Untersuchung und Charakterisierung des Lichtsammelkomplexes (LHPP) in etiolierten Pflanzen

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1 Ausführliche Zusammenfassung

Chlorophyll ist eines der verbreitetsten und häufigsten organischen Moleküle auf der Erde. Als eine essentielle Komponente der photosynthetischen Lichtreaktion ist dieses Molekül an der Lichtabsorption und am Energietransfer absorbierter Photonen von den Antennen ins Reaktionszentrum der Photosysteme I und II beteiligt als auch im Reaktionszentrum als primärer Elektronendonator sowie -akzeptor vertreten (von Wettstein et al., 1995). Voraussetzung für diese Funktion ist das umfangreiche delokalisierte π-Elektronensystem des Porphyrinrings, welches durch Absorption von Lichtquanten angeregt werden kann. Allerdings können diese angeregten Moleküle nicht nur mit weiteren Chlorophyllmolekülen reagieren, sondern können diese Anregungsenergie auch auf andere Moleküle übertragen wie zum Beispiel Sauerstoff, wodurch hochreaktiver Singulett-Sauerstoff entsteht (op den Camp et al., 2003; Rebeiz et al., 1988). Dieser Singulett-Sauerstoff hat, wie auch andere reaktive Sauerstoff-Spezies (ROS), eine schädigende Wirkung auf Pflanzen, welche sich in vorzeitiger Alterung des Gewebes und der Auslösung von programmiertem Zelltod niederschlägt (op den Camp et al., 2003).
Aufgrund dieser negativen Folgen haben Pflanzen mehrere Mechanismen zur Vermeidung solcher ROS entwickelt, so dass unter normalen Wachstumsbedingungen ein Gleichgewicht zwischen Entstehung und Entschärfung von ROS besteht (Foyer und Nocter, 2000). So kommen Chlorophyllmoleküle beispielsweise nicht als freie Pigmente vor, sondern sind zusammen mit Carotinoiden an bestimmte chlorophyllbindende Proteine von Photosystem I und II in der Thylakoidmembran der Chloroplasten gebunden. Dadurch wird unter anderem das Risiko einer möglichen Interaktion der angeregten Pigmente mit Sauerstoff vermindert (von Wettstein et al., 1995).

Chlorophyllsynthese in Angiospermen: NADPH:Protochlorophyllid-Oxidoreduktase und ein Lichtsammelkomplex in etiolierten Pflanzen

Chlorophyll und andere Tetrapyrrolkomponenten des Stoffwechsels werden über den sogenannten C5-Stoffwechselweg synthetisiert. Das erste Zwischenprodukt dieses Stoffwechselweges ist die C5-Verbindung 5-Aminolävulinsäure (von Wettstein et al.,

1995), welche aus der Umsetzung von Glutamyl-tRNA durch das Enzym Glutamyl-tRNA-Reduktase hervorgeht und aus der schließlich das Porphyrinringsystem aufgebaut wird (siehe Abb.1).

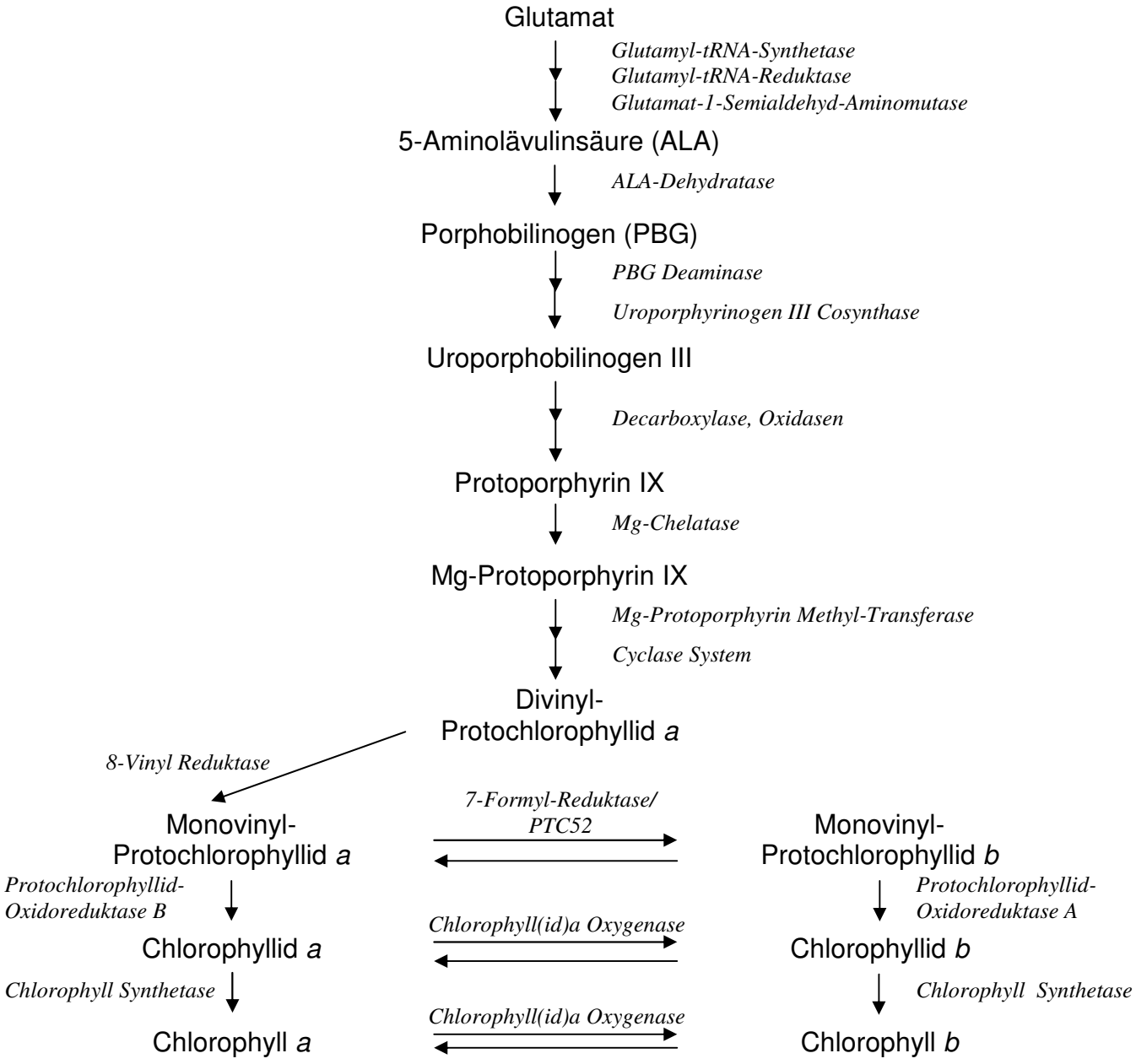

Abb.1: Biosyntheseweg von Chlorophyll in Pflanzen (vereinfacht). Enzyme der einzelnen Reaktionen sind kursiv dargestellt. Die Bildung von Monovinyl-Protochlorophyllid *b* aus Monovinyl-Protochlorophyllid *a* ist experimentell bewiesen, jedoch noch nicht veröffentlicht. Aus: Reinbothe und Reinbothe, 1996, und Rebeiz, C. A., http://w3.aces.uiuc.edu/nres/lppbp [25.09.2006], verändert.

Im Gegensatz zu den Endprodukten sind die meisten Zwischenprodukte dieses Stoffwechselweges in freier Form vorhanden und nicht an andere Komponenten, wie

Proteine, gebunden. Dies macht sie besonders zugänglich, nach Anregung mit Licht die aufgenommene Energie auf Sauerstoff zu übertragen (Matringe et al., 1989; Mock et al., 1998). Dementsprechend haben Angiospermen folgende Strategie entwickelt, um eine Akkumulation größerer Mengen solcher Zwischenprodukte zu vermeiden. Wenn Pflanzen im Dunkeln keimen, wird die Biosynthese von Chlorophyll auf der Stufe von Protochlorophyllid angehalten. Sobald eine bestimmte Menge dieses Zwischenproduktes angereichert ist, wird die Synthese von 5-Aminolävulinsäure abgeschaltet. Wenn nach Belichtung das angereicherte Protochlorophyllid zu Chlorophyllid umgesetzt wurde, wird die Blockade der 5-Aminolävulinsäuresynthese wieder aufgehoben (Reinbothe et al., 1996).

Das akkumulierende Zwischenprodukt der Chlorophyllsynthese, Protochlorophyllid, ist in etiolierten Keimlingen von Angiospermen mit dem Enzym NADPH:Protochlorophyllid Oxidoreduktase (POR) assoziiert und liegt zusammen mit dem Cosubstrat NADPH als ternärer Komplex vor (Griffiths, 1978; Apel et al., 1980). POR ist ein Zellkern-codiertes Protein, wird als Precursor im Cytosol synthetisiert und schließlich posttranslational in die Plastiden importiert. Dort katalysiert es die lichtabhängige Reduktion von Protochlorophyllid zu Chlorophyllid, welches die direkte Vorstufe des Chlorophylls darstellt (Griffiths, 1975; 1991). Im Prolamellarkörper aus Etioplasten von Gerste (und anderen Arten) existieren zwei Isoenzyme, PORA und PORB, welche sich in Expressionsmuster, mRNA-Stabilität und Importverhalten unterscheiden (Schulz et al., 1989; Benli et al., 1991; Armstrong et al., 1995; Holtorf et al., 1995; Reinbothe et al., 1995a; 1995b; 1995c): PORA wird ausschließlich in etiolierten Keimlingen exprimiert. Das Enzym wies eine Substratspezifität für Protochlorophyllid *b* auf. Dagegen wird PORB konstitutiv exprimiert und zeigte eine höhere Bindungsaffinität zu Protochlorophyllid *a* (Reinbothe et al., 1999). Einen weiteren interessanten Gesichtspunkt stellt das Importverhalten der beiden Isoenzyme dar. So wird PORB über den bekannten TIC/TOC-Importapparat (TIC/TOC = `Translocon of the inner/outer chloroplast envelope membrane´ (Schnell et al., 1997)) in die Plastiden eingeschleust (Reinbothe et al., 1995b; 1997). Demgegenüber erfolgt der Import der PORA-Vorstufe in die Plastiden über einen speziellen Importkomplex (PTC-Komplex = `Protochlorophyllide-dependent Translocon Complex´) und ist von seinem Substrat Protochlorophyllid *b* abhängig (Reinbothe et al., 1995a; 1996c; 1997).

Neben der Unterscheidung in Protochlorophyllid *a* und *b* wurde durch *in situ* Fluoreszenzspektroskopie eine weitere Einteilung in zwei dominierende unterschiedliche Arten von Protochlorophyllid unternommen. Diese wurden anhand ihrer Absorbtions- und Emissionswellenlängen charakterisiert und als photoaktives und photoinaktives Protochlorophyllid bezeichnet, je nachdem ob sie durch einen 1 ms-Lichtblitz zu Chlorophyllid umgesetzt werden konnten oder nicht (Litvin und Krasnovski, 1957; Shibata, 1957). Aufgrund dieser Beobachtung wurde angenommen, dass photoinaktives Protochlorophyllid (Protochlorophyllid 628/632) frei vorliegt, während photoaktives Protochlorophyllid (Protochlorophyllid 650/657) als Enzym- (POR-) gebunden galt (Griffiths, 1975). Eine andere Erklärung beruht auf der Beobachtung, dass Porphyrinmoleküle sich zu größeren Molekülaggregaten zusammenschließen können (Abrahams et al., 1994). Dabei wurde den Porphyrinaggregaten der Status des photoaktiven Protochlorophyllids zugeschrieben, den Monomeren der Status des photoinaktiven Protochlorophyllids (Böddi et al., 1998). Letztlich führten noch Crosslinking-Versuche mit isolierten Prolamellarkörpern zu der Vermutung, dass POR-Proteine sich zu Dimeren zusammenlagern (Wiktorsson et al., 1992; 1993).

Diese Beobachtungen ließen die Frage, inwieweit die beiden verschiedenen POR-Isoenzyme untereinander und mit ihrem Substrat bzw. mit photoaktivem oder photoinaktivem Protochlorophyllid interagieren, aufkommen. Des weiteren stellte sich die Frage nach der physiologischen Funktion zweier POR-Proteine als auch nach der Rolle des photoinaktiven Protochlorophyllids bei der Chlorophyllbiosynthese.

Bereits 1962 wurde ein hochmolekularer POR:Protochlorophyllid:NADPH-Komplex mit einem Molekulargewicht von ca. 600 kDa identifiziert und als Protochlorophyllid-Holochrom bezeichnet (Boardman et al., 1962). Diese Beobachtung wurde wieder aufgegriffen und in Anlehnung an den Lichtsammelkomplex der Photosynthese LHCII (Ide et al., 1987; Palsson et al., 1994; Kühlbrandt et al., 1994) ein Lichtsammelkomplex in etiolierten Keimlingen postuliert. *In vitro*-Assemblierungsversuche von Ternärkomplexen beider POR-Proteine mit ihrem jeweils spezifischen Substrat (es wurden die stabileren Zn-Analoga an Stelle der Mg-haltigen authentischen Verbindungen verwendet) und NADPH (d.h. PORA: Zn-Protophäophorbid *b*:NADPH und PORB:Zn-Protophäophorbid *a*:NADPH) hatten gezeigt, dass 5 ternäre PORA:Zn-Protophäophorbid *b*:NADPH-Komplexe mit einem PORB:Zn-Protophäophorbid *a*:NADPH-Komplex interagieren (Reinbothe et al.,

1999). Wurde dieser *in vitro* synthetisierte höhermolekulare Komplex mit einer Mischung aus Galakto- und Sulpholipiden aus Prolamellarkörpern inkubiert, so zeigte er die gleichen spektralen Eigenschaften wie isolierte Prolamellarkörper: Es kam zur Bildung von photoaktivem Zn-Protophäophorbid (650/657), das nach Belichtung mit einem 1 ms Blitz weißen Lichtes in Zn-Phäophorbid (684/690) umgewandelt werden konnte. Basierend auf dieser Übereinstimmung wurde die Existenz eines analogen Lichtsammelkomplexes in Etioplasten als wahrscheinlich angesehen. Dieser Komplex wurde als `Light-Harvesting-POR-Protochlorophyllide´ Komplex (LHPP) bezeichnet (Reinbothe et al., 1999). Wie kann man sich nun die Funktionsweise dieses Komplexes vorstellen? Analog zu LHCII wird dabei durch PORA-gebundenes Protochlorophyllid *b* in der Peripherie des Komplexes Licht absorbiert und auf den zentralen PORB:Protochlorophyllid *a*: NADPH-Komplex übertragen. Diese Energie wird dann von PORB zur Umsetzung von Protochlorophyllid *a* zu Chlorophyllid *a* genutzt, während zunächst PORA als Protochlorophyllid-reduzierendes Enzym inaktiv bleibt. Erst nach dem Zerfall der Struktur des Prolamellarkörpers wird PORA aktiv und setzt Protochlorophyllid *b* zu Chlorophyllid *b* um. Mit diesem Modell der Existenz eines Lichtsammelkomplexes in etiolierten Pflanzen lässt sich somit die Frage nach der Interaktion der beiden verschiedenen POR-Proteine erklären und es zeigt deren zwei verschiedenen Funktionen im Prolamellarkörper etiolierter Keimlinge. Des weiteren lässt sich die Existenz von sowohl photoaktivem als auch photoinaktivem Protochlorophyllid erklären, indem Protochlorophyllid *a* die Rolle des photoaktiven Moleküls, Protochlorophyllid *b* die der photoinaktiven Spezies einnimmt.

Um die Existenz dieses Lichtsammelkomplexes im Prolamellarkörper der Etioplasten zu beweisen und seine Struktur, Funktion und Biosynthese genauer zu charakterisieren, wurden im Rahmen dieser von der Deutschen Forschungsgemeinschaft unterstützten Arbeit folgende Aufgabenstellungen verfolgt:

1. Rekonstitution/Assemblierung von LHPP mit den authentischen (nativen), Mg-haltigen Pigmenten
2. Isolation und Nachweis des LHPP Komplexes aus Prolamellarkörpern
3. Herausstellung der Bedeutung von LHPP für den Wechsel eines Keimlings von Skotomorphogenese zu Photomorphogenese

4. Identifizierung essentieller Domänen in beiden POR-Proteinen aus Gerste für die Bindung der Pigmente als auch für die Aggregation der POR:Protochlorophyllid:NADPH Ternärkomplexe untereinander.

In vitro-Rekonstitution und *in vivo*-Nachweis von LHPP

Das Modell eines Lichtsammelkomplexes in etiolierten Pflanzen war zunächst starker Kritik ausgesetzt, da die *in vitro* Rekonstitution mit den Zn-enthaltenden Protochlorophyllid-Analoga (Zn-Protophäophorbid *a* bzw. *b*) durchgeführt worden war und LHPP *in vivo* nur indirekt (durch Übereinstimmung der spektralen Eigenschaften), nicht jedoch direkt als hochmolekularer Protein-Protochlorophyllid-Komplex identifiziert wurde (Armstrong et al., 2000).

Ziel der Untersuchungen, die zu Beginn dieser Doktorarbeit im Mittelpunkt des Interesses standen, waren daher der *in vivo*-Nachweis von LHPP und die *in vitro*-Rekonstitution des Komplexes mit den natürlichen Mg-haltigen Protochlorophylliden *a* und *b* (**Reinbothe et al., 2003b, siehe Manuskript 1**). Dazu wurden zunächst über chemische Synthese Protochlorophyllid *a* und *b* hergestellt und zusammen mit PORA bzw. PORB (aus einer gekoppelten *in vitro* Transkription/Translation) und NADPH inkubiert. Die entstandenen ternären Enzym-Substrat-Komplexe wurden auf ihre Fähigkeit zur Bindung von Protochlorophyllid *a* und *b* getestet. Dabei stellte sich eine etwa 10fach höhere Affinität von PORA zu Protochlorophyllid *b* im Vergleich zu Protochlorophyllid *a* heraus. Genau das umgekehrte Ergebnis erhielt man für PORB. Nach Umrechnung auf die Stoffmenge gebundenen Protochlorophyllids und bezogen auf die eingesetzte Enzymmenge ergab sich ein Verhältnis Protochlorophyllid:Enzym von 2:1. Das heißt, jedes Enzymmolekül hat zwei Moleküle Protochlorophyllid gebunden.

Mit den über Gelfiltration gereinigten ternären POR:Protochlorophyllid:NADPH-Komplexen wurde nun ein höhermolekularer Komplex gebildet, welcher ein Molekulargewicht von ca. 480 kDa aufwies. Der gereinigte Suprakomplex wurde nach SDS-Polyacrylamid-Gelelektrophorese (SDS-PAGE) und radioaktiver Intensitätsmessung der einzelnen Komponenten auf seine Zusammensetzung hin untersucht. Es ergab sich eine Stöchiometrie von 5 PORA:Protochlorophyllid *b*:NADPH-Komplexen zu einem PORB:Protochlorophyllid *a*:NADPH-Komplex.

Der gebildete gereinigte Suprakomplex wurde mit einer aus Prolamellarkörpern von Gerstenkeimlingen extrahierten Lipidmischung angereichert. Anschließend wurden fluoreszenzspektroskopische Messungen dieses Komplexes bei 77 K durchgeführt. Dabei konnten sowohl photoinaktives als auch photoaktives Protochlorophyllid nachgewiesen werden, letzteres konnte durch einen Lichtblitz von 1 ms zu Chlorophyllid umgesetzt werden. Analysen der extrahierten Pigmente des Komplexes mittels HPLC vor und nach der Belichtung ergaben, dass durch die Belichtung nur Chlorophyllid *a* gebildet wurde, während Protochlorophyllid *b* nicht zu Chlorophyllid *b* umgesetzt wurde. Das gleiche Ergebnis erhielt man aus Untersuchungen extrahierter Pigmente isolierter Prolamellarkörper vor und nach kurzer Belichtung von 1 ms. Weiterhin konnte in den Prolamellarkörpern auch 7-Hydroxy-Protochlorophyllid *a* nachgewiesen werden und zusätzlich nach der Belichtung auch höhere Mengen an Protochlorophyllid *a*, was auf die Tätigkeit des in Etioplasten vorkommenden Enzyms 7-Formyl-Reduktase bei der Umwandlung von Protochlorophyllid *b* zu Protochlorophyllid *a* über 7-Hydroxy-Protochlorophyllid *a* zurückzuführen sein kann (Scheumann et al., 1999; Reinbothe et al., 2003a).

Dieser *in vitro* gebildete Suprakomplex aus ternären POR:Protochlorophyllid:NADPH-Komplexen stimmte in seinen spektroskopischen Eigenschaften mit dem bereits früher postulierten nativen Komplex überein: Wurden isolierte Etioplasten einer nicht-wäßrigen Protein- und Pigmentextraktion und -fraktionierung mit Tetrachlorkohlenstoff/Heptan unterzogen, so zeigte der auf diese Weise aus den Etioplasten erhaltene Suprakomplex einen etwa 4-fachen Überschuss von PORA gegenüber PORB als auch einen 4,5-fachen Überschuss an extrahiertem Protochlorophyllid *b* gegenüber Protochlorophyllid *a*.

Nach Trennung durch nicht-denaturierende PAGE konnte der POR:Protochlorophyllid:NADPH-Suprakomplex aufgrund der Autofluoreszenz von Protochlorophyllid direkt bzw. nach Immunoblotting mit einem PORA-spezifischen Antikörper indirekt nachgewiesen werden. Dabei zeigte der *in vivo* isolierte Komplex in etwa die gleiche Größe wie der *in vitro* gebildete Komplex. Weiterhin zerfällt dieser Suprakomplex nach Belichtung in seine ternären POR:Protochlorophyllid:NADPH-Komponenten.

Über HPLC-Analyse der extrahierten Pigmente aus dem elektrophoretisch gereinigten nativen Komplex konnten vor Belichtung die Pigmente Protochlorophyllid *a*, *b* als auch 7-Hydroxy-Protochlorophyllid *a* nachgewiesen werden. Nach Belichtung

erschien ein neues, auf Chlorophyllid *a* zurückzuführendes Signal, welches zusammen mit PORB lief, während Protochlorophyllid *b* quantitativ unverändert blieb und zusammen mit PORA lief.

Struktur- und Reaktionsmodell von POR

Nachdem die Rekonstitution von LHPP mit den nativen Mg-haltigen Protochlorophylliden *a* und *b* erfolgreich war und die Existenz eines solchen Komplexes *in vivo* ebenso nachgewiesen werden konnte, wurde mit der weiteren Charakterisierung von PORA und PORB bezüglich deren Eigenschaften in diesem Komplex fortgefahren. Im Hinblick auf die Identifizierung katalytisch wichtiger Regionen von POR und die Beschreibung des Reaktionsmechanismus wurden in den letzten Jahren sehr große Fortschritte gemacht. Bereits vor annähernd 30 Jahren wurde von Griffiths die Beobachtung gemacht, dass das Enzym im Dunkeln einen ternären Komplex mit Protochlorophyllid und NADPH eingeht (Griffiths, 1978). Aufgrund der Präsenz von Protochlorophyllid ist dieser Komplex befähigt, Licht zu absorbieren, wobei diese Energie dazu verwendet wird, um Wasserstoff von NADPH auf das Protochlorophyllidmolekül zu übertragen (Lebedev und Timko, 1998; Schoefs und Franck, 2003; Masuda und Takamiya, 2004). Untersuchungen mit *4R* und *4S* ^{3}H-markierten NADPH-Isomeren ergaben einen Transfer des pro-S-Wasserstoffatoms des Nicotinamidrings auf C17 des Protochlorophyllids (Begley und Young, 1989). Die negative Ladung des Übergangszustandes führt schließlich zur Addition eines Protons an das C18. Dieses Proton stammt von einem konservierten Tyrosinrest im aktiven Zentrum des Enzyms, wobei ein benachbarter Lysinrest die Übertragung des Protons der Hydroxylgruppe des Tyrosins erleichtert, indem es dessen pK_a-Wert senkt (Wilks und Timko, 1995; Lebedev et al., 2001; Heyes und Hunter, 2002). In spektroskopischen Untersuchungen am Enzym-Substrat Komplex unter Verwendung von heterolog exprimiertem POR-Protein konnten verschiedene Schritte der Reaktion ohne den Einfluss von Protochlorophyllid-Aggregation oder Protein-Lipid-Interaktion analysiert werden (Lebedev und Timko, 1998; Schoefs und Franck, 2003; Masuda und Takamiya, 2004). Dabei stellte sich heraus, dass sich der katalytische Mechanismus der Reaktion aus einem initialen Licht-gesteuerten Schritt (Heyes et al., 2002) und

mehreren darauffolgenden sogenannten Dunkelreaktionen zusammensetzt (Heyes et al., 2003; Heyes und Hunter, 2004).

Durch Vergleich der Aminosäuresequenzen von PORs mit denen aus Datenbanken verfügbaren anderer Proteine ergab sich eine Klassifizierung von POR in die sogenannte `RED´ Familie, die sich aus Reduktasen, Epimerasen und Dehydrogenasen zusammensetzt (Baker, 1994; Wilks und Timko, 1995; Heyes und Hunter, 2005). Für viele Enzyme dieser Familie wurde deren Struktur mit Hilfe der Röntgenstrukturanalyse gelöst und die daraus gewonnenen Erkenntnisse dazu verwendet, eine 3D-Struktur von POR aus *Synechocystis* zu entwerfen (Townley et al., 2001). In diesem Modell sind zentral 7 β-Faltblätter parallel angeordnet und umgeben von 9 α-Helices.

Um ein 3D-Strukturmodell von PORA und PORB aus Gerste zu entwerfen, bezog man sich auf Röntgenstrukturdaten der 7-Hydroxysteroid-Dehydrogenase aus *E. coli*. Mit Hilfe des Programms Swiss PdBViewer version 3.7b2 konnte durch Homologievergleich ein Modell aufgestellt werden, welches Ähnlichkeit mit den bereits aus *Synechocystis* und Erbse aufgestellten Modellen aufweist und auf ein globuläres Protein mit 6 α-Helices und 7 β-Faltblättern hindeutet (**Buhr et al., eingereicht zur Publikation, siehe Manuskript 3**).

Neben dem bereits oben beschriebenen konservierten Tyrosin- und Lysinrest im aktiven Zentrum des Enzyms haben alle Mitglieder der `RED´-Familie eine konservierte Bindungsstelle für den Cofaktor NADPH. Dieses glycinreiche GXXXGXG-Motiv findet sich in der aminoterminalen Region des Enzyms im sogenannten `Rossmann-fold´ (Townley et al., 2001). Dagegen unterscheiden sich alle POR-Proteine von den meisten anderen Enzymen der `RED´-Familie durch eine 33 Aminosäuren umfassende Insertion zwischen der 5. und der 6. α-Helix, welche als `Extra loop´ Eingang in die Literatur fand (Wilks und Timko, 1995, Birve et al., 1996). Aufgrund seiner Zusammensetzung aus vorwiegend hydrophoben Aminosäureresten wurde angenommen, dass diese Struktur für eine Verankerung des Enzyms in der Lipidmembran verantwortlich sein könnte (Birve et al., 1996). Aus weiterführenden Untersuchungen ging hervor, dass dieser `Extra loop´ eine bedeutende Rolle bei der Wechselwirkung zwischen PORA und PORB zum Aufbau hochmolekularer LHPP-Komplexe einnimmt (Reinbothe et al., 2003c).

Weiterhin besitzen alle POR-Proteine von der Blaualge *Synechocystis* über Moose bis hin zu höheren Pflanzen über die Evolution hin hochkonservierte Cysteinreste

(Reinbothe und Reinbothe, 1996). Dabei wurde gemutmaßt, dass diese (oder manche von ihnen) eine wichtige Aufgabe in der Katalyse oder der Bindung von Substrat und/oder Cosubstrat übernehmen (Oliver und Griffiths, 1981), da durch eine Alkylierung der Thiolgruppen der Cysteine durch das Reagenz N-Phenylmaleinimid die enzymatische Reaktion inhibiert werden konnte (Oliver und Griffiths, 1981). Auch aus dem aufgestellten 3D-Strukturmodell (siehe oben) lässt sich auf eine eventuelle Aufgabe der Cysteinreste schließen.

Die mögliche Beteiligung der hochkonservierten Cysteinreste an der Substratbindung (und katalytischen Funktion) sollte im Rahmen dieser Doktorarbeit detailliert am Beispiel von PORA und PORB aus Gerste untersucht werden. Während sich Cys39 und Cys202 aus PORA und Cys104 und Cys276 aus PORB nahe am aktiven Zentrum befinden und so an einer Protochlorophyllidbindung beteiligt sein können, liegt Cys229 von PORA bzw. Cys303 von PORB im β-Faltblatt-reichen Ende auf der Außenseite des globulären Proteins, d.h. entfernt vom aktiven Zentrum. Für Cys91 von PORA und Cys166 von PORB fehlt ein Counterpart in POR aus Erbse, dementsprechend scheint eine Beteiligung dieser Cysteinreste am katalytischen Geschehen und anderen Wechselwirkungen unwahrscheinlich. Um die Funktion der Cysteinreste testen zu können, wurden auf cDNA-Basis die Nucleotidfolgen durch `site-directed mutagenesis´ so verändert, dass die codierten POR-Proteine anstelle eines Cysteinrestes einen Alaninrest aufwiesen. Die aus diesen Veränderungen hervorgehenden Proteine mit jeweils einem Cys→Ala Austausch wurden dann auf Kriterien wie Postimport-Stabilität, Fähigkeit zur Bindung von Protochlorophyllid und NADPH, enzymatische Aktivität und Aggregierung zu LHPP-Komplexen geprüft (**Reinbothe et al., 2006; Buhr et al., 2006, eingereicht zur Publikation, siehe Manuskript 2 und 3**).

Die Bedeutung von Cysteinresten in PORB

Bei Betrachtung von PORB ergaben sich unter Einbeziehung der oben genannten Untersuchungskriterien folgende Beobachtungen: Der Austausch der Cysteinreste Cys104 und Cys166 äußerte sich in keinem veränderten Importverhalten bei Verwendung isolierter Chloroplasten aus Gerste verglichen mit dem nativen PORB. Beide mutierten Proteine wurden sowohl in mit Protochlorophyllid supplementierte

als auch in Protochlorophyllid-freie Chloroplasten aufgenommen. Erfolgte der Import der Enzyme im Dunkeln, waren diese resistent gegen eine aus dem Stroma isolierte Proteasemischung, was auf einen intakten ternären Enzym-Substrat Komplex zurückzuführen ist. Bei Import unter Lichtbedingungen konnte dagegen kein reifes Protein detektiert werden, obwohl die Konzentration der Proteinvorstufe im Inkubationsmedium abnahm. Dies ist dadurch zu erklären, dass das Protein aufgenommen wurde und in einem aktiven Ternärkomplex sein Substrat zu Chlorophyllid umgesetzt hat, wobei der entstandene Enzym-Produkt-Komplex anfällig gegen eine stromale POR-Protease ist und dementsprechend das Enzym proteolytisch abgebaut wurde. Weiterhin wurde festgestellt, dass die Cys104→Ala- und Cys166→Ala Mutanten pro Enzymmolekül 2 Protochlorophyllid-Moleküle binden. Durch Inkubation der *in vitro* rekonstituierten Ternärkomplexe mit Saccharose und anschließender Gelfiltration ließen sich diese beiden Bindungsstellen für Protochlorophyllid in eine schwach-bindende und eine stark-bindende unterscheiden (Saccharose stabilisiert Interaktionen zwischen Proteinen und Membranen) (Crowe und Crowe, 1984; Crowe et al., 1984). Der Austausch von Cys104 und Cys166 zeigte auch keinen Einfluss auf die Funktionalität des Enzym-Substrat Ternärkomplexes. Untersuchung der nach Belichtung der *in vitro* vorliegenden Ternärkomplexe gebildeten extrahierten Pigmente mittels Fluoreszenzspektroskopie ergab eine Umsetzung von Protochlorophyllid zu Chlorophyllid. Schließlich wurde für diese Mutantenproteine noch deren Fähigkeit zur Bildung hochmolekularer LHPP-Komplexe *in vitro* geprüft. Dabei stellte sich heraus, dass der Austausch von Cys104 und Cys166 die Zusammenlagerung zu LHPP nicht beeinträchtigt. Es konnte ein hochmolekularer Komplex isoliert werden, welcher nach nicht-denaturierender PAGE über Autoradiographie, Western Blotting mit einem PORA-Antikörper sowie Autofluoreszenz von Protochlorophyllid als LHPP identifiziert werden konnte. Ähnliche Ergebnisse wurden für einen isolierten Komplex nach *in organello* Import von pPORB (dem Vorstufenprotein von PORB), Cys104→Ala(pPORB) und Cys166→Ala(pPORB) in Chloroplasten einer PORB$^-$-Mutante erhalten. Diese Mutante ist homozygot und hat eine T-DNA Insertion im *porB*-Gen. Dies hat zur Folge, dass kein PORB-Protein mehr gebildet wird. Aus den Ergebnissen dieser Untersuchungen ließ sich die Schlussfolgerung ziehen, dass sowohl Cys104 als auch Cys166 nicht in die Bindung von Protochlorophyllid involviert sind und auch keine Auswirkung auf Funktionalität von LHPP und katalytischer Reaktion haben.

Dagegen erhielt man ein anderes Bild bei der Untersuchung der Cys276→Ala- und der Cys303→Ala Mutanten:

Im Falle der Cys276→Ala-pPORB Mutante wurde zwar auch dieses Protein in Chloroplasten mit oder ohne Protochlorophyllid importiert, jedoch erfolgte dieser Import nicht so effizient wie im Falle von nativem PORB oder der Cys104→Ala- oder Cys166→Ala Mutanten. Ein weiterer Unterschied zeigte sich im Import in Chloroplasten unter Lichtbedingungen. Während bei pPORB und den Cys104→Ala- oder Cys166→Ala Mutanten dabei kein reifes POR-Protein aufgrund des Abbaus des Enzym-Produkt Komplexes nach Umwandlung von Protochlorophyllid *a* zu Chlorophyllid *a* detektierbar war, konnte im Falle der Cys276→Ala Mutante eine geringe Menge an Enzym nachgewiesen werden. Diese Beobachtung deutete auf eine nicht stattgefundene Reaktion hin, der Enzym-Substrat Komplex blieb bestehen und müsste also vor Abbau durch POR-Proteasen geschützt sein. Stabilitätstests mit den *in vitro* gebildeten Ternärkomplexen ergaben allerdings eine Instabilität selbst der Enzym-Protochlorophyllid-Komplexe gegenüber stromalen POR-Proteasen: Sowohl unter Dunkelheit als auch nach Belichtung wurde der Enzym-Substrat Komplex langsam abgebaut. Eine Bestätigung der Hypothese, dass die katalytische Umsetzung von Protochlorophyllid *a* nicht ausgeführt wurde, lieferte ebenfalls der Funktionalitätstest der *in vitro* gebildeten ternären Komplexe. Fluoreszenzspektroskopie extrahierter Pigmente der ternären Komplexe nach Belichtung ergab kein Chlorophyllid *a*. Dennoch war durch Fluoreszenzspektroskopie Protochlorophyllid nachweisbar, aber nur bei Extraktion der Pigmente von im Dunkeln rekonstituierten und gehaltenen Ternärkomplexen. Im Vergleich zu nativem PORB oder den Cys104→Ala- und Cys166→Ala-Mutanten war bei Präsenz von Saccharose im Inkubationsmedium jedoch nur ein Molekül Protochlorophyllid *a* pro Enzymmolekül vorhanden, bei Abwesenheit von Saccharose konnte kein gebundenes Protochlorophyllid *a* detektiert werden. Die Cys276→Ala Mutante besaß also nur schwach gebundenes Protochlorophyllid *a*, während die starke Bindungsstelle offensichtlich durch die Mutation zerstört wurde. Aufgrund der ausgebliebenen katalytischen Reaktion kann gefolgert werden, dass diese starke Bindungsstelle, aufgebaut durch Cys276, im aktiven Zentrum des Enzyms platziert ist. Das schwach gebundene Protochlorophyllid *a* wird nicht zu Chlorophyllid umgesetzt, seine Bedeutung liegt dementsprechend in einer anderen Aufgabe.

Die Fähigkeit zur Bildung von LHPP-Komplexen blieb trotz der Cys276→Ala Mutation bestehen. Bei Einsatz äquimolarer Mengen an Ternärkomplexen wie bei Wildtyp-PORB oder den Cys104→Ala- und Cys166→Ala PORB-Mutanten nahm allerdings die Menge an aufgebautem LHPP ab. Dies könnte auf eine eventuelle geringfügige Konformationsänderung des mutierten Proteins hindeuten, ausgelöst durch den Aminosäureaustausch im aktiven Zentrum oder verursacht durch das fehlende Protochlorophyllidmolekül, was eine eventuell verminderte Affinität des Ternärkomplexes zu anderen Ternärkomplexen zum Aufbau von LHPP zur Folge hat. Das gleiche Bild einer geringeren Menge an gebildetem LHPP wie bei den *in vitro* Rekonstitutionsversuchen wurde auch bei den *in organello* Importansätzen gezeichnet.

Bei Betrachtung des Importverhaltens der Cys303→Ala Mutante in isolierte Chloroplasten ergab sich eine etwa gleich hohe Aufnahmerate wie bei nativem pPORB oder den Cys104→Ala- und Cys166→Ala pPORB-Mutanten. Erfolgte der Import in Chloroplasten im Dunkeln, war danach allerdings eine geringere Menge an prozessiertem PORB im Vergleich zu PORB oder den Cys104→Ala- und Cys166→Ala Mutanten zu finden, was auf einen Abbau selbst des Enzym-Substrat Komplexes hindeutet. Diese Annahme wurde durch Stabilitätstests verstärkt. *In vitro* gebildete Enzym-Substrat Ternärkomplexe wurden selbst bei Dunkelinkubation von stromalen Proteasen proteolytisch angegriffen.

Bei Import des mutierten Proteins unter Belichtung glich die Beobachtung der von pPORB oder den Cys104→Ala- und Cys166→Ala Mutanten, es konnte kein prozessiertes Protein detektiert werden. Dies lässt wiederum annehmen, dass eine Umsetzung zu Chlorophyllid stattgefunden hat und der Enzym-Produkt Komplex abgebaut wurde. Tatsächlich konnte auch in fluoreszenzspektroskopischen Messungen extrahierter Pigmente belichteter Ternärkomplexe die Bildung von Chlorophyllid *a* verfolgt werden. Bei der Fluoreszenzspektroskopie extrahierter Pigmente unbelichteter Ternärkomplexe konnte wie bei der Cys276 Mutante eine Stöchiometrie von gebundenem Protochlorophyllid zu Enzym von 1:1 sowohl in Anwesenheit als auch in Abwesenheit von Saccharose im Inkubationsmedium festgestellt werden. Das bedeutet, dieses Protochlorophyllid ist fest an das Protein gebunden und befindet sich aufgrund der Umsetzung zu Chlorophyllid im aktiven Zentrum des Enzyms. Dagegen ist mit dem Austausch von Cys303 gegen Alanin die schwache Bindungsstelle für Protochlorophyllid zerstört worden. Wurden nun die

entsprechenden Ternärkomplexe zum Aufbau von LHPP miteinander inkubiert, so konnte weder über Autofluoreszenz von Protochlorophyllid noch durch Western Blotting oder Autoradiographie eine Bildung von LHPP verfolgt werden. Auch der *in organello*-Assay des Imports von (Cys303→Ala)pPORB in PORB$^-$-Chloroplasten erbrachte keinen Nachweis von LHPP nach nicht-denaturierender PAGE. Aus diesen Beobachtungen lässt sich eine bedeutende Rolle der durch Cys303 von PORB gebildeten schwachen Protochlorophyllid-Bindungsstelle für die Assemblierung der Ternärkomplexe zu LHPP ableiten.

Auch eine Cys(276+303)→Ala PORB-Doppelmutante wurde untersucht. Aufgrund des Austausches sowohl von Cys276 als auch von Cys303 gegen Alanin war bei diesem Protein keine Bindungskapazität für Protochlorophyllid feststellbar. Das veränderte Protein wurde zwar in Etioplasten importiert, es waren jedoch trotz gleicher Aufnahmeraten wie bei nativem PORB geringere Mengen an prozessiertem PORB-Mutantenprotein nachweisbar. Da in Etioplasten noch keine stromale POR-Protease vorhanden ist (Reinbothe et al., 1995d), wird die geringere Menge an importiertem Mutanten-PORB auf einen unspezifischen Abbau des Proteins zurückgeführt, verursacht durch eine geringere Stabilität/veränderte Proteinstruktur aufgrund der Doppelmutation. In Bezug auf die Fähigkeit zum Aufbau von LHPP konnte bei der Doppelmutante wie auch schon bei der Cys303→Ala Mutante kein gebildeter LHPP Komplex nachgewiesen werden.

Die Bedeutung der Cysteinreste in PORA

Zur Untersuchung der Rolle von Cysteinresten in PORA wurde heterolog in *E. coli* exprimierte mutierte PORA-Proteine, die einzelne gegen Alanin ausgetauschten Cysteinreste aufwiesen, *in vitro* auf ihre Protochlorophyllid *b*-Bindungskapazität und Fähigkeit zur Synthese von Chlorophyllid *b* getestet. Weiterhin wurde die Stabilität der *in vitro* gebildeten Ternärkomplexe gegenüber POR-Proteasen sowie deren Potential zur Bildung hochmolekularer Aggregate (LHPP) über Western Blotting und Autofluoreszenz von Protochlorophyllid und dessen Funktionalität über Fluoreszenzspektroskopie geprüft.

Wie bereits für PORB gezeigt, konnten auch für PORA zwei Protochlorophyllid-Bindungsstellen pro Enzymmolekül identifiziert werden, eine schwach- und ein

starkbindende, welche sich mittels Gelfiltration der ternären Komplexe in einer Saccharose-haltigen oder -freien Inkubationslösung unterscheiden lassen. Diese zwei Protochlorophyllid-Bindungsstellen waren bei den Cys39→Ala- und Cys91→Ala Mutanten intakt, analog zum nativen PORA wurden zwei Protochlorophyllidmoleküle pro Enzym in den gebildeten Ternärkomplexen nachgewiesen, von denen ein Molekül in Abwesenheit von Saccharose im Medium abgelöst wurde. Ebenso konnten wie auch bei nativem PORA aus den aus Cys39→Ala- und Cys91→Ala PORA-Mutanten hervorgehenden Ternärkomplexen durch Inkubation mit PORB-Pigment-NADPH Komplexen hochmolekulare Aggregate gewonnen werden. Diese konnten nach nicht-denaturierender PAGE durch Autofluoreszenz der Pigmente und Western Blotting mit POR-spezifischen Antikörpern als LHPP identifiziert werden. Fluoreszenzemissionsspektroskopie der mit Galakto- und Sulpholipiden angereicherten Komplexe ließ zwei vorliegende Protochlorophyllidspezies, photoaktives Protochlorophyllid 650/657 und photoinaktives Protochlorophyllid 628/632, erkennen. Nach Belichtung verschwand das photoaktive Protochlorophyllid und eine neue Pigmentbande mit einer emittierten Wellenlänge von 690 nm, Chlorophyllid entsprechend, erschien. Dagegen blieb die Menge an photoinaktivem Protochlorophyllid unverändert. Wurden die belichteten Ansätze schließlich durch nicht-denaturierende PAGE aufgetrennt, ließ sich ein Zerfall der LHPP-Komplexe in seine Untereinheiten, die PORA-Pigment- und PORB-Pigment-Komplexe, feststellen. Pigmentanalysen ergaben an PORA gebundenes Protochlorophyllid *b*, während bei PORB Chlorophyllid *a* gefunden wurde. Diese Ergebnisse sprechen für die Bildung eines funktionsfähigen LHPP Komplexes durch die Cys39→Ala- und Cys91→Ala Mutantenproteine. Absorbierte Lichtenergie wird von PORA gebundenen Protochlorophyllid *b*-Molekülen auf PORB gebundenes Protochlorophyllid *a* zu dessen Umsetzung weitergeleitet, während PORA als reduzierendes Enzym zunächst inaktiv bleibt. Dieses PORA-Molekül ist, wenn mit Protochlorophyllid *b* komplexiert, geschützt gegen proteolytischen Abbau durch eine POR-Protease. Erst nach Umsetzung zum Enzym-Produkt Komplex (Enzym-Chlorophyllid *b* Komplex) nach weiterer Belichtung erfolgt dessen Abbau (Reinbothe et al, 1995d).

Der Austausch von Cys202 gegen Alanin resultierte im Verlust einer Protochlorophyllid-Bindungsstelle. Nur noch ein Pigmentmolekül, welches sich durch Gelfiltration in saccharosefreiem Medium ablösen ließ, wurde gebunden. Das bedeutet, durch die Mutation von Cys202 wurde die Bindungsstelle mit der starken

Wechselwirkung des Pigments mit dem Enzym zerstört. Die Beobachtung, dass das gebundene Protochlorophyllidmolekül im Ternärkomplex nicht durch Belichtung zu Chlorophyllid reduziert werden kann, lässt folgern, dass Cys202 die Pigmentbindungsstelle im aktiven Zentrum des Enzyms einnimmt. Trotz des Wegfalls dieser Protochlorophyllidbindungsstelle konnte durch Inkubation des Enzym-Substrat Komplexes des veränderten PORA-Proteins mit dem von PORB ein höhermolekularer Komplex isoliert werden. Dieser besaß die gleiche Größe wie der natives PORA-Protein enthaltende Komplex und wurde nach nicht-denaturierender PAGE durch Western Blotting als auch durch Autofluoreszenz und Autoradiographie nachgewiesen. Anders jedoch als der mit nativem PORA oder Cys39→Ala-PORA und Cys91→Ala-PORA aufgebaute LHPP-Komplex enthielt dieser Komplex kein typisches photoaktives oder photoinaktives Protochlorophyllid. Im Fluoreszenzemissionsspektrum war nur ein breites Signal, bestehend aus vier einzelnen Emissionssignalen zu erkennen. Auch nach Belichtung ergab sich keine Änderung des Emissionsspektrums, es war nur eine geringe Menge an gebildetem Chlorophyllid *a* nachweisbar, was darauf hinweist, dass LHPP nicht funktionstüchtig war. Als weiterer Unterschied zu LHPP mit nativem PORA oder Cys39→Ala-PORA und Cys91→Ala-PORA dissoziierte der Komplex nach Belichtung nicht. Eine Analyse der gebundenen Pigmente der PORA-Pigment- und PORB-Pigment- Subkomplexe ergab für PORA Protochlorophyllid *b* und für PORB Protochlorophyllid *a*, ein weiterer Hinweis auf nicht stattgefundenen Energietransfer von PORA-Protochlorophyllid *b* auf PORB-Protochlorophyllid *a*. Die Mutation von Cys202 zeigte schließlich auch Auswirkungen auf die Stabilität des Enzym-Substrat-Ternärkomplexes. Selbst mit gebundenem Protochlorophyllid ergab sich ein langsamer proteolytischer Abbau des Komplexes durch POR-Proteasen, obwohl dieser eigentlich davor geschützt sein sollte.

Ebenso wie bei der Cys202→Ala Mutante bindet das (Cys229→Ala)PORA-Protein nur ein Protochlorophyllid *b* pro Enzymmolekül und zeigt bereits als Enzym-Substrat Komplex eine beginnende Proteolyse durch POR-Proteasen. Allerdings besteht der Unterschied, dass dieses Protochlorophyllidmolekül in starker Wechselwirkung mit dem Protein interagiert: Selbst bei Fehlen von Saccharose im Medium wurde es durch Gelfiltration nicht abgespalten. Der Austausch von Cys229 gegen einen Alaninrest bewirkte also das Verschwinden der Protochlorophyllid schwach bindenden Bindungsstelle des Enzyms. Dennoch war das Enzym noch aktiv. Bei

Belichtung von rekonstituierten ternären Enzym-Substrat Komplexen wurde laut Fluoreszenzspektroskopiedaten sämtliches gebundene Protochlorophyllid in Chlorophyllid umgewandelt. Im Versuch jedoch, mit den ternären Enzym-Substrat Komplexen LHPP aufzubauen, konnte kein höhermolekularer Komplex isoliert werden. Nach nicht-denaturierender PAGE ergab sich weder durch Western Blotting noch durch Autoradiographie oder Pigment-Autofluoreszenz ein entsprechendes Signal. Lediglich die Ausgangsprodukte wurden nachgewiesen. Cys229 nimmt also, wahrscheinlich über das daran gebundene Protochlorophyllid, große Bedeutung ein bei der Aggregation von POR-Pigment-NADPH Ternärkomplexen zu LHPP.
Auch wenn der Cys229→Ala Mutante die Fähigkeit zur Bildung von LHPP-Komplexen fehlt, sollten die einzelnen Ternärkomplexe ihr Substrat bei Belichtung zu den jeweiligen Chlorophyllid-Spezies umsetzen können. Wenn jedoch (Cys229→Ala)PORA-Pigment-NADPH und PORB-Pigment-NADPH Komplexe im Verhältnis von 5:1 (analog dem Verhältnis in LHPP) zusammen mit Lipiden aus dem Prolamellarkörper inkubiert wurden, war kein photoaktives Protochlorophyllid nachweisbar. Es wurde nur ein breites Fluoreszenzsignal bei 632 nm gemessen, welches sich auch nach Belichtung nicht änderte. Dementsprechend wurde weder Chlorophyllid *a* noch Chlorophyllid *b* gebildet, was auch durch PAGE und anschließende HPLC-Analyse der extrahierten Pigmente bestätigt wurde. Getrennte Enzym-Substrat Ternärkomplexe sind in der Lipidmembran demnach inaktiv, was die Bedeutung eines aktiven LHPP Komplexes bei der Ergrünung von Pflanzen nur noch unterstreicht.

Die Biosynthese des LHPP Komplexes: Eine essentielle Voraussetzung für die Ergrünung etiolierter Keimlinge

Der Prozess der Ergrünung oder der De-etiolierung stellt einen kritischen Punkt im Lebenszyklus vieler Pflanzen dar. Beim hypogäischen Typ der Keimung wächst der Keimling zu Beginn seiner Entwicklung in unterirdischer Dunkelheit in Richtung Erdoberfläche. Dabei zeigt er die Merkmale eines als Skotomorphogenese bezeichneten Entwicklungsprogramms: Auf einem langgestreckten Hypokotyl mit einem apikalen Haken sitzen bei dikotylen Pflanzen aneinanderliegende, kleine und gelb-bleiche Keimblätter, deren Zellen Etioplasten enthalten (siehe Abb. 2). Sobald

der Keimling jedoch die Erdoberfläche durchdringt, untersteht seine Weiterentwicklung einem einschneidenden Wandel. Von nun an wird seine Morphologie und Physiologie durch das Licht bestimmt. Dies beinhaltet zunächst einmal eine Verringerung des Zellstreckungswachstums der Hypokotylzellen, die Entfaltung der Keimblätter und deren Wachstum, die Weiterentwicklung von Etioplasten zu Chloroplasten und damit einhergehend die Akkumulation von Chlorophyll (siehe Abb. 2) (Kendrick und Kronenberg, 1994).

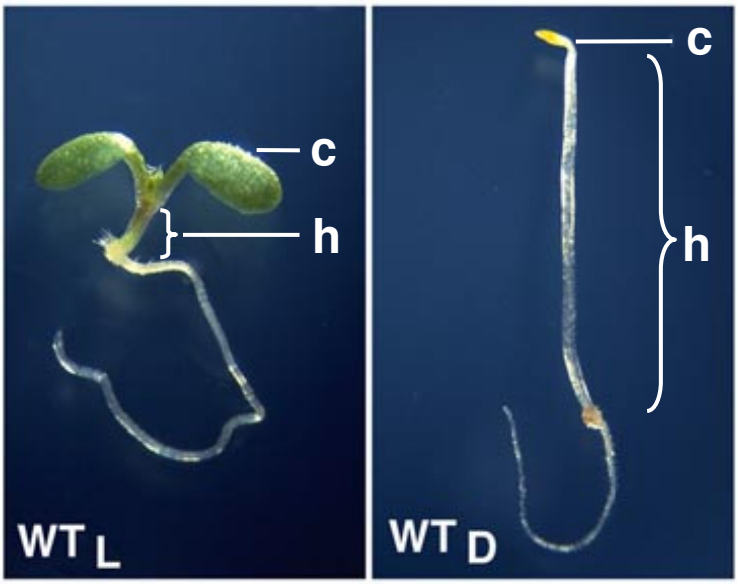

Abb. 2: Keimlinge von *Arabidopsis thaliana* gewachsen unter verschiedenen Lichtbedingungen. Im Licht gewachsene Keimlinge (WT_L) besitzen ein kürzeres Hypokotyl (h) sowie entfaltete, grüne Keimblätter (c). Etiolierte Keimlinge (Anzucht im Dunkeln, (WT_D)) zeigen ein verlängertes Hypokotyl (h) und gelbliche aneinandergelegte Keimblätter (c). Nach: http://www.yale.edu/denglab/What's_Arabidopsis.html [07.10.2006], verändert.

Dieser Abschnitt im Lebenszyklus einer Pflanze mit hypogäischer Keimung, die Umstellung auf Photomorphogenese, ist von besonderer Tragweite und unterliegt genauesten Steuerungsvorgängen. Dazu gehört unter anderem auch die Bereitstellung einer bestimmten Menge an Protochlorophyllid, der Vorstufe von Chlorophyll, um einen schnellen Aufbau des Photosyntheseapparates bei beginnender Belichtung zu ermöglichen. Dieser Protochlorophyllid-Pool muss jedoch stöchiometrisch der vorhandenen Menge an Protochlorophyllid-Reduktasen entsprechen, an die es mit NADPH in Form der Ternärkomplexe gebunden ist und welche schließlich in Form von LHPP miteinander interagieren. Dessen Existenz sichert bei beginnender Belichtung die Chloropyhll *a*-Synthese als auch die

Dissipation absorbierter überschüssiger Lichtenergie durch zunächst nicht-photoreduzierbares Protochlorophyllid *b* (Reinbothe et al. 1999).
Die Regulation der Protochlorophyllid-Synthese unterliegt einem Rückkopplungs-Regelkreis (`feedback-regulation´), der beim Erreichen einer bestimmten Protochlorophyllid-Konzentration zu einer Abschaltung der Synthese von 5-Aminolävulinsäure (siehe oben) führt. Ist dieser Regelkreis unterbrochen, wie zum Beispiel bei der *Arabidopsis flu*-Mutante, so akkumuliert freies Protochlorophyllid in den Etioplasten dunkel gewachsener Keimlinge (Meskauskiene et al., 2001). Offensichtlich korreliert in dieser Mutante die Menge an gebildetem Protochlorophyllid nicht mit den für die Bindung zur Verfügung stehenden POR-Molekülen. Bei anschließender Belichtung werden diese freien Pigmente energetisch angeregt und geben diese Energie auf Sauerstoff ab, wodurch ROS entstehen. Dies führt zu letalen photooxidativen Schäden (z.B. Ausbleichen der Keimblätter). Der Protochlorophyllid-Pool wird aber auch durch bestimmte Photorezeptoren aus der Gruppe der Cryptochrome und Phytochrome reguliert. So äußert sich der Ausfall des `Phytochrome interacting factor´ 1 in *Arabidopsis thaliana* ebenfalls in der Akkumulation von freiem Protochlorophyllid (Huq et al., 2004). Solche *pif1*-Mutanten zeigen wie *flu*-Mutanten bei Anzucht in Dunkelheit und anschließender Belichtung ebenfalls einen ausbleichenden Phänotyp.
Aber nicht nur der Ausfall von Faktoren, welche unmittelbar in den Chlorophyll-Stoffwechsel involviert sind, können zur Anreicherung von freiem Protochlorophyllid in Plastiden führen, wie in den folgenden Abschnitten dargestellt werden soll.

Der Import des PORA-Vorstufenproteins (pPORA) in Plastiden ist substratabhängig und wird durch einen spezifischen Importapparat vermittelt

Wie bereits erwähnt, werden PORA und PORB aus Gerste als höhermolekulare Vorstufenproteine (pPORA bzw. pPORB) im Cytosol synthetisiert und müssen anschließend posttranslational in die Plastiden importiert werden. Dort erfolgt dann die Prozessierung, d.h. die Abspaltung des jeweiligen Transitpeptids (einer N-terminalen Verlängerung, die für den Transport zum bzw. Import in den Plastiden verantwortlich ist) und das intraplastidäre Targeting. Interessanterweise unterscheiden sich beide Oxidoreduktasen in ihrem Importverhalten: Während der

Import von pPORB in Chloroplasten und Etioplasten mit gleicher Effizienz erfolgte, wurde pPORA nur in Etioplasten aufgenommen. Es konnte gezeigt werden, dass dieses unterschiedliche Importverhalten durch die Menge an Protochlorophyllid im jeweiligen Plastidentyp verursacht wird. Etioplasten akkumulieren spektroskopisch nachweisbare Mengen an Protochlorophyllid, Chloroplasten hingegen nicht. Allerdings war es möglich, isolierte Chloroplasten durch Fütterung von 5-Aminolävulinsäure zur Protochlorophyllidsynthese anzuregen und auf diese Weise die Importkapazität für pPORA wieder herzustellen (Reinbothe et al., 1995a).

Der Substrat-abhängige Import konnte nicht nur mit isolierten Plastiden von Gerste und anderen Pflanzenarten, also *in vitro*, sondern auch *in vivo* in *Arabidopsis thaliana* demonstriert werden, wobei er in diesem Fall auf die Kotyledonen beschränkt zu sein schien (Kim und Apel, 2004). Chloroplasten aus grünen Blättern adulter Pflanzen importierten ein pPORA-GFP-Fusionsprotein auch in Abwesenheit von Protochlorophyllid (Kim und Apel, 2004). Die Aufklärung der Diskrepanzen in der Aussage des *In-vivo-* und des *In-vitro*-Ansatzes waren und sind Gegenstand zahlreicher experimenteller Arbeiten, die hier nicht weiter beschrieben werden sollen. An dieser Stelle sei nur darauf hingewiesen, dass zum einen der physiologische Entwicklungszustand des analysierten Blattmaterials (Gerstenprimärblätter zur Isolation der Chloroplasten und Rosettenblätter von *Arabidopsis thaliana* zur GFP-Analyse) und zum anderen die verwendete Species (Gerste als monokotyle und *Arabidopsis thaliana* als dikotyle Pflanze) von Bedeutung für die beobachteten Unterschiede im Importverhalten der Plastiden sind (unveröffentlichte Daten, C. und S. Reinbothe, persönliche Mitteilung).

Durch welche Faktoren wird der Protochlorophyllid-abhängige Import von pPORA bestimmt?

1. Zum einen ist es das sogenannte Transitpeptid, das im Falle von pPORA eine Protochlorophyllid-Bindungsstelle aufweist und mit spezifischen Komponenten in der äußeren und inneren Plastidenhüllmembran interagiert, um die Bindung und anschließende Protochlorophyllid-abhängige Aufnahme von pPORA zu ermöglichen. Wurde das Transitpeptid von pPORA z.B. mit dem sogenannten `reifen´ Teil von PORB oder einem Reporterprotein (DHFR = Dihydrofolatreduktase, ein normalerweise cytosolisches Protein aus Maus) gekoppelt, so erfolgte der Import beider Proteine in isolierte Chloroplasten Protochlorophyllid-abhängig (Reinbothe et al., 1997).

2. Zum anderen sind es Multiprotein-Komplexe in der äußeren und inneren Plastidenhüllmembran, die spezifisch mit pPORA interagieren, nicht jedoch mit anderen bislang untersuchten, im Cytosol synthetisierten, plastidären Vorstufenproteinen. Die Komponenten dieser Multiprotein-Komplexe konnten partiell identifiziert und charakterisiert werden. Es handelt sich dabei um Proteine, die nicht zur TIC/TOC-Maschinerie (siehe oben), also nicht dem Standard-Importapparat der Chloroplasten gehören (Reinbothe et al., 2000). In Anlehnung an die Terminologie wurden diese schließlich als PTC-Komplex (`Protochlorophyllide-dependent Translocon Complex´) bezeichnet. In der äußeren Plastidenhüllmembran handelte es sich dabei um ein 130 kDa großes Protein (PTC130), ein 16 kDa großes Protein (PTC16) und ein 33 kDa großes Protein (PTC33)(Reinbothe et al., 2004a, 2004b). Um deren mögliche Funktion im pPORA-Import herauszufinden, muss man sich zunächst dem Import plastidärer Vorstufenproteine genauer zuwenden. Dieser setzt sich prinzipiell aus mehreren Schritten zusammen, die sich durch ihren Bedarf an GTP und ATP unterscheiden: 1. Der unspezifischen und reversiblen Interaktion des Vorstufenproteines mit (vermutlich Lipid- und Protein-)Komponenten der Plastidenhülle, 2. Die spezifische Interaktion des Transitpeptids mit einem Rezeptorprotein (Bindung), 3. Der partiellen Insertion des Vorstufenproteins in den Translokationskanal und 4. Der vollständigen Translokation durch diesen Kanal ins Stroma, wo die Abspaltung der Transitsequenz erfolgt (Keegstra und Cline, 1999). Basierend auf der zeitlichen Sequenz der Interaktion des pPORA-Vorstufenproteins mit den verschiedenen PTC-Komponenten während der verschiedenen Stadien des Importprozesses wurde vermutet, dass PTC 130 den Rezeptor darstellt, PTC33 als Modulator-GTPase für das `Einfädeln´ in den Translokationskanal verantwortlich ist und PTC16 als der eigentliche Translokationskanal fungiert (Reinbothe et al., 2004a, 2004b).

Das Fehlen von Komponenten des PORA-Importapparates verhindert die Ausbildung von LHPP und Prolamellarkörpern in Etioplasten und führt zu photooxidativen Schäden bei der Belichtung von etiolierten Keimlingen

Wenn pPORA eine spezifische Funktion bei der Assoziation von LHPP und dem Aufbau der Prolamellarkörper hat und diese strukturelle Organisation essentiell für den erfolgreichen Übergang etiolierter Keimlinge zum photomorphogenetischen Wachstum ist, dann sollte sich eine Behinderung des pPORA-Importes dramatisch auf die Etablierung des internen Membransystems der Etioplasten und den Ergrünungsvorgang auswirken. Um diese Hypothese zu testen, wurden zwei Knock-out Mutanten von *Arabidopsis thaliana* untersucht: Eine PTC33 Knock-out Linie (*ppi1*, Jarvis et al., 1998)) und eine PTC16 Knock-out Linie.

Es konnte gezeigt werden, dass *Arabidopsis thaliana ppi1*-Mutanten, denen TOC33 fehlt, pPORA tatsächlich nicht importieren (Reinbothe et al., 2005), und wie vermutet einen geringeren Gehalt an PORA sowie einen reduzierten Prolamellarkörper in ihren Etioplasten aufweisen (Jarvis et al., 1998; Reinbothe et al., 2005). Die *ppi1*-Mutante ist jedoch im Rahmen dieser Zusammenfassung eher von untergeordneter Bedeutung.

Anstelle dessen soll die Untersuchung der PTC16 Knock-out Linie im Mittelpunkt des Interesses stehen (**Pollmann et al., siehe Manuskript 4**). PTC16 ist ortholog zu OEP16 aus Erbse und *Arabidopsis thaliana* (Baldi et al., 1999; Pohlmeyer et al., 1997) und verwandt mit Aminosäure- und Präprotein-Translokatoren bekannt von Bakterien, Mitochondrien und Chloroplasten (Reinbothe et al., 2004b). Diese Mutante enthält eine T-DNA Insertion in Exon 1 des Gens, wodurch kein Transkript und damit auch kein Protein mehr gebildet wird. Etiolierte Keimlinge der Mutante wiesen einen 4,5fach höheren Gehalt an Protochlorophyllid auf. Wurden diese anschließend einer Dauerbelichtung ausgesetzt, wirkte die erhöhte Menge an Protochlorophyllid als `Photosensitizer´. Die Keimlinge nahmen photooxidativen Schaden und starben ab.

In vitro-Importversuche ergaben, dass der Import vieler zellkerncodierter plastidärer Proteine (z.B. kleine Untereinheit von RuBisCO, Chlorophyll *a*/*b* bindendes Protein von LHCII, Ferredoxin) und Aminosäuren nicht über OEP16 und damit über den PTC-Komplex vermittelt wird. Allerdings konnte in einem Importversuch mit dem Präkursor von PORA (pPORA) kein prozessiertes, also in den Plastiden aufgenommenes PORA detektiert werden. Sich anschließende chemische

*Crosslinking*experimente ergaben zwar eine Bindung von pPORA an den Plastiden, jedoch nicht dessen Aufnahme. Dies lässt den Schluss zu, dass OEP16 speziell in den Import von PORA involviert ist und den Translokationskanal bildet.
Das Fehlen von PORA in Etioplasten der OEP16 Knock-out Mutante äußerte sich neben dem erhöhten Protochlorophyllidgehalt auf chemischer Ebene auch im teilweise vollständigen Verlust der Prolamellarkörper auf elektronenmikroskopischer Ebene. Weiterhin war auch, verglichen mit *Arabidopsis thaliana* Wildtyp, kein hochmolekularer Lichtsammelkomplex etiolierter Pflanzen (LHPP) bestehend aus PORA/B:Protochlorophyllid *b*/*a*:NADPH Ternärkomplexen weder durch Fluoreszenz gebundener Pigmente noch über Western Blotting mit POR-Antikörpern nachweisbar. Demzufolge spielt die Anwesenheit von PORA eine essentielle Rolle in der Formation von LHPP und damit indirekt am Aufbau des Prolamellarkörpers bei der Deetiolierung und somit dem Überleben des Keimlings bei beginnender Belichtung.

Im Rahmen dieser Doktorarbeit konnte ein Lichtsammelkomplex etiolierter Pflanzen *in vitro* mit den natürlichen Mg-haltigen Substraten Protochlorophyllid *a* und *b* rekonstituiert und seine Existenz *in vivo* nachgewiesen werden. Dieser Suprakomplex ist essentiell für die Pflanze bei Wechsel von Skotomorphogenese zu Photomorphogenese. Ferner konnte gezeigt werden, dass zumindest zwei der evolutionär konservierten Cysteinreste in PORA und PORB von Bedeutung im Hinblick auf die Bindung von Protochlorophyllid sind: Zum einen für die Reaktion des Enzyms, also die Synthese von Chlorophyllid, zum anderen für die Assemblierung der ternären POR:Protochlorophyllid:NADPH-Komplexe zu LHPP.

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Manuscript 1

In Vitro Reconstitution of Light-harvesting POR-Protochlorophyllide Complex with Protochlorophyllides *a* and *b*

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In vitro-reconstitution of LHPP with protochlorophyllides *a* and *b*

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SUMMARY

NADPH:protochlorophyllide oxidoreductase (POR, EC 1.1.33.1) is a key enzyme for the light-induced greening of angiosperms. In barley, two POR proteins exist, termed PORA and PORB. These have previously been proposed to form higher molecular weight light-harvesting complexes in the prolamellar body of etioplasts (C. Reinbothe, N. Lebedev & S. Reinbothe (1999) *Nature* 397: 80-84). Here we report the *in vitro*-reconstitution of such complexes from chemically synthesized protochlorophyllides (Pchlides) *a* and *b* and galacto- and sulpholipids. Low temperature (77K) fluorescence measurements revealed that the reconstituted, lipid-containing complex displayed the same characteristics of photoactive Pchlide 650/657 as the presumed native complex in the prolamellar body. Moreover, Pchlide F650/657 was converted to chlorophyllide (Chlide) 684/690 upon illumination of the reconstituted complex with a 1-msec flash of white light. Identification and quantification of acetone-extractable pigments revealed that only the PORB-bound Pchlide *a* had been photoactive and was converted to Chlide *a*, whereas Pchlide *b* bound to the PORA remained photoinactive. Non-denaturing PAGE of the reconstituted Pchlide *a*/*b*-containing complex further demonstrated a size similar to that of the presumed native complex *in vivo*, suggesting that both complexes may be identical.

Abbreviations: Chl(ide), chlorophyll(ide); HPLC, high performance liquid chromatography; LHC, light-harvesting chlorophyll *a*/*b* binding protein; LHPP, light-harvesting POR-Pchlide complex of etiolated plants; Pchlide, protochlorophyllide; POR, NADPH:protochlorophyllide oxidoreductase; PAGE, polyacrylamide gel electrophoresis; ZnPP, Zn protopheophorbide; ZnPheo, Zn pheophorbide.

INTRODUCTION

NADPH:protochlorophyllide oxidoreductase (POR) is a key enzyme for the light-induced greening of etiolated angiosperm plants. It catalyzes the only known light-dependent step of chlorophyll biosynthesis, the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide)(1-3). In barley, two POR proteins have been identified, termed PORA and PORB (4). Both are light- and NADPH-dependent enzymes, which remarkably differ in their expression patterns during plant development. PORA appears only transiently in dark-grown seedlings, while PORB is expressed in etiolated, illuminated and light-adapted plants (4). The partial overlap in expression suggests that both PORA and PORB may be needed for efficient seedling de-etiolation. We proposed that in the prolamellar body of etioplasts, the PORA and PORB may cooperate in terms of a novel "light-harvesting POR:Pchlide *a*/*b*" complex termed LHPP (5).

In vitro-reconstitution experiments with synthetic Zn analogs of Pchlide *a* and Pchlide *b*, termed Zn protopheo-phorbides (ZnPP) *a* and *b*, respectively, indeed supported such a model. PORA-ZnPP*b*-NADPH and PORB-ZnPP*a*-NADPH ternary complexes were found to form oligomers (5). We observed that light, which was absorbed by ZnPP*b*, was transferred onto ZnPP*a* (5). This, by virtue of PORB, was reduced to Zn pheophorbide *a* (5), the Zn analog of Chlide *a* (6). The existence of analogous higher molecular weight light harvesting structures *in vivo* was inferred from previously reported energy transfer reactions, taking place from so-called photoinactive Pchlide to photoactive Pchlide and from photoinactive Pchlide to Chlide, in prolamellar bodies before and after flash light-illumination (7-12).

Previous critique questioned the existence of a Pchlide *a*/*b*-containing light-harvesting complex *in vivo*, based on the following main arguments (13). First, previous work seemed to indicate a lack of Pchlide *b* in etiolated plants (14). Second, respective *in vitro*-reconstitution experiments had thus far not been presented for Pchlide *a* and Pchlide *b*, which – according to the LHPP model (5) – should be cognate substrates of the PORB and PORA, respectively. Third, neither the reconstituted nor the presumed authentic complex had been resolved under native conditions as higher molecular weight, lipid-containing structures.

In the present study, we addressed these important questions and performed *in vitro*-reconstitution experiments. We demonstrate that the PORA and PORB

display the same stringent substrate specificities for Pchlide *a* and Pchlide *b* as those reported previously for ZnPP*a* and ZnPP*b* (5). We further show that reconstituted PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes establish higher molecular mass structures, the spectroscopic and physicochemical properties of which are very close, in most aspects even indistinguishable, from those of the presumed native complex.

MATERIALS AND METHODS

Cloning procedures **-** Double-stranded DNAs encoding the mature parts of the PORA and PORB proteins of barley, were generated by a polymerase chain reaction-based approach (15). The following primer pairs were used:
Primer 1 (5'AACTGCAGAT-GGGCAAGAAGACGCTGCGGCAG3') plus 2 (5'AACTGCAGGGTGGATCATAGTCCGAC-GAGCTT3'), and primers 3 (5'AACTGCAGATGGGCAAGAAGACTGTCCGCACG3') plus 4 (5'AACTGCAGTGATCATGCGAGCCCGACGAGCTT3'), as well as cDNA clones A7 (16) and L2 (4), respectively, as templates. After subcloning into the *Pst*I site of pUC19 (New England BioLabs), the DNAs for PORA and PORB were cut out with *Bam*HI and *Hin*dIII and inserted into identically treated pSP64 vectors (Promega)(17). The identity of the different clones was confirmed by DNA sequencing, using a T7 DNA sequencing kit (Promega) and the gel system described in Ref. 18.

Preparation of pigments – Chemical synthesis and characterization of Zn- and Mg-Pchlides *a* and *b* was performed as described in Refs. 6 and 14. HPLC was carried out either on a C18 reverse phase silica gel column (Macherey-Nagel Co., 250 x 4.6 mm, Nucleosil ODS 5 µm)(6) or a C30 reverse phase column (YMC Inc., Willmington, NC, USA, 250 x 4.6 mm, 5 µm)(19), using a Varian ProStar model 410 apparatus and a ProStar model 240 pump and a ProStar 330 photodiode array detector. In some experiments, a C18 reverse phase silica gel column (HyPurity^{TM}, 250 x 4.6 mm, Hypersil ODS 5 µm) and a Dynamax absorbance detector model UV-1 and a Dynamax SD-200 pump were used.

In vitro transcription/translation and reconstitution of POR-pigment complexes - Radiolabeled PORA and PORB molecules were synthesized by coupled *in vitro* transcription/translation (20) of the recombinant clones specified above and purified as described previously (3). Equal amounts of the PORA and PORB, as determined by counting their radioactivities and correcting the rates of incorporation for the different methionine contents (21), were supplemented with NADPH (0.5 mM final concentration) and synthetic Pchlide *a*, Pchlide *b*, ZnPP*a* or ZnPP*b*. In all cases, 10 µM final porphyrin concentrations were used. After a 15-min incubation in the dark, the assay mixtures were subjected to gel filtration on Sephadex G15 equilibrated in assay buffer (22). Enzyme-pigment complexes running in the flowthrough were extracted with acetone (see below) and pigments quantified in a spectrometer LS50B (Perkin Elmer Corp., Norwalk, CT)(23).

For the reconstitution of LHPP, equimolar amounts of the reconstituted PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes were incubated in the dark, as described previously (5). Then, the resulting high molecular weight complexes were separated from free, non-assembled POR-pigment-NADPH complexes by gel filtration on Sephadex G100 (5) or Superose 6 (Amersham Pharmacia Biotech, www.apbiotech.com). Fractions containing PORA-PORB-supracomplexes were identified by radioactivity measurements, pooled and in turn supplemented with a mixture of galacto- and sulpholipids containing monogalactosyl diacylglycerol, digalactosyl diacylglycerol, and sulphoquinovosyl diacylglycerol (58:36:6 mol%, see Ref. 5). The sample was then cooled to - 196°C and analyzed by fluorescence emission measurements at an excitation wavelength of 440 nm. For flash light experiments, the sample was warmed to about – 25°C (24), exposed to a single, 1-msec flash of white light, and immediately dipped into liquid nitrogen. Then, the spectroscopic measurements were repeated. For the experiment described in Fig. 8, two parallel samples were prepared, of which one was exposed to flash light as above, whereas the other was kept in darkness before non-denaturing electrophoresis (25).

Protein analyses – Three different methods were employed to prepare and analyze POR-pigment complexes, that of Ryberg and Sundqvist (26), that of Klement et al. (27), and a modified version of that of Gerhardt and Heldt (28).

In the first case, etioplasts were isolated from 5-d-old dark-grown barley plants by differential centrifugation (see Ref. 26, for details). One aliquot of the final etioplast suspension was extracted with an excess of 100% acetone containing 0.1 % (vol/vol) diethyl pyrocarbonate, protein recovered by centrifugation and subsequently used for Western blot analysis (29), using an antiserum against the PORA of barley (16). In three additional samples, the plastids were lysed hypotonically in a buffer containing 1 mM $MgCl_2$, 1 mM EDTA, 20 mM TES [N-tris(hydroxymethyl-2-aminomethane-sulfonic acid], 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.2, and etioplast inner membranes comprising prolamellar bodies and prothylakoids prepared by homogenization in a glass homogenizer. All samples were then centrifuged at 7,700 *g* for 15 min. For one sample, proteins found in the resulting pellet and supernatant, respectively, were extracted with acetone (see above), sedimented and used for subsequent Western blot analysis (see above). In case of the second sample, the obtained pellet was resuspended in the buffer described before but containing 50% sucrose, sonicated 3 x 5 sec with a Branson Sonifier (Danburry, USA), microtip, medium tune, and the homogenate placed at the bottom of a continuous 10-50% (w/w) sucrose gradient. In case of the third sample, essentially the same procedure was followed, except for the fact that the buffer used for ultrasonication lacked sucrose and that the etioplast inner membranes were loaded from the top onto the gradient.

After centrifugation of the gradients at 25,400 *g* for 2 h, several different bands were seen. For the bottom-loaded gradient, these corresponded to byoant densities of ca. 1.17 g cm^{-3} and ca. 1.21 g cm^{-3}, respectively. In case of the top-loaded gradient, also the lighter band was obtained, but not the heavier. Instead a novel, smeary band spreading over at least 3 fractions (designated T1-3 in Fig. 6) was recovered in the uppermost parts of the gradient. According to Ryberg and Sundqvist (26), band T1 as well as the band at 1.21 g cm^{-3} should represent prothylakoids, while the 1.17 g cm^{-3} band should be identical with prolamellar bodies. All of the different bands were retrieved from the gradients, diluted 4-fold with the buffer described above, and finally centrifuged at 42,500 *g* for 2h. Proteins found in the resulting pellet fractions were then extracted with acetone (see above) and analyzed by Western blotting (29).

According to Klement et al. (27), etioplasts were prepared from etiolated barley plants by Percoll density gradient centrifugation, retrieved, diluted and re-sedimented by centrifugation. The resulting etioplast pellet was resuspended in a buffer

containing 50 mM Tricine/KOH, pH 7.2, 20 % glycerol and four equal parts loaded onto sucrose buffer containing 0,5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM Tricine, 10 mM HEPES, pH 7.2. After a step of ultracentrifugation at 80,000 *g* for 20 min, the resulting pellet was resuspended in the same buffer as described before but containing 2.5 mM *n*-octyl-ß-D-glucoside and 5 % glycerol. After gentle shaking for 30 min, the assays were re-centrifuged as described previously and the obtained pellet treated with the same detergent buffer, but containing 15 mM *n*-octyl-ß-D-glucoside and 30% glycerol. The suspension was again gently shaken for 30 min and subjected to ultracentrifugation at 200,000 *g* for 20 min. The resulting supernatant was loaded onto a column (9 x 32 mm) of DEAE-cellulose (Sigma, Deisenhofen, Germany) equilibrated with a buffer containing 5 mM *n*-octyl-ß-D-glucoside, 10% glycerol, 0.3 mM $MgCl_2$, 0.3 mM EDTA, 10 mM Tricine, 10 mM HEPES, pH 7.2. Protein was eluted from the column by applying 12 mL of a gradient consisting of 5-20 mM *n*-octyl-ß-D-glucoside dissolved in equilibration buffer. Fractions of 0.5 mL were taken and analyzed for the presence of POR by Western blotting with the PORA antiserum described before.

As the third method, we adapted the non-aqueous fractionation technique of Gerhardt and Heldt (28) to isolated intact etioplasts. Briefly, etioplasts were isolated by differential centrifugation and Percoll density gradient centrifugation and further purified on cushions of Percoll as described (30). Each plastid sample was divided into two equal parts, of which one was exposed to a single flash of white light, whereas the other was kept in darkness. Etioplasts were then re-sedimented and immediately quenched and ground under liquid nitrogen. The etioplast powder was then lyophilized at –50°C. About 200-300 mg of the dry plastid material were transferred at –35°C into a mixture of heptane-carbon tetrachloride [C_7H_{16}/CCl_4 66:34 (v/v), density 1.28 g/cm^3]. The suspension was in turn ultrasonicated at –70°C with 10 5-sec-pulses in a Branson Sonifier (see above) and poured through a layer of quartz wool contained in a filter, to remove any remaining coarse material. The flowthrough was diluted 3-fold with heptane and centrifuged for 2 min at 3000 *g*. The clear supernatant was discarded, and the sediment resuspended in 3 mL of a CCl_4/C_7H_{16} mixture of the same density as that described before. Two 200-µL aliquots were withdrawn for determination of Pchlide *a* and Pchlide *b* levels by HPLC, and enzyme activities (see below). The remainder was loaded onto a freshly prepared, exponential 1.28-1.50 g/cm^3 density gradient of CCl_4/C_7H_{16}. After

centrifugation at 25,000 *g* for 2.5 h, during which time the material distributed isopycnically in the gradient, 1.2-mL fractions were removed, starting from the top of the gradient, and subsequently divided into three equal portions. One-third was used for determination of marker enzymes (NADPH:glycerinaldehyde-phosphate dehydrogenase, plastid stroma; phosphoenolpyruvate carboxylase, cytosol; α-mannosidase, vacuole), the second for SDS-PAGE, and the third portion for assay of Pchlide *a* and Pchlide *b* levels. All three divided portions and the two aliquots taken from the original sample (see above) were diluted 3-fold with heptane and centrifuged for 8 min at 18,000 *g*. The supernatant was discarded, except for the last 200 µL, which were used to resuspend the sediment by swirling it with a calcined quartz. All samples were then dried for 18 h in a desiccator, and then processed for electrophoresis, enzyme assays or pigment measurements. All manipulations were performed under a dim green light. Moreover, any step, which was expected to potentially lead to trapping of condensing water vapor, was carefully avoided (28).

Electrophoresis – SDS-PAGE was performed in 10-20 % (w/v) gradients of polyacrylamide as described (31). Non-denaturing, analytical PAGE was performed in 3 mm thick 7.5 % (w/v) polyacrylamide gels and the gels run using a discontinuous buffer system (25).

RESULTS

Our previous reconstitution experiments had shown that the PORA and PORB proteins of barley are able to form higher molecular weight light-harvesting complexes, if complexed with ZnPP*b* and ZnPP*a*, respectively, plus NADPH (5). As a first step to establish such complexes also with their presumed natural substrates, we synthesized Pchlides *a* and *b* chemically (6, 14). The isolated pigments are characterized in the accompanying paper (32). They were added to PORA and PORB polypeptides, which had been synthesized from corresponding cDNA clones by coupled *in vitro* transcription/translation (20). Pigment binding was tested in the following manner. Different amounts of the PORA and PORB were added to an excess of NADPH and isolated pigment, incubated for 15 min in darkness, and subsequently separated from the excess of non-bound pigments by gel filtration on Sephadex G15 (22). POR-

pigment complexes running in the flowthrough were then extracted with 100% acetone containing 0.1 % (vol/vol) diethyl pyrocarbonate, and pigments identified and quantified by HPLC and room temperature absorbance and fluorescence measurements.

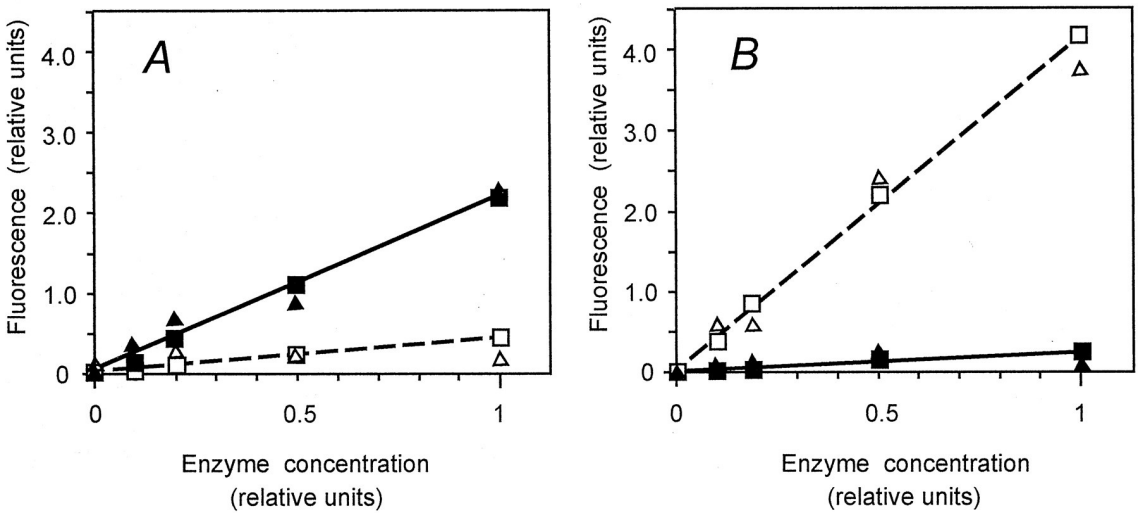

FIG. 1. Pigment binding characteristics of the PORA (A) and PORB (B). PORA and PORB proteins were produced by coupled *in vitro* transcription/translation of respective recombinant clones, purified, and 1, 0.2, 0.1 and 0.02 POR protein equivalents subsequently incubated for 15 min in the dark with Pchlide *a* (Δ---Δ), Pchlide *b* (▲—▲), ZnPP*a* (□---□) and ZnPP*b* (■—■), respectively. After a step of gel filtration on Sephadex G15, POR-bound pigments were extracted with a solution of practically pure acetone containing 0.1 % (vol/vol) diethyl pyrocarbonate. Pigments were identified and quantified by either HPLC analyses and subsequent absorbance measurements or by fluorescence spectroscopy, taking into account previously published absorption and emission coefficients of isolated pigments (6, 14). The plots show the amounts of PORA-bound and PORB-bound pigments versus the PORA and PORB protein concentrations in the assays. 1 POR protein equivalent corresponded to 2.78 pmoles of the PORA and 2.64 pmoles of the PORB, respectively.

Figure 1 shows a plot of the amount of Pchlide *a* and Pchlide *b* bound to the PORA or PORB *versus* the enzyme concentrations. From the linear relationships, it turned out that 1 µg of the PORA bound approximately 34.11 ng of Pchlide *b* and 31.95 ng of ZnPP*b*, but only 3.5 ng of Pchlide *a* and 3.28 ng of ZnPP*a*, respectively. For the PORB, just the opposite binding preferences were seen: 1 µg of the PORB bound approximately 33.2 ng of Pchlide *a* (31.1 ng ZnPP*a*) and only 3.2 ng of Pchlide *b* (3.02 ng ZnPP*b*).

Given that the PORA and PORB displayed the same stringent substrate specificities for Pchlides *a* and *b* as those reported previously for ZnPP*a* and ZnPP*b* (5), we next established reaction conditions that would allow the generation of higher molecular weight Pchlide *a*/*b*-POR light-harvesting complexes. Equimolar amounts of reconstituted PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes were mixed and, after a 15-min incubation in darkness, subjected to a further step of gel filtration on Sephadex G100 (5) or size-fractionated on a Superose 6 column (see *Materials and Methods*).

Figure 2A shows a size-fractionation on Superose 6. It revealed that the pigment-complexed PORA and PORB indeed gave rise to a higher molecular weight complex. Its size of ca. 480 kDa was similar to that of the so-called Pchlide holochrome of bean (33). Free, non-assembled PORA- and PORB-pigment ternary complexes were eluted at much later time points (Fig. 2A).

To determine the stoichiometry of the PORA and PORB in the recovered supracomplexes, fractions containing the different PORs were pooled (see Fig. 2A), separated by SDS-PAGE, and the gel subjected to autoradiography. This revealed that in the recovered higher molecular weight complex approximately 5 PORA-Pchlide *b*-NADPH ternary complexes interacted with just 1 PORB-Pchlide *a*-NADPH complex (Fig. 2B, fraction 5).

The oligomeric PORA-PORB protein complex contained in fraction 5 was subsequently supplemented with a lipid mixture containing monogalactosyl diacylglycerol, digalactosyl diacylglycerol, and sulphoquinovosyl diacylglycerol (58:36:6 mol%), which had been prepared from pigment-free prolamellar bodies of barley etioplasts (5). Then low temperature measurements were performed at 77K, in a Perkin Elmer spectrometer LS50B (23).

Figure 2C (solid line) demonstrates that two fluorescence peaks could be seen at an excitation wavelength of 440 nm: one at 657 nm and the other at 632 nm. Because these two peaks corresponded to photoactive Pchlide 650/657 and photoinactive Pchlide F628/632, known from the prolamellar body of etioplasts (e.g., Ref. 5), we exposed the lipid-containing complex to a saturating 1-msec flash of white light. As shown in Fig. 2C (dashed line), this gave rise to the quantitative conversion of Pchlide F650/657 to Chlide 684/690.

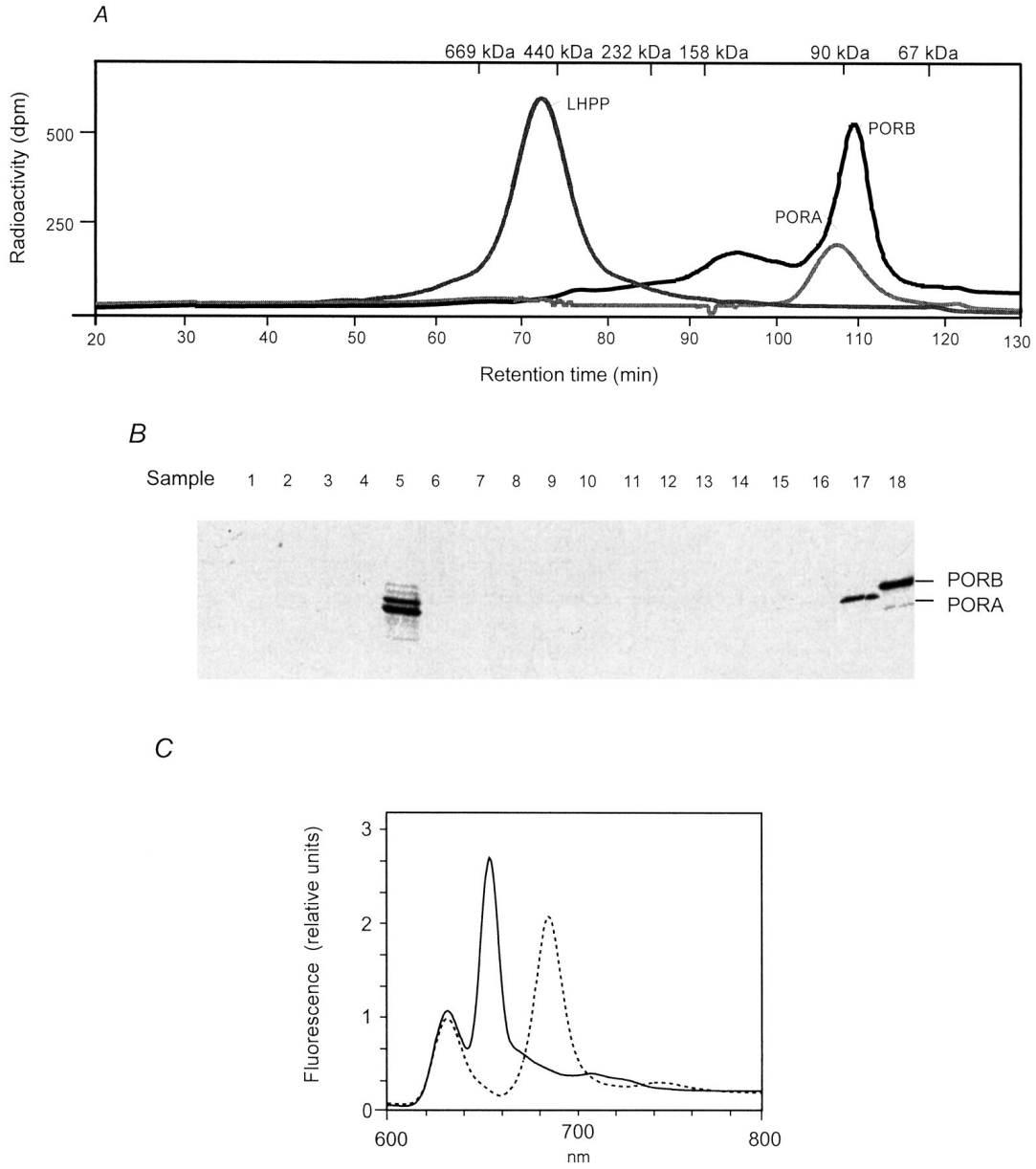

FIG. 2. Reconstitution of a photoactive light-harvesting POR-pigment complex (LHPP) *in vitro*. PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes which had been reconstituted and purified as described in Fig. 1, were mixed and incubated in equimolar concentrations for 15 min in the dark. Then, the assays were subjected to gel filtration. Individual fractions were harvested every min and aliquots taken for radioactivity measurements in a liquid scintillation counter (A). Migration of marker proteins is indicated on top. For tests on the presence of higher molecular weight complexes (B), fractions were pooled as follows: samples 1-4, fractions 1-60, containing 15 consecutive fractions each; sample 5, fractions 61-80; samples 6-16, fractions 81-104, containing 2 consecutive fractions each; sample 17, containing fractions 105-110; sample 18, containing fractions 112-116. In all cases, protein was recovered from the pooled fractions by precipitation with TCA and analyzed by SDS-PAGE. Fraction 5, containing the pooled PORA-PORB supracomplexes, was analyzed further and supplemented with a mixed galacto- and sulpholipid fraction isolated from pigment-free prolamellar bodies of barley etioplasts (C). The curves show low temperature spectra at –196°C (77K) at an excitation wavelength of 440 nm before (solid line) and after (dashed line) a single, 1-msec flash of white light. *Note* the quantitative conversion of Pchlide F650/657 to Chlide F684/690.

We next extracted pigments from the flashed sample with acetone (see above) and run HPLC analyses, as described in Ref. 14. Separation on a C18 column is shown in Fig. 3A. As demonstrated in the accompanying paper (32), the pigment peak eluting at ca. 12.5 min is identical with Pchlide *b*, while the peak eluting at ca. 15 min is identical with Pchlide *a* (Fig. 3A, panel a). Upon flash light-illumination, a novel peak appeared at ca. 14 min (Fig. 3A, panel b). Based on our previous *in vitro*-reconstitution experiments, we assumed that this peak might be due to Chlide *a.* To demonstrate this, another type of HPLC analysis was performed. Taking into account a recent paper of Fraser et al. (19), a C30 column was used. Synthetic Chlides *a* and *b* were prepared by the chlorophyllase reaction (34) and used as standards. Figure 3B (panel c) shows that Chlide *a* and Chlide *b* were well resolved on the C30 column and also separated from Chl *a* and Chl *b*. When the pigments, eluting at ca. 14 min on the C18 column (see Fig. 3A), were applied to the C30 column, the only detectable pigment was Chlide *a* (Fig. 3B, panel d). Thus only Chlide *a* had been produced upon flash light-illumination of the reconstituted complex (Table 1). By contrast, Pchlide *b*, which was ca. 5-fold more abundant than Pchlide *a*, did not seem to be photoconvertible at all under the tested conditions. Indeed, no Chlide *b* was formed (Table 1).

TABLE I

Quantification of pigments before and after flash light illumination of the reconstituted, lipid-containing complex

Pigments were extracted from the flashed and nonflashed sample with 100% acetone containing 0.1% (v/v) diethylpyrocarbonate and separated and identified by HPLC as described under „Materials and Methods“. Pigment levels refer to the sum of all detected pigments, set as 100. ND, nondetectable pigment levels.

Pigment	Pigment (percentage of total)	
	Nonflashed	Flashed
	%	%
Pchlide *a*	22	4
Pchlide *b*	78	76
Chlide *a*	ND	20
Chlide *b*	ND	ND

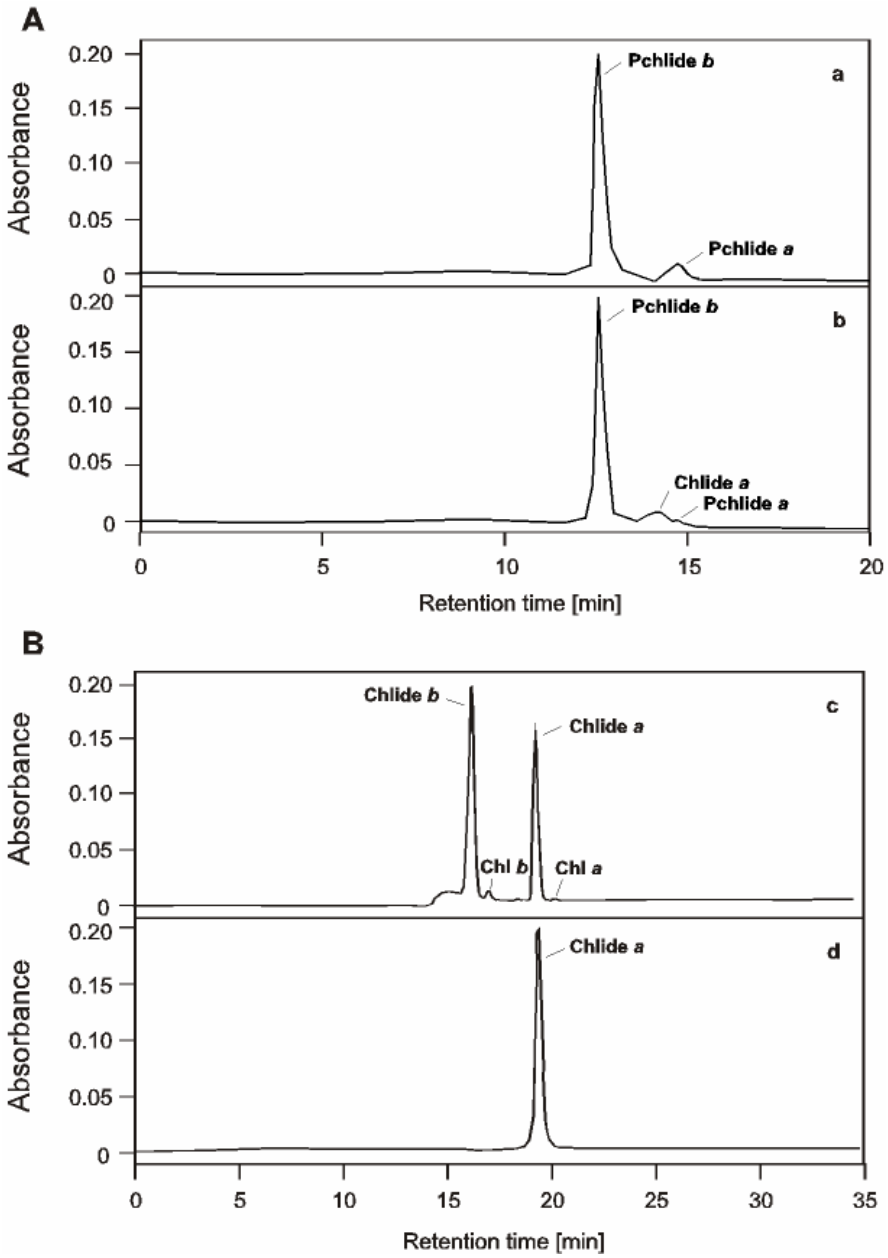

FIG. 3. Photoconversion of Pchlide *a*, but not Pchlide *b*, in the reconstituted complex. Higher molecular weight POR-pigment complexes were reconstituted and incubated with a mixed galacto- and sulpholipid fraction of barley prolamellar bodies as described in Fig. 2. The lipid-containing complex in turn was subjected to a single, saturating flash of white light. Pigments were extracted with 100% acetone containing 0.1 % (vol/vol) Diethylpyrocarbonate and analyzed by HPLC. (A) Separation of natural pigments on a C18 column. Absorbance measurements were made at 455 nm, which corresponds to the Soret band of Pchlide *b*. The chromatograms show pigments before (a) and after (b) light exposure. The pigment eluting at ca. 12.5 min corresponds to Pchlide *b*, while Pchlide *a* has a retention time of ca. 15 min (32). (B) Identification of Chlide *a* by HPLC on a C30 column. Synthetic standards (panel c: Chlide *b*, 16.25 min, Chl *b*, 17.5 min, Chlide *a*, 19.5 min, and Chl *a*, 21 min) were prepared as described under *Materials and methods* and separated on a C30 column. The pigment eluting at 14 min on the C18 column (A, panel b) was run in parallel (panel d); it corresponds to Chlide *a*.

An explanation for these findings could be that the PORA was *per se* inactive with Pchlide *b* and thus unable to convert the pigment to Chlide *b*. If so, no Chlide *b* should be produced also *in situ*. To test this hypothesis, we first analyzed pigments that were formed in isolated prolamellar bodies upon flash light-illumination by HPLC. Figure 4 shows a representative separation of pigments before (A) and after (B) a saturating 1-msec flash of white light. In addition to Pchlide *a* and Pchlide *b*, eluting at 12.5 min and 15 min, respectively (Fig. 4A), also 7-hydroxy-Pchlide *a* could be detected (peak 1)(see Ref. 32, for details). Upon flashing the sample, a novel pigment peak appeared (Fig. 4B, peak 4), the retention time and absorption properties of which were indistinguishable from those of Chlide *a* identified previously (data not shown, but see Fig. 3). At the same time, increasing amounts of Pchlide *a* (peak 3) were detectable. As shown in the accompanying paper (32), barley etioplasts contain an enzyme called 7-formyl reductase which converts Pchlide *b* to Pchlide *a* via 7-hydroxy-Pchlide *a* (see also Ref. 14). Upon resolution of pigments contained in peak 4 by subsequent HPLC on a C30 column, indeed only Chlide *a* but no Chlide *b* was observed (data not shown).

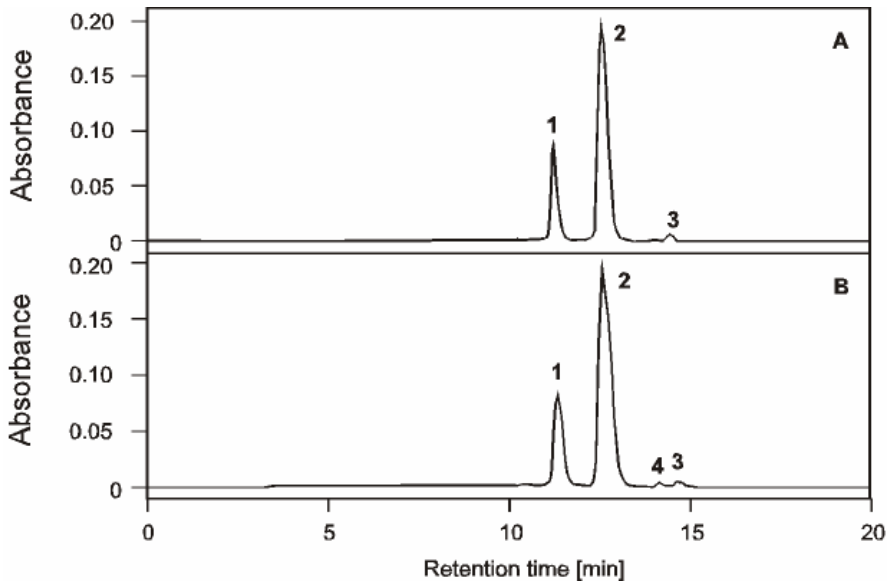

FIG. 4. Photoconversion of Pchlide *a*, but not Pchlide *b,* in isolated prolamellar bodies. Prolamellar bodies were isolated from barley etioplasts and either kept in darkness (A) or exposed to a 1-msec flash of white light (B). Pigments were separated by HPLC as described in Fig. 3A and identified using synthetic 7-hydroxy-Pchlide *a* (peak 1), Pchlide *b* (peak 2), Pchlide *a* (peak 3) and Chlide a (peak 4) as standards (not shown, but see Ref. 32). *Note* the limited amount of Chlide *a* in the illuminated sample, and the relative increase in the level of Pchlide *a*, which is indicative of 7-formyl reductase activity present in barley etioplasts (see also accompanying paper, Ref. 32).

As a second approach, the *in vitro*-synthesized PORA was incubated with ZnPP*b* (which is chemically more inert than Pchlide *b*), separated from non-bound pigment by gel filtration, and POR-pigment complexes runnig in the flowthrough exposed to white light. Parallel samples were kept in darkness. As a control, the PORB was used.

Figure 5 shows representative room temperature fluorescence emission spectra of PORA- and PORB-bound pigments after their extraction with acetone. They revealed that the PORA indeed converted ZnPP*b* to Zn pheophorbide (ZnPheo) *b* (Fig. 5B). By analogy, also ZnPP*a* was converted to ZnPheo *a* (Fig. 5A). Also with the PORB the same principal results were obtained (Fig. 5C and D, respectively). For either POR protein, a strict correlation was observed between the amounts of products formed and bound substrates, regardless of whether ZnPP*a* and *b* or Pchlides *a* and *b* had been used (Fig. 5 and unpublished data).

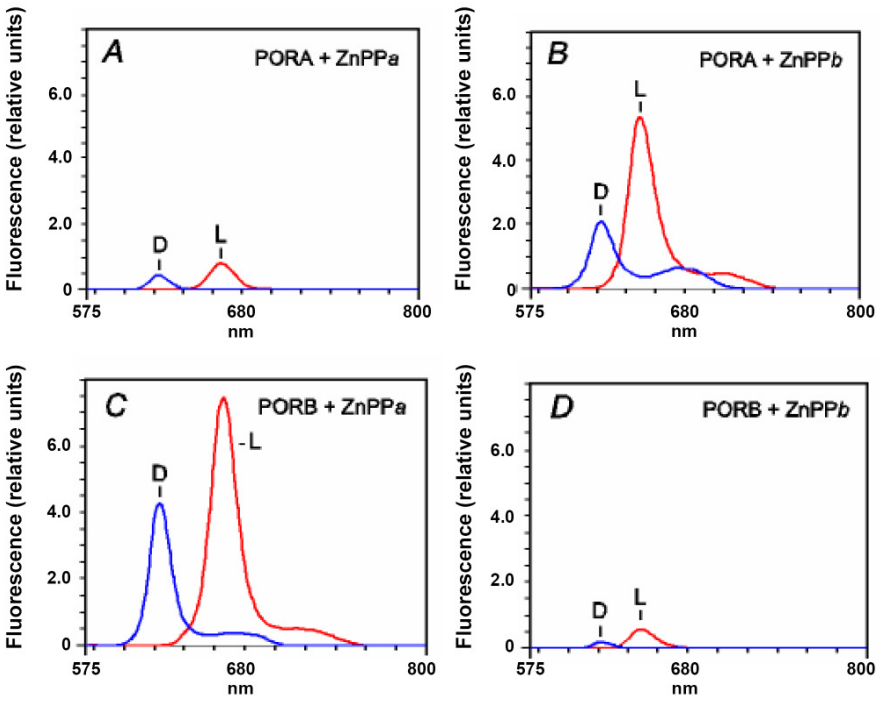

FIG. 5. Photoconversion of synthetic ZnPP*a* and ZnPP*b* by virtue of the PORA and PORB. *(*A-D*)* PORA-ZnPP*a*-NADPH (A), PORA-ZnPP*b*-NADPH (B), PORB-ZnPP*a*-NADPH (C) and PORB-ZnPP*b*-NADPH (*D*) complexes were reconstituted *in vitro* and purified by gel filtration as described in Fig. 1. The different ternary complexes were then exposed to white light (L) or kept in darkness (D) for 15 min. POR-substrate and POR-product complexes were extracted with acetone, and the released pigments identified and quantified by fluorescence emission measurements at an excitation wavelength of 440 nm. The products of photoconversion, Zn-pheophorbide *a* and Zn-pheophorbide *b*, have emission maxima at 665 nm and 652 nm, respectively.

All results presented thus far implied that the reconstituted, lipid-containing Pchlide *a*/*b*-POR protein complex may be identical with the presumed native complex in the prolamellar body of etioplasts. But, except for the spectroscopic data, no other line of evidence seemed to exist to support this notion. We consequently sought to identify the native complex by classical biochemical approaches and to compare its properties with those of the reconstituted complex.

Ryberg and Sundqvist (26) had shown that isolated etioplast inner membranes from wheat can be resolved into different sub-fractions, designated prolamellar bodies and prothylakoids, respectively, based on their different buoyant densities in sucrose gradients. We re-addressed this previous work for barley etioplasts and analyzed the abundance of the PORA and PORB after various steps of the plastid work-up procedure (see *Materials and methods*). As shown in Fig. 6, already during the very first step of isolation of the so-called etioplast inner membranes (presumed to comprise prolamellar bodies and prothylakoids, Ref. 26), a major part of the PORA became soluble. The same effect was seen for oat and wheat etioplasts, which were analyzed in parallel (data not given). In all cases, subsequent steps of prolamellar body and prothylakoid separation turned out to correlate with a further solubilization of the PORA. This is shown for barley in Fig. 6. With etioplast inner membranes which had been diluted with a buffer lacking any additives for membrane stabilization, the PORA was quantitatively released and then co-migrated in the uppermost fractions of a top-loaded 10-50% (w/w) sucrose density gradient. With barley etioplast inner membranes, which had been sonicated in a buffer containing 50% sucrose and loaded from the bottom, both the PORA and PORB appeared to be recovered in the lower parts of the gradient, containing prolamellar bodies. However, the approximately equimolar amounts of the PORA and PORB, which were at variance with their original stoichiometries in intact etioplasts, and the fact that both POR proteins migrated to slightly different positions in the gradient argued against working further with these samples.

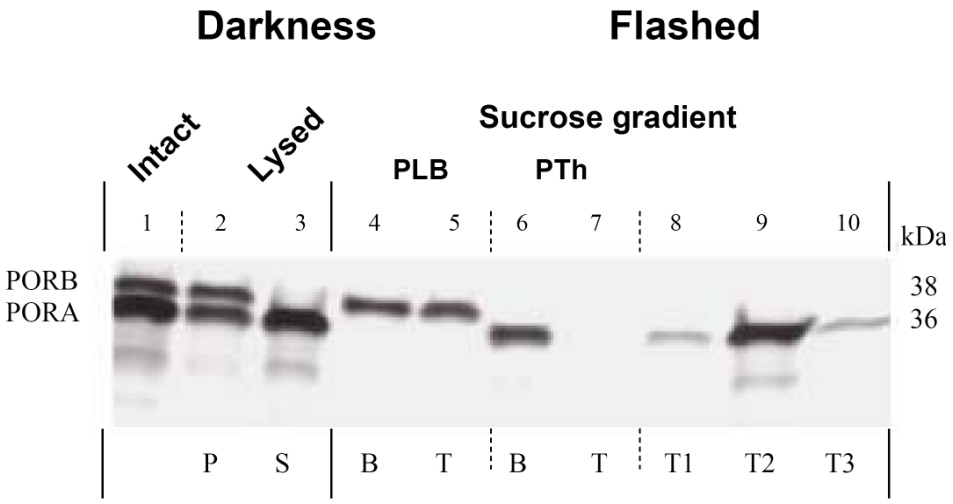

FIG. 6. Western blot analysis of POR-related proteins. Plastids were isolated and fractionated according to Ryberg and Sundqvist (26). The blot shows POR-related proteins in intact etioplasts (*Intact*, lane 1), the pellet (P) and supernatant (S) fractions of lysed etioplasts (*Lysed*; lanes 2 and 3), and in the indicated prolamellar body (PLB) and prothylakoid (PTh) fractions of a top-loaded (T) or bottom-loaded (B) gradient. *Note* the successive solubilization of the 36 kDa PORA protein during the various steps of the procedure and that the so-called prolamellar body fraction contains only the 38 kDa PORB protein, regardless of whether the gradients were top- or bottom-loaded. *Also noteworthy* is that the prothylakoid fraction of the bottom-loaded gradients contains the PORA protein (lane 6), while the corresponding fraction of the top-loaded gradient is devoid of this polypeptide (lane 7). In this case, the PORA smears across the uppermost parts of the gradient (lanes 8-10, fractions T1-T3).

In a recent paper, Klement et al. (27) reported the isolation of a pigment-free POR protein from oat. Such a preparation seemed particularly interesting to us because it would allow testing the pigment binding properties of the PORA and PORB in a more natural environment than in the *in vitro*-system. In principal, the method of Klement et al. (27) employs differential detergent solubilization of POR from the prolamellar body.

When we reproduced the published protocol and followed the fate of the PORA and PORB by Western blotting (Fig. 7), again drastic losses of the PORA during successive steps of the membrane preparation and solubilization procedure became apparent. In the final supernatant, approximately equal levels of the PORA and PORB were seen, which were at variance with the determined 5:1 protein stoichiometry in intact etioplasts. Moreover, subsequent chromatography on DEAE cellulose gave rise to at least 3 different POR protein bands, representing the PORA, the PORB and a slightly smaller degradation product (Fig. 7). To which extent these proteins would contribute to pigment binding could not be estimated.

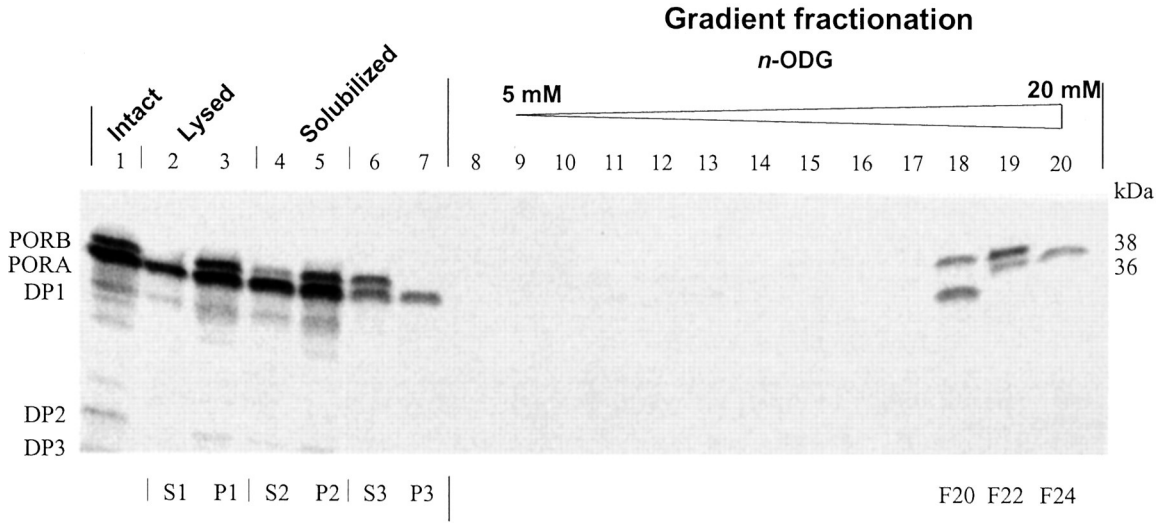

FIG. 7. Western blot analysis of POR-related proteins during the plastid work-up procedure of Klement et al. (27). The blot shows POR-related proteins in intact etioplasts (*Intact*, lane 1), lysed etioplasts (*Lysed*, lane 2 and 3), and in all of the different fractions obtained by stepwise detergent solubilization with *n*-octyl-ß-D-glucoside (*Solubilized*, lanes 4-7). *P* and *S* define respective pellet and supernatant fractions each. For DEAE-cellulose chromatography (*Gradient fractionation*), fraction S3 was used and POR-related proteins eluted with a 5-20 mM *n*-octyl-ß-D-glucoside (*n*-ODG) gradient (lanes 9-20). Lane 8 shows the flowthrough. *Note* the progressive solubilization of the bulk of the 36 kDa PORA protein during the plastid work-up procedure and that some PORA appears in the pellet fraction P3. This may be due to the high detergent concentration, favouring membrane lipid disorganization. The identity of the lower band in lane 7 is unknown, but it may be generated by limited proteolysis of residual PORB. *Noteworthy* in this context is also the occurrence of several other degradation products of POR, designated DP1-DP3. In the ultimate POR protein preparation (pooled fractions F20-24), at least three different POR proteins are found: PORA; PORB; and DP1, the pigment binding capacity of which is unknown.

Given this uncertainty and the fact that neither the method of Ryberg and Sundqvist (26) nor that of Klement et al. (27) allowed the recovery of an intact PORA-PORB protein complex, we sought for alternative methods. Taking into account a paper of Gerhardt and Heldt (28) on enzyme and metabolite measurements in different subcellular compartments, we adapted non-aqueous protein and pigment extraction and fractionation to isolated etioplasts. The method employed is based on the fact that the proteins and metabolites of a given compartment aggregate together upon lyophilization. Because each compartment has a characteristic protein, lipid, carbohydrate and ion complement, different fractions are obtained in non-aqueous gradients of carbon tetrachloride/heptane. These and an original etioplast sample were analyzed with respect to the PORA and PORB protein abundances as well as Pchlide *a* and Pchlide *b* levels. Moreover, we used non-denaturing, analytical PAGE to directly visualize POR-pigment complexes in etioplasts prior to fractionation (25).

Table 2 shows the determined PORA and PORB as well as Pchlide *a* and Pchlide *b* levels. It turned out that PORA is approximately 4.2-fold more abundant than PORB. Quantification of pigments showed that etioplasts contain an approximately 4.5-fold excess of Pchlide *b* relative to Pchlide *a*. Of the total Pchlide, only 18% was photoreducable. This photoconvertible Pchlide turned out to be identical with Pchlide *a* (Table 2).

TABLE II

Quantification of POR and pigment levels in isolated etioplasts before and after flash light illumination

Proteins and pigments were extracted from lyophilized etioplasts before and after flash light illumination and analyzed by SDS-PAGE and HPLC, respectively, as described under „Materials and Methods“. Levels are given in percentage of total, set as 100. ND, nondetectable levels.

	Sample	
	Nonflashed	Flashed
Pigment		
Pchlide *a*	18	ND
Pchlide *b*	82	82
Chlide *a*	ND	18
Chlide *b*	ND	ND
POR		
PORA	81	81
PORB	19	19

Figure 8A (panel a, lane *D*) shows a non-denaturing, analytical PAGE of the presumed natural POR-pigment complex. Based on the red-light-induced autofluorescence of Pchlide F650/657, this complex could directly be visualized by fluorography. Western blot analyses confirmed that it contained POR (Fig. 8A, panel b, lane *D*). Flash light-illumination and subsequent mild detergent treatment prior to electrophoresis (32), dissociated this total POR into two sub-fractions (Fig. 8A, FL). Upon scaling up the procedure 1000-fold, these could be identified as PORA and PORB by protein sequencing (data not shown). Their approximate stoichiometry was similar to that determined from the carbon tetrachloride/heptane gradients (Table 2) and also matched that expected from our previous *in vitro*-reconstitution experiments (Fig. 2). Indeed, when the *in vitro*-reconstituted, lipid-containing complex was subjected to non-denaturing PAGE, a similar, though slightly smaller, complex could be seen (Fig. 8B). This complex contained both PORA and PORB and displayed the same type of

autofluorescence as the presumed native complex. Moreover, it was rapidly dissociated into the two POR proteins upon flash light-illumination.

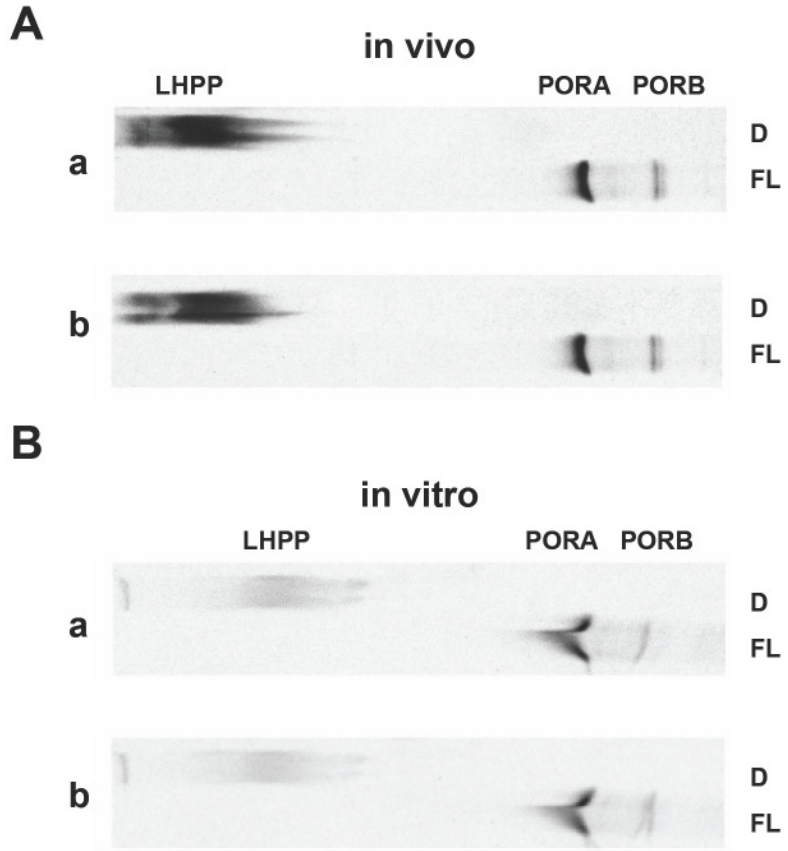

FIG. 8. Non-denaturing, analytical PAGE of the presumed native and reconstituted lipid-containing light-harvesting complexes. The presumed native complex was isolated from the prolamellar body of barley etioplasts (A) and compared with the reconstituted complex (B). In either case, the autoradiograms show parallel gel strips containing flashed (FL) and non-flashed (D) samples. Prior to analysis, half of the samples was illuminated with red light and immediately exposed to X-ray films at –70°C (panel a), whereas the other halves were blotted onto nitrocellulose and probed with the PORA antiserum (panel b). Higher molecular mass light-harvesting complexes indicative of LHPP are highlighted. These complexes dissociate into the respective PORA-pigment-NADPH and PORB-pigment-NADPH sub-complexes upon flash light illumination.

An HPLC analyses of pigments re-extracted from the electrophoretically resolved native POR-pigment complex is shown in Fig. 9. It demonstrated that the complex contained both Pchlide *b* and Pchlide *a* (Fig. 9A, peak 2 and 3, respectively). In addition, also substantial amounts of 7-hydroxy-Pchlide *a* could be seen (Fig. 9A, peak 1).

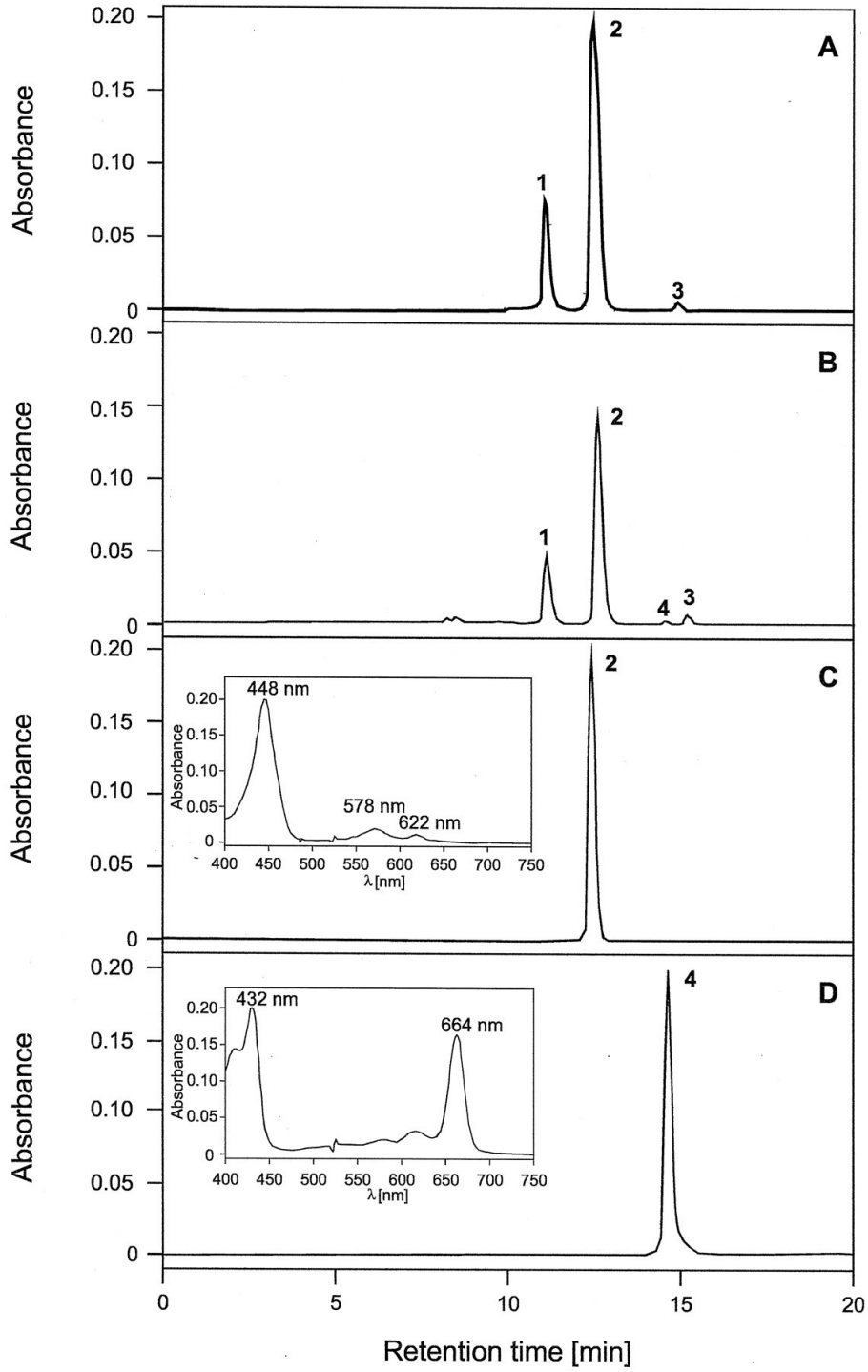

FIG. 9. Identification of pigments contained in the electrophoretically resolved native light-harvesting complex before and after its light-induced dissociation. Higher molecular weight POR-pigment complexes were isolated from prolamellar bodies of barley etioplasts as described in Fig. 8. The lipid-containing complexes in turn were resolved by non-denaturing PAGE. Part of the samples was subjected to a single, saturating flash of white light prior to electrophoresis (see Fig. 8), in order to induce the dissociation of the complex. After separation, individual gel slices were homogenized and extracted with acetone, and pigments analyzed by HPLC on a C18 column. Absorbance measurements at 455 nm show pigments recovered from the native light-harvesting complex (LHPP) before (A) and after flash light illumination (B) and from the detergent-released PORA- (C) and PORB-containing (D) sub-complexes. The insets in (C) and (D) show absorption spectra of the recovered pigments.

Upon flash light-illumination, correlating with the disintegration of the complex (see Fig. 8A), only Chlide *a* was produced (Fig. 9B, peak 4). It co-migrated with the PORB protein band (Fig. 9D). Protochlorophyllide *b*, by contrast, remained quantitatively unchanged (Fig. 9, compare B *versus* A) and co-migrated with the PORA protein band (Fig. 9C). This result not only confirmed the previously determined substrate specificities, but also that only PORB's bound pigment, i.e., Pchlide *a*, had been converted to Chlide *a*. The bulk of the pigment, corresponding to Pchlide *b*, remained non-photoconvertible.

DISCUSSION

In the present study, we addressed three different questions. First, would the PORA and PORB display the same stringent substrate specificities for their presumed natural substrates - Pchlide *b* and Pchlide *a*, respectively - as reported previously for their Zn counterparts (5)? Second, would the resulting PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes be able to establish higher molecular weight light-harvesting structures with galacto- and sulpholipids, as proposed previously (5)? Third, would similar, Pchlide *b*-containing complexes exist *in vivo*?

The answers to all of these questions were positive. We were able to demonstrate that PORA binds ca. 10-fold higher amounts of Pchlide *b* (ZnPP*b*) relative to Pchlide *a* (ZnPP*a*). PORB, by contrast, was specific for Pchlide *a* (ZnPP*a*) and bound ca. ten-fold lower levels of Pchlide *b* (ZnPP*b*). Either POR protein likewise converted these compounds into their respective products *in vitro*. However, if PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes were mixed and reconstituted to higher molecular weight complexes, only the PORB remained active. In the presence of galacto- and sulpholipids, the reconstituted Pchlide *a*/*b*-POR complex displayed the features of Pchlide F650/657. This Pchlide F650/657 was converted to Chlide F684/690 upon flash light-illumination. Indistinguishable spectral pigment species and pigment conversions have been described for isolated prolamellar body membranes of etioplasts (*see Introduction*). Moreover, we were able to resolve the lipid-containing structure both from the prolamellar body and after *in vitro*-reconstitution into similar higher molecular weight complexes under native conditions. Based on all of these

findings, we conclude that the reconstituted and analyzed authentic complexes may be structurally and functionally identical.

How is LHPP made *in vivo*? A key aspect related to this question refers to the origin of Pchlide *b*. The existence of this pigment has long been a matter of dispute (see literature cited in Ref. 14). As shown in this and the accompanying paper (32), the pigment is present in etiolated barley plants, but is rapidly converted to Pchlide *a*, if no precautions are taken. 7-formyl reductase presumably responsible for this conversion is highly active in barley etioplasts. It was for a long time implicated in Chl *b* to Chl *a* conversion, but it appears that 7-formyl reductase plays a more general role in fine-tuning the levels of both porphyrins and chlorins in dark-grown and illuminated plants (14, 32).

Enzymes, which may synthesize Pchlide *b*, have not been identified. The most likely candidates are proteins that could display (P)Chlide *a* oxygenase activity. Previous work has shown that there is a family of related proteins, which may exhibit such an activity (35-40). Tanaka et al. (36) cloned a *Chlamydomonas reinhardtii* cDNA for a putative Chlide *a* oxygenase (CAO). Later studies by Espineda et al. (37) and Tomitani et al. (38) showed that highly related CAO sequences also occur in *Arabidopsis thaliana* and other plant species. The *Arabidopsis* protein was expressed in bacteria and suggested to display Chlide *a*, but not Pchlide *a*, oxygenase activity (39). Recent work by Xu et al. (40), however, highlighted that heterologous expression of the *Arabidopsis* CAO in cyanobacteria leads to Pchl(ide) *b* accumulation. Although this demonstrates that CAO is well able to bind and convert Pchlide *a* to Pchlide *b*, it is not yet solved whether CAO is expressed in etiolated plants or not. If it would not occur, another enzyme should exist which drives Pchlide *b* synthesis.

Recent, yet unpublished work for barley shows that there is indeed a protein related to CAO (see Refs. 35 and 36, for sequence comparisons) which is able to convert Pchlide *a* to Pchlide *b*. It is part of the substrate-dependent import machinery in the plastid envelope through which the cytosolic precursor of the PORA is imported into the organelle (21, 22, 30). We were able to demonstrate that Pchlide *a* formed upon feeding isolated plastids the Pchlide precursor 5-aminolevulinic acid, is converted to Pchlide *b*. Concomitantly, the envelope-bound PORA precursor was chased into the plastids and processed to mature size. These findings imply that Pchlide *b* synthesis is directly coupled to the import step and that the novel Pchlide *a* oxygenase is located in the plastid envelope. Consistent with such an idea are also previous findings that

isolated envelope membranes of spinach chloroplasts contain Pchlide (41, 42). Work is in progress to further characterize the novel Pchlide *a* oxygenase.

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3

Manuscript 2

In vitro-mutagenesis of NADPH:protochlorophyllide oxidoreductase B: two distinctive protochlorophyllide binding sites participate in enzyme catalysis and assembly

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In vitro-mutagenesis of NADPH:protochlorophyllide oxidoreductase B: Two distinctive protochlorophyllide binding sites participate in enzyme catalysis and assembly

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Abstract

NADPH:protochlorophyllide oxidoreductase (POR) B is a key enzyme for the light-induced greening of etiolated angiosperm plants. It is nucleus-encoded, imported into the plastids posttranslationally, and assembled into larger light-harvesting POR:protochlorophyllide complexes termed LHPP (C. Reinbothe, N. Lebedev & S. Reinbothe (1999) *Nature* 397: 80-84). An *in vitro*-mutagenesis approach was taken to study the role of the evolutionarily conserved Cys residues in pigment binding. Four Cys residues are present in the PORB of which two, Cys276 and Cys303, established distinct pigment binding sites, as shown by biochemical tests, protein import studies, and *in vitro*-reconstitution experiments. While Cys276 constituted the Pchlide binding site in the active site of the enzyme, Cys303 established a second, low affinity pigment binding site that was involved in the assembly and stabilization of imported PORB enzyme inside etioplasts.

Keywords Chlorophyll biosynthesis – NADPH:protochlorophyllide oxidoreductase (POR) B – *In vitro* mutagenesis – Homology modelling – LHPP

Abbreviations: AHI, 7-α-hydroxysteroid dehydrogenase : 5-ALA, 5-aminolevulinic acid; EPR, electron paramagnetic resonance; FRET, fluorescence resonance energy transfer; LHPP, light-harvesting POR-Pchlide complex; Pchlide, protochlorophyllide; Chlide, chlorophyllide; POR, NADPH:protochlorophyllide oxidoreductase; PAGE, polyacryl-amide gel electrophoresis; SCD, short chain dehydrogenase; ZnPP, Zn protopheophorbide.

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Introduction

In angiosperms, the first step toward the establishment of the photosynthetic apparatus is strictly controlled by light and catalyzed by the NADPH:protochlorophyllide oxidoreductase (POR, EC 1.3.1.33)(Griffiths, 1975; Griffiths, 1978; Apel et al., 1980). POR itself is a light-dependent enzyme which catalyzes a hydrogen transfer from NADPH to protochlorophyllide (Pchlide), giving rise to the formation of the chlorophyll precursor chlorophyllide (Chlide). In etiolated barley plants, two closely related POR enzymes, termed PORA and PORB, have been identified (Holtorf et al., 1995; Reinbothe et al., 1999). Both POR enzymes are nuclear gene products that are synthesized as larger precursors in the cytosol and imported into the plastid post-translationally (Apel, 1981; Reinbothe et al., 1995; Reinbothe et al., 1995a; Reinbothe et al., 1995c). Interestingly, PORA and PORB displayed different substrate specificities with regard to Pchlide *a* and Pchlide *b* both *in vitro* and *in vivo* (Reinbothe et al., 1999; Reinbothe et al., 2003a; Reinbothe et al., 2003b). PORA was found to be specific for Pchlide *b*, whereas PORB preferred Pchlide *a*. Moreover PORA and PORB interacted with each other to form larger complexes (Reinbothe et al., 1999; Reinbothe et al., 2003a) indicative of the presence of the Pchlide holochrome described previously (Boardman, 1962). We re-named this complex Light-Harvesting POR-Pchlide complex, LHPP (Reinbothe et al., 1999). Based on *in vitro*-reconstitution experiments, using PORA and PORB expressed from respective cDNA clones and synthetic pigments (Pchlide *a* and Pchlide *b* or their Zn counterparts, Zn protopheophorbide *a* and Zn protopheophorbide *b*, respectively), NADPH, as well as galacto- and sulfolipids, we put forth that the Pchlide holochrome may consist of ring-like structures comprising 5 PORA-Pchlide *b*-NADPH and 1 PORB-Pchlide *a*-NADPH ternary complexes embedded into the lipid bilayers of the prolamellar body (Reinbothe et al., 1999; Reinbothe et al., 2003a).

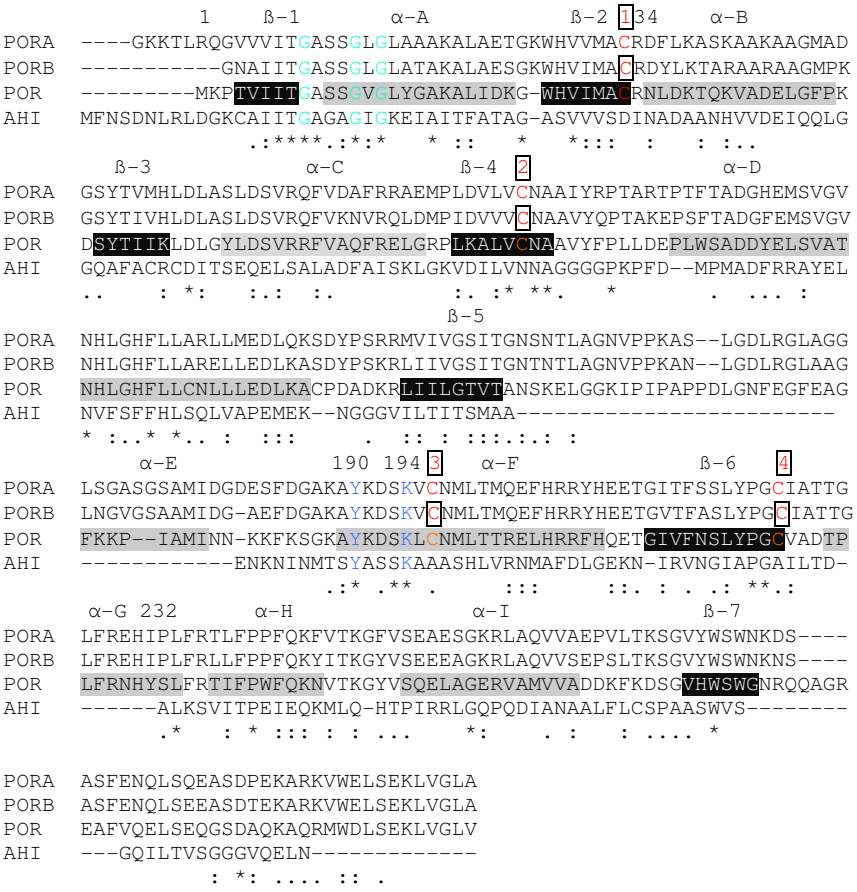

Fig. 1 Primary amino acid sequences of the PORA and PORB of barley as well as POR of *Synechocystis* and 7-α-hydroxysteroid dehydrogenase (AHI) of *E. coli*. α-helices (marked α-A-I) and ß-strands (marked ß-1-7) are given in grey and black boxes, respectively, and refer to POR of *Synechocystis* (see Townley et al., 2001, for details). The GXXXGXG motif (light blue colour) and YXXXK motif (dark blue colour) implicated in NADPH binding, as well as the conserved Cys residues (boxed in red and referred to as Cys1, Cys2, Cys3 and Cys4) are highlighted. Numbering of key amino acid residues refers to the cyanobacterial enzyme (Arg34, Tyr190, Lys194; Townley et al., 2001). Tyr190 and Lys194 correspond to Tyr275 and Lys279 of the pea enzyme (Lebedev et al., 2001). Asterisks mark conserved amino acid residues, dots and double points give column consensus, and dashes indicate gaps introduced during sequence alignment. Sequence comparison was made using standard computer programs.

Crystallographic data are not available for either the PORA or PORB nor for the resulting LHPP complex. Detailed comparisons of cDNA-based predicted amino acid sequences have unveiled a weak relationship of the barley PORA and PORB, pea POR, as well as POR of *Synechocystis* (synPOR) to the family of short chain dehydrogenases (SCDs) however (Baker, 1994; Dahlin et al., 1999; Townley et al., 2001)(Fig. 1). For the SCDs, X-ray structures have been obtained, allowing homology models to be constructed and tested by *in vitro*-mutagenesis. It could be demonstrated that all POR enzymes studied thus far share the presence of the

universally conserved Rossmann fold representing the adenine binding cleft with the SCDs. Amino acid residues already established as being necessary for structural and functional reasons in the SCDs are also conserved in POR (Fig. 1) and have proven roles in enzyme catalysis (Wilks & Timko, 1995; Lebedev & Timko, 1999; Lebedev et al., 2001)). It has been shown for example that two highly conserved Tyr and Lys residues (Tyr 190 and Lys 194 in synPOR, corresponding to Tyr275 and Lys279 in pea POR) establish part of the catalytic pocket and participate in the proper coordination of NADPH (Wilks & Timko, 1995; Lebedev & Timko, 1999; Lebedev et al., 2001). During the catalytic mechanism, Tyr190/275 donate a proton to the C17-C18 double bond at ring D of the macrocycle once the hydrogen has been perceived from NADPH. To allow deprotonation, Lys194/279 is presumed to lower the pK_a of Tyr190/275 (Lebedev & Timko, 1999; Lebedev et al., 2001).

The question of how the pigment substrate Pchlide is bound to the POR polypeptide is still unresolved. Previous inhibitor experiments using SH-modifying agents such as *N*-phenylmaleimide have implicated a role of 1 or 2 Cys residues in pigment binding (Griffiths, 1975; Oliver & Griffiths, 1981; Townley et al., 2001). Four Cys residues are present in the mature PORA and PORB of barley which are placed at equivalent positions of the polypeptide chains (Fig. 1). This conservation is suggestive of their involement in pigment binding and/or catalysis and motivated us to carry out a detailed mutagenesis analysis. The cDNA encoding PORB was modified to engineer point mutations in the different Cys residues. The resulting cDNAs were used to produce protein by coupled transcription/translation. In turn, the various generated PORB mutant proteins were tested with regard to pigment binding, *in vitro*-import into isolated plastids, as well as the establishment of higher molecular mass complexes. We demonstrate that two distinctive Pchlide binding sites are present in PORB: one in the catalytic cleft of the enzyme, and the other at the periphery of the predicted globular structure where it participates in PORA:PORB interactions and thereby protects the imported enzyme from proteolysis.

Materials and methods

Plant growth

Seeds of *Hordeum vulgare* L. cultivar Carina were germinated on moist vermiculite under continuous illumination provided by fluorescent bulbs for 5 days. Seeds of the PORB-deficient ($PORB^-$) line of *Arabidopsis thaliana* (Salk At4g27440)(Alonso et al., 2003) were purchased from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/) and germinated on agar medium in darkness for 4.5 days. Isolation and genetic characterization of this line is described in Fig. 9 (see supplementary material).

Construction of (Cys_x→Ala)-PORB mutant proteins

DNAs encoding the different (Cys_x→Ala)-PORB mutant proteins lacking their transit peptides for plastid import were generated in a Promega GeneEditor™ *in vitro* site-directed mutagenesis system using the following primers: Oligo-S: 5'-pCCGCGAGACCCACCCTTGGAGGCTCCAGATTTATC-3' plus M1 (Cys1→Ala)-PORB): 5'-pGTCATCATGGCGAGCCGCGACTACCTC-3'; Oligo-S plus M2 (Cys2→Ala)-PORB): 5'-pGACGTCGTCGTCAGCAAGGCCGCCGTG-3'; Oligo-S plus M3 (Cys3→Ala)-PORB): 5'-pGACAGCAAGGTGAGCAACATGCTGACC-3'; Oligo-S plus M4 (Cys4→Ala)-PORB): 5'-pCTCTACCCGGGGAGCATCGCCACCACG-3'. Generation of [(Cys3+4)]-PORB double mutant protein was achieved by restriction enzyme digestion and religation of DNA fragments of the clones encoding the (Cys3→Ala)-PORB and (Cys4→Ala)-PORB mutant proteins. For construction of transit peptide-containing proteins, the amplified DNAs were fused to DNA coding for transB, the transit peptide of pPORB, which had been produced as described previously (Reinbothe et al., 1997). The identity of all of the different clones was confirmed by DNA sequencing, using the gel system described by Sanger et al. (1977), or by GATC Biotech (Constance, Germany).

In vitro protein import into isolated plastids

^{35}S-precursors were synthesized in a TNT wheat germ coupled *in vitro* transcription/translation system (Promega) using the different generated clones (Krieg & Melton, 1984). Precursors were denatured with 8 M urea and diluted to a final 0.2 M urea concentration immediately before use. Protein import was studied as described previously (Reinbothe et al., 1995a; Reinbothe et al., 1995b; Reinbothe et al., 1995c) using either barley etioplasts and chloroplasts, respectively, or *Arabidopsis* etioplasts of the PORB^{-} line described previously. For each of the different precursors, 10 samples were prepared and run in parallel: 5 samples were incubated in darkness and 5 in white light. In either case, 1 assay was immediately stopped at time point zero by the addition of either one third volume of 20% (v/v) trichloroacetic acid (TCA) or an equal volume of doubly-concentrated SDS sample buffer (Laemmli, 1970). Two each out of the four remaining dark and light samples, respectively, were incubated in the presence of phosphate-buffered 5-aminolevulinic acid (5-ALA), whereas the others were mock-incubated with phosphate buffer for 15 min. After import, intact plastids were repurified on Percoll. One each of the two identical samples was treated with thermolysin (Cline et al., 1984), whereas the other was left untreated. Protein was extracted with TCA, washed with acetone, ethanol and diethylether, and separated by SDS-PAGE (Laemli, 1970). Protein detection was made by autoradiography.

Reconstitution of POR-pigment complexes

Equal amounts of the different ^{35}S-labeled (Cys1→Ala)-PORB, (Cys2→Ala)-PORB, (Cys3→Ala)-PORB, (Cys4→Ala)-PORB as well as ([Cys3+4]→Ala)-PORB mutant proteins were supplemented with 0.5 mM NADPH and 10 µM Pchlide *a* (Reinbothe et al., 2003a). By analogy, ^{35}S-PORA was incubated with Pchlide *b* and NADPH and reconstutued into the ternary state (Reinbothe et al., 2003a). POR-pigment-NADPH complexes formed during a 15-min dark incubation were depleted of non-protein-bound pigments by gel filtration on Sephadex G15 equilibrated in assay buffer (50 mM Hepes-KOH, pH 7.2, 2.5 mM $MgCl_2$, 1 mM EDTA and 8.5 mM methionine) either containing or lacking 350 mM sucrose (Reinbothe et al., 1990). POR-pigment

complexes eluted with the flowthrough in turn were kept in darkness or exposed to white light for 15 min. Enzymatic product formation occurring during this incubation was monitored fluorimetrically using acetone-extracted pigments and established procedures (Reinbothe et al., 2003a).

In vitro and in organello-assembly of PORA:PORA supracomplexes

For the reconstitution of higher molecular mass complexes containing the various PORB mutant proteins, equimolar amounts of the reconstituted ^{35}S-PORA-Pchlide *b*-NADPH and ^{35}S-PORB-Pchlide *a*-NADPH or (Cys_X→Ala)-PORB pigment complexes were incubated in the dark for 15 min and subjected to gel filtration on Sephadex G100 (Reinbothe et al., 1999)(Amersham Pharmacia Biotech, www.apbiotech.com). PORA:PORB-supracomplexes eluted with the flowthrough were identified by radioactivity measurements and were in turn supplemented with a mixed galacto- and sulfolipid fraction containing monogalactosyl diacylglycerol, digalactosyl diacylglycerol, and sulfoquinovosyl diacylglycerol (58:36:6 mol%, see Reinbothe et al., 1999). Lipid-containing ^{35}S-PORA:PORB supracomplexes were separated by non-denaturing polyacrylamide gel electrophoresis and detected by their blue light-induced pigment autofluorescence, autoradiography, and Western blotting using an alkaline phosphatase-based system (Reinbothe et al., 2003a). *In organello* assembly assays were performed in a similar way but after import of the respective ^{35}S-labeled precursor proteins into etioplasts of the PORB$^-$ line of *Arabidopsis thaliana*.

Protease treatment of POR-pigment complexes *in vitro*

Protease treatment of ^{35}S-POR-pigment-NADPH complexes was carried out as described (Reinbothe et al., 1995a; Reinbothe et al., 1995b). Briefly, ^{35}S-POR-substrate-NADPH complexes were reconstituted and gel-filtered in the presence of 350 mM sucrose. Aliquots of the flowthrough then were kept in darkness or exposed to white light. The different ^{35}S-POR-substrate-NADPH and ^{35}S-POR-product(-$NADP^+$) complexes subsequently were added to a plastid protease mixture prepared from isolated barley chloroplasts (Reinbothe et al., 1995c). After different time

intervals of incubation in darkness, aliquots were withdrawn and diluted with proteinase inhibitor cocktail containing 10 µg mL^{-1} antipain and 1 µg mL^{-1} pepstatin, which efficiently block the POR-degrading stromal protease (Reinbothe et al., 1995c). After precipitation with trichloroacetic acid, protein was washed, separated by SDS-PAGE and detected by autoradiography.

Results

PORB mutant proteins were engineered by *in vitro*-mutagenesis in which Cys104, Cys166, Cys276 or Cys303 (henceforth referred to as Cys1, Cys2, Cys3 or Cys4, respectively) were exchanged by Ala residues (Fig. 8A, see supplementary material). For each PORB mutant protein, two types of DNA were constructed: one encoding the precursor protein containing the transit peptide needed for chloroplast import, and one encoding the mature protein lacking its transit peptide. The resulting mutant DNAs were sequenced and subsequently used for coupled *in vitro* transcription/translation. Results in Figure 8 at the end of the manuscript show that the resulting ^{35}S-PORB mutant proteins displayed the expected molecular masses of 46 kDa (precursors, ^{35}S-pPORB) and 38 kDa (mature proteins, ^{35}S-PORB) upon denaturing SDS-PAGE.

The various constructed (Cys_x→Ala)-PORB mutant precursor proteins [(Cys_x→Ala)-pPORB)] were tested for their capability to be imported into isolated chloroplasts. Barley chloroplasts were isolated from light-grown plants and depleted of endogenous energy sources by keeping the organelles on ice for 1 h in darkness (Reinbothe et al., 1995b). The plastids in turn were added to the various ^{35}S-(Cys→Ala)-PORB precursors in assay mixtures supplemented with 2 mM Mg-ATP. Incubations were carried out either in the presence or absence of the Pchlide precursor 5-ALA. For each precursor, two different types of incubation were performed. Half of the assays was incubated in darkness, whereas the other halves were incubated in white light. We assumed that the light incubations would allow us testing the stability of imported proteins after they had bound and converted Pchlide and NADPH into their respective products. Previous work has shown that PORB binds Pchlide and NADPH shortly after import and forms protease-resistant PORB-Pchlide-NADPH ternary complexes in darkness (Reinbothe et al., 1995a). Upon

illumination, these complexes converted Pchlide to Chlide and were subsequently proteolytically degraded (Reinbothe et al., 1995a). A light-induced, multi-component plastid protease is involved in POR breakdown (Reinbothe et al., 1995c). This so-called POR degrading protease is absent from etioplasts and does not attack PORB-substrate complexes, such as PORB-Pchlide and PORB-Pchlide-NADPH, but efficiently degrades POR-product complexes formed in chloroplasts upon illumination (Reinbothe et al., 1995a; Reinbothe et al., 1995c). In the latter case, the rate of POR degradation even exceeded that measured for the naked POR polypeptide lacking its substrates and products (Reinbothe et al., 1995c).

Figure 2 shows the results of a representative import experiment. It revealed that non-mutagenized PORB was readily taken up by isolated barley chloroplasts (Fig. 2A). As shown previously (Reinbothe et al., 1995a; Reinbothe et al., 2000), PORB enters the plastids through the general protein import apparatus comprising core translocon proteins of 159, 75 and 34 kDa (called Toc159, Toc75 and Toc34, respectively; see Bauer et al., 2001, for review). Import was consequently not dependent on Pchlide and similar mature PORB protein levels were found in darkness in chloroplasts which contained or lacked Pchlide produced by 5-ALA feeding (Fig. 2A). In white light, no mature PORB protein was detectable in either case (Fig. 2B). At first glance this result suggested that white light inhibited import. However, upon careful inspection of the autoradiograms from several independent experiments we noted that the precursor levels had similarly declined as those observed in the dark samples. We therefore reinterpreted our results as evidence for the degradation of the imported, processed enzyme inside chloroplasts. Most likely, this degradation was triggered by enzymatic Chlide formation occurring after import.

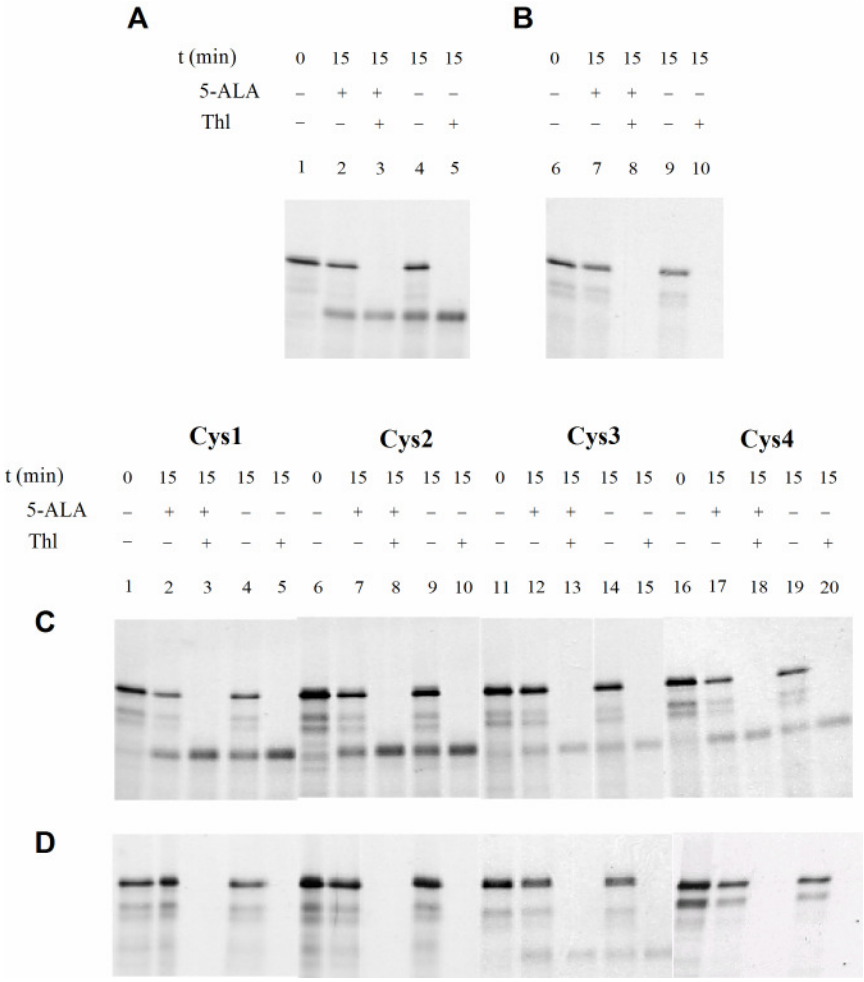

Fig. 2 Import of authentic pPORB and (Cys_xAla)-pPORB mutants into isolated barley chloroplasts. Chloroplasts were isolated from light-grown barley seedlings, energy-depleted and added to ^{35}S-labeled pPORB (A and B), (Cys1→Ala)-pPORB, (Cys2→Ala)-pPORB, (Cys3→Ala)-pPORB and (Cys4→Ala)-pPORB mutant proteins (C and D) which had been produced as described in Fig. 8B. Import was studied at 23°C in darkness (A and C) or white light (B and D) in the presence of 2 mM Mg-ATP in assay mixtures containing 5-ALA dissolved in phosphate buffer or phosphate buffer alone. After import, intact plastids were repurified on Percoll. Protein was extracted from intact plastids with trichloroacetic acid, washed and resolved by SDS-PAGE. Protein detection was made by autoradiography. Results refer to three independent experiments. The autoradiogram shows precursor and mature PORB protein levels in chloroplasts containing (+ 5-ALA) or lacking (- 5-ALA) the exogenous 5-ALA-derived Pchlide after their post-import treatment with (+ Thl) or without (- Thl) thermolysin. Lanes 1, 6, 11 and 16 show respective input standards.

Replacement of Cys1 or Cys2 by Ala residues had no detectable effect on the import of the engineered (Cys1→Ala)-PORB and (Cys2→Ala)-PORB mutant proteins in darkness. In either case, the precursor levels declined similarly during the incubation and the respective mature PORB proteins appeared (Fig. 2C, lanes 2 and 4 as well as 7 and 9). These results indicated that the precursors had been imported and

processed to mature size. That the mature (Cys1→Ala)-PORB and (Cys2→Ala)-PORB were localized inside chloroplasts was proven by their resistance against thermolysin (Fig. 2C, lanes 3 and 5 as well as 8 and 10). The finding that mature (Cys1→Ala)-PORB and (Cys2→Ala)-PORB accumulated in a form which was resistant against stromal proteases present inside chloroplasts confirmed that they were complexed with Pchlide and/or NADPH. We tentatively concluded that Cys1 and Cys2 were not involved in Pchlide and/or NADPH binding.

Substitution of Cys3 or Cys4 by Ala residues differentially influenced the import of the (Cys3→Ala)-pPORB and (Cys4→Ala)-pPORB mutant proteins (Fig. 2C, lanes 11-15 and 16-20, respectively; compare *upper bands*). In case of (Cys4→Ala)-pPORB, the precursor amount dropped to levels comparable to those of the wild-type PORB and the (Cys1→Ala)-pPORB and (Cys2→Ala)-pPORB mutant proteins. In case of (Cys3→Ala)-pPORB, the level of precursor declined less than that of wild-type PORB and the (Cys1→Ala)-pPORB and (Cys2→Ala)-pPORB mutant precursors. At the same time, less mature (Cys3→Ala)-PORB and (Cys4→Ala)-PORB were detectable inside chloroplasts after the import reactions (Fig. 2C, lanes 11-15 and 16-20, respectively; compare *lower bands*). Interestingly, tiny amounts of mature (Cys3→Ala)-PORB remained detectable in chloroplasts when the import reactions were performed in white light (Fig. 2D, lanes 11-15 *lower band*). By contrast, (Cys4→Ala)-PORB and the other tested mutant proteins vanished from chloroplasts after white light incubation as did wild-type PORB protein (Fig. 2D). Together the results suggested differential effects of the Cys3 and Cys4 mutations on import, substrate binding and/or the stability of the PORB enzyme inside chloroplasts.

In order to more precisely determine the stability of the (Cys3→Ala)-PORB and (Cys4→Ala)-PORB, *in vitro*-reconstitution experiments were performed. Mature (Cys3→Ala)-PORB and (Cys4→Ala)-PORB as well as (Cys1→Ala)-PORB and (Cys2→Ala)-PORB lacking their chloroplast transit peptides for import were produced from corresponding cDNA clones as described in Fig. 8C (see supplementary material) and incubated with Pchlide *a* and NADPH. Reconstituted (Cys_x→Ala)-PORB-pigment-NADPH complexes in turn were subjected to gel filtration on Sephadex G15 equilibrated in assay buffer either containing or lacking sucrose (see Materials and methods). We reasoned that inclusion of sucrose would allow identifying also low-affinity pigment binding sites. Sucrose and other sugars have documented stabilizing effects on protein and membrane assemblies *in vitro* and *in*

vivo (Crowe & Crowe, 1984; Crowe et al., 1984). Pilot experiments using the non-mutagenized PORB identified 2 Pchlide *a* binding sites per enzyme monomer: one high affinity binding site and one low affinity binding site (Table 1). Identical results were obtained for the (Cys1→Ala)-PORB and (Cys2→Ala)-PORB mutant proteins (Table 1). By contrast, only 1 Pchlide *a* molecule was present in case of the (Cys3→Ala)-PORB and (Cys4→Ala)-PORB monomers: a high affinity Pchlide molecule bound to (Cys4→Ala)-PORB and a low affinity pigment molecule bound to (Cys3→Ala)-PORB (Table 1).

Table 1. Pigment binding to (Cys_x)-PORB mutant proteins.

	Pigment [pmol per µg POR protein] [1)]	
	+ Sucrose	- Sucrose
PORB	52.2±0.15	26.1±0.04
(Cys1→Ala)-PORB	52.4±0.12	26.2±0.06
(Cys2→Ala)-PORB	52.0±0.16	26.0±0.12
(Cys3→Ala)-PORB	26.2±0.04	n.d.
(Cys4→Ala)-PORB	26.2±0.06	26.0±0.02

^{35}S-(Cys_x)-PORB-pigment-NADPH complexes were reconstituted and subjected to gel filtration on Sephadex G15 in the absence or presence of 350 mM sucrose. As control, the native, non-mutagenized PORB was used. ^{35}S-(Cys_x)-PORB-pigment-NADPH complexes eluted with the flow-through were extracted with acetone and quantified by HPLC or fluorescence spectroscopy at an excitation wavelength of 431 nm, using known absorption and emission coefficients (Reinbothe et al., 2003a; Reinbothe et al., 2003b). Standard deviations calculated from five independent experiments are indicated; n.d., defines not detectable pigment levels.
[1)]One µg of PORB corresponds to 26.26 pmoles of enzyme.

To further assess the functional state of bound pigments, PORB-pigment complexes that had been depleted of non-tightly-bound pigments by gel filtration in the absence of sucrose were exposed to white light. Parallel samples were kept in darkness. Pigments then were extracted with acetone and subjected to fluorescence spectroscopy. In Figure 3 are shown representative room temperature emission spectra of pigments extracted from the various (Cys_x→Ala)-PORB-substrate and (Cys_x→Ala)-PORB-product complexes after their light (L, red graphs) and dark (D, blue graphs) incubations. At an excitation wavelength of 431 nm, Pchlide *a* has a

fluorescence emission maximum at 627 nm, whereas Chlide *a* has an emission maximum at 665 nm. The spectra reveal that (Cys1→Ala)-PORB, (Cys2→Ala)-PORB and (Cys4→Ala)-PORB were all able to bind Pchlide *a* and NADPH (Fig. 3A, B and D, respectively, *Darkness*) and to convert Pchlide *a* to Chlide *a* upon illumination (Fig. 3A, B and D, respectively, *Light*). By contrast, (Cys3→Ala)-PORB was unable to establish photoactive ternary complexes with Pchlide *a* and/or NADPH, and consequently no Chlide *a* was produced (Fig. 3C, compare *Darkness* versus *Light*).

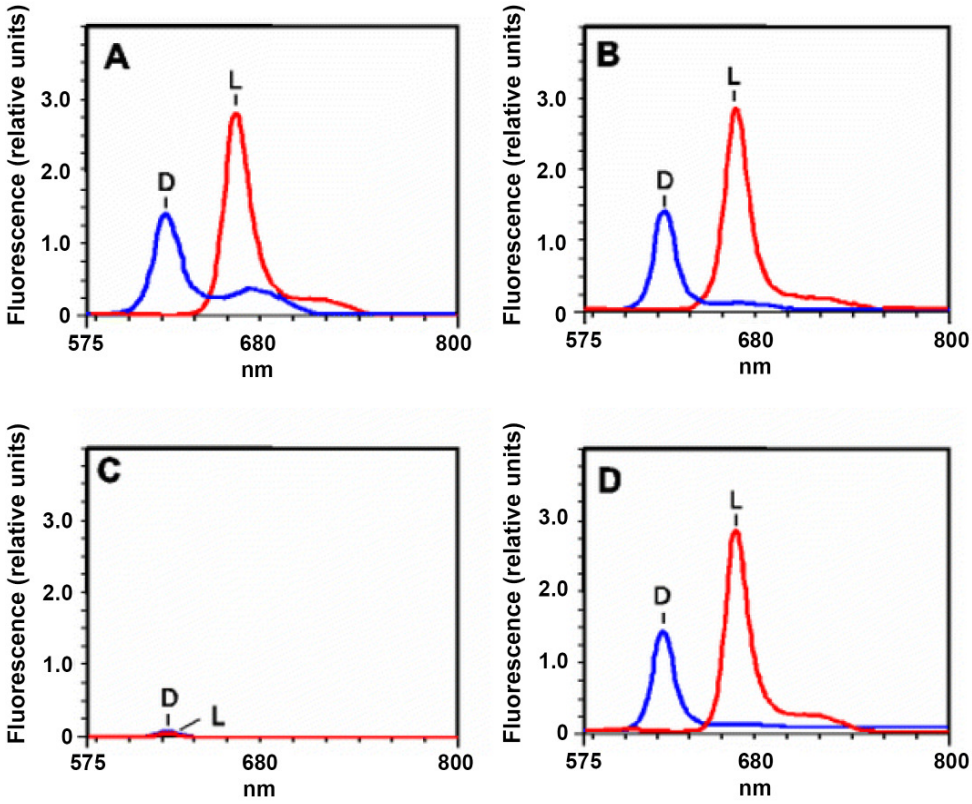

Fig. 3 Pigment binding and conversion characteristics of (Cys_x→Ala)-PORB mutant proteins. Mutant PORB-proteins were produced as described in Fig. 8C and reconstituted into PORB-pigment-NADPH ternary complexes with Pchlide *a* and NADPH. After a step of gel filtration on Sephadex G15 equilibrated in assay buffer lacking sucrose, aliquots of PORB-pigment-NADPH ternary complexes eluted with the flowthrough were kept either in darkness or exposed to white light. Then the assays were extracted with acetone and subjected to fluorescence spectroscopy at an excitation wavelength of 431 nm. (A) Fluorescence emission spectra of pigments extracted from the (Cys1→Ala)-PORB after incubation in darkness (*D*, *blue line*) or white light (*L*, *red line*). (B)-(D), as (A), but showing spectra of pigments extracted from (Cys2→Ala)-PORB, (Cys3→Ala)-PORB and (Cys4→Ala)-PORB, respectively. *Note* that the (Cys1→Ala)-PORB, (Cys2→Ala)-PORB and (Cys4→Ala)-PORB mutant proteins bind apparently the same amounts of Pchlide *a* as that bound by the non-mutagenized PORB (not shown, but see Table 1). By contrast, (Cys3→Ala)-PORB is unable to bind Pchlide *a* under the employed assay conditions. Results refer to five independent *in vitro*-reconstitution experiments.

To probe the conformation of the various (Cys_x→Ala)-PORB mutant proteins, protease digestion experiments were performed. POR-degrading protease was prepared from barley chloroplasts (Reinbothe et al., 1995c) and added to reconstituted and gel-filtered (Cys_x→Ala)-PORB-substrate and (Cys_x→Ala)-PORB-product complexes. In contrast to the previous experiment, all operations were carried out in the presence of sucrose in order to maintain also loosely bound pigments. As controls, the naked (Cys_x→Ala)-PORB-polypeptides devoid of any substrates and products were incubated and gel-filtered identically.

Figure 4 depicts time courses of PORB protein breakdown for the reconstituted (Cys_x→Ala)-PORB-substrate and (Cys_x→Ala)-PORB-product complexes, as well as naked (Cys_x→Ala)-PORB mutant proteins devoid of their substrates and products. The different curves highlight that (Cys1→Ala)-PORB (panel A) and (Cys2→Ala)-PORB (panel B) were stable in the presence of Pchlide and NADPH. After prior light incubations provoking enzymatic Chlide formation, both (Cys1→Ala)-PORB and (Cys2→Ala)-PORB were degraded. By contrast, (Cys3→Ala)-PORB (panel C) and (Cys4→Ala)-PORB (panel D) showed different behaviours. Even in the presence of Pchlide and NADPH either PORB mutant protein was degraded. White light exposure accelerated this decline for (Cys4→Ala)-PORB, but had no effect on (Cys3→Ala)-PORB.

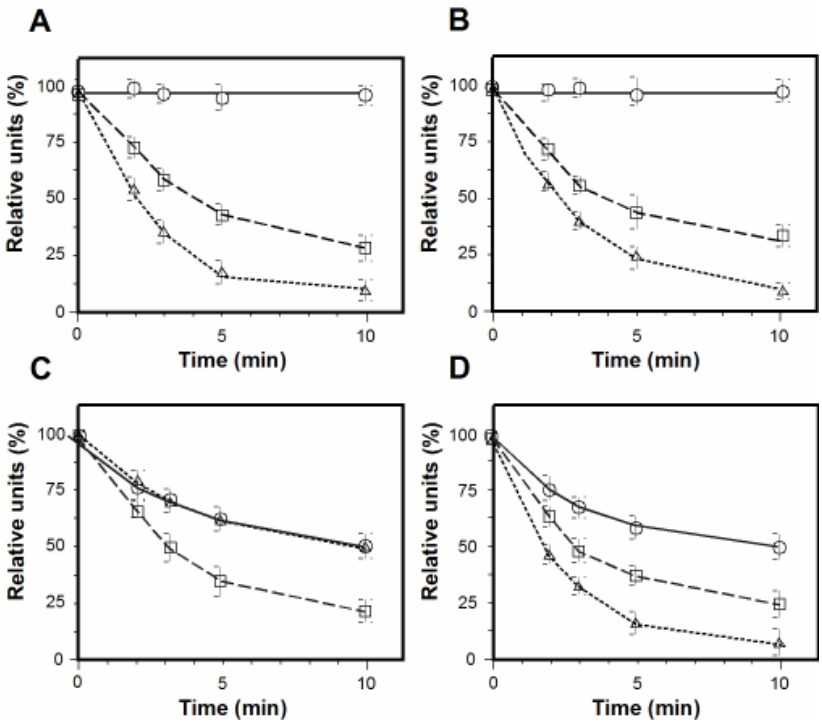

Fig. 4 Protease sensitivity of reconstituted POR-pigment complexes. PORB-Pchlide *a*-NADPH complexes that contained equal amounts of the different ^{35}S-labelled (Cys$_x$→Ala)-PORB mutant proteins were reconstituted as described in Fig. 3 and subjected to gel filtration on Sephadex G15 equilibrated in assay buffer containing 350 mM sucrose. After gel filtration, aliquots of the recovered PORB-Pchlide *a*-NADPH complexes were kept in darkness or exposed to white light, before being incubated with a stromal protease preparation from barley plastids. As controls, (Cys$_x$→Ala)-PORB mutant proteins devoid of substrates or products were produced and gel-filtered identically. After various time intervals of incubation in darkness, aliquots were withdrawn, protein precipitated with trichloroacetic acid, and ^{35}S-labeled proteins were run by SDS-PAGE, detected by autoradiography and quantified. Quantification refers to identical amounts of input radioactivity (8.000 dm), set as 100. Curves show time courses of the levels of the naked PORB proteins devoid of their substrates and products (dashed lines) as well as PORB contained in the respective PORB-Pchlide *a*-NADPH (solid lines) and PORB-Chlide *a*-NADP$^+$ complexes (dotted lines) for (Cys1→Ala)-PORB (A), (Cys2→Ala)-PORB (B), (Cys3→Ala)-PORB (C) and (Cys4→Ala)-PORB (D). Error bars derived from 3 independent experiments are indicated.

The ability of the (Cys$_x$→Ala)-PORB mutant proteins to assemble into larger PORA:PORB complexes (LHPP) was tested subsequently. Equimolar amounts of reconstituted ^{35}S-PORA-Pchlide *b*-NADPH and ^{35}S-PORB-Pchlide *a*-NADPH or (Cys$_x$→Ala)-PORB-Pchlide *a*-NADPH complexes were mixed and, after a 15-min incubation in darkness, subjected to a further step of gel filtration on Sephadex G100 (Reinbothe et al., 1999). Consistent with previous results (Reinbothe et al., 1999), this step led to a depletion of unassembled PORA- and PORB-pigment-NADPH ternary complexes (data not shown). PORA:PORB supracomplexes eluted with the flowthrough in turn were supplemented with a lipid mixture containing monogalactosyl

diacylglycerol, digalactosyl diacylglycerol, and sulfoquinovosyl diacylglycerol (58:36:6 mol%), which had been prepared from pigment-free prolamellar bodies of barley etioplasts (Reinbothe et al., 1999). The resulting lipid-containing ^{35}S-PORA:PORB supracomplexes were subjected to non-denaturing PAGE and detected by their blue light-induced pigment autofluorescence, Western blotting using POR antiserum, and autoradiography (Reinbothe et al., 2003a).

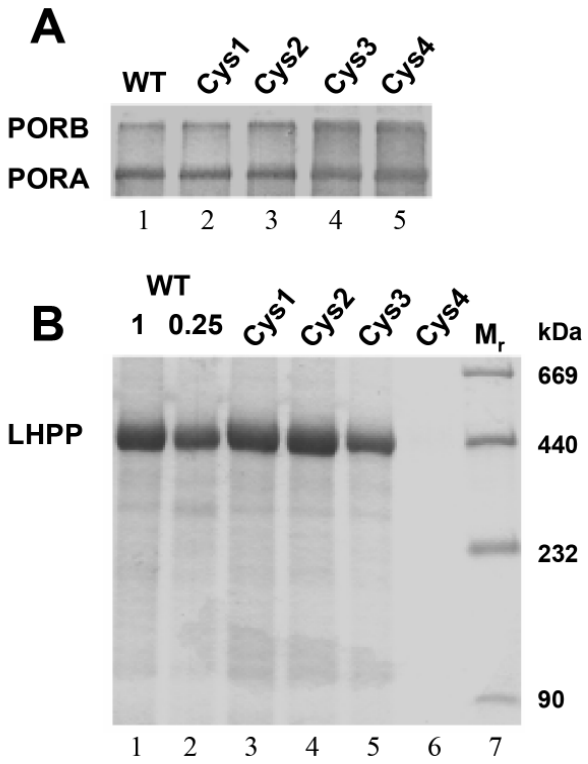

Fig. 5 *In vitro*-reconstitution of (Cys$_x$→Ala)-PORB-containing PORA:PORB supracomplexes. (Cys$_x$→Ala)-PORB-Pchlide *a*-NADPH complexes that contained equal amounts of the different ^{35}S-(Cys$_x$→Ala)-PORB mutant proteins were reconstituted and gel-filtered as described in Fig. 3 and mixed with equimolar amounts of reconstituted ^{35}S-PORA-Pchlide *b*-NADPH complexes (A). After 15-min incubations in darkness, the resulting PORA:PORB complexes were subjected to a second gel filtration step on Sephadex G100. Established supracomplexes eluted with the flowthrough were supplemented with a mixed galacto- and sulfolipid fraction from isolated, pigment-depleted prolamellar bodies of barley etioplasts. Lipid-containing PORA:PORB supracomplexes in turn were separated by non-denaturing PAGE and detected by autoradiography (B). (A) Autoradiogram showing reconstituted PORA-pigment-NADPH and PORB-pigment-NADPH ternary complexes after their assembly and segregation into larger PORA:PORB complexes but prior to the addition of the membrane lipids. (B) Autoradiogram of PORA:PORB supracomplexes established with PORA and the wild-type PORB (lanes 1 and 2) as well as the (Cys1→Ala)-PORB (lane 3), (Cys2→Ala)-PORB (lane 4), (Cys3→Ala)-PORB (lane 5) and (Cys4→Ala)-PORB (lane 6) mutant proteins in the presence of lipids. Lanes 1 and 2 contain aliquots corresponding to 1 and 0.25 protein equivalents of reconstituted supracomplexes containing the wild-type PORB; lane 7 depicts molecular mass standards. Identical results were obtained in 4 independent experiments.

Figure 5B shows higher molecular mass complexes containing ^{35}S-PORA-Pchlide *b*-NADPH and ^{35}S-PORB-Pchlide *a*-NADPH ternary complexes (lanes 1 and 2). Higher molecular mass complexes of similar size were detectable for the radioactive (Cys1→Ala)-PORB and (Cys2→Ala)-PORB mutant proteins (Fig. 5B, lanes 3+4, respectively). By contrast, less complex was established with ^{35}S-(Cys3→Ala)-PORB (Fig. 5B, lane 5), and in fact no complexes were detectable after *in vitro*-reconstitution with ^{35}S-(Cys4→Ala)-PORB (Fig. 5B, lane 6). Loading controls confirmed that approximately equal amounts of PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH as well as (Cys_x→Ala)-PORB-Pchlide *a*-NADPH ternary complexes had been used for and segregated into the respective ≈ 5:1 stoichiometry after *in vitro*-reconstitution (Fig. 5A, and data not shown).

In organello-assembly assays were carried out to substantiate our conclusion that Cys4 played a major role in the formation of PORA:PORB supracomplexes, i.e., LHPP assembly. According to previous work, LHPP is present only in etioplasts and rapidly dissociates upon illumination (Reinbothe et al., 1999) in conjunction with the dispersal of the prolamellar body (Virgin et al., 1963; Kahn, 1968). Superimposed on this effect is the rapid depletion of PORA which is due to cumulative negative effect of light on *porA* gene transcription, *porA* mRNA abundance and PORA protein import (see Reinbothe et al., 1996, for review). In addition, the light-induced POR-degrading protease turns over freshly formed PORA-Chlide *b*(-$NADP^+$) complexes (Reinbothe et al., 1995a). Hence, in light-adapted and green plants, no PORA protein is detectable (Holtorf et al., 1995), not permitting to study the assembly of the various (Cys_x→Ala)-PORB mutant proteins *in planta*.

As an alternative approach, ^{35}S-PORB mutant proteins were imported into isolated etioplasts of an *Arabidopsis* knock-out line which lacks PORB transcript because of a T-DNA insertion in the respective *PORB* structural gene (Salk At4g27440; see Fig. 9, supplementary material). This mutant displayed no visible phenotype in white light, consistent with results of Frick et al. (2003) using a similar $PORB^-$ mutant line. Light-adapted Arabidpsis plants express a third POR enzymes called PORC (Oosawa et al., 2000; Su et al., 2001; Pattanayak and Tripathy, 2002) that seems to functionally replace PORB in the knock-out line (Frick et al., 2003). Western blot experiments confirmed that similar levels of the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase were present in chloroplasts of $PORB^-$ and wild-type plants (Fig. 6A, lanes 1 and 2, *LSU* and *SSU*, respectively). By contrast, no

PORB protein was detectable in either etioplasts (Fig. 6A, lanes 1 and 2) or chloroplasts (data not shown). Interestingly, slightly elevated levels of PORA were observed in PORB$^-$ *versus* wild-type etioplasts (Fig. 6A, lanes 1 and 2).

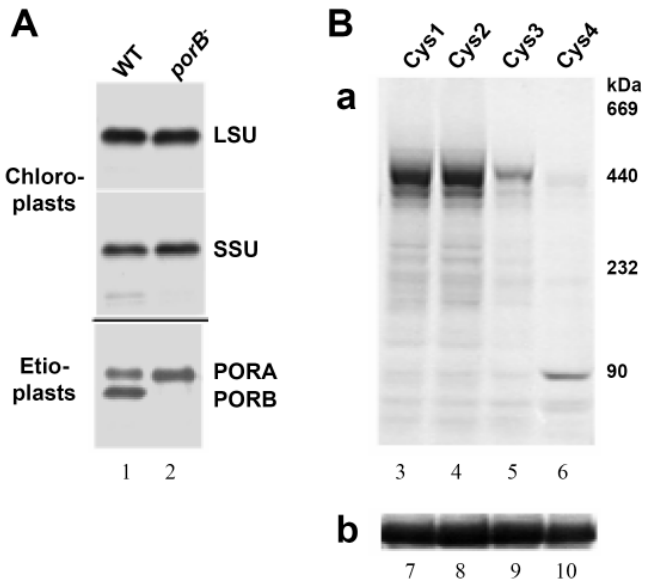

Fig. 6 *In organello*-assembly of (Cys_x→Ala)-PORB mutant proteins. Import reactions were performed for the various (Cys_x→Ala)-pPORB mutant precursor proteins as described in Fig. 2 but with etioplasts from a PORB-deficient *Arabidopsis* line. After import, the plastids were lysed and membrane fractions recovered by centrifugation and solubilized with 1.3 % decyl maltoside. Membrane proteins in turn were analyzed by non-denaturing PAGE and higher molecular mass complexes containing the different ^{35}S-PORB mutant proteins were detected by autoradiography (B, panel a). From a parallel batch of samples, total plastid protein was prepared and subjected to denaturing PAGE and autoradiography (B, panel b) or Western blotting (A). (A) Western blot the large subunit (LSU) and small subunit (SSU) levels of ribulose-1,5-bisphosphate carboxylase/oxygenase in chloroplasts and of POR-related proteins in etio-plasts of wild-type (lane 1) and PORB$^-$ (lane 2) plants. (B, panel a) Detection of ^{35}S-PORB: PORA supracomplexes in membrane fractions containing (Cys1→Ala)-PORB (lane 3), (Cys2→Ala)-PORB (lane 4), (Cys3→Ala)-PORB (lane 5) and (Cys4→Ala)-PORB (lane 6) mutant proteins. (B, panel b) Detection of ^{35}S-(Cys1→Ala)-PORB (lane 7), ^{35}S-(Cys2→Ala)-PORB (lane 8), ^{35}S-(Cys3→Ala)-PORB (lane 9) and ^{35}S-(Cys4→Ala)-PORB (lane 10) in total plastid samples comprising soluble and membrane proteins after SDS-PAGE. Molecular mass standards are indicated. Identical results were obtained in 3 independent experiments.

When the non-mutagenized pPORB was imported into etioplasts of the PORB$^-$ line, a larger ^{35}S-PORB-containing complex could be detected by non-denaturing polyacrylamide gel electrophoresis of solubilized membrane fractions (data not shown). For the (Cys1→Ala)-PORB and (Cys2→Ala)-PORB mutant proteins, higher molecular mass complexes of similar size were produced (Fig. 6B, panel a, lanes 3 and 4, respectively). In case of (Cys3→Ala)-PORB, lower amounts of such

complexes were present (Fig. 6B, panel a, lane 5). For (Cys4→Ala)-PORB almost no higher molecular mass complexes containing ^{35}S-PORB were detectable after import. Small amounts of a lower molecular mass ^{35}S-PORB protein band were found (Fig. 6B, panel a, lane 6) presumably representing assembly-incompetent but membrane-bound enzyme dimers. Autoradiography of SDS-PAGE-resolved total plastid proteins confirmed that approximately equal amounts of imported ^{35}S-(Cys$_x$→Ala)-PORB mutant proteins were present (Fig. 6B, lane b, lanes 1-10).

Townley et al. (2001) put forth that His232 in synPOR (see Fig. 1) may be involved in the complexation of Pchlide. If so, PORB double mutant proteins lacking both Cys3 and Cys4 should still be able to bind Pchlide. In order to test this hypothesis, the PORB cDNA was modified to engineer a (Cys3→Ala)- and (Cys4→Ala)-PORB double mutant protein ([Cys3+4]→Ala)-PORB). This protein in turn was tested for its Pchlide binding properties, import behaviour, as well as assembly competence. As control, the wild-type PORB was used.

Figure 7 indicates that no Pchlide binding was detectable for the ([Cys3+4]→Ala)-PORB (panel A, lane 2 *versus* lane 1). When the *in vitro* protease sensitivity of the double mutant protein was examined using isolated POR-degrading protease, a drastic increase was found with respect to the naked, non-substrate complexed wild-type PORB (Fig. 7B, dotted versus dashed line). Import assays carried out in either darkness or white light revealed that ([Cys3+4]→Ala)-pPORB was readily taken up by isolated etioplasts but, as compared to the wild-type PORB, less mature enzyme accumulated in either case (Fig. 7C, lanes 2 and 3). We interpret this result as evidence for a non-specific degradation of the ([Cys3+4]→Ala)-PORB double mutant protein by plastid proteases other than the POR-degrading protease, which is absent from etioplasts (Reinbothe et al., 1995a; Reinbothe et al., 1995c). This basal proteolytic activity seems to be also present in chloroplasts, as deduced from the the lower levels of mature ([Cys3+4]→Ala)-PORB versus wild-type PORB in dark-incubated chloroplasts (Fig. 7C, lanes 2 and 3, compare panels a and b). In fact in chloroplasts that were allowed to import the ([Cys3+4]→Ala)-pPORB during a 15-min preincubation in darkness and were subsequently shifted to white light, the mature ([Cys3+4]→Ala)-PORB was rapidly degraded (data not shown). Hereby the time course of protein degradation was virtually indistinguishable from that observed in Fig. 7B, underscoring the protease hypersensitivity of the ([Cys3+4]→Ala)-PORB double mutant protein.

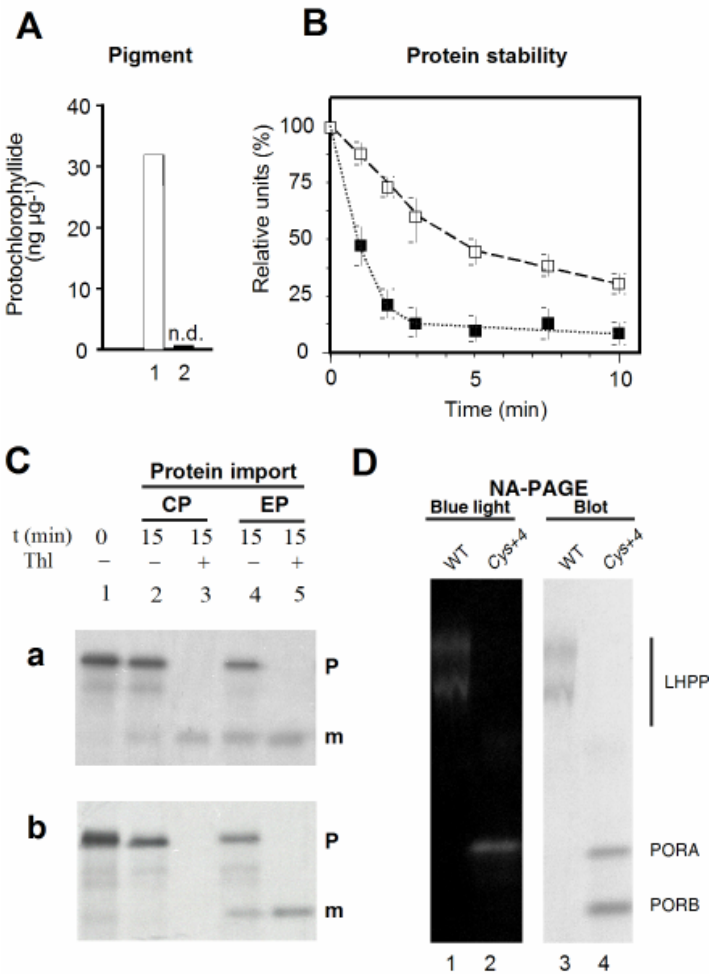

Fig. 7 Pigment binding, plastid import, and assembly competence of ^{35}S-([Cys3+4]→Ala)-PORB double mutant. A double mutant PORB protein was generated lacking both Cys3 and Cys4 and radiolabeled by coupled *in vitro*-transcription/translation. As control, wild-type PORB was produced identically. (A) Pigment binding to wild-type PORB (lane 1) and ([Cys3+4]→Ala)-PORB (lane 2), determined as described in Fig. 3. (B) Protease sensitivity of ^{35}S-([Cys3+4]→Ala)-PORB (dotted line) and wild-type PORB (dashed line). Protease protection assays were carried out as described in Fig. 4, using non-pigment-complexed proteins. (C) Import of ([Cys3+4]→Ala)-PORB into isolated barley chloroplasts (CP, lanes 2 and 3) and etioplasts (EP, lanes 4 and 5) in darkness (a) and white light (b), performed as outlined in Fig. 2. The autoradiogram shows precursor (P) and mature (m) PORB protein levels in reisolated plastids treated with (+ Thl) or without (- Thl) thermolysin. Lane 1 shows the respective input standard at time zero. (D) *In vitro*-assembly competence of ^{35}S-([Cys3+4]→Ala)-PORB (Cys3+4) and wild-type (WT) PORB. Assembly assays were carried out as described in Fig. 5, using wild-type PORB or the ^{35}S-([Cys3+4]→Ala)-PORB double mutant protein. For ([Cys3+4]→Ala)-PORB, no higher molecular mass complexes were produced and the PORA-Pchlide *b*-NADPH ternary complex and non-pigment-complexed PORB protein were supplemented with the galacto- and sulfolipid fraction before non-denaturing PAGE. POR-pigment complexes were detected by pigment autofluorescence under blue light and Western blotting, respectively, using a POR-specific antiserum. Note the lack of lipid-containing higher molecular ^{35}S-([Cys3+4]→Ala)-PORB:PORA supracomplexes and the simultaneous detection of unassembled ^{35}S-PORA-Pchlide *b*-NADPH ternary complexes and pigment-free mutant PORB. For comparison, the migration position of reconstituted higher molecular mass complexes containing wild-type ^{35}S-PORB-Pchlide *a*-NADPH and ^{35}S-PORA-Pchlide *b*-NADPH ternary complexes is highlighted (LHPP). Error bars derived from 4 independent experiments are indicated.

As a final experiment we performed *in vitro*-assembly tests with the ^{35}S-([Cys3+4]→Ala)-PORB and ^{35}S-PORA Pchlide *b*-NADPH ternary complexes. The results are shown in Figure 7D and indicated that ([Cys3+4]→Ala)-PORB was unable to establish larger complexes with PORA (lanes 2 and 4). Due to the lack of bound pigments, no autofluorescencing PORB-Pchlide *a*-NADPH ternary complexes were detectable under blue light on the non-denaturing polyacrylamide gel, although the protein was readily detectable on the respective Western blot (Fig. 7D, compare lanes 2 and 4). Unassembled PORA-Pchlide *b*-NADPH ternary complexes, by contrast, displayed a strong red pigment fluorescence and were likewise detectable on the immunoblot. Controls performed with the pigment-complexed wild-type PORB highlighted the reconstitution of fluorescing ^{35}S-PORA:PORB supracomplexes under the conditions employed (Fig. 7D, lanes 1 and 3).

Discussion

Several different techniques have been used to study the interaction of POR with its substrate Pchlide and co-substrate NADPH. For example, electron paramagnetic resonance (EPR) spectroscopy has allowed the identification of a semi-reduced radical species of Pchlide that is formed upon absorption of a single quantum of light at temperatures below -50°C (Lebedev & Timko, 1999). Recent applications of fluorescence resonance energy transfer (FRET) techniques have allowed detecting changes occurring to the POR polypeptide during pigment and NADPH binding (Townley et al., 2001). It could be demonstrated, for example, that a single Trp residue (out of 5) in POR of *Synechocystis* (synPOR) interacts with NADPH and transferred its excitation energy onto the co-substrate (Townley et al., 2001). In the proposed homology model of synPOR, a unique Trp (Trp27) is located in the hydrophobic core of the protein and thus in a region suited for binding NADPH as well as Pchlide. Furthermore, this Trp residue is proximal to the nucleotide-binding residue Arg34 and is around 18 Å from the nicotineamide group of NADPH, well within the Förster radius required for efficient FRET (Townley et al., 2001).

In a second study, the structuring effect of the pea POR polypeptide on Pchlide was examined (Lebedev et al., 2001). Using Pchlide dissolved in Tris buffer and Pchlide bound to POR, it was observed that Tyr- and Trp-dependent changes

occurred in the excitation spectrum of Pchlide, which demonstrates energy transfer to occur between either amino acids and the porphyrin ring system (Lebedev et al., 2001).

Comparable FRET studies are not possible for Cys because this aliphatic amino acid does not absorb in the UV and blue light regions of the spectrum. As an alternative approach we used *in vitro*-mutagenesis to replace Cys1, Cys2, Cys3, Cys4 or Cys3+Cys4 by Ala residues in the PORB of barley. Import studies revealed that none of these Cys residues was necessary for the import of pPORB into isolated barley chloroplasts. Consistent with previous results obtained for the native, non-mutagenized PORB (Reinbothe et al., 1995a), (Cys1→Ala)-PORB, (Cys2→Ala)-PORB and (Cys4→Ala)-PORB were imported with similar rates into chloroplasts containing or lacking Pchlide produced by 5-ALA feeding (Fig. 2). In case of (Cys3→Ala)-PORB and ([Cys3+4]→Ala)-PORB, a slight depression of import occurred with either plastid type (Figs. 2 and 7) the reasons of which are unknown.

Replacement Cys1 or Cys2 by Ala residues had no detectable impact on the stability of imported (Cys1→Ala)-PORB and (Cys2→Ala)-PORB mutant proteins (Fig. 2). Either protein was stable in darkness, demonstrating their complexation with Pchlide and/or NADPH. Reconstitution experiments identified (Cys1→Ala)-PORB and (Cys2→Ala)-PORB to be catalytically active and to convert Pchlide *a* to Chlide *a* in a light- and NADPH-dependent fashion (Fig. 3, and data not shown). The resulting (Cys1→Ala)-PORB-Chlide(-$NADP^+$) and (Cys2→Ala)-PORB-Chlide(-$NADP^+$) complexes *in vitro* were degraded by added stromal POR-degrading protease (Fig. 4). Also *in organello*, (Cys1→Ala)-PORB and (Cys2→Ala)-PORB were rapidly turned over in white light (Fig. 2), i.e. under conditions when Chlide was produced. On the basis of these results we conclude that neither Cys1 nor Cys2 played a role in import, Pchlide and/or NADPH binding, as well as catalysis. Moreover, it is tempting to hypothesize that conformational changes similar to those occurring in the native PORB destined (Cys1→Ala)-PORB and (Cys2→Ala)-PORB to degradation.

In contrast to Cys1 and Cys2, substitution of Cys3 had a profound effect on the enzyme activity (Fig. 3). (Cys3→Ala)-PORB was unable to tightly bind and convert Pchlide *a in vitro*. Gel filtration experiments highlighted (Cys3→Ala)-PORB to be impaired in reconstituting the photoactive enzyme state with Pchlide *a* and NADPH (Fig. 3). Nevertheless the enzyme was able to establish one non-photoconvertible, low-affinity pigment binding site (Table 1).

In the structural model of synPOR, Cys196 (corresponding to Cys3 of barley PORB) is located in helix α-F (Townley et al., 2001). This region of the POR polypeptide is highly conserved in all POR enzymes (Fig. 1) and therefore may establish a similar α-helical stretch in the PORB. Secondary structure predictions made for higher plant PORs however suggest that the YxxxK motif and nearby Cys3 may not directly be part of an α-helix but just be flanked by two adjacent α-helices (Birve et al., 1996). Regardless of what the exact 2D- structure in the POR polypeptide might be, it contains Tyr190 and Lys194, the counterparts of which in the pea enzyme have established roles in catalysis (Wilks & Timko, 1995; Lebedev & Timko, 1999; Lebedev et al., 2001). We hypothesize that the flat porphyrin molecule may encounter substantial structural deformations in order to properly fit into the catalytic enzyme pocket and expose the sulfhydryl group of Cys3 to the Mg central atom of Pchlide. Data of Lebedev et al. (2001) and previous inhibitor experiments with SH-modifying compounds (Griffiths, 1975; Townley et al., 2001; Oliver & Griffiths, 1981) indeed support this view. For example, Lebedev et al. (2001) observed an initial loose binding of NADPH and Pchlide to pea POR and a subsequent induced fit to give rise to the photoactive enzyme state. In addition to being responsible for the stabilization of the co-substrate and porphyrin pigment in the photoactive state, Tyr190 donates a proton and thereby actively participates also in dark conversion of the unstable radical intermediate into the final product, Chlide *a* (Lebedev & Timko, 1999; Lebedev et al., 2001).

Cys4 in PORB of barley is located in the very end of ß-strand ß-6 (see Fig. 1) and constitutes a second, low affinity Pchlide binding site. Gel filtration assays suggest Pchlide to bind to the surface of the predicted globular structure. This binding may be supported by hydrophobic interactions. *In vitro*- and *in organello*-assembly assays illustrated that Cys4 was involved in the formation of larger PORA-PORB complexes (Figs. 5 and 6). It is temting to hypothesize that Cys4-bound Pchlide may play a role in energy transfer between neighbouring POR-pigment complexes. Energy transfer is known for many decades to take place in the prolamellar body (Smith & Benitez, 1954; Kahn et al., 1970; Mathis & Sauer, 1972; Ignatov & Litvin, 1981) where the PORA and PORB are major constituents (Dehesh & Ryberg, 1995; Holtorf et al., 1995; Reinbothe et al., 1995a).

PORB double mutants lacking both Cys3 and Cys4 were unable to bind Pchlide *in vitro* (Fig. 7). At first glance this result seems to disprove the involvement of His232 and its counterparts in pigment binding. However, it cannot be excluded for the moment that the double mutation affected the 3D structure of PORB and thereby secondarily disturbed Pchlide binding to His232. The presented protease digestion experiments, showing a hypersensitivity of the ([Cys3+4]→Ala)-PORB double mutant protein (Fig. 7), may be interpreted in this way.

What is the functional state and role of PORB *in planta*? Franck et al. (2000) analyzed transgenic plants expressing cDNAs for the PORA and PORB in sense or anti-sense orientation. Using *in vitro* room and low temperature fluorescence spectroscopy the authors reported that there would be a 1:1 stoichiometry between the amount of PORA or PORB and the amount of photoactive Pchlide, i.e., the amount of POR assembled into POR-Pchlide-NADPH ternary complexes. On the other hand, part of the total Pchlide was reported to be present in a non-photoactive form and to establish an energy transfer unit. In such units, the photoactive:photoinactive Pchlide ratio was surprisingly estimated to range from 6:1 to 13:1, depending on the different transgenic lines used. The presented *in situ* fluorescence emission and excitation analyses were not substantiated by respective HPLC and mass spectroscopy data that would have allowed identifying and measuring the amount and spectral properties of implicated pigments. Care thus must be taken when interpreting the results of Franck et al. (2000) and other previous studies relying solely on *in situ* fluorescence measurements (see Armstrong et al., 2000, for references). On the other hand, no protein data were presented to confirm specific expression of the PORA and PORB in the different generated transgenic sense and anti-sense lines. Finally, the POR-to-pigment stoichiometries were not verified for the PORA and PORB by biochemical tests. In conclusion, it remains unclear whether species-specific differences in the presence and functional state of the PORA and PORB as well as in the composition and size of the Pchlide pool in etiolated plants may have influenced data interpretation with regard to the etioplast pigment-protein complexes and inner membrane architecture as well as pigment heterogeneity *in planta.* In contrast to Franck et al. (2000) our results clearly support the view that the PORA and PORB accomplish unique functions in etiolated plants. Because PORB is also expressed in light-adapted plants, our findings shed additional light onto the mechanism by which light-adapted plants keep their

chlorophyll homoeostasis (see Reinbothe et al., 1996, for review). Work is in progress to resolve the 3D-structures of the PORA and PORB and resulting LHPP complex and to dissect their functions in plants by genetic approaches.

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Supplementary material

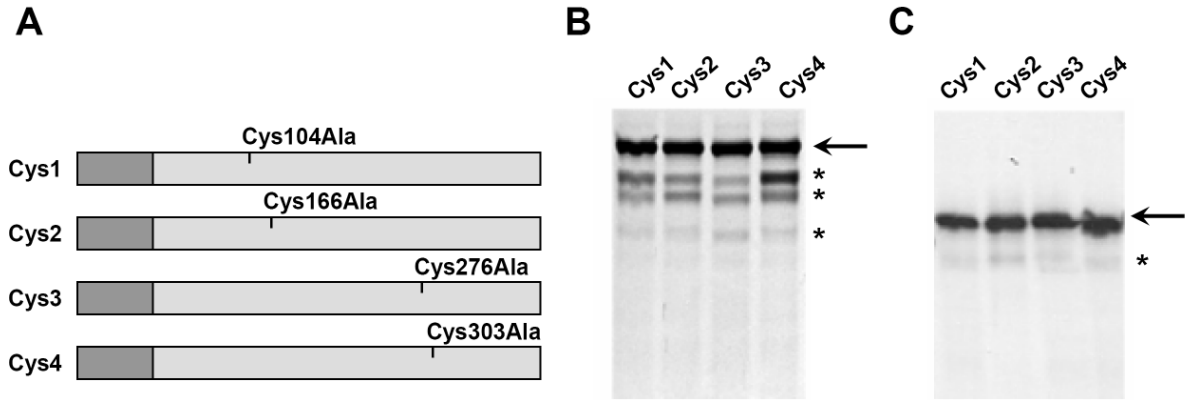

Fig. 8 Production of (Cys_x→Ala)-(p)PORB mutant proteins. (A) Schematic representation of the constructed (Cys_x→Ala)-(p)PORB mutant proteins. Dark grey and light grey columns illustrate NH_2-terminal presences (p) and mature enzymes, respectively. (B) *In vitro*-synthesis of (Cys_x→Ala)-pPORB mutant proteins by coupled transcription/translation of respective recombinant clones and detection of the radiolabeled products by SDS-PAGE and autoradiography. (C), as (B), but showing mature (Cys→Ala)-PORB mutant proteins lacking their respective transit peptides which had been produced from another set of respective DNA clones. Arrows mark main bands, whereas asterisks indicate proteins of lower sizes that may be due to either proteolysis or translation initiation at internal ATG codons.

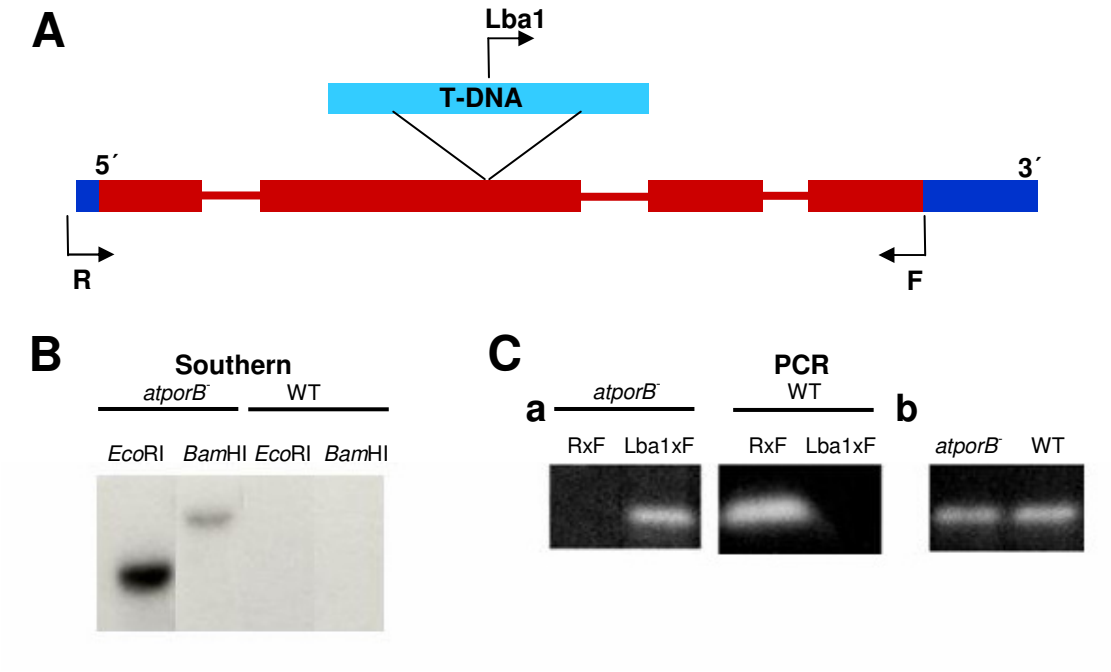

Fig. 9 Identification of the *AtporB*⁻ T-DNA insertion line (Salk At4g27440). (A) Diagram of At*porB* gene and T-DNA insertion. 5'- and 3'-untranslated regions are in blue, exons and introns are marked by boxes and solid lines, respectively. The 4.5 kb T-DNA insertion (not drawn to scale) is shown in green. R and F as well as Lba1 mark primers for PCR analyses. (B) DNA gel blot analysis of the *AtporB*⁻ line. Genomic DNA (10 µg) from homozygous *porB*⁻ plants or wild-type plants was digested with *Eco*RI and *Bam*HI, respectively, and the filter-bound DNA fragments hybridized to a DNA probe corresponding to the kanamycin-resistance gene of the T-DNA. (C) Confirmation of the T-DNA insertion by PCR, using the indicated primers (Lba1 x F, only detectable in the T-DNA insertion line: R x F, only detectable in the wild-type; apt, encoding adenine phosphoribosyl transferase (Moffat et al. (1994) Gene 143:211-216) detectable with wild-type and *AtporB*⁻ DNA).

4

Manuscript 3

Site directed mutagenesis of NADPH:protochlorophyllide oxidoreductase A: Novel insights into the structure, function and assembly of LHPP

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Site directed-mutagenesis of NADPH:protochlorophyllide oxidoreductase A: Novel insights into the structure, function and assembly of LHPP

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Summary

NADPH:protochlorophyllide oxidoreductase (POR; E.C. 1.3.33.1) belongs to the family of short chain dehydrogenases (SCDs) for which X-ray structures have been obtained. Using 7—α-hydroxysteroid reductase (AHI) as template, a homology model was constructed for PORA of barley and examined by site-directed mutagenesis. Accordingly, PORA is predicted to be a globular protein consisting of 6 α-helices and 7 ß-strands. Amino acid residues already established in AHI and other SCDs as being involved in NADPH binding and catalysis are highly conserved in PORA. The model predicts that one Cys residue may be needed for the proper coordination of protochlorophyllide (Pchlide). Here we report that two highly conserved Cys residues constitute distinctive Pchlide bindings sites. One Pchlide binding site was found in the active cleft of the enzyme where it participated in the establishment of the photoactive enzyme state. The second Pchlide binding site is most likely surface-exposed and was involved in PORA:PORB protein interactions, allowing energy transfer to take place between neighbouring POR-pigment-NADPH ternary complexes. Light absorbed by PORA-bound Pchlide *b* was transferred onto PORB-bound Pchlide *a*. Catalysis-induced changes in PORB conformation during subsequent Pchlide *a* to Chlide *a* reduction then led to the dissociation of the reconstituted light-harvesting POR:Pchlide (LHPP) supracomplex. Our results shed new light onto the structure and function of LHPP and suggest a mechanism for the light-induced disintegration of the prolamellar body.

INTRODUCTION

Plant development can proceed in two fundamentally different ways. In the presence of light, plants undergo a series of temporally and spatially coordinated changes known as photomorphogenesis (Kendrick and Kronenberg, 1994). During this process leaves expand, stems undergo radial enlargement, pigments accumulate within developing chloroplasts, and numerous metabolic processes associated with photosynthesis become operational (Ma et al., 2001; Tepperman et al., 2001; Schroeder et al., 2002). In the absence of light, a radically different growth pattern is triggered, called skotomorphogenesis. Dark-grown seedlings display an apical hook, closed and unexpanded cotyledons, and elongated hypocotyls. Dark-grown angiosperm seedlings are devoid of chlorophyll (Chl) and are incapable of photosynthetic function (Kendrick and Kronenberg, 1994). If left to grow in the dark, they will eventually die. However, upon emergence from the soil or from underneath fallen leaves, the seedlings undergo de-etiolation, i.e., cotyledons open, expand, and begin to photosynthesize. At the same time, hypocotyl elongation is inhibited and cell differentiation is initiated in vegetative meristems. Proplastids present in leaf meristems differentiate into other forms, such as etioplasts in dark-grown plants and chloroplasts in plants undergoing photomorphogenesis. Etioplasts transform into chloroplasts once the seedling de-etiolates (Virgin et al., 1963; Kahn, 1968; Kahn et al., 1970; Kirk and Tiley-Basset, 1978).

Chlorophyll and heme needed for the assembly and function of the photosynthetic apparatus are synthesized via the C5-pathway that is well established in most organisms (Beale and Weinstein, 1990; von Wettstein et al., 1995; Reinbothe and Reinbothe, 1996b; Suzuki et al., 1997; Beale, 1999; for reviews). In higher plants and algae, the C5-pathway takes place in plastids and involves both soluble and membrane-associated enzymes (Beale and Weinstein, 1990).

Tetrapyrrole synthesis is regulated by light primarily at two points in the C5-pathway: the synthesis of 5-aminolevulinic acid (5-ALA) and the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) (Reinbothe and Reinbothe, 1996b; Reinbothe et al. 1996a). In dark-grown angiosperms, Chl synthesis leads only to the formation of Pchlide, the immediate precursor of Chlide. Once a critical level of Pchlide has been reached, 5-ALA synthesis is rapidly switched off (Granick, 1950). Only after illumination when Pchlide has been photoreduced to Chlide by virtue of the

NADPH:Pchlide oxidoreductase (POR) 5-ALA synthesis does resume (Reinbothe et al., 1996a). Feedback control of Chl biosynthesis has been attributed to inhibition of 5-ALA synthesis. In analogy to its regulatory role in animals and yeast (Andrew et al., 1990; Labbe-Bois et al., 1990), heme has been proposed to act as a feedback inhibitor in plants as well (Pontoppidan and Kannangara, 1994). An additional factor was discovered in *Arabidopsis thaliana* termed FLU (FLUORESCENT) that operates independently of heme and selectively inhibits only the Mg^{2+} branch of the C5-pathway (Meskauskiene et al., 2001). In a recent report, another factor was described that negatively regulates the chlorophyll biosynthetic pathway in *Arabidopsis* (Huq et al., 2004). This factor is a bHLH transcriptional regulator related to the previously discussed phytochrome-interacting factor (PIF) 3 (Quail 2002a, b). It was proposed that PIF1 represses the expression of key enzymes operating in the C5-pathway in the dark and that this activity would be negatively regulated by light (Huq et al., 2004). Because PIF1 does not affect the expression of the FLU protein, additional compounds must be present to modulate the rate of chlorophyll biosynthesis in response to the light environment.

The second regulatory point in the Mg branch of the C5-pathway occurs at the step of Pchlide reduction. In angiosperms, Pchlide reduction is a light-dependent reaction catalyzed by POR (EC 1.3.33.1)(Griffiths, 1975, 1978; Apel et al., 1980) (Fig. 1). Both, POR and its pigment substrate Pchlide accumulate to high levels in the leaves of dark-grown plants (Boardman, 1962). Dark-stable POR-Pchlide-NADPH ternary complexes are poised such that absorption of a photon by the pigment itself leads to its immediate reduction, resulting in the formation of Chlide *a* (see Lebedev and Timko, 1998, for a review). Chlide *a* is subsequently esterified and further modified to produce Chls *a* and *b* in conjunction with the formation of functional photosynthetic membrane complexes (reviewed by Sundqvist and Dahlin, 1997).

Eukaryotic POR is nucleus-encoded. It is synthesized as a larger precursor polypeptide in the cytosol (Apel, 1981). In etiolated angiosperms, POR is localized primarily in the prolamellar body (PLB) of etioplasts (Dehesh and Ryberg, 1985; Ryberg and Dehesh, 1986; Shaw et al., 1985). In light-grown seedlings, much smaller amounts of POR are present (Barthélemy et al., 2000; Forreiter et al., 1990). Despite its light requirement for catalysis, POR activity and amount rapidly decline as a result of proteolysis (Forreiter et al., 1990). At a stage when Chl accumulation reaches its maximum rate, only trace amounts of POR are detectable.

In angiosperms such as barley (Holtorf et al., 1995), *Arabidopsis* (Armstrong et al., 1995; Oosawa et al., 2000), tobacco (Masuda et al., 2002) and *Amaranthus tricolor* (Iwamoto et al., 2001), as well as gymnosperms such as pine species (Forreiter and Apel, 1993; Skinner and Timko, 1998; Spano et al., 1992), *por* gene families were identified which encode highly conserved POR polypeptides. PORA represents the negatively light-regulated POR enzyme whose level drops as a result of the concerted effect of light at the levels of transcription, mRNA stability and plastid protein import (Reinbothe et al., 1995a-c, 1996a). PORB, the second POR protein identified in barley and *Arabidopsis* (Armstrong et al., 1995; Holtorf et al., 1995), is constitutively expressed in dark-grown, illuminated and light-adapted plants (Reinbothe et al., 1996a). A third, light-induced *por* gene was recently discovered the function of which is unknown (Oosawa et al., 2000; Su et al., 2001; Pattanayak and Tripathy, 2002).

PORA and PORB establish larger light-harvesting POR:Pchlide (LHPP) complexes in the prolamellar body of etioplasts (Reinbothe et al., 1999, 2003a-c). In several aspects LHPP resembles the major light-harvesting complex of photosystem II, LHCII (Kühlbrandt et al., 1994). By analogy to LHCII, which is composed of Chl *a* and Chl *b* (Kühlbrandt et al., 1994), LHPP contains two different types of pigments: Pchlide *a* and Pchlide *b* (Reinbothe et al., 1999, 2003a, 2003b). In contrast to LHCII, these two pigments are bound to two different polypeptides however: Pchlide *a* to PORB and Pchlide *b* to PORA (Reinbothe et al., 1999, 2003a, 2003b). In case of LHCII, a single polypeptide chain accustoms both Chl *a* and Chl *b* (Kühlbrandt et al., 1994). Monomeric LHCII further assembles into trimers (Dreyfuss and Thornbeer, 1994). Energy transfer between Chl *b* and Chl *a* can take place because of the different energy contents and life times of their excited states (Ide et al., 1987; Palsson et al., 1994). For the same reasons, energy transfer is possible in the prolamellar body comprising LHPP and bound Pchlide *b* and Pchlide *a* (Smith and Benitez, 1954; Kahn et al., 1970; Mathis and Sauer, 1972; Vaughan and Sauer, 1974; Ignatov and Litvin, 1981; Fradkin et al., 1993). LHPP additionally contains NADPH as well as galacto- and sulfolipids that alter the spectral properties of bound pigments. Membrane lipids shift the absorption maximum from ≈ 630 nm to ≈ 650 nm (Reinbothe et al., 1999; 2003a). In this wavelength region of the spectrum, the Pr form of phytochrome has its main absorption maximum, such that Pchlide *a* photoreduction and phytochrome action are tied during seedling development.

In the present work, site-directed mutagenesis was used to identify putative Pchlide binding sites in PORA and to study their impact on the structure, function and assembly of LHPP. Previous work had implicated a role of Cys residues in binding of the tetrapyrrole pigment (Griffiths, 1975; Oliver and Griffiths, 1981). A homology model was constructed for barley PORA on a template from the tyrosine-dependent oxidoreductase family. In this model, one Cys residue (Cys202) is predicted to be involved in the formation of the enzyme's photoactive state. When Cys202 was exchanged by an Ala residue, indeed PORA activity dropped to undetectable levels, validating the structural model. In addition, a second Pchlide binding Cys residue (Cys229) was discovered in the carboxy-terminal part of the PORA polypeptide. This Cys residue is most likely surface-exposed and participated in PORA:PORB interactions leading to the formation of LHPP. In the reconstituted, lipid-containing LHPP, PORA was catalytically inactive and Pchlide *b* bound to either Cys residue transferred its excitation energy onto PORB-bound Pchlide *a*. Catalysis-induced change in PORB conformation during Pchlide *a* to Chlide *a* conversion then led to the dissociation of LHPP. We suppose that this mechanism could provide the trigger for the light-induced disintegration of the prolamellar body. Collectively, our results unveil hitherto unknown aspects of the structure, function and assembly of LHPP and highlight its unique role for plant greening.

RESULTS

Homology modelling of barley PORA and PORB

POR is a light-dependent enzyme which catalyzes a hydrogen transfer from NADPH to Pchlide, giving rise to the formation of the Chl precursor Chlide (see also Fig. 1).

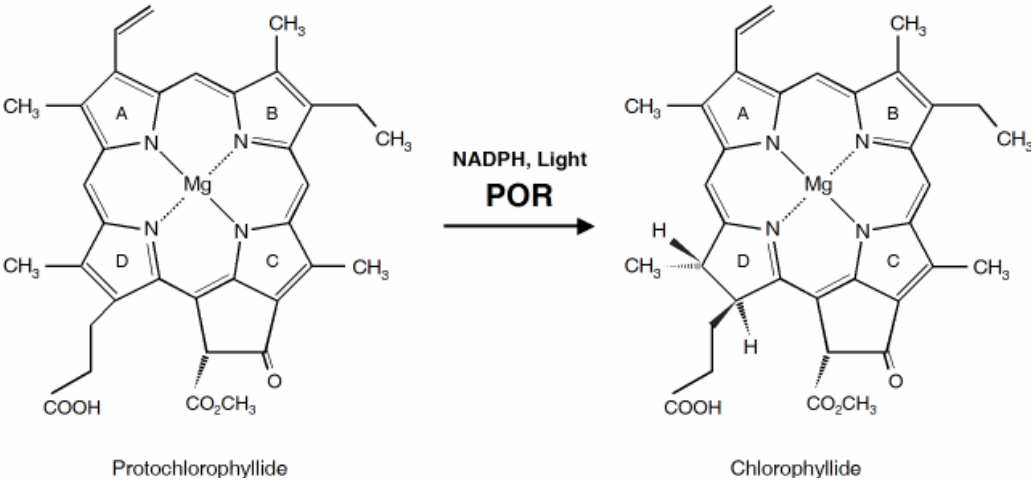

Figure 1. POR reaction.

Thus far, little was known about the exact 3D-structures of the PORA and PORB and resulting LHPP complex. In order to obtain first insights into these structures, a 3D-modelization of the PORA and PORB polypeptides was performed. The approach taken was based on the finding that POR is related to the group of short chain dehydrogenases (Baker, 1994; Dahlin et al., 1999; Townley et al., 2001, see Fig. 2 for a sequence comparison). For these enzymes, X-ray structures have been obtained (Ghosh et al., 1991 ; Varughese et al., 1992 ; Tsigelny & Baker, 1995), allowing homology models to be constructed using the *Swiss PdBViewer* version 3.7b2 programme and 7-α-hydroxysteroid dehydrogenase (ahi) from *E. coli* as template. The PORA and PORB structural models are highlighted in Fig. 3. The proposed structural models confirm and extend previous models developed for POR of *Synechocystis* (synPOR)(Townley et al., 2001) and pea (Dahlin et al., 1999).

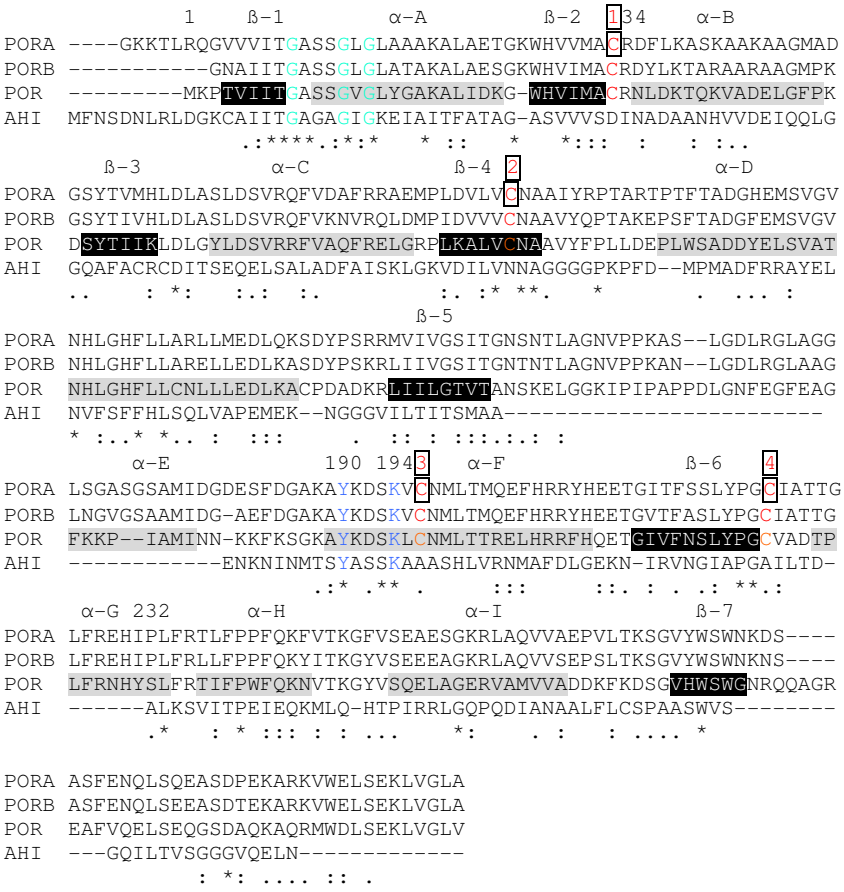

Figure 2. Primary amino acid sequences of the PORA and PORB of barley as well as POR of *Synechocystis* and 7-α-hydroxysteroid dehydrogenase (AHI) of *E. coli*.
α-helices (marked α-A-I) and ß-strands (marked ß-1-7) are given in grey and black boxes, respectively, and refer to POR of *Synechocystis* (see Townley et al., 2001, for details). The GXXXGXG motif (light blue colour) and YXXXK motif (dark blue colour) implicated in NADPH binding, as well as the conserved Cys residues (boxed in red and referred to as Cys1, Cys2, Cys3 and Cys4) are highlighted. Numbering of key amino acid residues refers to the cyanobacterial enzyme (Arg34, Tyr190, Lys194; Townley et al., 2001). Tyr190 and Lys194 correspond to Tyr275 and Lys279 of the pea enzyme (Lebedev et al., 2001). Asterisks mark conserved amino acid residues, dots and double points give column consensus, and dashes indicate gaps introduced during sequence alignment. Sequence comparison was made using standard computer programs.

Accordingly, the PORA and PORB of barley are presumed to consist of 6 α-helices and 7 ß-strands. Similar α-helices and ß-strands are present in POR of Synechocystis (synPOR) and are marked α-A - I and ß-1 - 7, respectively, in Fig. 2. Amino acid conservation in synPOR and the barley PORA and PORB is almost perfect in the so-called Rossmann fold representing the adenine binding cleft in the SCDs. Amino acid residues already established as being necessary for structural and functional reasons in the SCDs are also conserved in synPOR and the barley PORA and PORB (Figs. 2 and 3). It is assumed that the highly conserved Tyr and Lys

residues in the YXXXK motif, in conjunction with the GASSGXG motif, establish the enzyme catalytic pocket and participate in the proper coordination of NADPH. The ε-amino group of the Lys side chain hereby is supposed to interact with the hydroxyl group of Tyr and to lower its pK_a such that a proton can be donated during the catalytic mechanism. In addition, both amino acid residues are implicated in the formation of the POR photoactive state with Pchlide (Wilks & Timko, 1995; Lebedev & Timko, 1999; Lebedev et al., 2001; Townley et al., 2001).

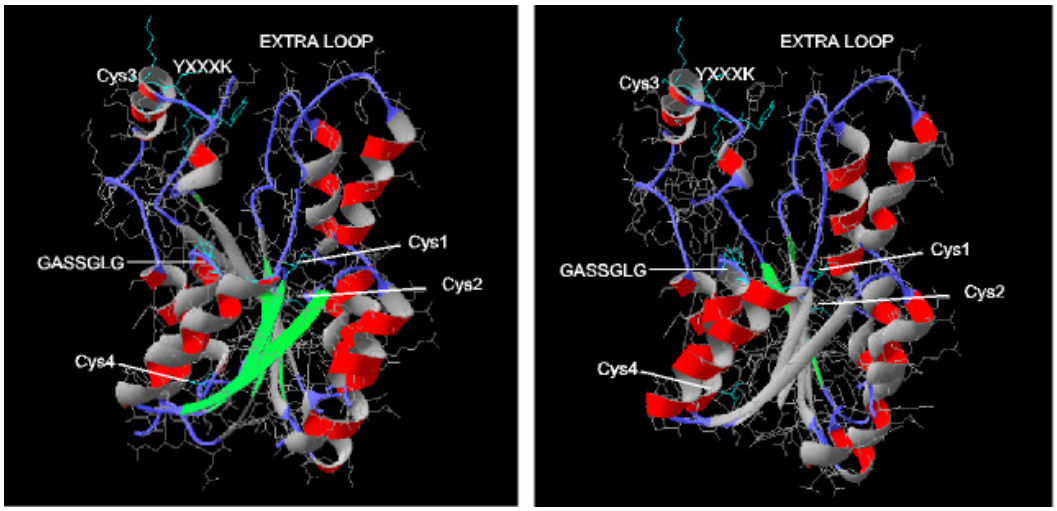

Figure 3. Homology model of barley PORA (A) and PORB (B).
α-Helices and ß-strands are shown in red and green colour in the ribbon representation, respectively. Presumed active site residues such as Tyr and Lys in the YXXXK motif as well as the conserved Gly residues in the GASSGLG motif (see Fig. 2) are highlighted. In addition, the 4 evolutionarily conserved Cys residues (referred to as Cys1-4) are given. The extra loop, establishing an oligomerization domain of the PORA and PORB, was conceptually incorporated as helix-turn strand. The models depict the non-substrate- and non-co-substrate-complexed states of the PORA and PORB.

A role of one or two Cys residues in Pchlide binding has been proposed. SynPOR contains 6 Cys residues of which Cys-37, Cys89, Cys199 and Cys226, henceforth referred to as Cys1, Cys2, Cys3 and Cys4, respectively, have counterparts in the PORA and PORB of barley (see also Fig. 2):

	Cys 1	Cys 2	Cys 3	Cys 4
POR *Synechocystis*	37	89	199	226
PORA	39	91	202	229
PORB	32	84	194	221

Evidence for an involvement of Cys residues in catalysis has come from chemical modification of Cys residues with *N*-phenylmaleimide and other sulfhydryl group-modifying compounds (Oliver and Giffiths, 1981). For example, upon treatment of synPOR with these agents, a drop in activity was observed (Townley et al., 2001). In the proposed structural models (Fig. 3), at least Cys1 and Cys3 in the PORA and PORB are in close vicinity to the active centre and thus could be involved in Pchlide binding. In either case, Cys1 is facing the GASSGLG motif, whereas Cys3 is juxta-positioned to the YXXXK motif participating in the catalytic mechanism. Cys2 has no counterpart in POR of pea and was therefore disregarded as Pchlide binding site. Cys4 is present in the ß-strand-rich ends of either predicted globular structure and thus well apart from the presumed catalytic sites.

Bacterial expression and purification of barley PORA and PORB

To identify which Cys residue in the PORA could provide a Pchlide binding site, an *in vitro*-mutagenesis approach was taken. DNAs encoding (CysX⟶Ala)-PORA (X=1, 2, 3 or 4) mutant proteins were generated by PCR, sequenced, and subcloned into the pQE30 vector. Cloning was made in a way such that the resulting proteins would no longer contain their transit peptides for plastid import but to bear hexa-histidine [$(His)_6$] tags at their NH_2-termini (see Materials and Methods). After transformation into *E. coli* strain XL1blue, expression of the PORA and PORB proteins was induced with IPTG. After 3 h, bacteria were harvested by centrifugation, lysed, and the PORA and PORB contained in the soluble fraction purified by differential centrifugation and chromatography on Ni-NTA agarose. The final protein fractions were approximately 95% pure and contained only minor contaminants of bacterial proteins (Fig. 4A). Western blot analysis using respective anti-POR and anti-$(His)_6$ antisera confirmed that PORA and PORB displayed the expected molecular masses of 36 kDa and 38 kDa, respectively (Fig. 4B).

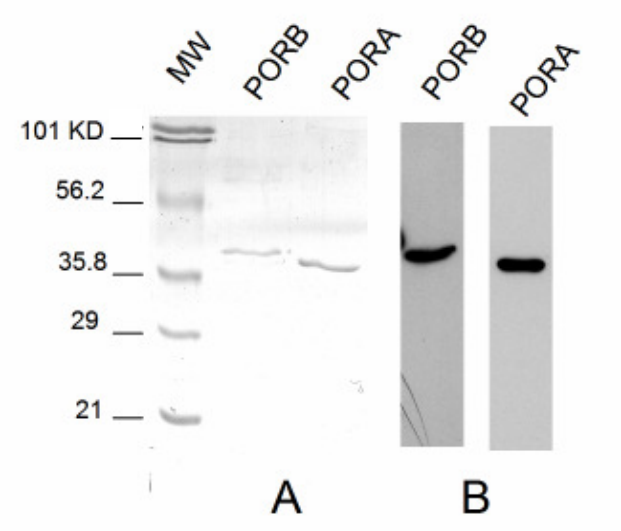

Figure 4. Bacterial expression of barley PORA and PORB.
After expression and purification the ≈ 95% pure enzymes were subjected to Coomassie staining (A) or Western blotting using an anti-$(His)_6$ antiserum (B). Molecular mass standards are given (MW).

Substrate binding to PORA and PORB as well as PORA mutant proteins

We first tested binding of Pchlide *a* and Pchlide *b* to the bacterially expressed and purified PORA and PORB proteins. Different amounts of either POR protein were incubated with Pchlides *a* or *b* and NADPH in the dark for 15 min before being subjected to a step of gel filtration. PORA-pigment-NADPH and PORB-pigment-NADPH ternary complexes eluted with the flowthrough were extracted with acetone. Pigments identification and quantification were made by HPLC as well as room temperature absorbance and fluorescence measurements (Reinbothe et al., 2003b). Figure 5A and B show plots of the amounts of Pchlide *a* and Pchlide *b* bound to the PORA or PORB *versus* the enzyme concentrations. From the linear relationships, it turned out that 1 µg of PORA bound ≈ 34.12 ng of Pchlide *b* and ≈ 3.5 ng of Pchlide *a*. This corresponded to 27.72 pmol of PORA, 54.3 pmol of Pchlide *b* and 5.7 pmol of Pchlide *a* and confirmed previously reported 1:2 versus 1:0.2 stoichiometries of PORA to pigment in the recovered PORA-pigment-NADPH complexes (Reinbothe et al., 2003a,b). By contrast, 1 µg (26.6 pmol) of PORB bound ≈ 33.2 ng (corresponding to 54.2 pmol) of Pchlide *a* and only ≈ 3.2 ng (5.1 pmol) of Pchlide *b*, consistent with previous results (Reinbothe et al., 2003a,b).

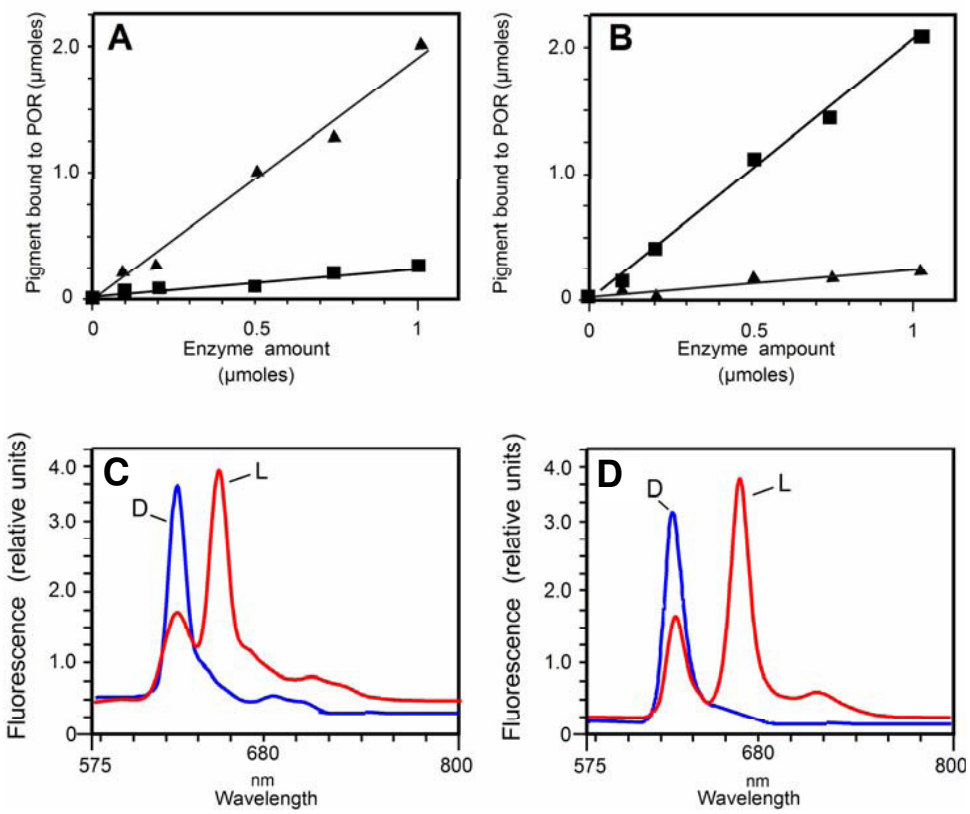

Figure 5. Pigment binding and conversion characteristics of bacterially expressed barley PORA and PORB.
Different amounts of PORA and PORB were reconstituted into POR-pigment-NADPH ternary complexes with Pchlide *a* (■—■) and Pchlide *b* (▲—▲) and NADPH and depleted of non-protein-bound pigment by gel filtration on Sephadex G15. Aliquots of ternary POR-pigment-NADPH complexes eluted with the flowthrough in turn were kept in darkness or exposed to white light. After 15 min, pigments were extracted with acetone and identified and quantified by HPLC and subsequent absorbance measurements or by fluorescence spectroscopy, respectively. (A, B) Plots showing the amounts of POR-bound pigments *versus* the PORA and PORB protein concentrations. (C, D) Pigment conversion analyzed by fluorescence spectroscopy. The curves show emission spectra of pigments extracted from the PORA (C) and PORB respectively (D) after respective dark (*D*, blue line) and white light (*L*, red line) incubations. The excitation wavelength was at 440 nm.

The catalytic properties of the PORA and PORB were tested as follows. Assays containing PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes were reconstituted as described, subjected to gel filtration and subsequently split. Halves of the incubation mixtures were exposed to white light, whereas the other halves were kept in darkness. After incubation, pigments were extracted with acetone and subjected to room temperature fluorescence emission spectroscopy. Figure 5C and D show that either POR protein converted only half of its bound pigment to the respective product. At the chosen excitation wavelength of

440 nm, Pchlides *a* and *b* have emission maxima at 627 nm and 629 nm, respectively, whereas Chlide *a* and Chlide *b* emit at 665 nm and 652 nm.

Table 1. Pigment binding to bacterially expressed PORA and PORB.

	Pigment [pmol per µg POR protein] [1)]	
	+ Sucrose	- Sucrose
PORA		
Pchlide *a*	5.72±0.05	2.62±0.02
Pchlide *b*	54.30±0.30	27.15±0.05
PORB		
Pchlide *a*	52.40±0.12	26.20±0.20
Pchlide *b*	5.20±0.20	2.10±0.02

^{35}S-PORA and ^{35}S-PORB were reconstituted into their respective substrate- and co-substrate-bound states in assay buffer containing or lacking 350 mM sucrose and subjected to gel filtration on Sephadex G15. ^{35}S-POR-pigment-NADPH complexes eluted with the flow-through in turn were extracted with acetone. Pigment quantification was made by HPLC and absorbance measurements or by fluorescence spectroscopy, using known absorption and emission coefficients (Reinbothe et al., 2003a,b). Excitation was made at 431 nm. Standard deviations calculated from three independent experiments are indicated.
[1)]One µg of PORA corresponds to 27.72 pmoles and 1 µg of PORB corresponds to 26.62 pmoles of enzyme.

The observed partial conversion of bound substrate is due to the presence of two distinct Pchlide binding sites. We have recently shown that wheat germ-translated PORB contains two Pchlide binding sites and that these can be distinguished by gel filtration in the presence or absence of 350 mM sucrose (Reinbothe et al., 2006b). While tightly bound pigments remain attached to the POR polypeptide chain, pigments that are only loosely bound to protein are released. These loosely bound pigments are retained in the presence of 350 mM sucrose. Table 1 shows that also for the bacterially expressed and purified PORB the same observations were made. In addition, our experiments unveiled the existence of two Pchlide *b* binding sites for PORA: one high affinity binding site and one low affinity binding site (Table 1). Only Pchlide bound to the former is photoconvertible, whereas Pchlide bound to the latter is not.

In a next step, pigment binding was studied with the different (Cys→Ala)-PORA mutant proteins. Figure 6 shows room temperature fluorescence emission spectra of pigments extracted from the (Cys1→Ala)-PORA, (Cys2→Ala)-PORA, (Cys3→Ala)-PORA and (Cys4→Ala)-PORA mutant proteins after gel filtration in the presence of sucrose and subsequent dark and light incubations, respectively.

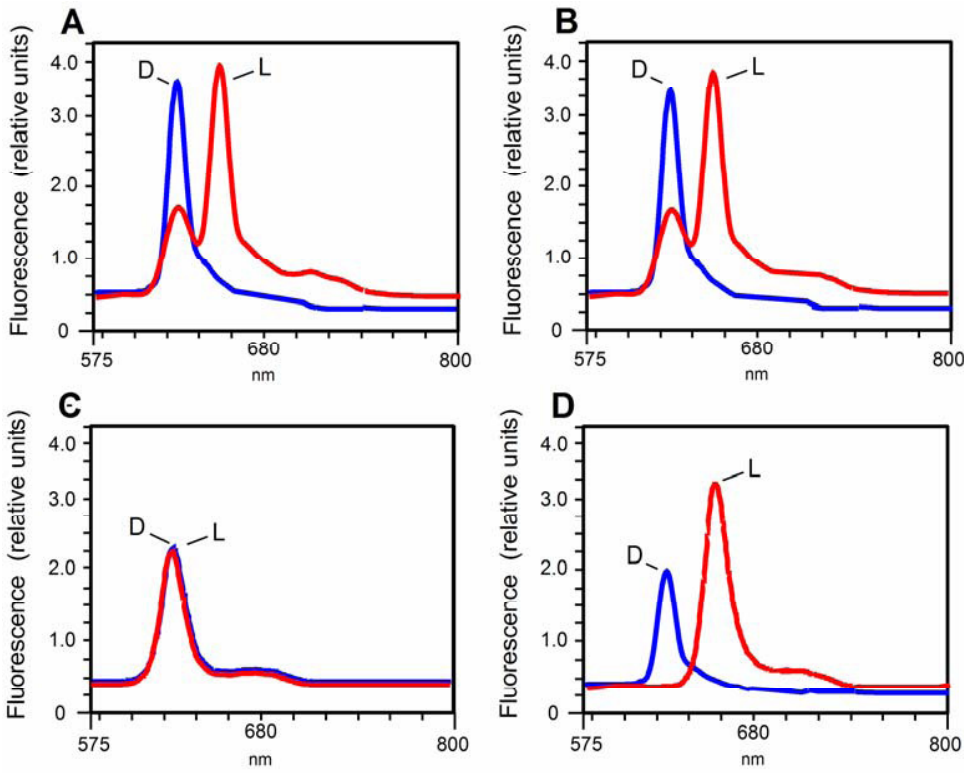

Figure 6. Pigment binding and conversion characteristics of (Cys→Ala)-PORA mutant proteins. PORA mutant proteins were reconstituted into their pigment-bound states as described in Fig. 5 and purified by gel filtration. After respective dark and light incubations, pigment conversion was followed by fluorescence spectroscopy. (A-D) Fluorescence emission spectra of pigments extracted from the (Cys1→Ala)-PORA (A), (Cys2→Ala)-PORA (B), (Cys3→Ala)-PORA (C) and (Cys4→Ala)-PORA (D) after respective dark (*D*, blue line) and white light (*L*, red line) incubations. *Note* that the (Cys3→Ala)-PORA (C) and (Cys4→Ala)-PORA (D) bind only half of the amount of Pchlide *b* as compared to (Cys1→Ala)-PORA and (Cys2→Ala)-PORA (B) and that (Cys3→Ala)-PORB-bound (C) pigment is non-photoconvertible.

Quantification of the binding data is presented in Table 2. Collectively, the data revealed that the (Cys1⟶Ala)-PORA and (Cys2⟶Ala)-PORA each bound 2 Pchlide *b* molecules per enzyme monomer, only one of which was photoconvertible and transformed into Chlide *b* (Fig. 6A and B, and Table 2). By contrast, (Cys3⟶Ala)-

PORA and (Cys4⟶Ala)-PORA were able to bind only one Pchlide *b* molecule per enzyme monomer (Fig. 6C and D, and Table 2). While Pchlide *b* attached to (Cys3⟶Ala)-PORA was non-photoconvertible, Pchlide *b* bound to (Cys4⟶Ala)-PORA was readily converted to Chlide *b* upon illumination (Fig. 6C and D, and Table 2).

Table 2. Pigment binding and conversion determined for (CysX→Ala)-PORA mutant proteins.

	Pchlide *b* [pmol per µg POR protein] [1)]			
	+ Sucrose		- Sucrose	
	Dark	Flash	Dark	Flash
(Cys1→Ala)-PORA	54.40±0.20	27.20±0.04	27.20±0.04	n.d.
(Cys2→Ala)-PORA	54.20±0.15	27.30±0.16	27.20±0.04	n.d.
(Cys3→Ala)-PORA	27.20±0.04	27.30±0.18	n.d.	n.d.
(Cys4→Ala)-PORB	27.25±0.06	n.d.	27.40±0.24	n.d.

^{35}S-(CysX→Ala)-PORA-pigment-NADPH complexes were reconstituted in the presence or absence of 350 mM sucrose as described in Table 1 and subjected to gel filtration on Sephadex G15. POR-pigment-NADPH complexes eluted with the flowthrough were then either kept in the dark or exposed to a saturating, 1-msec flash of white light. After extraction with acetone, pigments were identified and quantified by fluorescence spectroscopy. Standard deviations were calculated from five independent experiments. n.d. defines not detectable Pchlide *b* levels.
[1)]One µg of PORA mutant protein corresponds to ≈ 27.72 pmoles of enzyme.

Assembly of the PORA and PORB as well as PORA mutant proteins into larger complexes

The assembly competence of the PORA and PORB and (Cys_x⟶Ala)-PORA mutant proteins was assessed in subsequent experiments. Equimolar amounts of reconstituted PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes were mixed and, after a 15-min incubation in darkness, size-fractionated on a Superose 6 column equilibrated in assay buffer containing sucrose (see *Methods*).

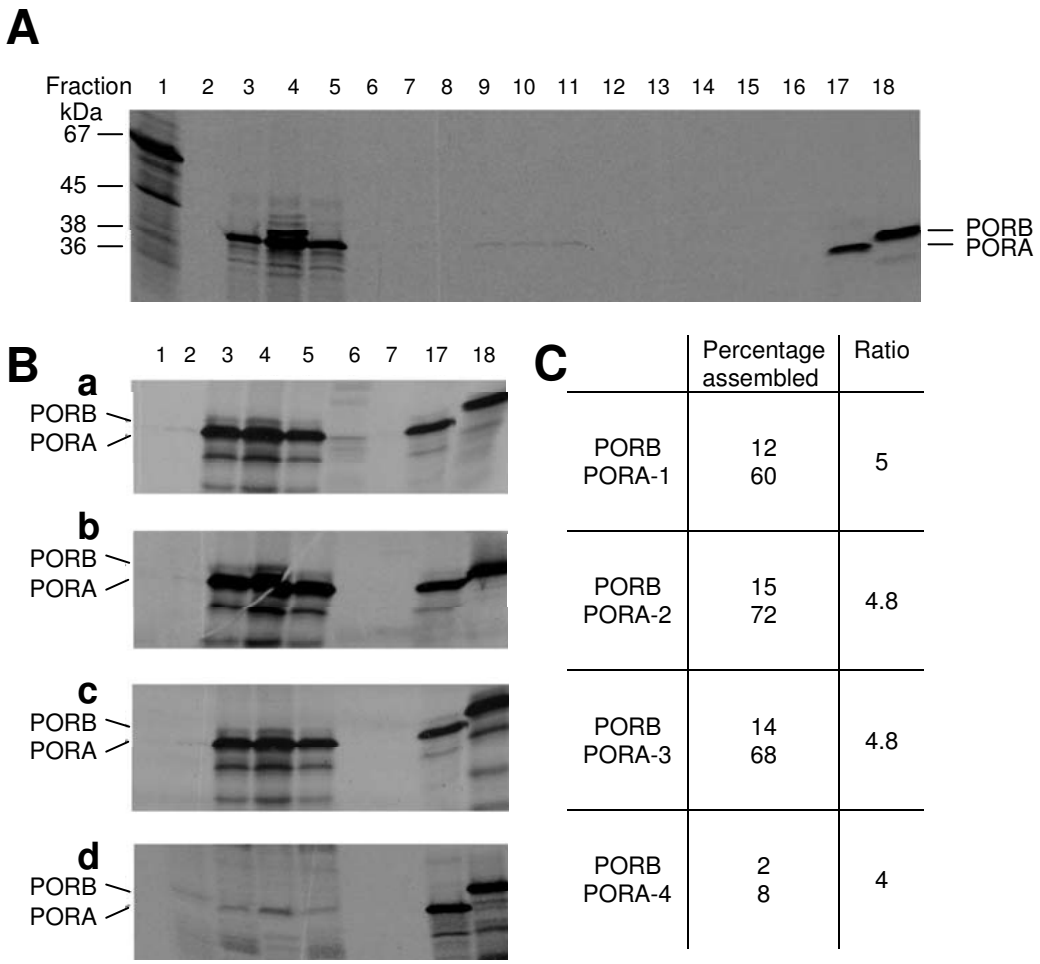

Figure 7. Assembly of (Cys→Ala)-PORA mutant proteins with PORB.
Equal amounts of reconstituted PORA-Pchlide *b*-NADPH, (Cys→Ala)-PORA-Pchlide *b*-NADPH, and PORB-Pchlide *a*-NADPH ternary complexes were incubated for 15 min in the dark. Then the assays were subjected to gel filtration on Superose 6. Individual fractions were harvested and aliquots taken for SDS-PAGE and Western blotting using an ECL system. (A) Autoradiogram of established higher molecular mass PORA:PORB complexes containing wild-type PORA and PORB. Fractions 17 and 18 show unassembled PORA-pigment-NADPH and PORB-pigment-NADPH complexes. (B) Autoradiogram of higher molecular mass PORA:PORB complexes containing PORB and the (Cys1→Ala)-PORA (a), (Cys2→Ala)-PORA (b), (Cys3→Ala)-PORA (c) and (Cys4→Ala)-PORA (d) mutant proteins. (C) Quantification of mutant PORA (numbered 1-4) and PORB in the established higher molecular mass complexes. Percentages refer to the total amount of signal obtained on the films, including assembled and non-assembled POR-pigment complexes. The ratio indicates the relative amounts of the PORA mutant proteins to PORB in the recovered higher molecular mass complexes.

Figure 7 shows the results obtained for the wild-type PORA and PORB as well as combinations of PORB and the different (Cys⟶Ala)-PORA mutant proteins. Panel A highlights complex formation for the wild-type PORA with PORB. Free, non-assembled PORA- and PORB-pigment ternary complexes were eluted at much later time points (Fig. 7A, lanes 17 and 18, respectively). When assembly assays were carried out for

the (Cys1⟶Ala)-PORA, (Cys2⟶Ala)-PORA and (Cys3⟶Ala)-PORA, similar results were obtained as for wild-type PORA. All three mutant proteins established larger complexes similar in size to those found for wild-type PORA (Fig. 7, compare B, panels a, b and c, versus A). By contrast, (Cys4⟶Ala)-PORA produced drastically less PORA:PORB supra-complexes that, in most experiments, were at the limit of detection (Fig. 7B, panel d).

Energy transfer in the established PORA:PORB complexes

The results presented thus far implied that Cys3 and Cys 4 in the PORA accomplished distinct roles as Pchlide binding sites: Cys3 as part of the active site of the enzyme, and Cys4 as a second, low affinity pigment binding site. Taking into account findings that tetrapyrroles have the capacity to arrange into stacks capable of energy transfer, we supposed that Pchlide *b* bound to Cys4 could play a role in PORA:PORB interactions and subsequent energy transfer between neighbouring POR-pigment complexes. LHPP in the prolamellar body interacts with galacto- and sulfolipids and establishes two spectral forms of Pchlide: Pchlide-650/657 and Pchlide-628/632 (the first number indicates the absorption maximum, the second the respective emission maximum). Pchlide-650/657 is also called photoactive Pchlide because it can be converted to Chlide-684/690 with a single, 1-msec flash of white light. Pchlide 628/632, by contrast, is non-photo-convertible and named photoinactive Pchlide. Energy transfer takes place from photoinactive Pchlide to photoactive Pchlide and from photoinactive Pchlide to Chlide in the prolamellar body (Smith and Benitez, 1954; Kahn et al., 1970; Mathis and Sauer, 1972; Vaughan and Sauer, 1974; Ignatov and Litvin, 1981; Fradkin et al., 1993).

Oligomeric PORA-PORB protein complexes reconstituted as described were supplemented with a lipid mixture containing monogalactosyl diacylglycerol, digalactosyl diacylglycerol, and sulphoquinovosyl diacylglycerol (58:36:6 mol%) which had been prepared from pigment-depleted prolamellar bodies of barley etioplasts (Ryberg et al., 1983). Then the samples were analyzed further in two ways. One aliquot was subjected to non-denaturing, analytical polyacrylamide gel electrophoresis (NA-PAGE) to monitor the presence of lipid-containing higher molecular mass complexes (Reinbothe et al., 2003b). The other aliquots were subjected to fluorescence emission spectroscopy at 77K at an excitation wavelength

of 440 nm (Lebedev et al., 1995). For the (Cys4→Ala)-PORA, which is assembly-incompetent, pigment- and NADPH-complexed PORA and PORB were directly added at a 5:1 stoichiometry to the lipid mixture and analyzed identically.

Figure 8 depicts low temperature fluorescence emission spectra of PORA:PORB supra-complexes that had been reconstituted with PORB and the different (Cys→Ala)-PORA mutant proteins. Apparently, (Cys1→Ala)-PORA and (Cys2→Ala)-PORA were capable of reconstituting Pchlide-650/657 (Fig. 8A and B, blue line). In either case, the established Pchlide-650/657 was photoactive and converted to Chlide-684/690 upon flashing the samples (Fig. 8A and B, red line). As expected, the level of Pchlide-628/632 remained unchanged.

For (Cys3⟶Ala)-PORA and (Cys4⟶Ala)-PORA, a different result was obtained (Fig. 8C and D, blue line). The (Cys3⟶Ala)-PORA-containing complex produced a broad fluorescence peak emitting at ≈635 nm (Fig. 8C, blue line). A more precise spectroscopic analysis based on the second derivative and Gaussian deconvolution resolved the 635 nm band to consist of four Pchlide forms, with fluorescence emission maxima at 628 nm, 632 nm, 635 nm, and 642 nm, designated Pchlide-F628, Pchlide-F632, Pchlide-F635, Pchlide-F642, respectively. Upon flash light illumination, almost no visible change occurred in the low-temperature Pchlide fluorescence emission spectrum, except for a minor decrease in the Pchlide-F635 and Pchlide-F642 peaks (Fig. 8C, red line). In addition, a new pigment peak appeared emitting at 672 nm. Pigment analysis in acetone at room temperature by fluorescence spectroscopy and HPLC on C30 RP columns showed that Chlide *a* had been produced (data not shown).

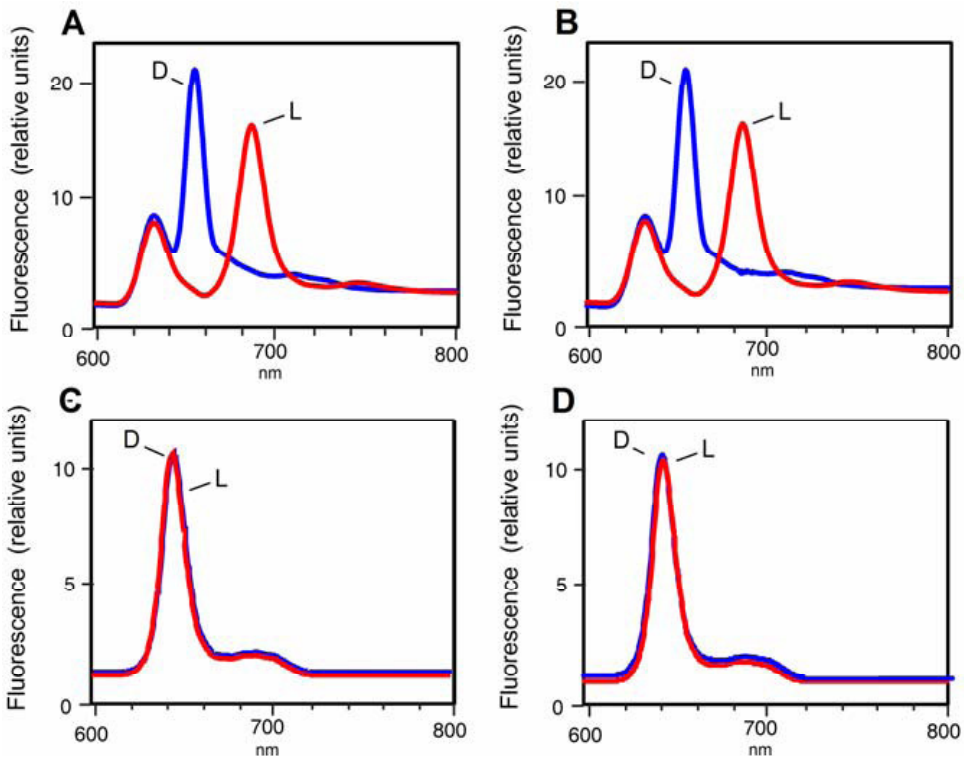

Figure 8. Low temperature fluorescence analysis of reconstituted, lipid-containing PORA:PORB supra-complexes before and after flash light illumination.
PORA:PORB supra-complexes containing the different pigment-complexed (Cys→Ala)-PORA mutant proteins were reconstituted as described in Fig. 7 and supplemented with a mixed galacto- and sulpholipid fraction isolated from pigment-depleted prolamellar bodies of barley etioplasts. Then the samples were cooled to 77K and analyzed by fluorescence emission spectroscopy at an excitation wavelength of 440 nm. (A-D) The curves show spectra obtained for (Cys1→Ala)-PORA- (A), (Cys2→Ala)-PORA- (B), (Cys3→Ala)-PORA- (C) and (Cys4→Ala)- PORA (D)-containing assays before (blue lines) and after (red lines) a saturating 1-msec flash of white light, respectively.

Light-induced dissociation of PORA:PORB supra-complexes

The presence and functional states of the PORA and PORB was studied further by NA-PAGE. PORA:PORB supra-complexes that had been reconstituted into their lipid-bound states as before were subjected to non-denaturing PAGE and subsequently detected by blue light-induced pigment fluorescence and Western blotting, respectively.

Figure 9 shows that (Cys1⟶Ala)-PORA, (Cys2⟶Ala)-PORA and (Cys3⟶Ala)-PORA all were present in terms of larger complexes (A) exhibiting strong blue light-induced pigment fluorescence (C). By contrast, no fluorescing

supra-complexes were found for (Cys4⟶Ala)-PORA (Fig. 9A) that had previously been demonstrated to be assembly-incompetent (see Fig. 7). Nevertheless, (Cys4⟶Ala)-PORA was present in a pigment-complexed form, as evidenced by the appearance of a respective fluorescence band under blue light (Fig. 9C). At the same time a fluorescing PORB band appeared indicative of the presence of pigment (Fig. 9C). Electrophoresis of aliquots of the incubation mixtures revealed similar amounts of the different (Cys⟶Ala)-PORA mutant proteins (Fig. 9E and F).

Upon exposure to the 1-msec white light flash, PORB: (Cys1⟶Ala)-PORA and PORB:(Cys2⟶Ala)-PORA supra-complexes dissociated into fluorescing PORA-pigment and PORB-pigment subunits (Fig. 9, panels B and D). In case of the PORB:(Cys2⟶Ala)-PORA supra-complex, this dissociation seemed incomplete and small amounts of the higher molecular mass complex remained detectable. Strikingly, no comparable dissociation occurred for the PORB:(Cys3⟶Ala)-PORA higher molecular mass complex (Fig. 9B and D) and only trace amounts of fluorescing PORA-pigment and PORB-pigment complexes were found (Fig. 9B and D). In case of (Cys4⟶Ala)-PORA no supra-complexes were detectable at all and only fluores-cing PORA-pigment and PORB-pigment subunits lighted up on the gels (Fig. 9A-D).

Interestingly, slight differences in the migration behaviour became apparent for the resolved PORB-pigment complex. PORB was present in terms of a slightly faster and slightly slower migrating band, designated PORB-I and PORB-II, respectively. While PORB-II was abundant in incubation mixtures containing (Cys3⟶Ala)-PORA and (Cys4⟶Ala)-PORA, PORB-I was present only in mixtures containing the (Cys1⟶Ala)-PORA and (Cys2⟶Ala)-PORA. HPLC analyses performed with pigments extracted from these two different PORB protein bands highlighted PORB-I to contain Chlide *a* and PORB-II to contain Pchlide *a*. Pigments extracted from the (Cys⟶Ala)-PORA protein bands in all four cases provided Pchlide *b* as the only detectable pigment. When we extracted pigments from the non-dissociable PORB:(Cys3⟶Ala)-PORA higher molecular complex, Pchlide *a*, Pchlide *b* and Chlide *a* were resolved in a stoichiometry 1:2.5:0.01.

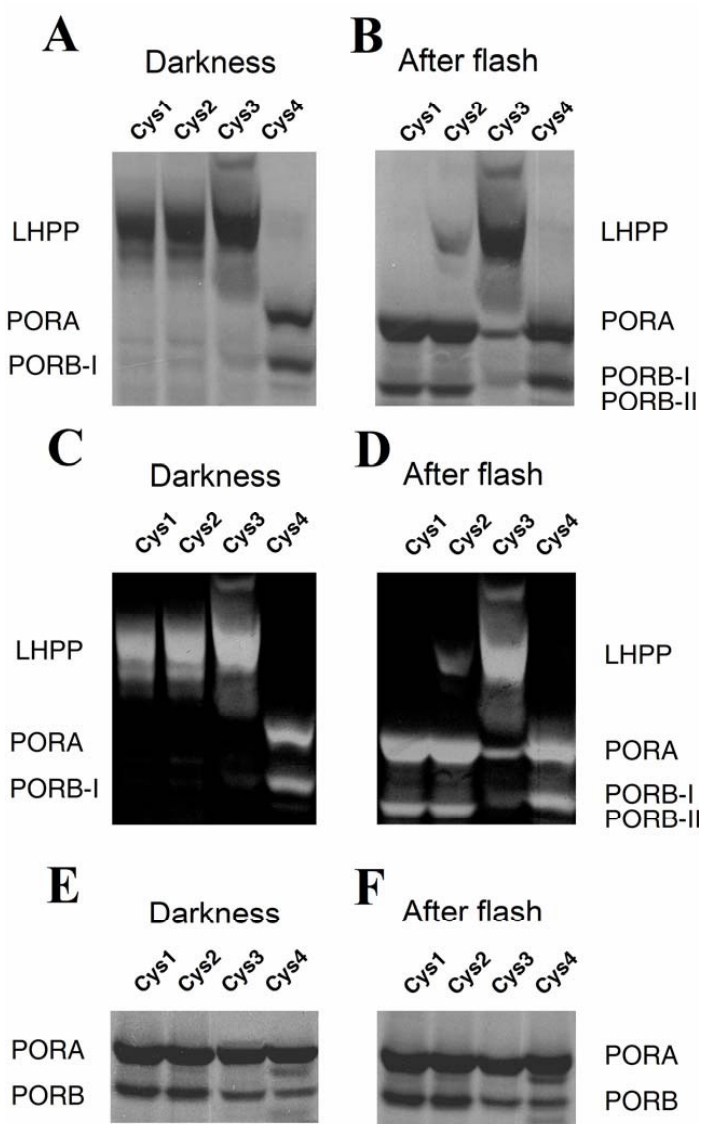

Figure 9. Non-denaturing, analytical PAGE of lipid-containing PORA:PORB supracomplexes. Pigment-complexed PORB and (Cys→Ala)-PORA mutant proteins were reconstituted into their lipid-bound states as described in Fig. 8. Then aliquots of the assays were directly loaded onto a non-denaturing, analytical polyacrylamide gel or flashed before electrophoresis. Aliquots of the different samples were analyzed by Western blotting or by blue light-induced pigment fluorescence. (A, B) Western blot of POR-related proteins in assays containing PORB and the (Cys1→Ala)-PORA (Cys1), (Cys2→Ala)-PORA (Cys2), (Cys3→Ala)-PORA (Cys3) and (Cys4→Ala)-PORA (Cys4) mutant proteins in non-flashed (A) and and flashed (B) samples. (C, D) Pigment fluorescence of the same POR-pigment complexes shown in (A) and (B), respectively. (E, F) Western blots of proteins corresponding to those in (A) and (B), respectively, after denaturing electrophoresis.

These results confirmed that only minor traces of Chlide *a* had been produced. The fact that no Chlide *b* was detectable in our experiments underscored that supracomplex formation and membrane binding inactivated PORA as Pchlide *b*-reducing enzyme.

Light-induced changes in POR conformation in relation to the disintegration of the prolamellar body

The prolamellar body of etioplasts rapidly dissociates upon illumination (Virgin et al., 1963; Kahn, 1968; Kahn et al., 1970). We hypothesized that catalysis-induced changes in PORA and/or PORB protein conformation could trigger the dissociation of LHPP and dispersal of the prolamellar body. To examine this supposed mechanism, two types of experiments were performed. In the first experiment, *in vitro*-reconstitution experiments were carried out using different combinations of PORA/B-substrate and PORB-Chlide *a*(-$NADP^+$) and PORA-Chlide *b*(-$NADP^+$) complexes. This type of experiment unveiled that POR-product complexes are unable to establish larger complexes (data not shown).

In the second type of experiment, the different (Cys⟶Ala)-PORA mutant proteins were used to probe changes in PORA conformation occurring during substrate binding and catalysis. ^{35}S-Met-labelled (Cys⟶Ala)-PORA containing Pchlide *b*, Pchlide *b* plus NADPH, or Chlide *b*(-$NADP^+$) was reconstituted as described and added to isolated POR-degrading stromal protease. As shown previously, the POR-degrading protease is a light-induced, Mg-ATP- and metal ion-dependent multi-component stromal protease that degrades POR-product complexes but not POR-substrate complexes (Reinbothe et al., 1995c, d). After different time intervals, aliquots were taken and protein precipitated with trichloroacetic acid, separated by SDS-PAGE and detected by autoradiography. Figure 10 highlights time courses of PORA breakdown obtained for the different PORA-substrate and PORA-product complexes. The data revealed that (Cys1⟶Ala)-PORA (panel A) and (Cys2⟶Ala)-PORA (panel B) were stable in the presence of Pchlide and NADPH. After light incubation causing enzymatic Chlide *b* formation, both (Cys1⟶Ala)-PORA and (Cys2⟶Ala)-PORA were rapidly degraded. By contrast, (Cys3⟶Ala)-PORA (panel C) and (Cys4⟶Ala)-PORA (panel D) showed a different behaviour. Even in the presence of Pchlide and NADPH either PORA mutant protein was turned over. In case of (Cys3⟶Ala)-PORA, the rate of PORA breakdown was apparently unaffected

by white light pre-treatment, whereas that of (Cys4⟶Ala)-PORA was enhanced after illumination of the ternary complex.

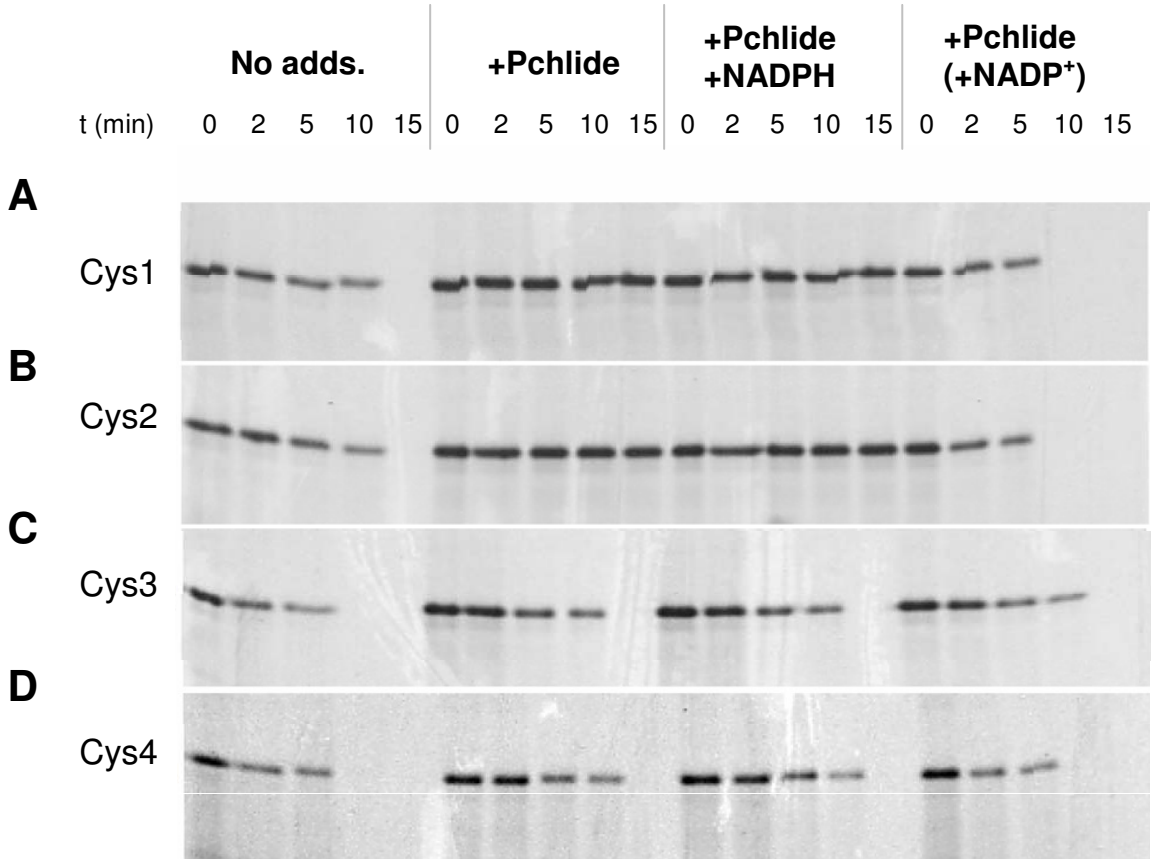

Figure 10. Protease sensitivity of reconstituted POR-pigment complexes.
(A-D) PORA-pigment complexes containing the different (Cys→Ala)-PORA mutant proteins were reconstituted with Pchlide *b* or Pchlide *b* plus NADPH as described in Fig. 5 and depleted of non-protein-bound pigments by gel filtration. Aliquots were subjected to white light treatment to induce enzymatic chlorophyllide *b* formation or kept in darkness. As control, (Cys→Ala)-PORA mutant proteins devoid of substrates or products were synthesized *in vitro*, subjected to gel filtration and mock-incubated. Subsequently, POR-degrading protease was added and the mixtures incubated in the dark for variable periods. Protein was extracted with trichloroacetic acid, washed, and run by SDS-PAGE. Protein detection was made by autoradiography. The autoradiograms show time-courses of POR breakdown depending on the presence or absence of substrates and products for the (Cys1→Ala)-PORA (A), (Cys2→Ala)-PORA (B), (Cys3→Ala)-PORA (C) and (Cys4→Ala)-PORA (D) mutant proteins.

DISCUSSION

In the present work, a structure-function study was performed for PORA of barley. PORA represents the negatively light-regulated POR enzyme that rapidly disappears from etiolated plants upon illumination. Its expression and role is confined to etiolated plants, whereas PORB persists in illuminated and light-adapted plants.

Structure-function studies identify Cys202 and Cys229 as distinctive Pchlide binding sites

Using an *in vitro*-mutagenesis approach, two distinctive Pchlide binding sites were identified in the PORA. Out of the four evolutionarily conserved Cys residues, Cys202 (operationally defined as Cys3) and Cys229 (operationally defined as Cys4) were shown to participate in the establishment of the photoactive enzyme state and LHPP assembly, respectively. Remarkably, PORA assembled into larger complexes with PORB was inactive as Pchlide-reducing enzyme. Protochlorophyllide *b* bound with NADPH to PORA instead operated as an accessory pigment and transferred the excitation energy onto PORB-bound Pchlide *a*. In the reconstituted complex, 5 PORA-Pchlide *b*-NADPH ternary complexes interact with just 1 PORB-Pchlide *a*-NADPH ternary complex. Similar POR and pigment stoichiometries as well as pigment interconversions have been determined for the prolamellar body of etioplasts, and an even larger PORA:PORB supracomplex was resolved (Reinbothe et al., 2003a,b; 2004a,b). The native complex additionally contained galacto- and sulpholipids that are presumed to shift the absorption maximum of bound pigments from ≈ 630 nm to ≈ 650 nm. Similar spectral changes were observed in this work for the *in vitro*-reconstituted complex, consolidating the LHPP model (Reinbothe et al., 1999).

The structural model depicted in Figure 3 suggests that Cys202 (Cys3) may be in close contact with the NADPH binding site. Tyr275 and Lys279 of pea POR have documented roles in Pchlide and NADPH binding. Lebedev et al. (2001) reported that substrate and co-substrate binding to POR occurs in two steps: an initial loose association of Pchlide and NADPH, and some sort of ″induced fit″ to give rise to the photoactive enzyme state. In the structural model depicted in Fig. 3, the YXXXK motif comprising the universally conserved Tyr and Lys residues as well as the adjacent Cys202 (Cys3) are predicted to be readily accessible to their ligands. A

as yet undetermined POR conformational change then could lead to the proper coordination of all reactands. Gel filtration experiments highlight recovery of tightly folded, non-dissociable PORA-Pchlide *b*-NADPH ternary complexes (Reinbothe et al., 1995a). Determined K_D values for Pchlide and NADPH are further supportive of almost irreversible binding of substrate and co-substrate to the POR polypeptide chain (Lebedev et al., 2001).

Direct evidence for structural changes occurring to the POR polypeptide upon substrate and co-substrate binding has been provided by fluorescence resonance energy transfer (FRET) (Lebedev et al., 2001; Townley et al., 2001) and protease protection experiments (this study). For example, Lebedev et al. (2001) and Townley et al. (2001) analyzed FRET from protein Trp and Tyr residues to Pchlide and NADPH during substrate and co-substrate binding. In the proposed homology model of synPOR, a single Trp is located in the hydrophobic core of the protein and thus in an environment well suited for binding the co-substrate and pigment. Furthermore, this Trp residue is proximal to the nucleotide-binding residue Arg34 and around 18 Å from the nicotineamide group of NADPH, at distances well within the Förster radius required for FRET. Protease digestion experiments add to these previous findings. We observed that Pchlide *b* and NADPH stabilize PORA against added POR-degrading stromal proteases. PORA mutant protein in which Cys202 (Cys3) had been exchanged by an Ala residue bound only half of the pigment level measured for the wild-type and was protease hyper-sensitive.

Cys4 constitutes a second, low-affinity Pchlide *b* binding site. We observed that Pchlide *b* bound to Cys4 participates in PORA:PORB interactions. Replacement of Cys4 by an Ala residue halved the level of bound pigment per PORA enzyme monomer and gave rise to assembly-incompetent, protease hyper-sensitive PORA molecules.

Both Cys3 and Cys4 are involved in energy transfer. This is apparent from the lack of Pchlide-650/657 in assays containing the (Cys3⟶Ala)-PORA and (Cys4⟶Ala)-PORA mutant proteins. In either case bright pigment peaks at 630-635 nm were detectable by low temperature fluorescence spectroscopy. (Cys3⟶Ala)-PORA established higher molecular mass complexes similar in size to those established with wild-type PORA and the (Cys1⟶Ala)-PORA and (Cys2⟶Ala)-PORA. But these complexes were largely photoinactive and non-

dissociable upon treatment with flashes of white light. Trace amounts of Chlide *a* were produced, suggesting that PORB was present in terms of pseudo-LHPP structures displaying very weak activity in Pchlide *a*-reduction. Because *in vitro* tests performed in the absence of PORA and galacto- and sulpholipids showed that PORB is *per se* catalytically active, we conclude that membrane binding leads to a drastic reduction of PORB's activity as Pchlide-reducing enzyme. *Vice versa*, assembly of PORA-Pchlide *b*-NADPH ternary complexes into LHPP gave rise to an inactivation of PORA that was no longer photoactive but operated as light scavenger. Last but not least, PORA-Pchlide *b*-NADPH ternary complexes added to the lipid mixture of the prolamellar body were incapable of producing Chlide *b*, highlighting one more level of control of PORA activity.

Catalysis-induced changes in POR conformation in relation to the dissociation of LHPP, dispersal of the prolamellar body, and greening

Catalysis-induced changes in PORB conformation upon Pchlide *a*-to-Chlide *a* conversion were sufficient to trigger the dissociation of the established lipid-containing PORA:PORB supracomplexes. It is tempting to speculate that this light effect may correlate with the rapid disintegration of the prolamellar body that is observed in etiolated plants upon illumination (Kahn, 1968; Virgin et al., 1973). *In vitro*-reconstitution experiments revealed that PORB-Chlide *a*(-$NADP^+$) complexes no longer assembled with PORA into larger complexes. On the other hand, also PORA-Chlide *b*(-$NADP^+$) complexes were found to be assembly-incompetent. Given that PORA is inactive in terms of Pchlide *b* reduction in the reconstituted complex, only catalysis-induced changes in PORB conformation can be responsible for the dissociation of LHPP. It is attractive to hypothesize that this dissociation initiates the disintegration of the prolamellar body. Once released from the dissociated supracomplex, PORA could regain its activity and operate as Pchlide *b*-reducing enzyme. This mechanism could ensure that no re-assembly of LHPP takes place in the early hours of greening where the photosynthetic apparatus needs to be established without interfering light-trapping reactions.

Recent biophysical studies have provided compelling evidence for major light-dependent reorganization events affecting both membrane protein assembly and the protein-lipid interface (Kóta et al., 2002). Kóta et al. (2002) reported on gross

alterations in the secondary structure of membrane constitutents, affecting the conformation, composition and dynamics of lipid acyl and polypeptide chains. We observed that PORA and PORB as constituents of the prolamellar body (e.g., Dehesh & Ryberg, 1986; Reinbothe et al., 2003b) undergo changes in their conformations during their assembly and disassembly. Superimposed on these effects is the phytochrome- and cryptochrome-mediated light control of nuclear and plastid photosynthetic gene expression. Chlorophyll(ide) *a* has a major role for the assembly of the plastid-encoded photosynthetic reaction centers and core antenna, whereas Chl(ide) *b* is involved in the regulated import and stabilization of the nucleus-encoded chlorophyll(ide) *b* binding proteins (Reinbothe et al., 2006a). Chlorophyllides *a* and *b* produced during the disintegration of LHPP may bind to water-soluble chlorophyll binding proteins, thereby lowering the amount of phototoxic chlorophyll precursors (Reinbothe et al., 2004a, b). Presumably the sum of all of these tightly regulated processes allows newborn seedlings to de-etiolate under a variety of different light intensities.

Genetic evidence for a photoprotective role of PORA

Genetic evidence strongly supports the major conclusion of the present work that PORA plays a photoprotective role during greening. This is illustrated by the work of Lebedev et al. (2001) and more recent work of Frick et al. (2003). Lebedev et al. (1995) used the *det340/cop1* mutant of *Arabidopsis thaliana* that is depleted of PORA protein because of constitutive depression of *porA* gene transcription to study the greening process. The authors observed that etiolated *det340/cop1* seedlings were light hyper-sensitive and impaired in chlorophyll *a* and, more significantly, chlorophyll *b* synthesis. Spectroscopic evidence was obtained for a route of chlorophyll *a* synthesis not involving Pchlide F-657 but instead depending on Pchlide-F642. Interestingly, similar Pchlide species were reproduced in this work for PORB:(Cys3-Ala)-PORA supracomplexes reconstituted with membrane lipids. We interpret this result as evidence for a partial protection of PORB by the (Cys3-Ala)-PORA mutant protein.

A breakthrough towards an understanding of PORA and PORB function during greening has recently been achieved. Frick et al. (2003) isolated a double knock-out mutant of *Arabidopsis* lacking both PORB and PORC because of DNA insertions in

their respective structural genes. PORC has been identified by sequence homology as a consequence of the *Arabidopsis* genome project (Oosawa et al., 2000; Su et al., 2001; Pattanayak and Tripathy, 2002). Unlike PORA and PORB, PORC is expressed at barely detectable levels in dark-grown plants and is positively light-regulated during greening (Oosawa et al., 2000; Su et al., 2001; Frick et al., 2003). The spatial expression pattern of PORC appears similar to that of PORB in older seedlings and adult plants but its function remains obscure (Armstrong et al., 1995; Frick et al., 2003).

The phenotype of the *porB-porC* double mutant provides the first, though indirect, evidence for a photoprotective role of PORA in light-dependent chlorophyll biosynthesis. Germinating *porB-porC* double mutants initially resemble wild-type seedlings at a time point when the wild type is known to transiently express PORA (Armstrong et al., 1995), but develop first *chlorina* (chlorophyll *b*-free) and then *xantha* (chlorophyll *a*- and chlorophyll *b*-free, white) phenotypes as photomorphogenesis progresses and PORA expression is turned off (Frick et al., 2003). PORA's function thus must lie in conferring photoprotection on PORB and not in driving Chlide *b* synthesis. Taken together these findings convincingly support the LHPP concept and highlight that PORA and PORB do not accomplish redundant roles *in planta*.

The results summarized in this study show that homology modelling in combination with *in vitro*-mutagenesis is a powerful tool to examine the structure of the PORA and PORB. However, no answers can be obtained for the LHPP complex in which the 3D-structures of the PORA and PORB are different from those of the enzymes in solution. The situation is even more complex given that membrane lipids drastically affect the structures and catalytic properties of either enzyme. Work is therefore needed to resolve the 3D-structure of LHPP by crystallography and X-ray analysis.

METHODS

Primers

Oligo-S: 5'-pCCGCGAGACCCACCCTTGGAGGCTCCAGATTTATC-3';
M1 (Cys39→Ala)-PORA: 5'-pCACGTCGTCATGGCGGCGCGCGACTTCCTCAAG-3';
M2 (Cys91→Ala)-PORA: 5'-pCTGGATGTGCTGGTCGCGAACGCCGCCATCTAC-3';
M3 (Cys202→Ala)-PORA: 5'-pAAGGACAGCAAGGTGGCCACATGCTGACCATG-3';
M4 (Cys229→Ala)-PORA: 5'-pTCGCTCTACCCGGGCGCCATCGCCACGGGG-3'.

Production of (Cys_x→Ala)PORA mutant proteins

DNAs encoding the different (Cys→Ala)-PORA mutant proteins lacking their transit peptides for plastid import were generated with clone L2 (Holtorf et al., 1995) as template and the Promega GeneEditor™ *in vitro* site-directed mutagenesis kit. The following primer combinations were employed: Oligo-S plus M1, Oligo-S plus M2, Oligo-S plus M3, and Oligo-S plus M4. The resulting DNAs were sub-cloned into the pSP64 vector (Promega GmbH, Mannheim, Germany).

For construction of DNAs encoding PORA proteins bearing hexahistidine ($[His]_6$)-tags at their NH_2-termini, the different DNA inserts were removed from the generated clones with *Bam*HI and *Hin*dIII and inserted into *Bam*HI- and *Hin*dIII-pretreated pQE30 vector (Qiagen, Hilden, Germany) using T4 DNA-ligase (Promega). By the same procedure, a $(His)_6$-PORB cDNA was constructed. All of the different DNAs in turn were transformed into *Escherichia coli* strain XL1blue. Positive clones were selected either by a PCR-based approach (5' 94°C ; 30x [30'' 94°C ; 30'' 55°C ; 2' 30''72°C]) using primers corresponding to the pQE30 vector and Taq DNA polymerase (Sigma) (2.5 units per 25 µL) or by DNA mini preparation and diagnostic restriction enzyme digestion. DNA sequencing confirmed the identity of the generated clones; it was performed by standard procedures, using the gel system described by Sanger et al. (1977) and a multicapillar ABI3100 (36 cm/POP4) sequencer, or by GATC Biotech (Constance, Germany).

Bacterial expression of PORA and PORB proteins in *E. coli*

Single positive colonies of *E. coli* were picked and propagated in 20 mL of LB medium containing 100 µg/ml ampicillin, diluted to 1 L with the same medium, and cultivated overnight at 37°C. Protein expression was induced at 30°C by adding IPTG to a final 0.5 mM concentration. After 3 hr, bacteria were sedimented by centrifugation at 6.000 *g* for 30 min, briefly washed with lysis buffer (50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole, pH 8.0), and resuspended in 25 mL of lysis buffer containing 20 µM PMSF, 100 µM leupeptin, and 1 µM pepstatin. After disruption in a French press cell (Thermo Spectronic) at 1000 Psi, bacterial remnants were removed by centrifugation at 12.000 *g* for 30 min at 4°C. The resulting supernatant was re-centrifuged at 50.000 *g* for 1 h, and the cleared supernatant purified on a *HiTrap Chelating®, Ni-NTA-agarose* affinity column. Proteins bound to the affinity matrix were eluted with lysis buffer containing 300 mM imidazol. Elution was monitored by Western blotting using either anti-POR (Schulz et al., 1989) or anti-($[His]_6$) antisera. Final protein preparations were passed over a *HiTrap Desalting®* column equilibrated in assay buffer.

Reconstitution of POR-pigment-NADPH complexes

Either bacterially expressed or wheat germ-translated, purified proteins were used. Pulse-labelling of proteins in *E. coli* with ^{35}S-sulfate was performed as described by Scharf and Nover (1982), whereas *in vitro*-protein synthesis in the presence of ^{35}S-methionine was carried out in a TNT wheat germ coupled *in vitro* transcription/translation system (Promega). ^{35}S-PORA and ^{35}S-PORB as well as ^{35}S-(Cys1→Ala)-PORA, ^{35}S-(Cys2→Ala)-PORA, ^{35}S-(Cys3→Ala)-PORA and ^{35}S-(Cys4→Ala)-PORA molecules that had been produced from the recombinant clones described above were purified and resolved by SDS-PAGE (Laemmli, 1970). After electrophoresis and autoradiography of an aliquot of the different protein samples, the radioactivity bands were cut out and counted in a liquid scintillation counter. Equal amounts of the different proteins were supplemented with 5 mM NADPH and incubated with chemically pure Pchlide *a* or Pchlide *b* (Reinbothe et al., 2003a), each added to 1 mM final concentrations to the assays. POR-pigment-NADPH complexes formed during a 15-min dark incubation were depleted of non-

protein-bound pigments by gel filtration on Sephadex G15, equilibrated either in assay buffer containing 350 mM sucrose or assay buffer lacking sucrose (Reinbothe et al., 1995a,b). POR-pigment-NADPH ternary complexes eluted with the flowthrough in turn were kept in darkness or illuminated with white light for appropriate periods. Enzymatic product formation occurring during the light incubations was monitored fluorometrically, using pigments extracted with acetone from an aliquot of the reconstituted POR-product complexes (see below).

Assembly assay of LHPP

Gel-filtered, ^{35}S-methionine-labeled (Cys1→Ala)-PORA-Pchlide *b*-NADPH, (Cys2→Ala)-PORA-Pchlide *b*-NADPH, (Cys3→Ala)-PORA-Pchlide *b*-NADPH, and (Cys4→Ala)-PORA-Pchlide *b*-NADPH complexes as well as PORA-Pchlide *b*-NADPH complexes were mixed with an equimolar amount of ^{35}S-PORB-Pchlide *a*-NADPH complexes and incubated in the dark for 15 min. One aliquot of the reaction mixtures was immediately precipitated with trichloroacetic acid and used for radioactivity measurements using a liquid scintillation counter. Another aliquot was subjected to gel filtration on Superose 6 (Amersham Pharmacia Biotech, www.apbiotech.com) equilibrated in assay buffer containing 350 mM sucrose. Individual fractions were harvested, and aliquots were withdrawn for radioactivity measurements. Alternatively, non-radiolabeled proteins were used and detected by Western blotting, using POR-specific antiserum (Schulz et al., 1989). In either case, appropriate fractions were pooled and protein precipitated with trichloroacetic acid, processed with acetone, ethanol and diethyl ether and resolved on 10-20% polyacrylamide gradients containing SDS (Laemmli, 1970). After electrophoresis, ^{35}S-PORA:PORB higher molecular weight complexes and non-assembled ^{35}S-PORA, ^{35}S-PORB and ^{35}S-PORA derivatives were visualized by autoradiography or, in experiments where non-radiolabeled proteins had been used, by Western blotting.

Pigment measurements

HPLC was performed on either C18 reverse phase (RP) silica gel columns (Macherey-Nagel Co., 250 x 4.6 mm, Nucleosil ODS 5 µm)(Scheumann et al., 1999) or C30 RP columns (YMC Inc., Willmington, NC, USA, 250 x 4.6 mm, 5 µm)(Fraser

et al., 2000), using established procedures and a Varian ProStar model 410 apparatus, ProStar model 240 pump, and ProStar 330 photodiode array detector. In some experiments, Chlides *a* and *b* were separated on RP18 Gromsil columns (Grom, Herrenberg, Germany), using a gradient of 100% acetone, applied for 3 min, and 60% acetone/40% acetic acid-supplemented water, pH 6.5, reached within 20 min (Oster et al., 2000). Low-temperature luminescence spectroscopy was performed at 77K at excitation wavelengths of either 440 nm or 470 nm, in a Life Sciences spectrometer LS50 (Perkin Elmer Corp., Norwalk, CT)(Lebedev et al., 1995).

Protease treatment of POR-pigment complexes *in vitro*

Protease treatment of reconstituted ^{35}S-POR-pigment complexes was carried out as described (Reinbothe et al., 1995b). Incubation mixtures contained 7.5 µL of doubly-concentrated assay buffer (Reinbothe et al., 1995a), 1 µL of a 25 mM stock solution of Mg-ATP, pH 7.0, 5 µL of a plastid protease mixture prepared from barley chloroplasts (Reinbothe et al., 1995c), and 1.5 µL of bidistilled water. After 15-min incubations in darkness, the assays were terminated by the addition of 1 µL of protease inhibitor cocktail containing 10 µg mL^{-1} antipain and 1 µg mL^{-1} pepstatin, which efficiently block the POR-degrading protease (Reinbothe et al., 1995c).

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Manuscript 4

Plastid import of NADPH:protochlorophyllide oxidoreductase A is essential for seedling survival

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Plastid import of NADPH:protochlorophyllide oxidoreductase A is essential for seedling survival

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Summary

The synthesis of chlorophyll from protochlorophyllide in plastids is catalyzed by two closely related light-activated enzymes, NADPH:protochlorophyllide oxidoreductases PORA and PORB. As nucleus-encoded proteins, PORA and PORB use different translocons for plastid import, and PORA is transported through a protochlorophyllide-dependent translocon (PTC). Here we used reverse genetics to determine the role of one PTC protein, PTC16, which is a homologue of the outer envelope protein OEP16. Loss-of function mutation in the *ptc/oep16* gene causes a conditional seedling lethal phenotype related to defects in pPORA import and excess protochlorophyllide accumulation in darkness. Our results underscore the essential role of pPORA plastid import for seedling survival during the transition of emerging seedlings from heterotrophic to photoautotrophic growth.

In plants, light provides an important environmental signal and trigger for the production of photosynthetically active chloroplasts. In dark-grown angiosperms, plastid development is arrested at a state that leads only to the formation of so-called etioplasts (1). These organelles are devoid of chlorophyll and incapable of photosynthetic function. Once dark-grown plants are illuminated, they begin to synthesize chlorophyll and to assemble the photosynthetic apparatus (1).

In angiosperms the biosynthesis of Chl is a light-dependent reaction (2). NADPH:protochlorophyllide oxidoreductases PORA and PORB (3, 4) are present in the prolamellar body of etioplasts and establish larger light-harvesting POR:Pchlide complexes designated LHPP (5). Dark-stable PORA:PORB-Pchlide-NADPH supracomplexes are poised such that absorption of a photon by Pchlide *b* leads to energy transfer onto Pchlide *a* and its subsequent reduction, resulting in the formation of Chlide *a* (5, 6). As other free tetrapyrroles, Pchlide not bound to POR could operate as photosensitizer (7-9). Angiosperm plants, therefore, have evolved efficient mechanisms to keep the level of these potentially phototoxic compounds low (10-13). One such mechanism is feedback control of Pchlide synthesis by heme and Pchlide (11, 12). Another factor is the FLU (FLUORESCENT) protein that depresses Pchlide synthesis in darkness (13). LHPP itself contributes to photoprotection

because it dissipates excess light energy in terms of fluorescence or heat once the seedlings de-etiolate (5, 6, and S. Reinbothe, unpublished results). Interestingly, LHPP then dissociates and PORA, in conjunction with the dispersal of the prolamellar body (14), gains activity as a Pchlide *b*-reducing enzyme such that another part of the light energy is quenched in a non-hazardous way (5, 6).

Being encoded in the nucleus, PORA and PORB are synthesized as larger precursors (pPORA and pPORB)(3,4) and imported into the plastids through specific protein translocon complexes in the outer and inner envelope membranes (15-17). While pPORB enters the plastids via the general protein import apparatus comprising the presequence receptor TOC159 and translocation channel protein TOC75 (16, 17), pPORA uses a Pchlide-dependent translocon named the PTC complex which is distinctive from the general protein import site (15). Among the identified PTC proteins was a 16 kDa protein related to a group of amino acid and preprotein transporters found in free-living bacteria, and endosymbiotic mitochondria and chloroplasts (16). Several lines of evidence verified that the identified barley *ptc16* gene is an ortholog of the previously characterized pea and *Arabidopsis oep16* (At2g28900) gene (16).

At2g28900 contains 6 exons and 5 introns (Fig. S1A, see supplemental information) and we used reverse genetics to determine its role in *Arabidopsis*. A respective *Arabidopsis* mutant was purchased from the Salk Institute Genomic Analysis Laboratory collection (18) carrying a T-DNA insertion (Salk_018024.50.90.X) that disrupts the *AtOEP16-1:1* locus (Fig. S1B, see supplemental information). Expression studies showed that neither *oep16* transcript nor OEP16 protein were detectable in *Atoep16-1* plants and thus the mutant was null with respect to the *Atoep16-1:1* gene (Fig. S1C, see supplemental information).

Depending on the growth conditions, *Atoep16-1* seedlings exhibited different phenotypes (19)(Fig. 1). If grown in darkness and exposed to white light, the mutant rapidly bleached and finally died (Fig. 1B). In plants kept under continuous white light right from the beginning of germination, no phenotype was detectable and the plants looked like the wild-type (Fig. 1, compare C and F). Etiolated *Atoep16-1* plants examined under blue light with a Leica MZ12 fluorescence microscope showed a strong red fluorescence indicative of the presence of free porphyrin pigments in darkness (Fig. 1A, compare with the wild-type shown in D). These results were reminiscent of findings reported for the *flu* mutant of *Arabidopsis* that contains

elevated levels of red fluorescing Pchlide in darkness (13). Pchlide is present in a free form in *flu* plants and caused rapid cell death as a result of singlet oxygen formation that was triggered upon illumination (13). When dark-grown *Atoep16-1* seedlings were exposed to white light, very similar defects were observed including bleaching and cell death (Fig. 1B versus E).

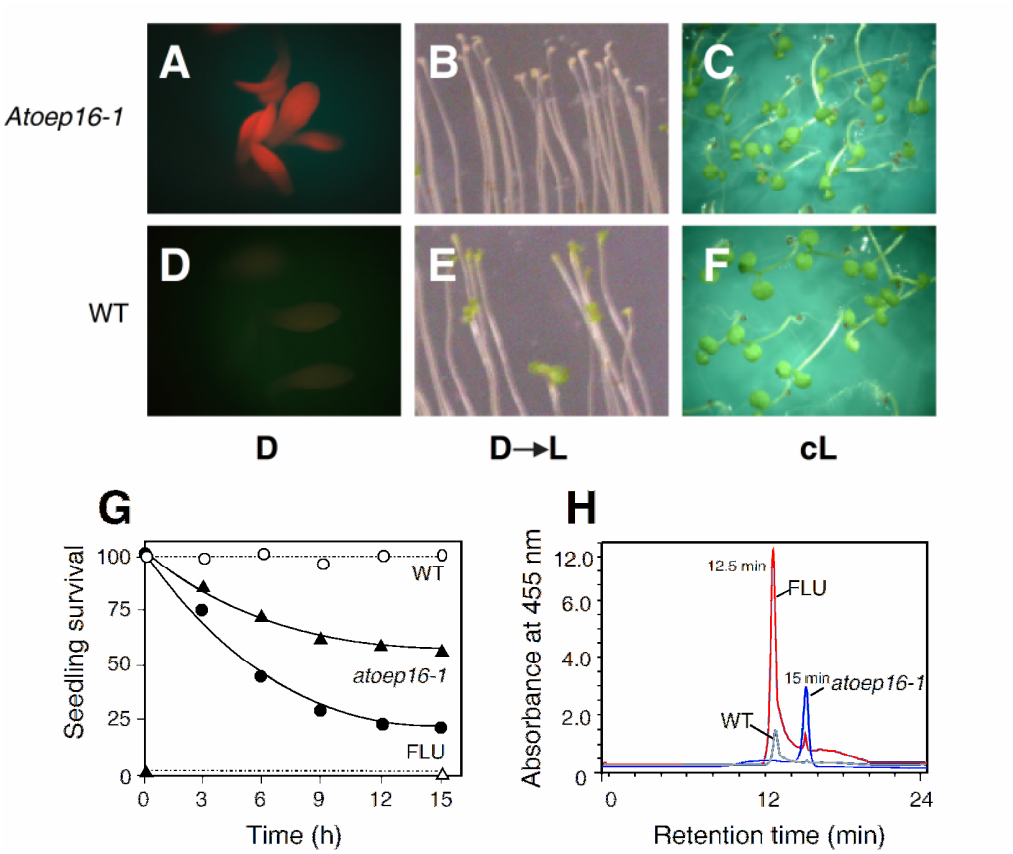

Fig. 1. Cell death phenotype of the *Atoep16-1* mutant in relation to growth conditions and porphyrin pigment accumulation.
(A-F) *Atoep16-1* (A-C) and wild-type (D-F) plants were grown in the dark (A and D), under a non-permissive dark-to-light shift (B and E), or under permissive continuous white light (C and F) and inspected under a microscope. Accumulation of free, red-fluorescing Pchlide was monitored under blue light (400-500 nm)(A and D). Free, excited Pchlide molecules present in etiolated *Atoep16-1* (A) but not in wild-type (D) plants caused photooxidative damages, as a result of which the seedlings died (B versus E, respectively).
(G) Seedling survival rates of wild-type (circles), *Atoep16-1* (triangles) and *flu* (dots) plants during a non-permissive dark-to-light shift. Out of a number of 300 seedlings in three independent experiments, the indicated percentages survived, whereas the remainder died as a result of pigment-sensitized photooxidation.
(H) Pigment accumulation in etiolated wild-type (graph in black colour), *Atoep16-1* (in blue) and *flu* (in red) plants. The different peaks were identified as Pchlide *b* (peak eluting at 12.5 min) and Pchlide *a* (peak eluting at 15 min), using synthetic standards and absorbance measurements as well as mass spectrometry (Fig. S1).

However, the time courses shown in Fig. 1G revealed some differences in cell death progression that, as we assumed, may be reflective of the actual level and/or composition of free porphyrin pigment(s) in *Atoep16-1* versus *flu* plants. Pigment analyses (20) indeed showed that while *flu* plants accumulate ca. 8.5 higher levels of total Pchlide than wild-type plants, *Atoep16-1* plants contained only ≈ 4.5-fold higher pigment levels. Interestingly, also the composition of pigments was altered in *Atoep16-1* versus *flu* and wild-type plants (Fig. 1H). While *Atoep16-1* plants accumulated Pchlide *a*, *flu* plants contained elevated levels of Pchlide *b* (Fig. 1H; for details of pigment identification, see Fig. S2, supplemental information).

Principally, the OEP16 protein could play a role in amino acid transport (21) or polypeptide transport (16). When we performed radioisotope labelling studies with ^{14}C-glutamate, ^{14}C-glutamine and ^{14}C-glycine and isolated chloroplasts and etioplasts (22), no difference in amino acid uptake was found for *Atoep16-1* versus wild-type plants (Fig. S3A, supplemental information, and data not shown) and also no difference was apparent in the patterns of plastid-encoded, ^{35}S-methionine-labelled (23) proteins (Fig. S3B, see supplemental information). On the other hand, the red fluorescence phenotype in darkness confirmed that *Atoep16* plants were not impaired in glutamate- and 5-ALA-dependent Pchlide synthesis. If OEP16 would be involved in amino acid uptake, an inhibition, but not an elevation, of glutamate-dependent synthesis of 5-ALA and Pchlide should have occurred in etiolated *Atoep16-1* plants which was obviously not observed (see Fig. 1). We thus concluded that *Atoep16-1* was not affected in bulk amino acid uptake into plastids.

Alternatively, OEP16 could be involved in polypeptide transport (16). This idea was tested by performing *in vitro*-import experiments for etioplasts and chloroplasts of *Atoep16-1* and wild-type plants (24). Figure 2 and 3 show that except for pPORA no differences in import were detectable for both plant and plastid types. In fact similar amounts of the different tested precursors were taken up and processed to mature size (Fig. 2D and Fig. 3A). Only import of pPORA was blocked *in vitro*, as evidenced by the unaltered precursor levels and the concomitant lack of the mature PORA in etioplasts from *Atoep16-1* versus wild-type plants (Fig. 2A). Likewise, no import was detectable with 5-ALA-treated, Pchlide-containing *Atoep16-1* as compared with wild-type chloroplasts (Fig. 2B and C). Chemical crosslinking (16) revealed that pPORA was able to bind *Atoep16-1* plastids but did not enter a productive import pathway (Fig. 3B). Transient assays in leaf epidermis cells

expressing transit peptide fusions of PORA (transA) with green jelly fish protein (GFP)(transA-GFP)(25) indeed confirmed the import defect of *Atoep16* plants (Fig. S4, see supplemental information).

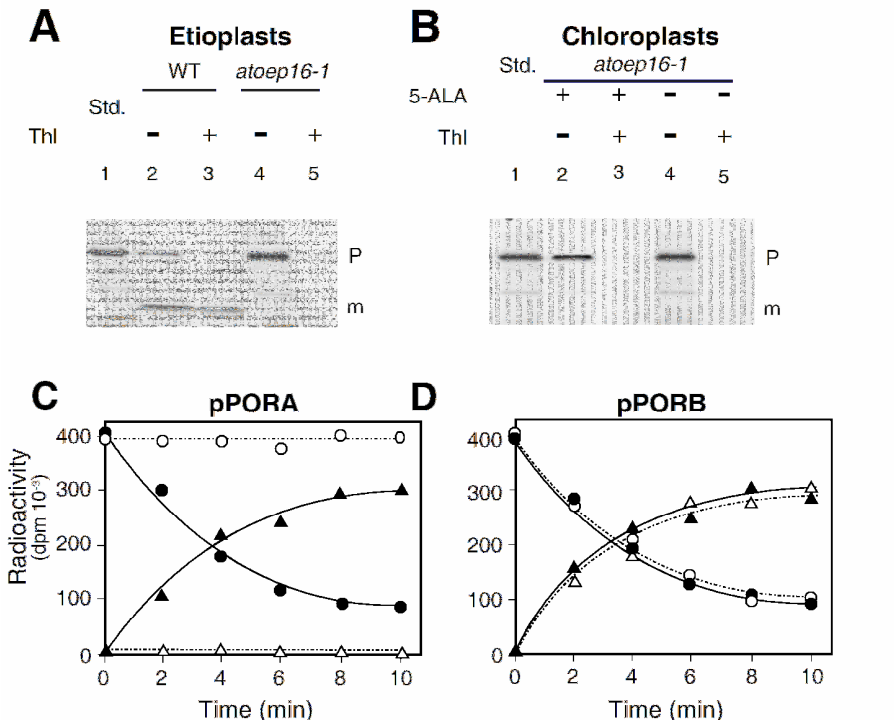

Fig. 2. *In vitro*-import of ^{35}S-precursors into plastids isolated from *Atoep16-1* and wild-type seedlings.
(A) Import of ^{35}S-pPORA into etioplasts of *Atoep16-1* and wild-type plants. P and m refer to precursors and mature proteins on the autoradiograms of SDS-PAGE-resolved proteins. Std defines input standards; Thl, thermolysin.
(B) as (A), but showing import data of ^{35}S-pPORA for 5-ALA-pretreated, Pchlide-containing and mock-incubated *Atoep16-1* chloroplasts.
(C) and (D), Time courses of import of ^{35}S-pPORA (C) and ^{35}S-pPORB (D) into 5-ALA-treated, Pchlide-containing wild-type (dots and filled triangles) and *Atoep16-1* (circles and open triangles) chloroplasts. Precursor and mature proteins are marked by circles and dots as well as open and filled triangles, respectively. Protein quantification was made using a PhosphorImager.

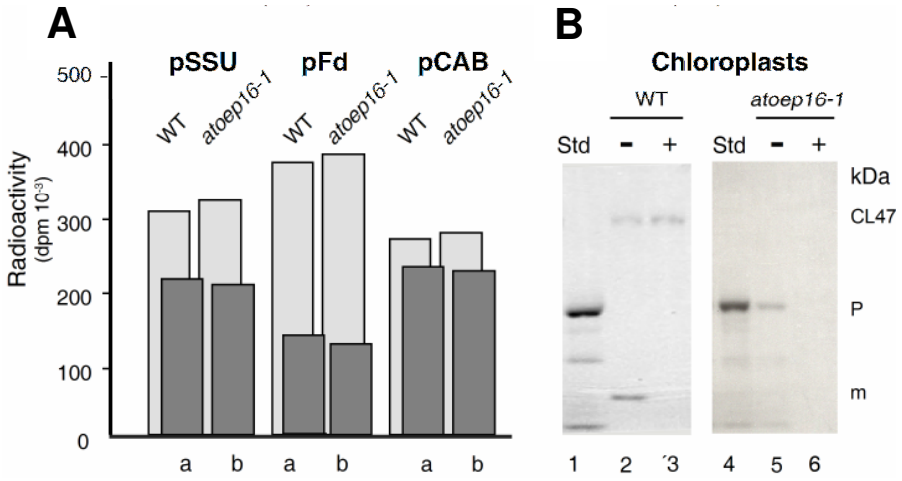

Fig. 3. *In vitro*-import of ^{35}S-precursors into plastids isolated from *Atoep16-1* and wild-type seedlings.
(A) Quantitaive import data for the small subunit precursor of ribulose-1,5-bisphosphate carboxylase/oxygenase, pSSU; precursor ferredoxin, pFd; and the chlorophyll *a*/*b*-binding protein precursor of photosystem II, pCAB. Light and dark grey columns refer to precursor and mature protein levels after 15-min import reactions into 5-ALA-treated, Pchlide-containing chloroplasts of wild-type (a) and *Atoep16* (b) plants.
(B) Crosslinking of 5,5'-dithiobis(2-nitrobenzoic acid (DTNB)-activated ^{35}S-transA-DHFR, consisting of the Pchlide-responsive transit peptide of pPORA (transA) and a dihydrofolate reductase (DHFR) reporter protein of mouse, to isolated 5-ALA-pretreated, Pchlide-containing wild-type (lanes 1-3) and *Atoep16-1* (lanes 4-6) chloroplasts. CL47 defines a 47 kDa products established between free sulhydryl groups of the 31 kDa precursor (P) and OEP16, whereas m stands for the imported, mature, 25 kDa DHFR (lane 2). Lane 3 shows a respective immunoprecipitation using OEP16 antiserum (16). Lanes 5 and 6 depict corresponding crosslink products and immunoprecipitates, respectively, obtained for *Atoep16* chloroplasts. In lanes 1 and 4 are shown input standards (Std). Note the lack of CL47 and imported, mature DHFR in *Atoep16* chloroplasts.

That knock-out in the *Atoep16-1:1* gene did not pleiotropically affect all of the different cytosolic precursors that need to be taken up by the plastid compartment is further supported by the apparent normal phenotype that is rescued in *Atoep16-1* plants grown in continuous white light (cf. Fig. 1C). Like pSSU, pFd, pCAB and pPORB, also the enzymes that are involved in 5-ALA synthesis and subsequent steps of Pchlide production were obviously not affected by the lack of the OEP16 protein. Otherwise no excess pigment would have accumulated in dark-grown *Atoep16-1* plants. All enzymes of the C5-pathway leading to chlorophyll are nucleus-encoded and imported posttranslationally from the cytoplasm (2).

The import defect in *Atoep16-1* seedlings is specific for pPORA. Protein gel blot analyses showed a lack of PORA and PORA-containing larger LHPP complexes in *Atoep16-1* versus wild-type plants (Fig. 4A, B). Electron microscopy (26)

highlighted a drastic reduction in the size, in most cases even a complete lack, of the prolamellar body in *Atoep16-1* as compared with wild-type plants (Fig. 4C). Together, these findings conclusively showed that PORA, as part of larger complexes in the prolamellar body, played an essential role for plant survival during seedling de-etiolation. Free Pchlide not bound to the PORA, by contrast, was phototoxic.

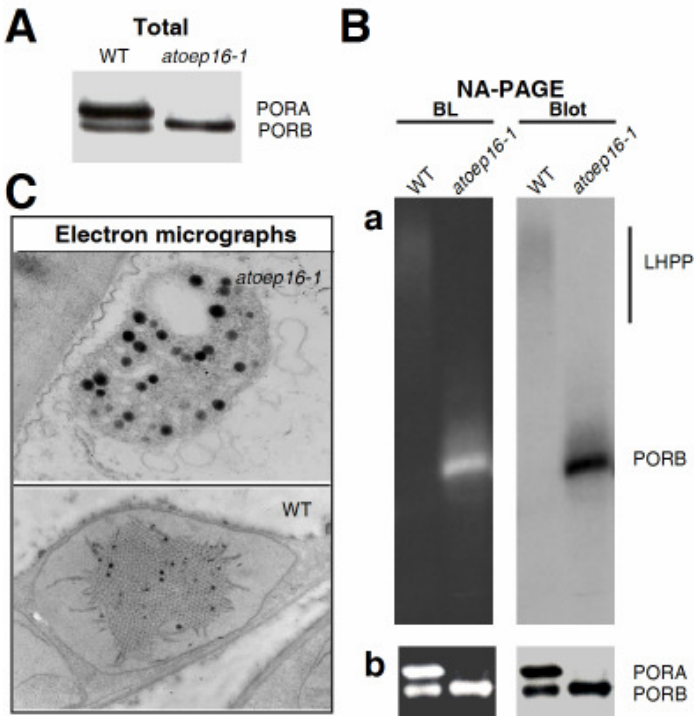

Fig. 4. POR expression in etiolated *Atoep16-1* and wild-type seedlings.
(A) PORA and PORB protein levels in *Atoep16-1* and wild-type etioplasts, determined by SDS-PAGE and autoradiography.
(B) Detection of light-dissociable PORA:PORB-Pchlide-NADPH supracomplexes indicative of LHPP in solubilized membrane fractions of *Atoep16-1* and wild-type etioplasts before (a) and after (b) a 1-msec flash of white light. Protein detection was made by non-denaturing (NA) PAGE and blue light (BL)-induced pigment autofluorescence (left hand panels) and protein gel blot (Blot) analysis (right hand panels) using a POR antiserum.
(C) Transmission electron micrographs of *Atoep16-1* and wild-type etioplasts. Bars indicated size markers.

A working model to explain the interaction of OEP16, PORA and FLU is shown in Fig. 5. Accordingly, PORA-Pchlide *b*-NADPH ternary complexes not assembled into PORA:PORB supra-structures after import through OEP16 could provide the link to the FLU protein and help regulate the size of the prolamellar body. In wild-type plants, PORA would sense the amount of Pchlide, and once enough

LHPP is made, excess PORA-Pchlide *b*-NADPH ternary complexes could bind to and block the activity of the FLU protein, thereby inhibiting glutamyl-tRNA reductase. By contrast, this negative feedback would no longer operate in the OEP16-deficient *Atoep16-1* mutant line because of the lack of imported PORA. Free, non-protein-bound pigment then would operate as photosensitizer and destruct the emerging seedlings once they are exposed to white light. Work is in progress to test this model by biochemical and genetic approaches.

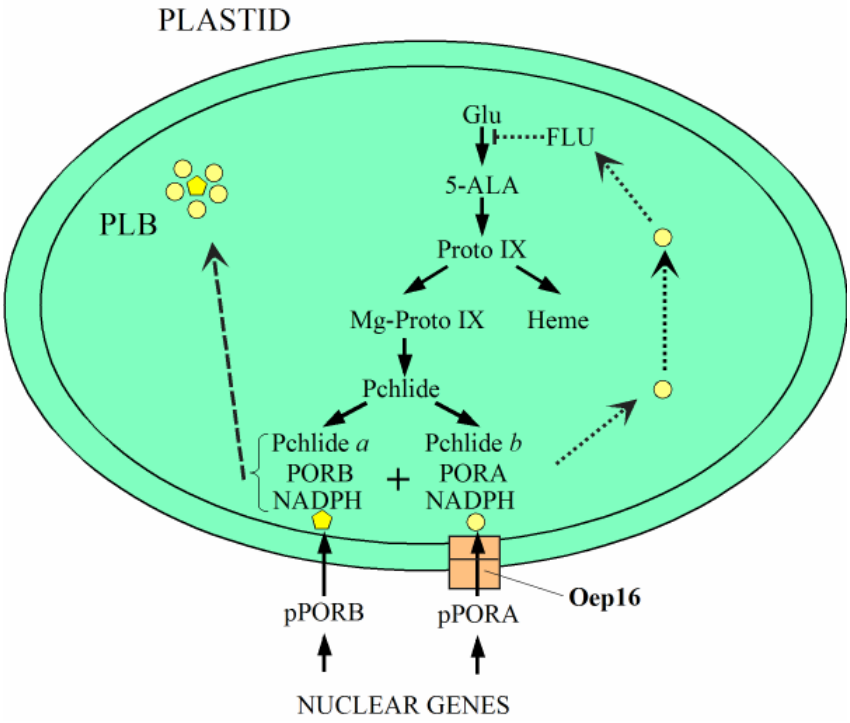

Fig. 5. Working model of pPORA import, LHPP assembly, and FLU-mediated regulation of Chl biosynthesis. In essence, the model suggests that free PORA-Pchlide *b*-NADPH ternary complexes not assembled into LHPP in the prolamellar body (PLB) exert a negative feedback loop on the FLU protein that in turn inhibits glutamyl-tRNA reductase and thereby restricts Pchlide synthesis. In the *Atoep16-1* mutant line lacking Oep16, no pPORA import occurs and Pchlide accumulation thus would no longer be feedback-inhibited, leading to Pchlide overproduction. Implicit in the model is another feedback loop exerted by Pchlide *b* on PTC52, operating as Pchlide *a*-oxygenase (not shown). Yellow circles and pentagons illustrate PORA-Pchlide *b*-NADPH and PORB-Pchlide *a*-NADPH ternary complexes, respectively. The ochre box highlights Oep16 as part of the Ptc complex. Abbreviations: Glu, glutamate,; 5-ALA, 5-aminolevulinic acid; Proto IX, protoporphyrin IX; Pchlide, protochlorophyllide.

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19. Seeds were germinated on half-concentrated Murashige-Skoog-agar medium on Petri dishes for 4.5 days. To trigger photooxidative damages, dark-grown seedlings were exposed to white light at 125 µE m^{-2}. Alternatively, seeds were germinated on soil and grown to maturity in continuous white light.
20. High performance liquid chromatography of acetone-extracted pigments was carried out using a C18 reverse phase silica gel column (Shandon, Hypersil ODS, 5 µm) and synthetic Pchlides *a* and *b* as standards. Pigments were detected and quantified at 455 nm, the Soret band of Pchlide *b* (see also Ref. 6).
21. Pohlmeyer, K., Soll, J., Steinkamp, T., Hinnah, S. & Wagner, R. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 9504-9509.
22. Uptake of ^{14}C-amino acids into isolated chloroplasts was monitored using the filter paper disc method of Mans & Novelli [*Arch. Biochem. Biophys.* **94**, 48-63 (1961)].
23. Pulse-labelling of chloroplast protein with ^{35}S-methionine (1.87 MBq per 50 µL-assay, 37 TBq/mmol, Amersham-Pharmacia) was carried out according to Mullet et al. [*Eur. J. Biochem.* **155**, 331-338 (1986)]. Protein was extracted with trichloro-acetic acid, depleted of chlorophyll, and washed and run on 10-20% SDS-polyacrylamide gradients [Laemmli, U.K. *Nature* **227**, 680-685 (1970)]. Protein detection was made by Coomassie staining and autoradiography or by Western blotting [see Fig. S3].
24. Protein import into isolated *Arabidopsis* chloroplasts and etioplasts was studied as described, using cDNA-encoded, wheat germ-translated, ^{35}S-precursors [Reinbothe et al., *Plant J.* **42**, 1-12 (2005)]. Plastids were treated with thermolysin [Cline et al. *Plant Physiol.* **75**, 675-678 (1984)] after import in order to degrade unimported precursors. Chemical crosslinking of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)-derivatized ^{35}S-pPORA and ^{35}S-pPORB was carried out as described (Ref. 16).
25. Transient expression of TransA-GFP derivatives was performed in *Arabidopsis* leaf epidermis cells. Ballistic bombardment was carried out using a pneumatic particle inflow gun according to Finer et al. [*Plant Cell Rep.* **11**, 323–328 (1992)]. The conditions of bombardment were adjusted to helium pressure of 6.5 bar, 12 cm target distance, with a disperse grid at 7 cm, using 1 µm of gold microcarriers (BIO-RAD). After bombardment, the plantlets were kept under

sterile conditions and incubated for 18 h in darkness. Confocal laser scanning microscopy was carried out utilizing a LSM 510 Meta microscope (ZEISS, Jena, Germany) with krypton/argon laser excitation at 488 nm and an emission wavelength window from 505 to 530 nm. LSM 510 Meta software release 3.2 (ZEISS) and Adobe Photoshop 7 (Adobe Systems, San Jose, CA) were used for image acquisition and processing.

26. Electron microscopy was carried out using ultrathin sections of leaf tissues prepared from etiolated plants using a ZEISS 109 electron microscope.
27. We are indebted to L. Reinbothe for editorial work. This study was supported by the French Ministry of Research and the CNRS. C.R. is a laureate of a Chaire d'Excellence at the UJF Grenoble.

Supplemental information

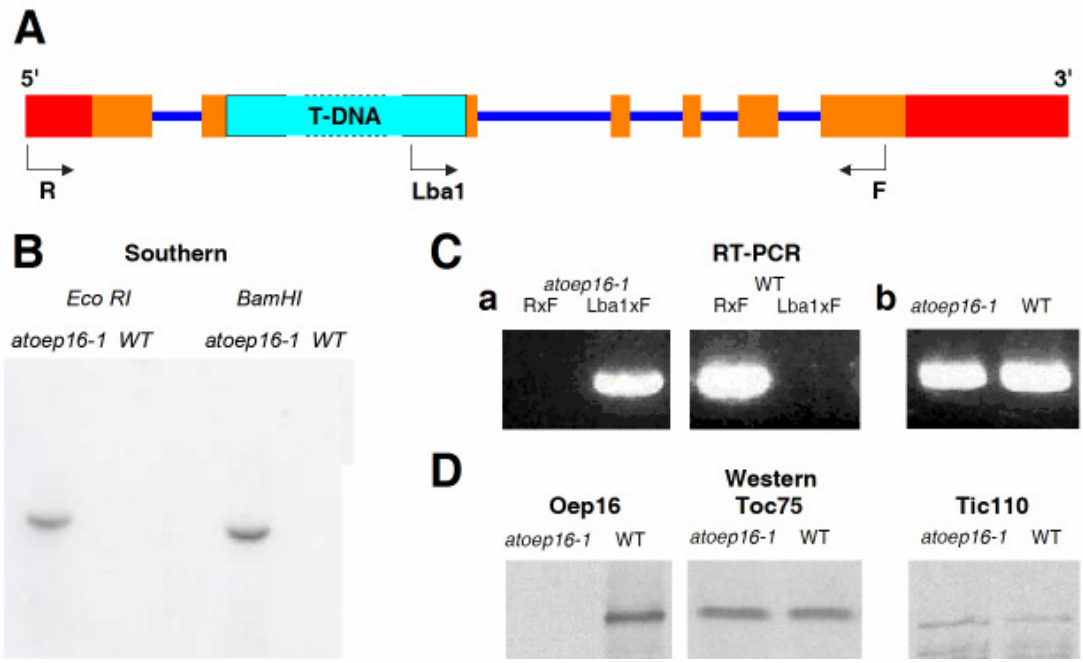

Fig. S1. Identification of the *Atoep16-1* T-DNA insertion line (SALK_024018.50.90.X).
(**A**) Diagram of the *Atoep16-1:1* gene and respective T-DNA insertion. 5'- and 3'-untranslated regions are in red, exons and introns in ochre and violet, respectively. The 4.5 kb T-DNA insertion (not drawn to scale) is shown in green. R and F as well as Lba1 mark primers used for PCR analyses.
(**B**) DNA gel blot analysis of the *atoep16-1* line. Genomic DNA (10 µg) from homozygous *Atoep16-1* plants or wild-type plants was digested with *Eco*RI and *Bam*HI, respectively, and the filter-bound DNA fragments were hybridized to a DNA probe corresponding to the kanamycin-resistance gene of the T-DNA using standard procedures (Ref. 1).
(**C**) Confirmation of the T-DNA insertion by PCR using the indicated primers. PCR was carried out according to Innis et al. (Ref. 2), using the following primers: Lba1: 5'-ATGGTTCACGTAGTGGGCCATCG-3', R (reverse primer): 5'-ATCCACCGTT-AAAAGCCCCTT-3', F (forward primer): 5'-AACGAACTGAGAAGCGGTTGC-3' (a). Panel b shows a PCR band obtained with primers specific for the adenine phosphoryl transferase gene of wild-type and *Atoep16-1* plants.
(**D**) Western blot analysis of OEP16 protein expression in chloroplasts of *Atoep16-1* and wild-type plants. Protein was prepared from isolated chloroplasts and subjected to Western blotting (Ref. 3), using antisera against OEP16 and the 75 kDa and 110 kDa proteins of the outer and inner envelope membrane translocases of chloroplasts, TOC75 and TIC110, respectively.

1. Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual* (Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY).
2. Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (1990) *PCR Protocols* (Academic Press, San Diego, CA).
3. Towbin, M. et al., *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354 (1979).

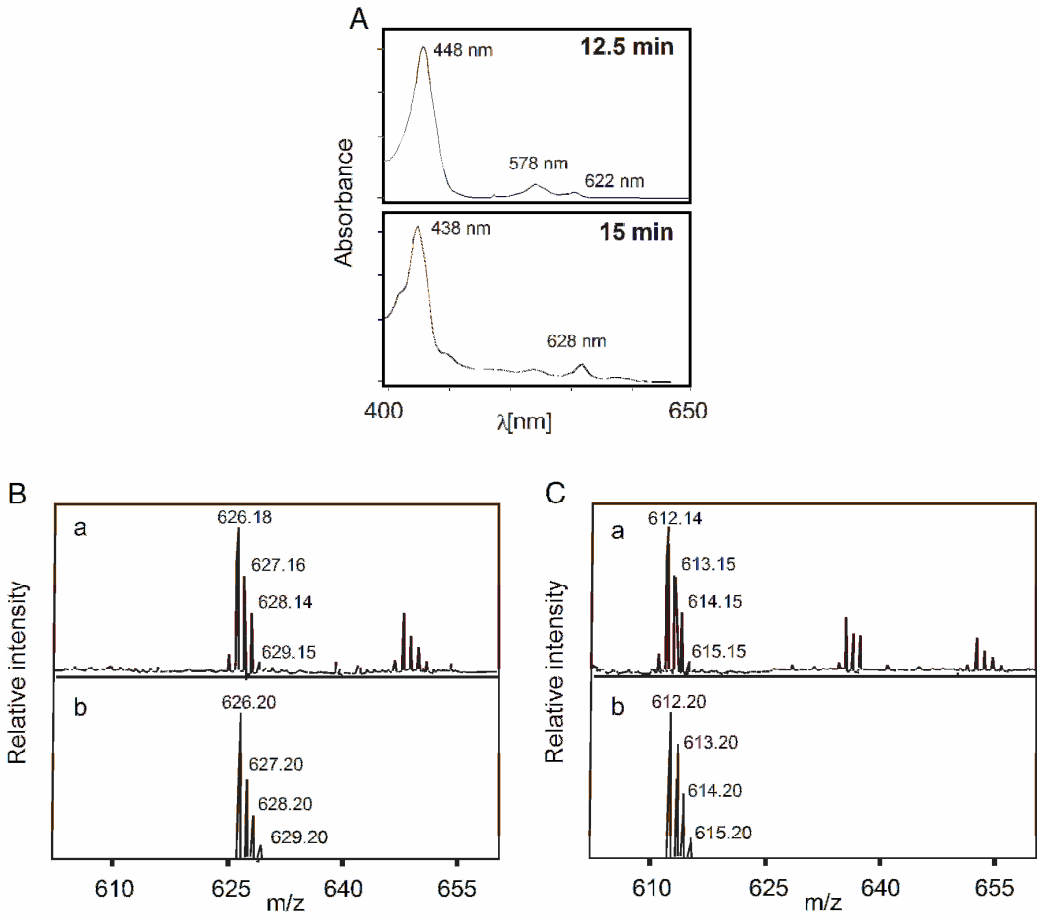

Fig. S2. Identification of pigments. Pigments were extracted from etiolated wild-type, *flu* and *Atoep16* plants, subjected to HPLC on a C18 reversed phase column as described in Fig. 1H, and the peaks eluting at 12.5 min and 15 min, respectively, in turn were subjected to absorbance measurements and mass spectrometry.
(**A**) The absorption spectrum of the peak eluting at 12.5 min is indicative of Pchlide *b*, whereas that eluting at 15 min is indicative of Pchlide *a*.
(**B**) Confirmation of the identity of Pchlide *b* eluting at 12.5 min by matrix-assisted laser desorption/ionization mass spectroscopy (a) and theoretical isotopic distribution of C35H30N4O6Mg corresponding to Pchlide *b* (b). The matrix used was terthiophene (molecular weight 248.4, not shown).
(**C**) Mass spectrometry data for Pchlide *a* eluting at 15 min (a) and theoretical isotopic distribution of C35H32N4O5Mg corresponding to Pchlide *a* (b). Note that the spectra are identical to those published by Schoch et al. (1), Scheumann et al (2) and Xu et al. (3).

1. Schoch, S., Helfrich, M., Wiktorsson, B., Sundqvist, C., Rüdiger, W. & Ryberg, M. (1995) *Eur. J. Biochem.* **229**, 291-298.
2. Scheumann, V., Klement, H., Helfrich, M., Oster, U., Schoch, S. & Rüdiger, W. (1999) *FEBS Lett.* **445**, 445-448.
3. Xu, H., Vavilin, D., Vermaas, W. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 14168-14173.

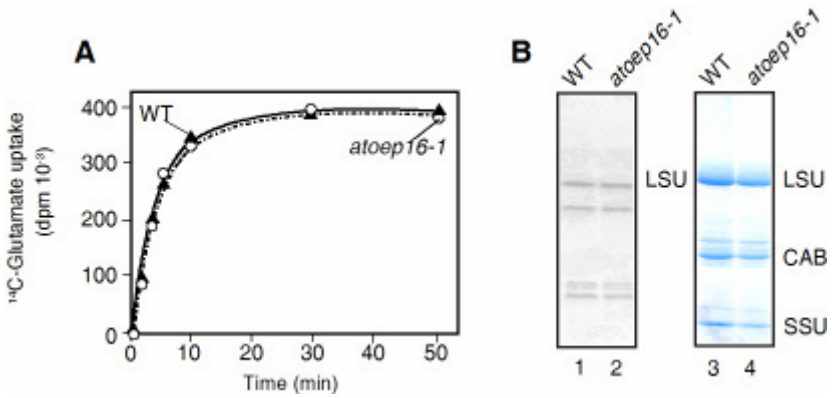

Fig. S3. ^{14}C/^{35}S-amino acid uptake into *Atoep16-1* and wild-type chloroplasts.
(**A**) Isolated chloroplasts were incubated with ^{14}C-glutamate for various time intervals and uptake of radioactivity was measured as specified in Ref. 23.
(**B**) After labelling isolated chloroplasts with ^{35}S-methionine for 5 min (lanes 1 and 2) or 20 min (data not shown), protein was extracted and precipitated with trichloroacetic acid, separated by SDS-PAGE and detected by either autoradiography (lanes 1 and 2) or Coomassie staining (lanes 3 and 4). LSU and SSU refer to the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase; CAB defines the chlorophyll *a*/*b*-binding protein of photosystem II.

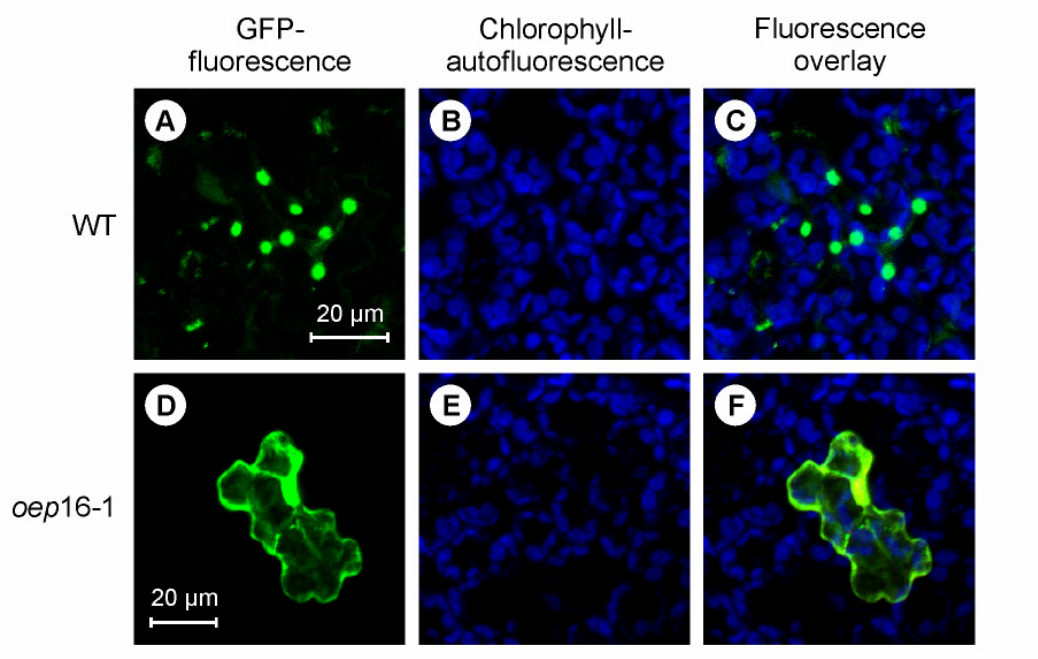

Fig. S4. Expression and plastid import of TransA-GFP in *Arabidopsis* leaf epidermis cells of *Atoep16-1* and wild-type plants.
DNA for transA-GFP, encoding transA and the green jelly fish protein (GFP), was transformed into leaf epidermis cells of *Arabidopsis* wild-type and *atoep16* plants. After 24 h in darkness, GFP (A,D) and chlorophyll (B,D) fluorescences were monitored between 505-530 nm and 575-605 nm, respectively, using an excitation wavelengths of 488 nm. Panels C and F show merges of A and B as well as D and E, respectively. As sub-cellular localization controls, the naked GFP moiety lacking a transit peptide attached to it (not imported into either *atoep16* and wild-type plastids) and transB-GFP (imported into both plastid types) were used (data not shown).
(**A-C**) Import of transA-GFP into wild-type chlorplasts.
(**D-F**) Lack of of transA-GFP import into *atoep16* chlorplasts.

6 Summary

The light harvesting complex of etiolated plants, shortly called LHPP (light-harvesting protochlorophyllide-oxidoreductase : protochlorophyllide complex) was further investigated with regard to its structure, function and biosynthesis.

Previous experiments have shown that such a high-molecular light-harvesting aggregate could be assembled of ternary PORA-Zn protopheophorbide *b*-NADPH- and PORB–Zn protopheophorbide *a*-NADPH-complexes *in vitro* in a stoichiometry of 5:1. These experiments were repeated with the chemically synthesized protochlorophyllide *a* and *b* (the authentic Mg-containing compounds) in the dark to form first of all the appropriate POR-Pigment-NADPH ternary complexes (manuscript 1, 2, 3). These complexes were further incubated with each other in the dark: A high molecular weight complex could be reconstituted that was identified to be LHPP because of its spectroscopic properties and by a POR-specific antibody (manuscript 1, 3). Furthermore, an analogous complex of approximately the same size could be isolated from the prolamellar bodies of etioplasts in barley and was characterized as presumed LHPP-complex using POR-specific antibodies (manuscript 1).

The *in vitro* reconstituted complex was supplied with a mixture of galacto- and sulfolipids isolated from prolamellar bodies. Fluorescence spectroscopy of the lipid-containing LHPP-complex at low temperature revealed the formation of photoactive protochlorophyllide 650/657 as well as photoinactive protochlorophyllide 628/632 in both the *in vitro* synthesized and the *in vivo* isolated complex (manuscript 1, 3). To demonstrate the function of the *in vitro* synthesized LHPP this reconstituted complex was illuminated with a 1 msec flash of white light. Thereafter, pigment analysis was carried out using fluorescence spectroscopy at low temperature (77 K): Photoactive protochlorophyllide 650/657 was reduced to Chlorophyllide 684/690 whereas photoinactive protochlorophyllide 628/632 was still detectable in the same amount as before illumination (manuscript 1, 3). The same observations were made for the isolated native LHPP-complex before and after flash-light illumination. HPLC analysis of acetone-extracted pigments combined with absorption spectroscopy or mass spectrometry was performed to identify the pigments in the native LHPP-complex. It was shown that only PORB-bound protochlorophyllide *a* was converted to chlorophyllide *a* by its enzyme, whereas protochlorophyllide *b* remained stable

(manuscript 1, 3). This finding confirmed the hypothesis of PORA-bound protochlorophyllide *b* to function as an antenna to harvest light and to transmit the energy onto protochlorophyllide *a* bound to PORB used to convert protochlorophyllide *a* to chlorophyllid *a*.

The cysteine residues in POR were assumed to play an important role in enzyme catalysis, since they are evolutionary conserved from cyanobacteria to higher plants. Previous work gave rise to the hypothesis that these residues are essential for pigment binding since alkylating of the thiol-groups with N-phenylmaleimide results in a loss of function of the enzyme. To proof this hypothesis, the cysteine residues of barley PORA and PORB were individually exchanged by alanine at the cDNA-level using site-directed mutagenesis. The altered cDNA´s were (heterologously) expressed and their capacity in pigment binding and ability to assemble into higher molecular-mass complexes was investigated (manuscript 2, 3).
In PORB two of the four cysteines (Cys276 and Cys303) could be identified to establish distinct pigment binding sites. Cys276 constitutes the protochlorophyllide binding site in the active site and is important for proper catalytic conversion of its substrate protochlorophyllide *a* (manuscript 2). This could be demonstrated by the assembly of PORB-protochlorophyllide *a*-NADPH-ternary complexes. Pigment analysis using fluorescence spectroscopy after flash-light illumination showed that (Cys276→Ala)PORB failed in pigment reduction (manuscript 2).
The second (low affinity) binding site of PORB was identified as Cys303 and can be found on the outer surface of the enzyme. This cysteine was shown to be essential for the interaction between the POR-protochlorophyllide-NADPH-ternary complexes to result in LHPP formation (manuscript 2). Furthermore, by binding its substrate it increases the stability of PORB after import into the plastids as was demonstrated in stromal-protease assays (manuscript 2).
In case of PORA also two of the four cysteines were involved in pigment binding: Cys202 and Cys229. As well as in PORB, one cysteine residue (Cys202) is responsible for binding a pigment in the active site of the enzyme, although in this case the pigment is a protochlorophyllide *b*-molecule (manuscript 3). Nevertheless this protochlorophyllide *b* remains photoinactiv in LHPP and can be photoconverted to chlorophyllide *b* only after disintegration of the LHPP complex (and the prolamellar bodies, respectively) (manuscript 3).

Cys229, the other pigment binding site of PORA, is placed at the periphery of the enzyme and was demonstrated to have a weak interaction with protochlorophyllide *b* (manuscript 3). Nevertheless, this protochlorophyllide molecule was proven to be necessary to allow the interaction of PORA/B-protochlorophyllide *b*/*a*-NADPH-ternary complexes to give rise to the LHPP complex. Therefore, Cys229 is essential for the biosynthesis of LHPP (manuscript 3) and for a functional energy transmittance by `fluorescence resonance energy transfer´ (FRET) onto the photoactive PORB-bound protochlorophyllide *a* in LHPP.

As mentioned before, PORA was a main constituent of LHPP. The uptake of this nucleus-encoded protein into the etioplast was shown to be dependent on a special import apparatus called PTC, from which OEP16 from *Arabidopsis thaliana* was identified to form the channel for the translocation of this protein (manuscript 4). Lacking of OEP16 in a knockout mutant of *Arabidopsis thaliana* did not interfere the import of other proteins or amino acids but led to the absence of PORA in the etioplast (manuscript 4). As a consequence no formation of prolamellar bodies and an increased content of protochlorophyllide compared to *Arabidopsis* wildtype etioplasts could be observed. Since also no LHPP complexes were detectable in the knockout mutant (manuscript 4), these effects lead to an increased sensitivity of etiolated seedlings for irradiation emphasizing the role of LHPP in seedling survival when exposed to light.

7 Zusammenfassung

In dieser Arbeit sollten Struktur, Funktion und Biosynthese von LHPP (`Light-harvesting Protochlorophyllide-Oxidoreduktase : Protochlorophyllide complex´), dem Lichtsammelkomplex etiolierter Pflanzen, näher untersucht werden.
In früheren Experimenten konnte ein solcher hochmolekularer Lichtsammelkomplex aus PORA-Zn Protopheophorbid *b*-NADPH- und PORB-Zn Protopheophorbid *a*-NADPH-Ternärkomplexen mit einer Stöchiometrie von 5:1 *in vitro* nachgewiesen werden. Unter Verwendung von chemisch hergestelltem Protochlorophyllid *a* und *b* wurde dieses Experiment zunächst durch Herstellung der entsprechenden Protein-Pigment-NADPH-Ternärkomplexe im Dunkeln und anschließender Dunkelinkubation dieser Ternärkomplexe wiederholt (Manuskript 1, 2, 3). Dabei konnte ein hochmolekularer Komplex isoliert werden, welcher durch Nachweis mit einem POR-spezifischen Antikörper und aufgrund seiner spektroskopischen Eigenschaften als LHPP identifiziert wurde (Manuskript 1, 3). Gleichzeitig konnte ein analoger Komplex von annähernd gleicher Molekulargewichtsgröße aus dem Prolamellarkörper von Gerstenetioplasten isoliert werden und durch einen POR-spezifischen Antikörper als vermutlicher LHPP-Komplex charakterisiert werden (Manuskript 1).
Der *in vitro* synthetisierte Komplex wurde gleichzeitig mit verschiedenen, vor allem für den Prolamellarkörper beschriebenen Lipidmolekülen, wie Galakto- und Sulfolipiden, komplementiert und eine Fluoreszenzmessung der gebundenen Pigmente durchgeführt. Dabei konnte gezeigt werden, dass, wie auch im mutmaßlichen nativen LHPP-Komplex, photoaktives Protochlorophyllid 650/657 neben dem photoinaktiven Protochlorophyllid 628/632 vorliegt (Manuskript 1, 3). Um die Funktion des *in vitro* gebildeten LHPP-Komplexes zu demonstrieren, wurde dieser durch einen 1 msec langen Weißlichtblitz belichtet und sowohl in einer Fluoreszenzanalyse als auch über HPLC die gebildeten Pigmente charakterisiert. Dabei stellte sich heraus, dass das photoaktive Protochlorophyllid 650/675 in Chlorophyllid 684/690 umgewandelt wurde, während das photoinaktive Protochlorophyllid auch nach dem Lichtblitz in unveränderter Menge vorlag (Manuskript 1, 3). Ähnliche Pigmentverteilungen vor und nach der Belichtung konnten auch in dem nativen LHPP-Komplex nachgewiesen werden. Bei differentieller Betrachtung der an PORA bzw. PORB gebundenen jeweiligen Pigmente wurde gezeigt, dass durch einen Lichtblitz nur an PORB gebundenes

Protochlorophyllid *a* zu Chlorophyllid *a* umgesetzt wurde, während an PORA gebundenes Protochlorophyllid *b* unverändert blieb (Manuskript 1, 3). Dieser Befund untermauert die Hypothese einer Antennenfunktion von PORA-gebundenem Protochlorophyllid *b*, um Lichtanregungsenergie zu sammeln, auf PORB-gebundenes Protochlorophyllid *a* zu übertragen und dessen Reduktion zu Chlorophyllid *a* zu vermitteln.

Eine bedeutende Funktion in der katalytischen Reaktion von NADPH:Protochlorophyllid-Oxidoreduktasen wird den Cysteinresten in den Aminosäuresequenzen des Enzyms zugeschrieben, da diese Cysteinreste evolutionär konserviert wurden und dementsprechend sowohl bei Cyanobakterien als auch höheren Pflanzen vorhanden sind. Schon in früheren Arbeiten wurde durch Blockierung der Thiolgruppen mit Alkylierungsreagenzien eine Funktion bei der Pigmentbindung als wahrscheinlich angesehen. Um diese Resultate weiter zu vertiefen, wurden bei beiden NADPH:Protochlorophyllid-Oxidoreduktasen aus Gerste durch „site-directed“ Mutagenese die jeweiligen vier Cysteinreste gegen Alaninreste auf cDNA-Ebene ersetzt, heterolog exprimiert und deren Fähigkeit zur Pigmentbindung in ternären Systemen als auch zur Komplexierung zu LHPP untersucht (Manuskript 2, 3).
In PORB konnten so von den vier existierenden Cysteinen zwei (Cys276 und Cys303) identifiziert werden, welche verschiedenartige Pigmentbindungsstellen im Enzym repräsentieren (Manuskript 2). Cys276 nimmt eine Pigmentbindungsstelle im aktiven Zentrum des Enzyms ein und spielt eine wichtige Rolle bei der katalytischen Umsetzung seines Substrats, Protochlorophyllid *a*. Dies wurde nach Assemblierung von PORB-Protochlorophyllid *a*-NADPH-Ternärkomplexen mit anschließender Belichtung und Fluoreszenzanalyse der gebildeten Pigmente als auch in Pigmentbindungsstudien mit (Cys276→Ala)PORB-Mutantenprotein gezeigt (Manuskript 2). Die zweite Pigmentbindungsstelle von PORB stellt das an der Enzymperipherie liegende Cys303 dar, wobei nur eine schwache Wechselwirkung des mit ihm assoziierten Protochlorophyllidmoleküls besteht. Dieses Cystein konnte als essentiell für die Interaktion der ternären POR-Protochlorophyllid-NADPH-Komplexen erwiesen werden, ist also an der Bildung von LHPP maßgeblich beteiligt (Manuskript 2). Des weiteren ist es durch die Bindung an sein Substrat für die

Stabilität von PORB nach dem Import in den Etioplasten verantwortlich (Manuskript 2).
Auch in PORA wurde von den vier im Enzym vorliegenden Cysteinresten von zweien deren Pigmentbindungsfähigkeit nachgewiesen. Es handelt sich dabei um die Cysteinreste Cys202 und Cys229. Wiederum steht ein Rest, Cys202, für die Bindung – diesmal eines Protochlorophyllid-*b*-moleküls – im aktiven Zentrum des Enzyms zur Verfügung. Dennoch ist dieses Protochlorophyllid *b* in LHPP photoinaktiv, und wird erst nach dem Zerfall von LHPP und weiterer Belichtung zu Chlorophyllid *b* umgesetzt (Manuskript 3). Der andere an der Pigmentbindung beteiligte Cysteinrest Cys229 liegt wieder an der Peripherie des Enzyms und zeigt nur eine schwache Bindungsaffinität zu Protochlorophyllid *b*. Die Bindung dieses Pigments an den Enzymkomplex ermöglicht jedoch erst die Bildung höhermolekularer Aggregate aus POR-Protochlorophyllid-NADPH-Ternärkomplexen (Manuskript 3). Damit ist Cys229 also sowohl an der Genese von LHPP beteiligt als auch für die funktionelle Energieübertragung durch „Fluorescence resonance energy transfer“ auf das das photoaktive Pigment enthaltende PORB-Protein verantwortlich.

Die essentielle Rolle eines funktionalen LHPP-Komplexes zeigte die Untersuchung einer OEP16 Knockout Mutante von *Arabidopsis thaliana* auf. OEP16 wurde in *Arabidopsis thaliana* als Translokationskanal des PTC-Komplexes, d.h. des speziellen Importapparates für PORA identifiziert. Das Fehlen dieses Translokationsproteins in der Plastidenhülle beeinträchtigte zwar nicht die Aufnahme anderer plastidärer, zellkerncodierter Proteine oder von Aminosäuren, bedingte jedoch die Abwesenheit von PORA in Etioplasten (Manuskript 4). Dies hatte zur Folge, dass Etioplasten dieser Mutante im Vergleich zu denen von Wildtyp-*Arabidopsis* keine Prolamellarkörper bilden und höhere Mengen an Protochlorophyllid akkumulieren. Weiterhin konnte in solchen Mutanten kein LHPP nachgewiesen werden (Manuskript 4). Diese Befunde gingen einher mit der Beobachtung, dass etiolierte Mutantenkeimlinge bei anschließender Dauerbelichtung photooxidative Schäden davontrugen und schließlich abstarben (Manuskript 4). Dies lässt die Schlussfolgerung zu, dass LHPP einen bedeutenden Eckpunkt bei der Deetiolierung, d.h. dem Wechsel der keimenden Pflanze von Skotomorphogenese zu Photomorphogenese darstellt.

8 Eigenanteil der Arbeiten in den Manuskripten

Manuskript 1:

Reinbothe, C., Buhr, F., Pollmann, S., Reinbothe, S. (2003b) *In vitro* reconstitution of LHPP with protochlorophyllides *a* and *b*. *J. Biol. Chem.* **278**: 807-815.

- Proteinsynthese von PORA und PORB nach klonierten cDNA´s durch gekoppelte *in vitro* Transkription/Translation (siehe Material und Methoden)
- *In vitro* Rekonstitution der ternären Komplexe aus PORA/PORB, Protochlorophyllid *b*/*a* und NADPH sowie deren Aufreinigung über Gelfiltration und Quantifizierung der gebundenen Pigmente durch Fluoreszenzspektroskopie (siehe Abb. 1)
- *In vitro* Rekonstitution von LHPP, dem Lichtsammelkomplex etiolierter Pflanzen aus den einzelnen gereinigten PORA/PORB:Protochlorophyllid *b*/*a*:NADPH Komplexen und dessen Charakterisierung durch SDS-Polyacrylamid-Gelelektrophorese (siehe Abb. 2)
- Isolierung des vermeintlichen nativen lipidhaltigen LHPP-Komplexes aus Prolamellarkörpern sowie dessen Nachweis und Charakterisierung nach nichtdenaturierender Polyacrylamid-Gelelektrophorese (siehe Abb. 8)

Manuskript 2:

Reinbothe, C., Buhr, F., Bartsch, S., Desvignes, C., Quigley, F., Pésey, H., Reinbothe, S. (2006) *In vitro* mutagenesis of NADPH:protochlorophyllide oxidoreductase B: Two distinctive protochlorophyllide binding sites participate in enzyme catalysis and assembly. *Mol. Gen. Genomics* **275**: 540-552.

- Konstruktion der (Cys→Ala)-*porB* cDNA´s durch `site-directed mutagenesis´. Daraus gingen vier mutierte cDNA´s hervor, bei denen jeweils ein Cysteincodon gegen ein Alanincodon ausgetauscht waren sowie eine Doppelmutante, bei der das Codon für Cys276 und Cys303 in *porB* gegen Alanincodons ausgewechselt waren. Die Mutationen wurden sowohl an cDNA´s mit als auch ohne Transitpeptidsequenz durchgeführt (siehe Material und Methoden). Diese Klonierungsschritte stellten die Basis für alle darauffolgenden Experimente dieser Studie dar.
- Proteinsynthese von PORB nach mutierten porb-cDNA´s als auch nach Wildtyp-*porb*-cDNA durch gekoppelte *in vitro* Transkription/Translation
- *In vitro* Rekonstitution der ternären Komplexe aus PORB/mutiertem PORB, Protochlorophyllid *a* und NADPH, deren Aufreinigung mittels Gelfiltration sowie Quantifizierung gebundener Pigmente durch Fluoreszenzspektroskopie (siehe Tab. 1)
- Isolierung einer Galakto- und Sulpholipidmischung aus Etioplasten
- *In vitro* Rekonstitution von LHPP aus PORA:Protochlorophyllid *b*:NADPH- und (Cys→Ala)PORB/PORB:Protochlorophyllid *a*:NADPH-Ternärkomplexen, sowie Aufreinigung und Charakterisierung der gebildeten Suprakomplexe durch nichtdenaturierende Polyacrylamid-Gelelektrophorese (siehe Abb. 5)
- Genetische/molekularbiologische Charakterisierung der homozygoten PORB knock-out Linie von *Arabidopsis thaliana* mittels Southern- und Westernblotanalyse (siehe Abb. 9)

Manuskript 3:

Buhr, F., el Bakkouri, M., Valdez, O., Tichtinsky, G., Reinbothe, S., Reinbothe, C. Site directed mutagenesis of NADPH:protochlorophyllide oxidoreductase A: Novel insights into the structure, function and assembly of LHPP. Eingereicht zur Veröffentlichung in *The Plant Cell*.

- Austausch der einzelnen Cysteinreste gegen Alanin auf Basis einer *porA*-cDNA durch `site-directed mutagenesis´. Es entstanden jeweils vier verschiedene *porA*-cDNA Klone mit und ohne Transitpeptidsequenz, in denen jeweils ein Cysteincodon gegen ein Alanincodon ausgewechselt wurde (siehe Material und Methoden). Diese Klonierungsschritte waren die Grundlage für alle folgenden Experimente
- Synthese von (Cys→Ala)PORA anhand der hergestellten mutierten cDNA-Klone als auch von Wildtyp-PORA aus *porA*-cDNA über gekoppelte *in vitro* Transkription/Translation (siehe Material und Methoden)
- *In vitro* Rekonstitution von Ternärkomplexen aus (mutiertem)PORA, Protochlorophyllid *b* und NADPH, Aufreinigung der entstandenen Komplexe und Quantifizierung der von ihnen gebundenen Pigmente durch Fluoreszenzspektroskopie (siehe Tab. 1)
- Isolierung einer Galakto- und Sulpholipidmischung aus Etioplasten
- *In vitro* Rekonstitution von LHPP-Suprakomplexen aus PORA/B:Protochlorophyllid *b*/*a*:NADPH-Ternärkomplexen, wobei entweder jeweils nichtmutiertes PORA oder mutiertes PORA in den jeweiligen Ternärkomplexen vorlag. Die gebildeten Suprakomplexe wurden gereinigt und mittels Polyacrylamid-Gelelektrophorese und Fluoreszenzspektroskopie untersucht

Manuskript 4:

Pollmann, S., Springer, A., Buhr, F., Lahroussi, A., Duret, A., Bonneville, J.-M., Samol, I., Reinbothe, C., Reinbothe, S. Plastid import of NADPH:protochlorophyllide oxidoreductase A is essential for seedling survival. Eingereicht zur Veröffentlichung in *Nature Cell Biology*.

- Isolierung und genetische Charakterisierung einer OEP16 Knock-out Linie von *Arabidopsis thaliana* (siehe Abb. S1)

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe. Des weiteren erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen. Weiterhin habe ich keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth, den 11. Oktober 2006

Frank Buhr