose PP2AA3 (At1g13320) as a reference gene and primers based on the previously determined expression stability in Arabidopsis across a variety of conditions (Czechowski et al., 2005). To compute the expression values for each gene of interest (GOI) we used the average reaction efficiency using the following formula $Expression_{GOI} = \frac{avgEff}{CT}$, where the Ct values were obtained with PCR base line subtracted curve fit setting of the iCycler IQ (Bio-Rad) for data analysis. The expression value for each GOI was normalized by the mean GOI of
the reference gene (Vandesompele et al., 2002). The normalized expression values for the target genes were compared by ANOVA (p<0.05).

**mRNA, protein, and plastid ultrastructural analyses**

Cotyledons from 3- and 5-d-old etiolated seedlings were harvested to obtain pooled material for gel blot analyses of their mRNA and protein contents or for plastid ultrastructural analysis by transmission electron microscopy. RNA analyses were performed essentially as previously described by Armstrong et al., (1995). The amounts of POR and Lhcb1 gene family transcripts were determined by hybridization of radioactively labeled Arabidopsis DNA probes prepared from primers 1.9/2.0 (porA) and AtPor2.1/AtPor2.2 (porB) (Paddock et al., 2008; Armstrong et al., 1995; Su et al., 2001), with gel blots containing 10 µg of total RNA per sample immobilized on a GeneScreen nylon hybridization transfer membrane (Life Science Products).

Total protein samples were prepared from frozen ground seedlings extracted with homogenization buffer (0.0625 M Tris-HCl, pH 6.8; 1% (w/v) SDS; 10% (v/v) glycerol; 0.5% (v/v) β-mercaptoethanol). Samples were incubated at 95°C for 10 min and thereafter centrifuged at 16,000 g for 10 min. Supernatant protein concentrations were determined as previously described by Armstrong et al., (1995). Protein extracts were concentrated using a methanol/chloroform/water mixture (Wessel and Flügge, 1984). Samples containing 40 µg total protein were separated electrophoretically under denaturing conditions by SDS-PAGE on a 10% gel and were electroblotted onto a
nitrocellulose membrane (Schleicher and Schuell). Western blot analysis of the immobilized antigens was performed using a polyclonal anti-Arabidopsis POR antiserum (Sperling et al., 1997) raised against a recombinant MalE::PORB fusion protein produced in *Escherichia coli*, combined with an Immun-Star™ chemiluminescence detection kit (Bio-Rad).

Cotyledons collected for plastid ultrastructure analysis were vacuum infiltrated at 10 psi for 16 h in a fixative composed of 0.1 M sodium phosphate buffer, pH 7.0, 3% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in the dark at room temperature. Fixed tissues were washed five times for 5 min in 0.1 M sodium phosphate buffer, pH 7.0, and post-fixed for 1 h in 1 M sodium phosphate buffer, pH 7.0 containing 1% (v/v) osmium tetroxide. Post-fixed tissues were washed 3 times for 5 min in 0.1 M sodium phosphate buffer, pH 7.0, then four times in distilled water, and stained for 2 h in 1% (v/v) uranyl acetate before being washed three times in distilled water. A gradual dehydration through an ethanol series over 1 h was followed by two 10 min washes in propylene oxide. Propylene oxide was gradually replaced with Spurr resin (Ted Pella) over the course of 12 h and then the cotyledons were embedded in 100% (v/v) Spurr resin. After polymerization, samples were sectioned and mounted on Formvar-coated copper grids, then stained with 2% (v/v) uranyl acetate and Reynolds lead citrate, and viewed on a Technai G2 Spirit transmission electron microscope.
**Quantitative pigment determinations by fluorescence microscopy**

Room temperature fluorescence emission analyses of the Pchlide, Chlide, Chl a and Chl b present in etiolated and light-treated etiolated seedlings, as well as older light grown seedlings, were performed. Total photosynthetic pigments were extracted for at least 48 h from 10 pooled seedlings per sample in 3 ml of 80% (v/v) acetone supplemented with 0.1 N ammonium hydroxide at 4°C. Fluorescence emission spectra of the pigment extracts were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian) with both the excitation and emission slit widths set at 5 nm. The pigment extracts were excited at 433 nm, which preferentially causes Chl a fluorescence emission at 670 nm, and at 455 nm, which preferentially causes Chl b fluorescence emission at 655 nm. The Pchlide and photoactive Pchlide amounts were determined by excitation of etiolated seedling and flash treated etiolated seedling pigment extracts at 433nm with measurement of emission bands at 634 and 672 respectively. The pigment contents were calculated according to Sperling et al., (1998). Pigment accumulation data were compared by ANOVA (p<0.05).

**3.5. REFERENCES**


Figure 3.1. **Organization of the Arabidopsis PORA gene structure, and identification of the porA-1 Dissociation element insertion mutation.**

White and black boxes indicate exons and introns, respectively. The gray triangle represents the porA-1 Ds element insertion. Arrows indicate the positions and orientations of oligonucleotide primers (see Materials and Methods). (B) Nucleotide sequence of the porA-1 Ds insertion site. The 5’end of the Ds element is indicated in bold typeface. The 5’-to-3’orientation of the element is shown by the arrow. The underlined nucleotide A is replaced by a C in the 5’ends of the porB-1 and porC-1 Ds element insertions within the Arabidopsis PORB and PORC genes, respectively (Frick et al., 2003). The italicized sequence located immediately downstream of the insertion site corresponds to primer 7.3. (C) Identification of heterozygous and homozygous porA-1 mutants by PCR. Genomic template genotypes are indicated at the top and expected product sizes for each primer pair with a given template are indicated at the bottom. PCRs shown in lanes 1, 3 and 5 were performed with the primer pair 1.9/5.8, and those presented in lanes 2, 4 and 6 were carried out with primers 1.9/Ds5. Lanes 1 and 2 represent the positive and negative controls with a wild type template, respectively. The absence of a PCR product in lane 5 indicates the homozygosity of the Ds element, whereas the products observed in lanes 4 and 6 confirm the presence of the porA-1 insertion.
Figure 3.2. *porA-1* seedlings exhibit arrested growth at the cotyledon stage in the absence of sucrose.

*porA-1* seedlings growth arrest and low light Chl production in *porB-1 porC-1* double mutants. Phenotypes of *porA-1* homozygous mutants within a segregating F2 population are illustrated. Bars = 0.5 mm. (A, B) Wild type siblings are shown on the left, *porA-1* homozygous seedlings on the right. (A) 14-d-old seedlings. The *porA-1* seedling has arrested at the cotyledon stage on MS agar without sucrose. (B) 21-d-old seedlings. Supplementation of the medium with sucrose allows heterotrophic growth. The *porA-1* seedlings grow as *chlorina* dwarfs on MS agar supplemented with sucrose after 7 days after transfer from MS. (C, D) *porA-1* mutant growth under photoperiodic conditions at a fluence rate of 50 µE m$^{-2}$ s$^{-1}$ on MS agar supplemented with sucrose is shown. (C) Typical 1-month-old seedlings grew to be 1.5 cm tall and (D) 4-month-old seedlings to be seedling shown is 6 cm tall. The *porB-1 porC-1* mutant growth on MS agar supplemented with sucrose. (E) after 2 months, (F) after 9 months, at light fluence of 12.5 µE m$^{-2}$ sec$^{-1}$ on under photoperiodic condition.
Figure 3.3. *porA-1* and *porB-1* are RNA and protein null mutants.
3-d-old etiolated seedlings (genotypes indicated from left to right) were used as the starting material. (A) Northern analysis. Gel blots were prepared with 2.5 µg of total RNA and probed for the transcripts indicated at the left. Ethidium bromide-stained RNA was used as a loading control. Electrophoretically-separated total protein and incubated with an anti-POR antiserum. The mobility and molecular mass in kDa of a marker protein are indicated. Similar results were previously obtained for light-grown *porB-1* mutant (Frick et al., 2003).
Figure 3.4. *PORA* transcript levels are dramatically and specifically reduced in *PORA* RNAi lines. Transcript accumulation was determined for all three *POR* genes in the *PORA* RNAi transgenic plants. The relative expression levels of *PORA*, *PORB* and *PORC* were examined by qRT-PCR analysis, using *PP2AA3* as an internal control, in 5-d-old etiolated wild type and transgenic seedlings. The values shown are the means of three independent biological replicates, with ±95% confidence intervals indicated by the error bars.
Figure 3.5. Plants with severely reduced or no detectable PORA expression are dwarfed and chlorotic.

Two PORA RNAi lines (Ln 70 and 75, see Materials and Methods) and the porA-1 mutant are shown, along with their respective Columbia and Landsberg erecta wild types. Bar = 0.5 mm. Seedlings were grown at a fluence rate of 125 µE m⁻² s⁻¹ for 9 days in photoperiodic conditions on (A, B) MS agar or (A) MS agar plus sucrose. (A) PORA RNAi lines 70 and 75 segregate two types of seedlings on MS agar. (B) The amount of Chl was determined in aqueous acetone-extracted total pigments. Chl quantification was based on room temperature fluorescence emission measurements of 5 independent pigment extracts of 10 seedlings each, with ±95% confidence intervals indicated by the error bars. (C) 7-week-old adult plants grown at a fluence rate of 125 µE m⁻² s⁻¹ in photoperiodic conditions. Columbia wild type is compared to the PORA RNAi lines 70 and 75.
Figure 3.6. *porA-1* and *porB-1* etioplasts both have reduced amounts of photoactive Pchlide.
Total Pchlide was calculated by its fluorescence emission in aqueous acetone pigment extracts from non-illuminated 5-d-old etiolated seedlings. Photoactive Pchlide present prior to illumination was calculated as the difference between total Pchlide and nonphotoactive Pchlide, which was directly measured from its fluorescence emission in pigment extracts from flash-illuminated 5-d-old etiolated seedlings. Each data point represents at least 10 replicates of 10 seedlings each, with error bars indicating ±95% confidence intervals.
Figure 3.7. *porA-1* etioplasts have reduced prolamellar body development. Ultrathin sections of cotyledon plastids from 5-d-old etiolated seedlings were examined by transmission electron microscopy. Arrows indicate prolamellar bodies found in typical etioplasts. Bars = 0.5 µm. (A) wild type. (B) *porA-1*.
Figure 3.8. The loss of **PORA** or **PORB** attenuates greening in a fluence-rate dependent manner.

5-d-old etiolated seedlings were removed from the dark and illuminated under two different conditions to examine their greening: (A-C) moderate light, 50 \( \mu \text{E m}^{-2} \text{s}^{-1} \) and (D-F) high light, 400 \( \mu \text{E m}^{-2} \text{s}^{-1} \). Eight replicates of 10 seedlings each were sampled after 2, 6, 9, 12, 24, 36 and 48 h of illumination. Chl was extracted with aqueous acetone and quantified by measuring the fluorescence emission of the pigment extracts. Error bars indicate \( \pm 95\% \) confidence intervals.
Figure 3.9. The growth and Chl accumulation phenotypes of the *porA-1* mutant are light- and sucrose-dependent.

(A-D) Columns indicate continuous growth in low light (LL), 5 µE m⁻² s⁻¹; moderate light (ML), 50 µE m⁻² s⁻¹; and high light (HL), 400 µE m⁻² s⁻¹. Each panel illustrates the growth of a representative wild type seedling on the left and a *porA-1* mutant seedling on the right. (A) Phenotypes of 7-d-old seedlings on MS, (B) on MS supplemented with sucrose; (C) Phenotypes of 14-d-old seedlings on MS, (D) on MS supplemented with sucrose. Size bars = 0.5 mm for rows A-B and rows C-D. Total Chl accumulated in 3-d-old etiolated seedlings shifted to very high light (1000 µE m⁻² s⁻¹) or (F) very low light (0.1µE m⁻² s⁻¹) for 3 d. 10 replicates of 10 seedlings each were extracted with aqueous acetone and Chl was quantified by measuring its fluorescence emission in the pigment extracts. Error bars indicate ±95% confidence intervals.
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Figure 3.10. **Primers used**
CHAPTER 4

ARABIDOPSIS LIGHT-DEPENDENT PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR) IS REQUIRED FOR PROLAMELLAR BODY FORMATION

ABSTRACT

Greening in angiosperms requires light because, in contrast to other photosynthetic organisms, they rely exclusively on a light-dependent mechanism to reduce protochlorophyllide during chlorophyll biosynthesis. In *Arabidopsis thaliana* three structurally related but differentially regulated NADPH:protochlorophyllide oxidoreductases, denoted PORA, PORB and PORC, mediate light-dependent Pchlide reduction. The *PORA* and *PORB* genes are strongly expressed early in seedling development, while primarily *PORB* and *PORC* are expressed in older plants. Although *PORA* is negatively light-regulated, a very low level of expression persists even in continuously illuminated plants in cauline leaves and at the shoot apex in response to low light. As recently demonstrated, either PORB or PORC suffices to maintain light-dependent chlorophyll biosynthesis throughout most of plant development. Here we have
characterized \textit{porA-1 porB-1} and \textit{porA-1 porC-1} null double mutants of Arabidopsis to investigate the contributions of each POR isoform to Chl biosynthesis and growth in green plants. Photoperiodically-grown \textit{porB-1} and \textit{porC-1} single mutants resemble the wild type, whereas the \textit{porA-1}, \textit{porA-1 porB-1} and \textit{porA-1 porC-1} mutants are light-green and seedling growth is arrested at the cotyledon stage. When supplied with sucrose, the \textit{porA-1}, \textit{porA-1 porB-1} and \textit{porA-1 porC-1} mutants develop as light-green dwarfs. Etioplast development in \textit{porA-1 porB-1} double mutant is defective with no detected prolamellar body formation or photoactive protochlorophyllide conversion; the \textit{porA-1 porC-1} double mutants etioplasts resembled those of the single \textit{porA-1} mutant. This work demonstrates that prolamellar body formation is dependent only on PORA and PORB, and PORA has unique roles not shared with the other isoforms.

\section*{4.1. INTRODUCTION}

In dark grown angiosperms, light activates the enzyme activity within the ternary complex of protochlorophyllide (Pchlide) oxidoreductase \textit{(POR, EC 1.3.1.33)}, Pchlide and NADPH; this complex is localized to the prolamellar bodies (PLB) of the etioplast (Schoef and Franck, 2003). \textit{POR} is the major protein constituent of the PLBs, other components include lipids, carotenoids and chlorophyll (Chl) precursors. The PLB ultrastructure displays a regular, honeycomb latticed structure, with (pro)thylakoids emanating (Rosinski and Rosen, 1972; Selstam and Sandelius; 1984). Upon absorption of a photon by the pigment, \textit{POR} catalyzes a reduction across the C17-C18 double bond.
of the D-ring of protochlorophyllide a to yield chlorophyllide a (Chlide), a Chl precursor (Heyes and Hunter, 2005). In light-grown angiosperms PLBs are gradually replaced by chloroplast containing thylakoids; POR is still associated with membranes in these plastids. Angiosperms only have light-dependent POR, in contrast to gymnosperms and non-vascular plants which have the unrelated light-independent POR, a three subunit (ChlL, ChlN and ChlB) plastid genome encoded enzyme. The loss of plastid-encoded light-independent POR in angiosperms, together with evolution of small POR gene families in numerous species, suggests an adaptive advantage for differential POR regulation (Masuda and Takamiya, 2004).

The three Arabidopsis POR isoforms, denoted PORA, PORB and PORC, are differentially expressed and therefore hypothesized to perform specialized functions (Masuda and Takamiya, 2004). PORA and PORB mRNA and protein are both readily detected in etiolated seedlings (Armstrong et al., 1995; Holtorf et al., 1995). The amount of PORC mRNA in etiolated seedlings is very low (Ossawa et al., 2000; Su et al., 2001). When transgenic antisense and overexpression constructs changed PORA or PORB protein accumulation, the total POR content correlated with photoactive Pchlide content, the ratio of photoactive to nonphotoactive pigment and PLB aggregation (Franck et al., 2000). In the porA-1 and porB-1 mutants there are partial reductions in PLB accumulation as well as photoactive Pchlide conversion (Frick et al., 2003; Masuda et al., 2003). A proposed function for PORA and PORB in etiolated seedlings is to bind Pchlide under dark grown conditions. When Pchlide is not bound to the active site of POR it is considered non-photoactive Pchlide. Non-photoactive Pchlide accumulation is
sensitizer for the production of singlet oxygen, which is implicated in photooxidative
damage (Op Den Camp et al., 2003). Tetrapyrrole biosynthesis control points comprise
of feedback regulation of 5-aminolaevulinic acid in addition to Mg$^{2+}$ ion insertion into
porphyrin macrocycle (Moulin and Smith et al., 2005). The accumulation of excess
Pchlide is prevented by negative feedback control in etiolated angiosperms
(Meskauskiene et al., 2001).

The regulation of POR import into the plastid is still unresolved. Recent studies
into the cytosolic precursor (pre) POR plastid import suggest a multifaceted model with
differential regulation between isoforms and in different organs. *Arabidopsis* cotyledons
demonstrate a Pchlide-requirement for the import of prePORA but not prePORB, while
import is independent of Pchlide in true leaves (Kim and Apel et al., 2004). Conflicting
studies exist on prePORA plastid import requirement for the outer envelope solute
channel protein Oep16. In an *Atoep16-1* mutant Philippar et al. (2007) verified wild type
PORA import and etioplast ultrastructure, while Pollmann et al. (2007) reported a PORA
import defect in addition to a seedling conditional Pchlide accumulation related lethal
phenotype. *In vivo* data suggests a putative member of the outer plastid envelope
translocon Toc33 is required for the import of PORB but not PORA in *Arabidopsis*
cotyledons. However, *in vitro* data indicates that in true leaves Toc33 is required for
PORA but not PORB import in the dark (Kim et al., 2005; Reinbothe et al., 2005).

During the first few hours of photomorphogenesis *PORA* is transiently expressed.
In adult plants *PORA* is expressed in cauline leaves, as well as in low light shifted young
rosette leaves at the shoot apex (Armstrong et al., 1995; Holtorf et al., 1995; Frick et al.,
The *porA-1* mutant has a lethal photoautotrophic growth defect at the cotyledon stage. The *porA-1* mutant can be partially rescued by sucrose supplementation, and maintains light fluency sensitive heterotrophic growth on supplemented media to establish dwarf bushy adult plants. Under light-grown conditions *PORB* is expressed with a circadian pattern and at lower levels than in etiolated seedlings. The *PORC* gene is positively regulated by light illumination in a fluency dependent manner (Ossawa et al., 2000; Su et al., 2001). Under normal laboratory light growth conditions no obvious phenotypes were observed in *porB-1* and *porC-1* null mutants, hence PORB and PORC provide for functionally redundant bulk Chl biosynthesis (Frick et al., 2003; Masuda et al., 2003). In *porB-1 porC-1* double mutants Chl gradually fades over the 3-4 days after germination to reveal a pale yellow chlorotic phenotype. The *porB-1 porC-1* double mutant can be maintained as a slow growing chlorotic dwarf plant under low light conditions with sucrose-supplemented growth media. When *PORA* is ectopically expressed in the *porB-1 porC-1* double mutant background, the ectopically accumulating PORA rescued the phenotype.

Here we characterize *porA-1 porB-1* and *porA-1 porC-1* mutants. Our results demonstrate that PORA and PORB are absolutely required for proper etioplast development in dark grown seedlings. The individual contributions, in plant growth and development, of PORB and PORC isoforms are revealed in the absence of the remaining POR gene family members. Additionally, even though some POR functions are redundant, the differential expression each isoform leads to unique contributions.
4.2. RESULTS

Generation of Arabidopsis double homozygous null mutants in the PORA, PORB and the PORA, PORC genes.

The Arabidopsis genome contains three genes encoding POR enzymes, PORA, PORB and PORC. In order to determine the biological significance of POR in Arabidopsis, all three genes must be examined. A collection of Arabidopsis mutants has been created using Dissociation (Ds) elements spread within genome (Parinov et al., 1999). Previous work with mutants from this collection isolated null alleles in these genes: porA-1, porB-1 and porC-1 (Frick et al., 2003). A cross was performed between the porA-1 and the porB-1 mutants and a second cross between the porA-1 and porC-1 mutants. PCR was used to isolate double mutant seedlings that amplify a fragment flanking PORA gene::Ds and the PORB or PORC genes::Ds insertions and the inability to amplify a wild-type fragment using primers flanking the insertion site (Figure 4.1A and B). The Ds element used is roughly 6.5kB, therefore standard PCR will not be able to amplify across the insertion sight (Parinov et al., 1999; Sundaresan et al., 1995). Preliminary examination of the double mutants found growth related phenotypes in the different mutant combinations.

Further confirmation of the absence of functional POR proteins was done using antibodies. A polyclonal anti-Arabidopsis POR antiserum reacts to all three Arabidopsis POR isoforms; two distinct POR-immunoreactive species are detected in protein extracts from light grown seedlings (Figure 4.1C). Specifically, the wild-type and porA-1 mutant
contained both light expressed isoforms, indicating they are PORB and PORC. The
*porB-1* and *porA-1 porB-1* double mutants are devoid of the upper POR isoform,
suggesting that the lower isoform is the PORC polypeptide. The *porC-1* and *porA-1*
*porC-1* mutant were devoid of the lower POR isoform, signifying that the upper isoform
is the PORB polypeptide. The double mutants are depleted of other POR isoforms thus
confirming the identity of the remaining PORB and PORC polypeptides.

*Growth in the porA-1 porB-1 and porA-1 porC-1 double mutants is deficient*

Homzygous *porA-1*, *porA-1 porB-1* and *porA-1 porC-1* mutants arrest growth at
the cotyledon stage when geminated on MS agar media (Figure 4.2A, C and E). The
effect of light intensity and sucrose supplementation on growth of the mutants was
examined. The three fluency treatments used were low-light (5 µE m⁻² s⁻¹; Figure 4.2A
and B), moderate-light (50 µE m⁻² s⁻¹; Figure 4.2C and D) and high-light (450 µE m⁻² s⁻¹;
Figure 4.2E and F). The difference in growth reduction among *porA-1*, *porA-1 porB-1*
and *porA-1 porC-1* mutants are most distinct on media without sugar under moderate
light growth conditions (Figure 4.2A, C and E). All genotypes became chlorotic when
grown under high light fluencies, whereas under the low-light treatment displayed stunted
cotyledons and reduced hypocotyl growth compared to wild-type, *porB-1* and *porC-1*.
The growth of the *porA-1*, *porA-1 porB-1* and *porA-1 porC-1* mutants on sucrose
supplemented media is reduced compared to wild type under all the treatments but not as
dramatically as on unsupplemented media (Figure 4.2B, D and E). The growth of *porA-
1*, *porA-1 porB-1* and *porA-1 porC-1* mutants on low and moderate-light treatments
resulted in reduced cotyledons and leaf sizes. The high-light treated wild-type and the \( porB-1 \) and \( porC-1 \) mutants greened similarly, whereas the \( porA-1 \), \( porA-1 \ porB-1 \) and \( porA-1 \ porC-1 \) mutants grew slowly with a chlorotic phenotype. The high-light treatment also induced red pigment formation that is most likely anthocyanin (Solfanelli et al., 2006; Steyn et al., 2002).

Homozygous \( porA-1 \), \( porA-1 \ porB-1 \) and \( porA-1 \ porC-1 \) null mutants display growth arrest when geminated on MS agar media (Figure 4.2A, C and E). When germinated on or transferred to sucrose supplemented media they grow as dwarfs but complete their lifecycle (Figure 4.3). Growth conditions were optimized for sterile propagation of viable \( porA-1 \), \( porA-1 \ porB-1 \) and \( porA-1 \ porC-1 \) mutants. Under optimized growth conditions the \( porA-1 \), \( porA-1 \ porB-1 \) and \( porA-1 \ porC-1 \) mutant bolt at a similar time after germination as wild type. The inflorescences and rosettes of the \( porA-1 \), \( porA-1 \ porB-1 \) and \( porA-1 \ porC-1 \) mutants are small compared to wild type (Figure 3A). The \( porA-1 \), \( porA-1 \ porB-1 \) and \( porA-1 \ porC-1 \) mutants grow with a similar dwarf phenotype (Figure 4.3B, C and D). Over time, the mutants produce several short inflorescences with reproductively competent flowers that produce a severely reduced seed set (Figure 4.3E, F and G).

Growth defects in the \( por \) mutants are presumably in part due to a deficiency in Chl accumulation. Contributions of each POR to \( Arabidopsis \) greening were evaluated by quantitative measurements of Chl accumulation in the \( POR \) mutants and wild type in 5-d-old etiolated seedlings after 48 h of illumination. Wild type, and the \( porB-1 \) and \( porC-1 \) single mutants demonstrate similar levels of Chl under high light (400 µE m\(^{-2}\) s\(^{-1}\)).
while in the *porA-1* mutant levels were reduced ~50% (Figure 4.4). The *porA-1 porB-1* and *porA-1 porC-1* double mutants had Chl accumulations that were reduced to ~30% of *porA-1* indicating independent contributions of PORB and PORC in greening in the absence of PORA accumulation.

**The prolamellar body is absent in porA-1 porB-1 mutants**

We hypothesized that the accumulation of prolamellar body in etiolated seedlings correlates with the accumulation of POR. The POR protein is sub-localized to the prolamellar body in the etioplast of dark grown seedlings; therefore observation of the ultrastructure of the etioplast will reveal any structural role of POR proteins. Ultrathin sections of cotyledons of various genotypes were examined by transmission electron microscopy. The plastid inner-membrane architectures were observed from at least three independent seedlings of each genotype, representative images are shown in Figure 4.5. In wild type etioplasts, prolamellar bodies with unstacked prothylakoids extending between them and throughout the stroma were observed (Figure 4.5A). These resemble the etioplasts reported in *porC-1* mutant seedlings (Frick et al., 2003; Masuda et al., 2003).

In contrast, loss of PORA and/or PORB correlates with a reduction of the prolamellar body. The most striking phenotype is seen in *porA-1 porB-1* double mutants, where no prolamellar body was detected, although there were prothylakoids throughout the etioplasts (Figure 4.5D). There were significant reductions in the extent of prolamellar body observed in the *porA-1* single mutant (Figure 4.5B) and the *porA-1*
porC-1 mutants (Figure 4.5E). Slightly less prolamellar bodies were observed in the porB-1 mutants (Figure 4.5C) than in the porA-1 and porA-1 porC-1 mutants, suggesting that the protein encoded by the PORB gene plays the larger role in organization of the prolamellar body.

**Accumulation of photoactive-Pchlide is reduced in por mutants**

In etioplasts, the POR protein is found in complex with its substrate, Pchlide, in the prolamellar body. The population of Pchlide found in such a complex is photoactive. Any Pchlide not in the complex with POR is not photoactive. Presumably, reduction in POR accumulation would lead to a reduction in photoactive-Pchlide. To evaluate this hypothesis, the total Pchlide and the photoactive-Pchlide contents present in the different genotypes were quantified by fluorescence measurements of pigment extracts prepared from flashed (non-photoactive-Pchlide) and non-flashed (total Pchlide) etiolated seedlings. Total Pchlide accumulations in wild-type, porA-1, porC-1 and porA-1 porC-1 mutants were similar, whereas the porB-1 and porA-1 porB-1 mutants accumulated roughly ~35% less total Pchlide (Figure 4.6). This suggests that PORB is necessary for normal accumulation of Pchlide, even the Pchlide not complexed with POR, but PORA and PORC are not necessary for this accumulation.

More differences were seen between wild type and the por mutants in accumulation of photoactive-Pchlide (Figure 4.6). The photoactive-Pchlide content was not significantly different between wild type and the porC-1 mutant, consistent with the absence of PORC accumulation in etiolated seedlings. There were reductions in
photoactive-Pchlide content in the *porA-1*, *porB-1* and *porA-1 porC-1* mutants of roughly 50-70% compared to wild type. The *porA-1 porB-1* double mutant did not significantly photo-convert any Pchlide. The percent reduction of photoactive POR (the ratio of photoactive-Pchlide to total Pchlide was normalized to wild type) is 40-60% in the *porA-1*, *porB-1* and *porA-1 porC-1* mutants, whereas the reduction in the *porA-1 porB-1* double mutant is essentially 100%. These reductions clearly demonstrate that PORA and/or PORB are required for photoactive-Pchlide formation. There was no statistical difference between the *porA-1* and the *porA-1 porC-1* genotypes in total Pchlide and photoactive-Pchlide, suggesting no active roll for PORC in this transition.

4.3. DISCUSSION

A current hypothesis proposes PORA and/or PORB protein accumulation correlates with photoactive Pchlide conversion and PLB aggregation. Several studies have addressed the expression and functions of POR in *Arabidopsis* seedlings (Franck et al., 2000; Runge et al., 1996; Sperling et al., 1998, 1997). For example, seedlings grown for several days under continuous far-red light contained little Chl and did not green even when transferred thereafter to white light, due to a phytochrome A-mediated block of photomorphogenesis (Runge et al., 1996). These seedlings were depleted of PORA and partially depleted of PORB, causing a reduction in PLB size. Similarly, no PORA and reduced amounts of PORB were detected in the constitutive photomorphogenic *cop1-18* mutant where phytochrome-dependent processes occur in the absence of light (Sperling
et al., 1998). When the cop1-18 mutant was germinated in the dark it displayed light-grown morphology. The cop1-18 mutants plastid ultrastructure resembled a pseudochloroplast with no PLBs or photoactive Pchlide. When PORA or PORB were ectopically expressed either in the cop1-18 mutant background (Sperling et al., 1998), or in far-red light-grown wild type plants the photoactive Pchlide and PLBs were restored (Sperling et al., 1997). Ectopic expression resulted in a partial rescue of the greening defects in POR-depleted cop1-18 mutants and far-red light-treated seedlings, thus suggesting functional redundancy of the PORA and PORB isoforms. Both approaches suggest interactions that adjusted POR protein content while leading to imprecise conclusions. When transgenic antisense and overexpression constructs were targeted towards PORA and PORB, the total POR content correlated with photoactive Pchlide content, the ratio of photoactive to nonphotoactive pigment (Franck et al., 2000) and PLB accumulation but POR isoform specific conclusions were confounded by overexpression, partial protein knockdowns and nonspecific gene silencing.

To reveal phenotypical defects caused by POR isoform specific depletion, complete knockouts of PORA, PORB and PORC were isolated. The use of single PORA and PORB mutants demonstrate a reduction in PLBs in addition to a decrease in photoactive Pchlide conversion linked with PORA and PORB isoform specific depletion, whereas PORC mutant etioplasts resemble wild type (Frick et al., 2003; Masuda et al., 2003). When PORA was ectopically expressed in the porB-1 porC-1 double mutant background it led to a phenotypical rescue that resembled wild type. To demonstrate PORA and PORB combine to act as contributors in PLB formation we created the porA-1
porB-1 double mutant. This double mutant clearly demonstrates PORA and PORB are required for normal etioplast development with complete elimination of PLBs as well as photoactive Pchlide conversion in their absence. Total Pchlide accumulation in the porA-1 porB-1 double mutant mirrored the total Pchlide accumulation in the porB-1 mutant. Excess Pchlide accumulation indicates a potential for photooxidation upon illumination, which would not be ameliorated due to Pchlide photoconversion by PORA, the remaining dark expressed POR in PORB mutants. The effect of this excess Pchlide accumulation in the porA-1 porB-1 double mutant on photomorphogenesis is the subject of ongoing investigation but does not lead to lethal photooxidative damage, demonstrated by cotyledon greening as a result of PORC catalytic activity in the porA-1 porB-1 double mutant. The survival of the porA-1 porB-1 double mutant with Pchlide not bound by POR contrasts a POR specific phenotypic defect observed in an Atop16, a PORA import defective mutant, which was shown to display a Pchlide dependent light conditional lethality (Pollmann et al., 2007). The similarity in photoactive Pchlide conversion and PLB accumulation in the porA-1 and the porA-1 porC-1 mutants confirm previous investigations conclusion that PORC is not active in etiolated seedlings (Oosawa et al., 2000; Su et al., 2001; Frick et al., 2003; Masuda et al., 2003).

Phenotypical abnormalities arise in seedlings which result from POR isoform specific depletion. A photoautotrophic growth defect in the PORA depleted (porA-1, porA-1 porB-1 and porA-1 porC-1) mutants is partially rescued with heterotrophic growth on sucrose supplemented media. There is a slight decrease in the early greening in the porA-1 porB-1 and porA-1 porC-1 double mutants compared to the porA-1 mutant
indicating a potential developmental lag where both PORB and PORC are required to maintain Chl levels when PORA is not accumulating. The level of light fluency increased the severity of the growth deficiency in PORA depleted mutants indicating possible photoprotective effects ameliorated by PORA. The LHPP model suggests the formation of a specific PORA:PORB protein complex as a prerequisite for deetiolation and greening in plants (Pollman et al., 2007; Reinbothe et al., 1999). PORB and PORC-dependent Chl synthesis in the absence of the other POR isoforms disagrees with the proposed LHPP model, which has been repeatedly questioned in the literature with serious doubts regarding its validity (Masuda et al., 2004; Armstrong et al., 2000). There is an interesting parallel between the porA-1, porA-1 porB-1 and porA-1 porC-1 mutant early growth and the porB-1 porC-1 double mutant growth. These POR depleted mutants all display growth blockage several days after germination on unsupplemented media. In PORA depleted mutants with PORB and (or) PORC accumulation Chl is maintained in the cotyledons, whereas the porB-1 porC-1 double mutants have xantha (highly chlorophyll-deficient) cotyledons. The porB-1 porC-1 double mutant is extremely sensitive to light preferring low light fluencies (15 µE m⁻² s⁻¹), whereas PORA depleted mutants prefer moderate light (50 µEm⁻² s⁻¹) for heterotrophic growth.

Heterotrophic growth on supplemented media reveals the requirement of PORA for normal growth and development. Low light PORA Chl synthesis in cauline leaves suggests an active role for PORA in light grown seedlings. The removal of PORA activity can be partially subverted by sucrose supplementation suggesting either a metabolic carbon deficit or systemic regulation block that reduces overall growth. The
addition of either the porB-1 or porC-1 mutations to the porA-1 mutation did not drastically alter the sucrose supplemented light grown seedling phenotype of the porA-1 mutant, thus suggesting redundancy between PORB and PORC catalytic activity. This result confirms previous observations of the porB-1 and porC-1 null mutants, under normal light growth condition, PORB and PORC provide for functionally redundant bulk Chl biosynthesis (Frick et al., 2003; Masuda et al., 2003) even in the porA-1 mutant background in adult plants. Heterotrophic growth on sucrose supplemented media produced reproductively competent dwarf bushy seedlings with Chl synthesis from either PORB or PORC demonstrating independent biologically significant of each of these POR isoforms. Alterations in seedling morphology due to the absence of PORA expression reveal the integration of overall plant physiology and development with POR gene regulation. The redundancy in the light accumulation of PORB and PORC emphasizes the requirement for the accumulation activity of a key biosynthetic enzyme required for photosynthesis.

4.4 MATERIALS AND METHODS

Generation of Transgenic Plants, Plant Growth Conditions and Sample Collection

Landsberg erecta stock number CS20 Arabidopsis seeds were obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. porB-1 and porC-1 alleles described in Frick et al. (2003). porA-1 allele described earlier. Heterozygous porA-1 mutant lines were used as the F₀ pollen donors and the
homozygous porB-1 and the porC-1 lines as the F₀ pollen acceptor followed by selection using PCR for both the PORA and PORB or PORC Ds insertions::genomic border.

Presence of the porA-1 allele and the porB-1 or porC-1 alleles was selected for in the F₁ generation. Homozygosity at the porA-1 locus and the presence of porB-1 or the porC-1 alleles were selected for in the respective F₂ lines. Lines homozygous for porA-1 and porB-1 were denoted porA-1 porB-1 and those homozygous for porA-1 and porC-1 were denoted porA-1 porC-1 in the F₃ generations. For propagation and analysis, the photoperiodically grown homozygous porA-1, porA-1 porB-1 and porA-1 porC-1 mutants were maintained on MS agar supplemented with 2% sucrose at a fluence rate of 50 µE m⁻² s⁻¹ under long day conditions.

Photoperiodically grown plants were maintained in long day conditions (16 h d/8-h night). Continuous white light conditions at 22°C were provided by a combination of TLD 36W/84 (4100 K) cool-white fluorescent lamps (Philips). F17T8/TL741 17 watt (4100 K) cool-white fluorescent lamps (Philips) and ES27 27 watt (2700 K) compact fluorescent bulbs (TCP). Seedlings were germinated by sowing surface-sterilized seeds on MS agar plates. To help synchronize germination, sowed seeds were placed in the dark at 4°C for a least 24 h to allow seed imbibition. Etiolated seeds were thereafter exposed at 22°C to 1-2 h white light at a fluence rate of 160 µE m⁻² s⁻¹. The illuminated imbibed seeds were returned to darkness at room temperature to produce etiolated seedlings. All fluence rates were measured with a Li250 light meter (Li-Cor). Plant material was harvested and either frozen in liquid nitrogen and stored at -80°C until its use for DNA and protein analysis or processed immediately for electron microscopy and
spectroscopic measurements. In flash illumination experiments, one flash per plate of seedlings was given using a 550FD (Vivitar) camera flash attachment to ensure full conversion of all photoactive-Pchlide to Chlide. Etiolated seedlings were harvested using a green safe light (Kodak 7B filter). Images of seedlings and plants were collected using a Sony DSC-S75 digital camera.

**DNA isolation, PCR, and protein ultrastructural analyses**

To determine the genotypes of individual plants grown on MS agar, total DNA was extracted and analyzed using REDExtract-N-Amp™ Plant PCR kit (Sigma). PCR reactions to assay for intact genomic sequences, and generate $Ds::$genomic border fragments were performed as follows: 1 cycle of 5 min at 95°C, 40 cycles of 30 sec at 95°C, 1 min at 55°C, 2 min at 72°C, and one cycle of 7 min at 72°C.

Whole 14-d-old seedlings grown in $80 \mu \text{E m}^{-2} \text{s}^{-1}$ were harvested to obtain pooled material for protein gel blot analyses. Total protein extracts were prepared essentially as in Sperling et al., (1997). Protein content was assayed using a modified Lowry et al. (1951) method (RC DC Protein Assay, Bio-Rad Laboratories). Samples containing 60 µg total protein were separated electrophoretically under denaturing conditions by SDS-PAGE on a 10% polyacrylamide gel and were electroblotted onto an Immun-Blot PVDF membrane (Bio-Rad Laboratories). Western blot analysis of the electroblotted antigens was performed using a 1:500 dilution of a polyclonal anti-\textit{Arabidopsis} POR antiserum (Sperling et al., 1997), combined with an Immun-Star™ Goat Anti-Rabbit IgG Detection
Kit (Bio-Rad Laboratories). An identical protein gel was stained with Coomassie Brilliant Blue R250.

5-d-old etiolated cotyledons harvested for transmission electron microscopy were vacuum infiltrated at 10 psi in a fixative composed of (3%) glutaraldehyde and (2%) paraformaldehyde in the dark. Post-fixation treatments of the samples included osmium tetroxide and uranyl acetate. A gradual dehydration with ethanol was followed by washes in propylene oxide. Propylene oxide was gradually replaced to 100% (v/v) Spurr resin (Ted Pella, Inc.). After polymerization, cotyledons ultrathin sections were mounted on Formvar-coated copper grids, then stained with uranyl acetate and Reynolds lead citrate, and viewed on a Technai G2 Spirit transmission electron microscope.

**Quantitative pigment determinations by fluorescence microscopy**

Room temperature fluorescence emission analyses of the Pchlide, Chlide, Chl $a$ and Chl $b$ present in etiolated and light-treated etiolated seedlings, as well as older light grown seedlings, were performed. Total photosynthetic pigments were extracted for at least 48 h from 10 pooled seedlings per sample in 3 ml of 80% (v/v) acetone supplemented with 0.1 N ammonium hydroxide at 4°C. Fluorescence emission spectra of the pigment extracts were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian) with both the excitation and emission slit widths set at 5 nm. The pigment extracts were excited at 433 nm, which preferentially causes Chl $a$ fluorescence emission at 670 nm, and at 455 nm, which preferentially causes Chl $b$ fluorescence emission at 655 nm. The Pchlide and Chlide amounts were determined by excitation of etiolated seedling
and flash treated etiolated seedling pigment extracts at 433nm with measurement of emission bands at 634 and 672 respectively. The pigment contents were calculated according to Sperling et al. (1998). Pigment accumulation data were compared by ANOVA (p<0.05).

4.5. REFERENCES


enhances seedling survival in white light and protects against photooxidative damage. *Plant J* 12:649-658.


Figure 4.1. **Confirmation of the genotype of putative porA-1 porB-1 and porA-1 porC-1 doubly homozygous mutants by PCR and protein accumulation.**

Genotypes of genomic DNA templates are given in the upper line. Primer locations and sequences are shown in Frick, et al., (2003), primer sets used are as follows: *Ds::POR A* present, lanes 1 and 5, 1.9/DS5; *POR A* intact lanes 2 and 6, (Panel A) *Ds::POR C* present, lanes 3 and 7, 1.7/DS3; *POR C* intact lanes 4 and 8, 5.1/5.2; (Panel B) *Ds::POR B* present, lanes 3 and 7, 2.7/DS3; *POR B* intact lanes 4 and 8, 2.7/6.0. Lanes 5-8 represent positive controls with a wild-type template. The absence of PCR products in lanes 2 and 4 indicates the homozygosity of the respective *Dissociation (Ds)* elements, whereas the products observed in lanes 1 and 3 confirm the presence of the insertions. (C) The *porA-1 porB-1* and *porA-1 porC-1* mutants lack the corresponding POR transcripts. A gel blot prepared using of electrophoretically-separated total protein from light grown seedlings was incubated with an anti-*Arabidopsis* POR antiserum. The bottom panel shows Coomassie blue staining of the membrane, revealing the ribulose-1,5-bis-phosphate carboxylase/oxygenase large subunit as a loading control.
Figure 4.2. Reduced growth in the \textit{porA-1 porB-1} and \textit{porA-1 porC-1} mutants at light intensities.

From left to right wild-type, \textit{porA-1}, \textit{porA-1 porB-1}, \textit{porB-1}, \textit{porA-1 porC-1}, \textit{porC-1}. Rows A and B demonstrate 14-d-old seedlings grown under continuous light treatments of 5 \(\mu\)E m\(^{-2}\) sec\(^{-1}\), Rows C and D 50 \(\mu\)E m\(^{-2}\) sec\(^{-1}\), and Rows E and F 400 \(\mu\)E m\(^{-2}\) sec\(^{-1}\). Growth on MS in demonstrated in Rows A, C and E and growth on MS supplemented with sucrose in Rows B, D and F. Size Bar = 0.5 mm.
Figure 4.3. Dwarf growth in the *porA-1 porB-1* and *porA-1 porC-1* Arabidopsis plants.

(B and E) *porA-1*, (C and F) *porA-1 porB-1* and (D and G) *porA-1 porC-1* mutant grown in photoperiodic conditions at a fluence rate of 50 µE m$^{-2}$ sec$^{-1}$ on MS agar supplemented with sucrose. 1-month-old wild-type seedling is 8 cm tall, 1-month-old seedlings (B-D) are approximately 2 cm tall, 2.5-month-old seedlings shown in E-G are approximately 3.5 cm tall.
Figure 4.4. Chlorophyll content of *porA-1 porB-1* and *porA-1 porC-1* is reduced under high light intensity.
The amount of total Chl was determined in acetone-extracted total pigments from 5-d-old etiolated seedlings grown under continuous light conditions at a fluence rate of 400 µE m⁻² sec⁻¹ for 48 h. Chl quantification based on room temperature fluorescence emission measurements of 5 pigment extracts of 10 seedlings each ±95% confidence interval indicated by error bars.
Figure 4.5. **Prolamellar bodies are absent from the etioplasts of the porA-1 porB-1 double mutant.**
Etioplast membrane ultrastructure of the porA-1 porB-1 and porA-1 porC-1 mutants is altered. Ultrathin sections of cotyledon plastids from 5-d-old etiolated seedlings were examined by transmission electron microscopy. Bars = 0.5 µm. (A) Wild-type. (B) porA-1 (C) porB-1, (D) porA-1 porB-1 arrow indicates prothylakoids and (E) porA-1 porC-1 arrow indicates prolamellar body.
Figure 4.6. **The porA-1 porB-1 double mutant contains no photoactive-Pchlide.**

Quantification of total Pchlide and photoactive-Pchlide in 5-d-old etiolated seedlings. Total Pchlide was calculated from the fluorescence emission band at 634 nm from non-illuminated seedling extracts. Photoactive-Pchlide was calculated from the fluorescence emission band at 672 nm from flash-illuminated seedling extracts. Each data point represents at least ten replicates of ten seedlings with error bars indicating ±95% confidence intervals.
CHAPTER 5 – FUTURE PERSPECTIVES

5.1 Further elucidate the Arabidopsis por mutant phenotypes during skotomorphogenesis and photomorphogenesis.

Rationale:
To complement earlier analysis, extensive phenotypic, and biochemical analyses of the homozygous porA, porB, porC mutants and their respective double mutants performed during photomorphogenesis, the effects of these mutations at the cellular, organellar, and molecular levels will be addressed. The phenotypes of etiolated seedlings during skotomorphogenesis and the early stages of light-induced, POR-mediated greening will be further studied. The evaluation of predicted PLB components, indicated by proteomic (Sundqvist et al., 2008) and biochemical analyses of etiolated seedlings during skotomorphogenesis, in por mutants containing altered PLB may provided clues to the function of this membrane lipid structure. Understanding the mechanism behind photoautotrophic PORA-depletion induced growth defects will reveal specialization of the PORA gene function.
Experimental approach:

The porA-1, porB-1, porC-1 mutants and their respective double mutants create a valuable set of POR depleted transgenic lines for the analysis of the effect of the absence of POR on etioplast development, photomorphogenesis and adult plant development. Pigments and proteins associated with photomorphogenesis can be tested during the shift from etiolated growth to either continuous illumination or photoperiodic conditions, or, alternatively, seedlings can be grown under these conditions from the onset. Photomorphogenesis can also be studied either in the presence of Chl production during growth in white light, or in the near absence of Chl production accompanied by nonphotoactive Pchlide overaccumulation during growth in continuous far-red light. This latter growth condition, followed by a shift to white light of different fluence rates, will be used to assay seedling sensitivity to Pchlide-induced photooxidative damage and killing. Greening and seedling growth under light stress conditions will be assayed by transferring etiolated seedlings to light of different fluence rates provided at various ambient temperatures, as it is known that cold treatment can inhibit the greening process and increase light sensitivity (Krol et al., 1999). The rationale is that by maximizing the range and severity of stresses to which por mutant seedlings are exposed the likelihood of exposing “cryptic” phenotypes under laboratory conditions will increase. Monitoring the response of oxidative stress indicators and antioxidants (ascorbate peroxidase, glutathione, malondialdehyde (MDA) (a lipid peroxidation marker), etc.) will help define the extent and nature of possible oxidative damage related to reduced POR accumulation or Chl accumulation during the various growth conditions tested.
Photosynthetic pigments will be monitored following their organic extraction and initial fractionation based on polarity using absorption measurements for the carotenoids and highly sensitive room temperature fluorescence excitation and emission measurements for the Mg-tetrapyrroles (ie. Mg-protoporphyrin IX, Pchlide a, Chlide a, Chl b) and protoporphyrin IX. For a more detailed characterization of the pigments, larger amounts of seedlings will be processed and the pigment extracts separated on reverse-phase columns coupled to a photodiode array HPLC system.

Many factors affect a seedlings response to light, including regulation by growth hormones such as auxin, cytokinins, brassinosteroids, abscisic acid, and ethylene (reviewed in Nemhauser and Chory, 2002). Observation of mutant seedling growth in the presence of exogenously applied phytohormones might reveal differential growth responses in the mutants versus the wild type. The effect of sugar supplementation on Arabidopsis growth has been shown to have profound effects on seedling development (reviewed by Gibson, 2004, 2005). By manipulating the sugar supplementation used, the PORA-depleted heterotrophic growth phenotype will be characterized more thoroughly. Careful analysis of photosynthetic parameters in the cotyledons at the point at which Chl accumulation is no longer normal, will help reveal key details of the PORA-dependent photoautotrophic growth defect.

PORA-depleted mutant seedlings display morphological and growth aberrations that can be investigated more closely, such as cotyledon and true leaf anatomy, and seedling viability. Specifically the porA-1 porB-1 double mutant has proved to be more difficult to propagate using the same tissue culture techniques as the porA-1 and the
*porA-1 porC-1* mutants suggesting possible defects that are exaggerated by due to combination of these two mutations. Initial observations indicate that sucrose supplemented growth conditions possibly produce slightly smaller *porA-1 porB-1* seedlings than *porA-1* and the *porA-1 porC-1* mutants. Seed viability is considerably reduced compared to the *porA-1* and the *porA-1 porC-1* mutants. Therefore more in-depth analysis of the differences in Chl accumulation kinetics needs to be performed to find the source of these isoform-specific phenotypic defects.

From transmission electron microscopy and PLB constituent analysis data it seems both the PORA and PORB enzymes can mobilize lipids to form the PLB. Plastid thylakoids and PLBs contain four classes of membrane lipids: monogalactosyldiacylglycerol, digalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol, and phosphatidylglycerol. A side effect of bleaching herbicides (carotenoid synthesis inhibitors) on etiolated barley seedlings was to cause significant differences in lipid accumulation as well as disorganization of the PLBs (Baccio et al., 2002). PLB aggregation is altered in the por mutants, therefore, we will use thin layer chromatography to measure alterations in plastid membrane lipid composition.

In Blomqvist et al (2008), a proteomic approach was used to study protein extracts from highly purified PLBs from dark-grown wheat leaves. The authors identified a set of proteins involved in pigment biosynthesis, the photosynthetic light reactions, the Calvin cycle proteins, chaperones, and plastid protein synthesis. Orthologs of this set of proteins will be identified in Arabidopsis and quantitatively evaluated for
transcriptional variations in the PLB-depleted *por* mutants. When available, antibodies will be requested or purchased for PLB protein accumulation studies.

In addition to the analysis of the *por* single mutants, it will be desirable to study many of the phenotypic parameters described above using *porA-1 porB-1*, *porA-1 porC-1* and *porB-1 porC-1* double mutant combinations, and potentially a *por* triple mutant (which would be expected to be seedling lethal). Photosynthetic pigment analysis is required in the double mutants to further establish the unmasked contribution to bulk pigment accumulation of each of the POR isoforms under a variety of growth conditions. Double mutant experiments may help to address questions of functional redundancy that arise, as in the case of the roles of PORB and PORC in green plants. Previous evidence indicated possible functional redundancy between PORB and PORC, and this study indeed described how the *porA-1 porB-1* and *porA-1 porC-1* double mutants display phenotypes that are dominated by the effects of the *porA-1* mutation. Furthermore, we demonstrated that the *porA-1 porB-1* and *porA-1 porC-1* mutants can be propagated as double homozygotes. These double mutants are a tremendous advantage for certain types of analyses, such as measurements of etiolated seedlings in which it is typically necessary to use a homogenous rather than a segregating seed population.

A portion of the above phenotypic analyses require relatively small seedling samples and can be readily performed with the homozygous *por* single and double mutants. Some experiments require larger numbers of seedlings, such as greening kinetics with duplicate samples collected at multiple time points, and HPLC analyses of the carotenoid species present, and might be difficult to perform with the *porA-1*
homozygote due to its relatively low seed set. Therefore, initial experiments can be performed with the *POR* RNAi transgenic lines because their seed set is more prolific.

### 5.2 Perform self-complementation/rescue and cross-complementation/rescue assays of the *por* mutants to identify POR-specific functions

**Rationale:**

The plastidic or whole plant phenotypic defects identified in the specific *por* single and double mutants, such as PLB deficiency and impaired greening, may be possible to rescue by ecotopic overexpression of either the same enzyme(s) or one of the other two Arabidopsis *POR* s. Furthermore, the creation of *por* mutant complementation constructs involving the swapping of the *POR* genomic promoter, plastid signaling peptide and mature protein encoding gene sequences from each isoform would create tools for evaluating the specificity of each portion of the POR protein sequences and their corresponding gene promoters.

**Experimental approach:**

The first approach has been to attempt rescue of the *porA-1 chlorina* dwarf phenotype both with a *POR* cDNA, as well as with *PORB* and *PORC* sequences. Self-rescue has been attempted by crossing the heterozygous mutant with well-characterized, homozygous ectopic *POR* -overexpressing lines (Sperling et al., 1997, 1998, 1999). Many lines were screened that contained both the ectopically expressed *POR* cDNA and
the porA-1 mutation but no phenotypically rescued lines were identified, although this search is ongoing. Analysis of porA-1 mutant crosses with ectopically expressing PORB and PORC plants is ongoing. Because the CaMV 35S promoter is used to drive POR cDNA expression in these lines, there is a possibility that the porA-1 complementation did not thus far succeed due to the cellular and developmental pattern of promoter activity.

An alternative complementation approach will involve the use of a tagged PORA gene under the control of its own promoter, which will be introduced into the heterozygous porA mutant by Agrobacterium-mediated in planta transformation. Successful complementation of the porA-1 mutant phenotype will be identified by PCR screening of the primary transformants and by subsequent analysis of their progeny. In this regard, a preliminary functional analysis of the endogenous PORA promoter has been completed and would be helpful in the design of plant transformation constructs (Armstrong, unpublished). A strategy to test the cross-complementation of the porA-1 mutant phenotype will be by introducing ectopic expressed PORB and PORC and then also examine isoform-specific phenomenon in their respective por double mutants. For example, the ectopic expression of PORC in the porA porB double mutant background would create a line designed to evaluate the potential for PORC-mediated PLB accumulation and photoactive Pchlide formation in etiolated seedlings. This line would also be used evaluate a rescue of the pleiotropic light-grown porA-1 mutant phenotype by ectopic PORC expression. Another intriguing, but difficult to isolate genotypic combination would result from the creation of a line with ectopic POR expression in a
porA porB porC triple mutant. Deregulating POR transcript accumulation would demonstrate the biological consequences of removal of the differential regulation to which the POR gene family is subject.

In analyzing the results of the cross-complementation experiments to test the functional redundancy of the Arabidopsis PORs several levels of complexity will have to be considered. Therefore if all three PORs are all catalytically equivalent, at what level does specificity occur? One possibility would be that the polypeptides themselves have intrinsically different biochemical and/or biophysical properties. However, the high degree of sequence conservation among mature POR polypeptides from higher plants, including Arabidopsis, suggests that alternative outcomes should be considered. It may be that any POR-specific phenotypes observed result not from differences in the intrinsic properties of the proteins, but rather from differences in their cellular patterns of gene expression. This hypothesis can be tested by producing chimeric transformation constructs containing, for example, the PORA promoter fused to the PORB cDNA or gene to be used for complementation studies with the porA-1 mutant. Here the preliminary data available from the deletion series of the POR promoter-GUS fusions would help to delineate the promoter fragment to use for a given chimeric construct (Armstrong, unpublished). Another level of complexity comes from differences between the POR protein plastid transit peptides. Pairwise amino acid sequence alignments for the Arabidopsis POR plastid transit peptides show that PORA and PORB share 75% identity with one another, whereas these sequences only share ~40% identity with the PORC transit peptide. POR isoform-specific plastid import has recently been a topic of
much interest and controversy (Kim and Apel 2004; Philippar et al., 2007; Pollmann et al., 2007; Kim et al., 2005; Reinbothe et al., 2005). For example, transgenic lines that carry the $PORA$ promoter fused to the $PORB$ cDNA transit peptide followed by the $PORA$ cDNA encoding only to mature protein gene with the transit could be used for rescue studies with the $porA-1$ and $porB-1$ mutants to reveal some of the specificity of $PORA$ import previously observed $in vivo$. 


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