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REVIEW

The light-dependent and light-independent reduction of protochlorophyllide *a* to chlorophyllide *a*

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Abstract

Two different pathways for protochlorophyllide *a* (Pchlide) reduction in photosynthetic organisms have been proved: one is strictly light-dependent whereas the second is light-independent. Both pathways occur in all photosynthetic cells except in angiosperms which form chlorophyll only through the light-dependent pathway. Most cells belonging to *Eubacteria* (*i.e.*, the anoxygenic photosynthetic bacteria) synthesize bacteriochlorophyll through the light-independent pathway. This review deals with the physiological, biochemical, and molecular biological features of molecules involved in both pathways of Pchlide reduction.

Additional key words: bacteriochlorophyll; bacteriochlorophyllide; cysteine; chlorophyll; greening; lysine; methionine; valine; tyrosine.

Introduction

The transformation of Pchlide to chlorophyllide (Chlide) is a reaction occurring in each eukaryotic and prokaryotic photosynthetic cell. The reaction consists of the *trans*-reduction of the double bond between the C_{17} and C_{18} at the D ring of Pchlide (Fig. 1). Two different pathways have been identified: the first one is light-dependent whereas the second one is not. Both occur in all photosynthetic organisms with the exception of angiosperms which lost the capacity to synthesize Chl in the dark during

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Abbreviations: Bchl: bacteriochlorophyll; Bchlide: bacteriochlorophyllide; C: cysteine; Chl: chlorophyll *a*; Chlide: chlorophyllide *a*; K: lysine; M: methionine; Pchl: protochlorophyll *a*; Pchlide: protochlorophyllide *a*; V: valine; Y: tyrosine
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evolution*. The most ancient group of photosynthetic organisms, *i.e.*, the anoxygenic bacteria, usually produce Bchl along the light-independent pathway (reviewed by Suzuki *et al.* 1997). Nonangiosperms failing to accumulate Chl in the dark are *Ginkgo biloba*, *Gnetum ula*, *Welwitschia mirabilis*, or *Euglena gracilis* (see Armstrong 1998 and references therein). Independent of the reduction pathway producing Chlide from Pchlide, all photosynthesizing organisms synthesize Pchlide from 5-aminolevulinic acid through the Beale pathway (reviewed by Schoefs and Bertrand 1997).

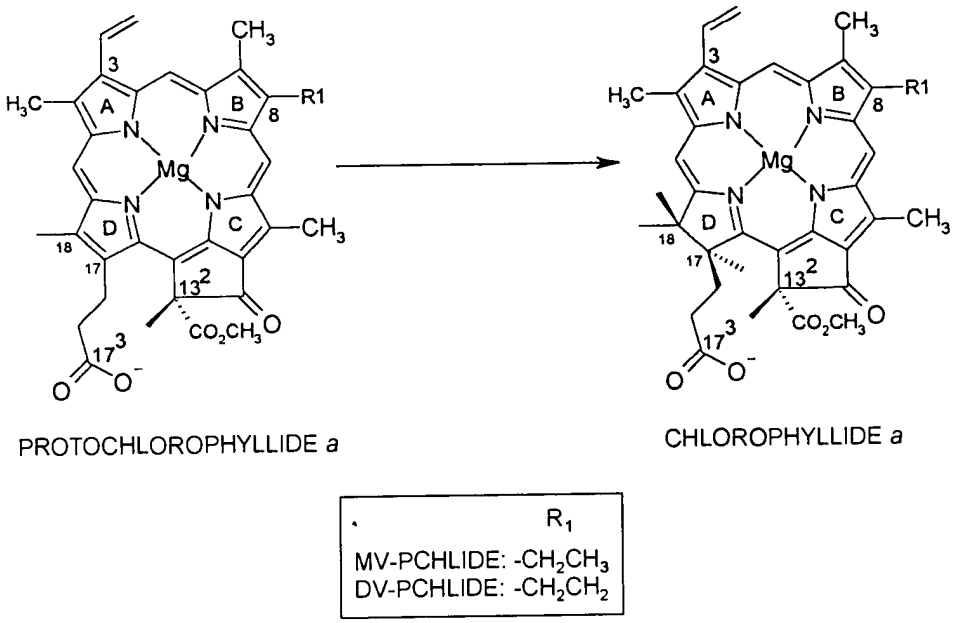


Fig. 1. Scheme of the reduction of protochlorophyllide to chlorophyllide. To unify the discussion, we use the IUPAC numbering system for chlorophyll carbon skeleton.

The light-dependent Pchlide reduction

The light-dependent reaction is studied more in higher plants than in the other branches of the plant kingdom probably because the light-independent pathway is absent in these organisms which greatly facilates the investigations.

*According to experimental reports, irradiated angiosperms replaced in the dark, after an irradiation, are in certain conditions able to increase their Chl content (reviewed by Adamson *et al.* 1997). This Chl accumulation occurs despite the fact that all the angiosperms examined so far lack the genes involved in the light-independent Pchlide reduction (see Table 1 in Armstrong 1998).

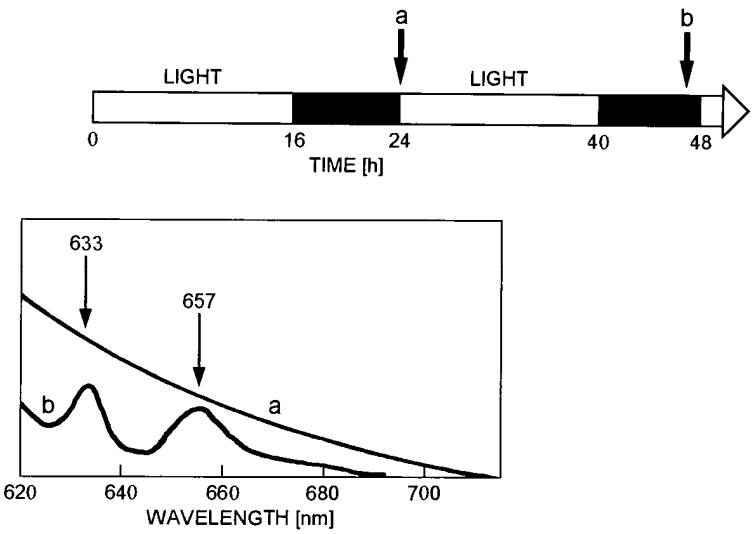


Fig. 2. 77 K fluorescence spectra of leaves recorded during the two first days of growth of bean seedlings under an irradiation regime of 16 h light and 8 h of darkness (*dark bar*). The positions of the emission maxima are slightly shifted to the longer wavelengths due to the baseline distortion.

In nature, plants germinate on the ground or near its surface and proplastids differentiate directly to chloroplasts (Whatley 1977). Tetrapyrrole biosynthesis starts very quickly after the imbibition. Using radiolabelled precursors, it was shown that the first Pchlide molecules are synthesized 12 h after the imbibition of cucumber cotyledons (Rebeiz *et al.* 1970). Pchlide was detected by 77 K fluorescence during the second light-dark cycle of greening in bean leaves (Fig. 2) and during the third one in bean cotyledons (Schoefs *et al.* 1994). A similar observation was reported with pea (He *et al.* 1994). However, *in situ* spectroscopic investigations, especially *in vivo* absorbance measurements, at these early developmental stages are difficult since the leaves are very small (Schoefs *et al.* 1992) and contain only traces of pigments. It is thus more convenient to use etiolated material from plants grown in complete darkness for a long period (1-2 weeks) which have accumulated larger amounts of Pchlide and NADPH:Pchlide oxidoreductase (POR) than younger leaves (Klein and Schiff 1972, Lancer *et al.* 1976, Savchenko *et al.* 1990, He *et al.* 1994, Schoefs *et al.* 1998).

Plant species have been classified in different groups on the basis of the chemical form of Pchlide (either monovinyl or divinyl) accumulated during the night and the chemical form of Chlide produced at the daybreak and later (El Mageed *et al.* 1997). The mechanism of Pchlide reduction is independent of the group to which a plant belongs (see below) and the transformation of 8-ethyl to 8-vinyl at the Pchlide ring B by a 8-vinyl Pchlide *a* reductase does not influence the spectral properties of the different Pchlide forms in the red spectral region (600-800 nm).

Chl formation in a model similar to that occurring in natural conditions: Spectroscopic analysis of Pchlide spectral forms of young nonirradiated leaves (*e.g.*, 2-d-old bean leaves) showed that they contain both photoactive and nonphotoactive Pchlide forms. Their ratio is in favour of the nonphotoactive form (Klein and Schiff 1972, Böddi *et al.* 1992, Schoefs *et al.* 1992, 1994, Schoefs and Franck 1993, Böddi and Franck 1997). Photoactive Pchlide is a multimeric aggregate of ternary complexes containing Pchlide, NADPH, and a photoenzyme, the POR (EC 1.6.99.1 or EC 1.3.1.33; for review see Ryberg *et al.* 1992). The pigments are organized in such a way that energy migration between them occurs (Kahn *et al.* 1970). Reconstitution experiments suggest that POR activity involves the formation of POR-dimers (Martin *et al.* 1997) which could correspond to the photoactive Pchlide $P_{638\text{-}645}$* observed *in vivo*. Aggregation of these dimers would form another spectral form of photoactive Pchlide observed *in vivo, i.e.*, $P_{650\text{-}657}$. These two forms of photoactive Pchlide could be in equilibrium (Schoefs and Franck 1997) as proposed initially by Kahn and Nielsen (1974). Since a separate analysis of behaviour of the two photoactive Pchlides is very difficult, they have been considered in this paper as a single entity denoted $P_{638,650\text{-}657}$.

Nonphotoactive Pchlide will be denoted as $P_{628\text{-}633}$. The biochemical state of nonphotoinactive Pchlide is less clear since it is spectrally and chemically heterogeneous (Bovey *et al.* 1974, Böddi *et al.* 1992, Schoefs *et al.* 1995, Böddi and Franck 1997). Among them, two different nonphotoactive Pchlide forms have been more characterized at the biochemical level: (*1*) free Pchlide, and (*2*) a complex similar to the photoactive Pchlide but with $NADP^+$ instead of NADPH ($P_{642\text{-}649}$, El Hamouri and Sironval 1979).

When a young dark-grown leaf (*e.g.*, 2-d-old) is irradiated by a short and saturating flash, $P_{638,650\text{-}657}$ is transformed to the Chlide absorbing at 678 nm and emitting fluorescence at 688 nm ($C_{678\text{-}688}$). $C_{678\text{-}688}$ is an aggregate similar to $P_{638,650\text{-}657}$ but containing Chlide and $NADP^+$ instead of Pchlide and NADPH (El Hamouri *et al.* 1981, Oliver and Griffiths 1982). The major part of Chlide is then liberated from the active site of the enzyme resulting in the formation of a new Chl(ide)** spectral form ($C_{670\text{-}675}$). The liberation of Chlide from the active site of the enzyme is indicated by the large simultaneous regeneration of photoactive Pchlide occurring during Chlide liberation (Schoefs and Franck 1993). In addition, energy migration measurements showed that $C_{670\text{-}675}$ was no longer in the aggregate after its formation (Schoefs and Franck 1993). If the spectroscopic properties of $C_{670\text{-}675}$ are well defined, its biochemical state remains to be determined precisely and it is not clear whether $C_{670\text{-}675}$ is released as free pigment or as a pigment-protein complex which in this case cannot be POR since it is involved in the regeneration of photoactive Pchlide. The remaining part of $C_{678\text{-}688}$ is transformed to another spectral form of Chlide ($C_{684\text{-}696}$). From the biochemical point of view, $C_{684\text{-}696}$ is similar to

*$P_{X\text{-}Y}$ and $C_{X\text{-}Y}$ means Pchlide and Chlide absorbing at X nm and emitting fluorescence at Y nm at 77 K.

**This term relates to either Chl and/or Chlide.

$C_{676\text{-}688}$ but contains NADPH instead of $NADP^+$ (El Hamouri *et al.* 1981, Oliver and Griffiths 1982). These events are summarized in Fig. 3*A*. Similar results were obtained with *Spirodela oligorrhiza*, a plant which does not develop etioplasts when cultivated in darkness (McCormac *et al.* 1996).

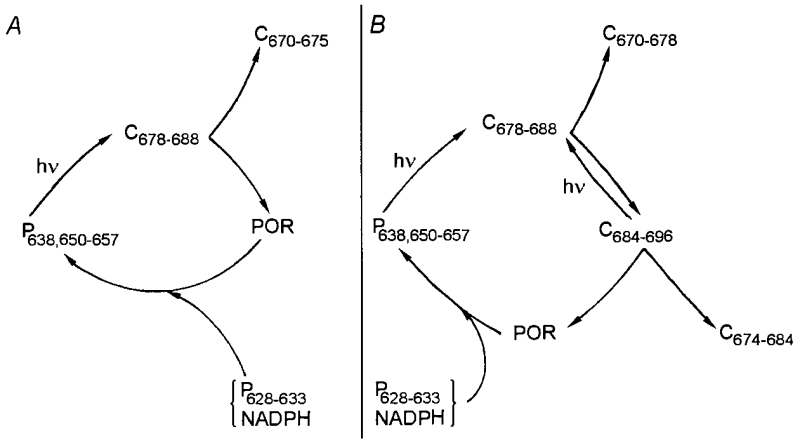
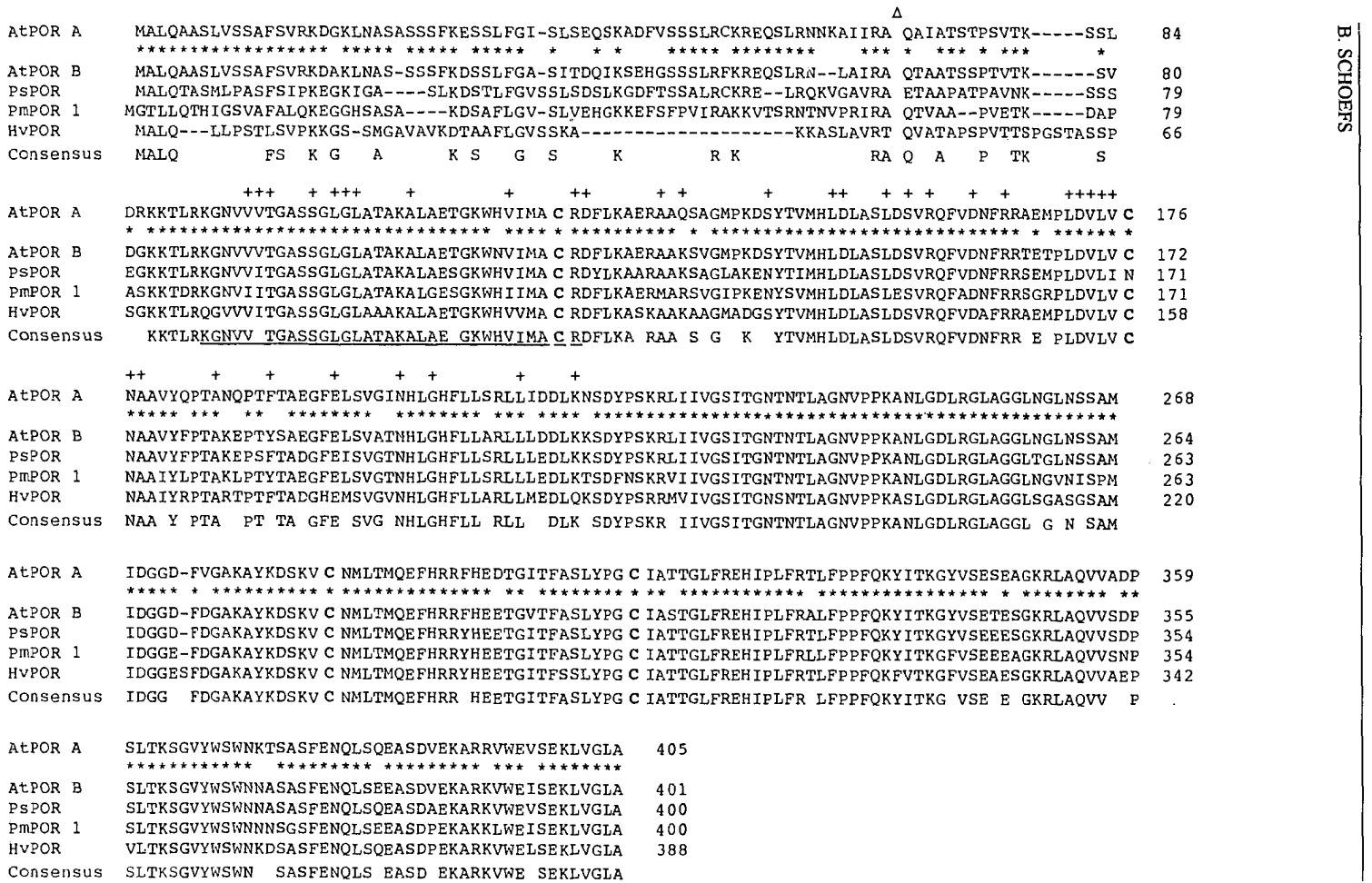


Fig. 3. The protochlorophyllide-chlorophyllide cycle in young (*A*) and old (*B*) leaves. To keep the figure clear, only the spectral forms whose role is clearly elucidated have been indicated.

Chl formation in plants cultivated for a long-time in the dark (*i.e.*, in etiolated leaves): During dark-growth, proplastids develop to etioplasts which are characterized by the presence of a prolamellar body and some single perforated membranes called prothylakoids (Whatley 1977). Simultaneously to the development of etioplast, photoactive Pchlide is accumulated (Klein and Schiff 1972, Schoefs *et al.* 1994) into the prolamellar body within which POR is by far the most abundant protein (Selstam *et al.* 1987, Lindsten *et al.* 1988). Etiolated leaves contain the same spectral forms of Pchlide as young leaves, *i.e.*, $P_{638,650\text{-}657}$ and $P_{628\text{-}633}$ (Klein and Schiff 1972). However, in this case the photoactive to nonphotoactive Pchlide ratio is in favour of the photoactive form (Schoefs and Franck 1993, He *et al.* 1994, Schoefs *et al.* 1994).

Similarly as in young leaves, irradiation triggers photoactive Pchlide transformation to $C_{678\text{-}688}$. The absorbance and fluorescence kinetics of the Pchlide photoreduction are monoexponential when the process is studied in the time-scale of seconds. The rate constants of the kinetics are similar in young and old leaves indicating that the photoreduction mechanism is identical regardless the leaf developmental stage (Schoefs *et al.* 1994). It was proposed that the photoreduction consists of two photochemical steps (Litvin *et al.* 1976). However, the monoexponential fit (Schoefs *et al.* 1994) together with assays about the quantum requirement of the photoreduction (Griffiths *et al.* 1996) contradict this view and favour a mechanism involving a single photochemical event. The formation of $C_{678\text{-}688}$ is preceded by the formation of several short-lived nonfluorescent intermediates whose chemical structure remains undetermined (reviewed by Griffiths 1991).


Δ
AtPOR A MALQAASLVSSAFSVRKDGKLNASASSSFKESSLFGI-SLSEQSKADFVSSSLRCKREQSLRNNKAIIRA QAIATSTPSVTK-----SSL 84
AtPOR B MALQAASLVSSAFSVRKDAKLNAS-SSSFKDSSLFGA-SITDQIKSEHGSSSLRFKREQSLRN--LAIRA QTAATSSPTVTK------SV 80
PsPOR MALQTASMLPASFSIPKEGKIGA----SLKDSTLFGVSSLSDSLKGDFTSSALRCKRE--LRQKVGAVRA ETAAPATPAVNK-----SSS 79
PmPOR 1 MGTLLQTHIGSVAFALQKEGGHSASA----KDSAFLGV-SLVEHGKKEFSFPVIRAKKVTSRNTNVPRIRA QTVAA--PVETK-----DAP 79
HvPOR MALQ---LLPSTLSVPKKGS-SMGAVAVKDTAAFLGVSSKA----------KKASLAVRT QVATAPSPVTTSPGSTASSP 66
Consensus MALQ FS K G A K S G S K R K RA Q A P TK S
AtPOR A DRKKTLRKGNVVVTGASSGLGLATAKALAETGKWHVIMA C RDFLKAERAAQSAGMPKDSYTVMHLDLASLDSVRQFVDNFRRAEMPLDVLV C 176
AtPOR B DGKKTLRKGNVVVTGASSGLGLATAKALAETGKWNVIMA C RDFLKAERAAKSVGMPKDSYTVMHLDLASLDSVRQFVDNFRRTETPLDVLV C 172
PsPOR EGKKTLRKGNVVITGASSGLGLATAKALAESGKWHVIMA C RDYLKAARAAKSAGLAKENYTIMHLDLASLDSVRQFVDNFRRSEMPLDVLI N 171
PmPOR 1 ASKKTDRKGNVIITGASSGLGLATAKALGESGKWHIIMA C RDFLKAERMARSVGIPKENYSVMHLDLASLESVRQFADNFRRSGRPLDVLV C 171
HvPOR SGKKTLRQGVVVITGASSGLGLAAAKALAETGKWHVVMA C RDFLKASKAAKAAGMADGSYTVMHLDLASLDSVRQFVDAFRRAEMPLDVLV C 158
Consensus KKTLRKGNVV TGASSGLGLATAKALAE GKWHVIMA C RDFLKA RAA S G K YTVMHLDLASLDSVRQFVDNFRR E PLDVLV C
AtPOR A NAAVYQPTANQPTFTAEGFELSVGINHLGHFLLSRLLIDDLKNSDYPSKRLIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLNGLNSSAM 268
AtPOR B NAAVYFPTAKEPTYSAEGFELSVATNHLGHFLLARLLLDDLKKSDYPSKRLIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLNGLNSSAM 264
PsPOR NAAVYFPTAKEPSFTADGFEISVGTNHLGHFLLSRLLLEDLKKSDYPSKRLIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLTGLNSSAM 263
PmPOR 1 NAAIYLPTAKLPTYTAEGFELSVGTNHLGHFLLSRLLLEDLKTSDFNSKRVIIVGSITGNTNTLAGNVPPKANLGDLRGLAGGLNGVNISPM 263
HvPOR NAAIYRPTARTPTFTADGHEMSVGVNHLGHFLLARLLMEDLQKSDYPSRRMVIVGSITGNSNTLAGNVPPKASLGDLRGLAGGLSGASGSAM 220
Consensus NAA Y PTA PT TA GFE SVG NHLGHFLL RLL DLK SDYPSKR IIVGSITGNTNTLAGNVPPKANLGDLRGLAGGL G N SAM
AtPOR A IDGGD-FVGAKAYKDSKV C NMLTMQEFHRRFHEDTGITFASLYPG C IATTGLFREHIPLFRTLFPPFQKYITKGYVSESEAGKRLAQVVADP 359
AtPOR B IDGGD-FDGAKAYKDSKV C NMLTMQEFHRRFHEETGVTFASLYPG C IASTGLFREHIPLFRALFPPFQKYITKGYVSETESGKRLAQVVSDP 355
PsPOR IDGGD-FDGAKAYKDSKV C NMLTMQEFHRRYHEETGITFASLYPG C IATTGLFREHIPLFRTLFPPFQKYITKGYVSEEESGKRLAQVVSDP 354
PmPOR 1 IDGGE-FDGAKAYKDSKV C NMLTMQEFHRRYHEETGITFASLYPG C IATTGLFREHIPLFRLLFPPFQKYITKGFVSEEEAGKRLAQVVSNP 354
HvPOR IDGGESFDGAKAYKDSKV C NMLTMQEFHRRYHEETGITFSSLYPG C IATTGLFREHIPLFRTLFPPFQKFVTKGFVSEAESGKRLAQVVAEP 342
Consensus IDGG FDGAKAYKDSKV C NMLTMQEFHRR HEETGITFASLYPG C IATTGLFREHIPLFR LFPPFQKYITKG VSE E GKRLAQVV P
AtPOR A SLTKSGVYWSWNKTSASFENQLSQEASDVEKARRVWEVSEKLVGLA 405
AtPOR B SLTKSGVYWSWNNASASFENQLSEEASDVEKARKVWEISEKLVGLA 401
PsPOR SLTKSGVYWSWNNASASFENQLSQEASDAEKARKVWEVSEKLVGLA 400
PmPOR 1 SLTKSGVYWSWNNNSGSFENQLSEEASDPEKAKKLWEISEKLVGLA 400
HvPOR VLTKSGVYWSWNKDSASFENQLSQEASDPEKARKVWELSEKLVGLA 388
Consensus SLTKSGVYWSWN SASFENQLS EASD EKARKVWE SEKLVGLA

In etiolated leaves, only a minor part of $C_{678\text{-}688}$ is transformed to $C_{670\text{-}675}$ (Schoefs and Franck 1993, Schoefs *et al.* 1994). The major part is transformed to $C_{684\text{-}696}$ which is an efficient fluorescence quencher at room temperature (Jouy and Sironval 1979). The $C_{684\text{-}696}$ formation which occurs readily in the dark after the initial phototransformation step, can be reverted to $C_{678\text{-}688}$ under irradiation (Fig. 3*B*), $C_{684\text{-}696}$ being the photoreceptor for this transformation (Franck and Inoue 1984). These reactions can be repeated and $C_{678\text{-}688}$ and $C_{684\text{-}696}$ form a cycle. *In vitro* experiments show that the $C_{678\text{-}688}$ to $C_{684\text{-}696}$ transformation requires NADPH (El Hamouri *et al.* 1981). Consequently, when the cycle is turning, NADPH is consumed. It is probably oxidized at each light-triggered $C_{684\text{-}696} \rightarrow C_{678\text{-}688}$ conversion (Franck and Schmid 1985). We showed recently that this cycle is involved in the photoprotection of newly formed Chlide against photooxidation (Franck *et al.* 1995, Schoefs and Franck 1995): when Chlide is in the $C_{678\text{-}688}$ conformation, it is readily photodestroyed while under the $C_{684\text{-}696}$ form it is not. Such a photoprotection mechanism is needed since carotenoids, although probably present in the photoactive Pchlide (Ouazzani Chahdi *et al.* 1998), do not exert a protective function at that state (Franck and Mathis 1980). Unprotected Chl(ide) molecules are very reactive with oxygen when irradiated and generate activated oxygen species which are able to destroy cellular and subcellular structures (reviewed by Bertrand and Schoefs 1999). The involvement of POR in this process was demonstrated as an increase of Chlide photoprotection in *Arabidopsis* overexpressing POR enzymes (Sperling *et al.* 1997).

When Chl(ide) aggregates dissociate, Chl(ide) is released from POR and is esterified during the Shibata shift (Shibata 1957, Ryberg *et al.* 1992). The final Chlide is $C_{674\text{-}684}$. During this shift, photoactive Pchlide regenerates (Shibata 1957). These events are summarized in Fig. 3*B*. Simultaneous to the different spectral shifts, the prolamellar body dissociates into vesicules which fuse together and with prothylakoids to form thylakoids (Shaw *et al.* 1985, Ryberg and Denesh 1986).

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Fig. 4. Structural features of higher plant NADPH:protochlorophyllide oxidoreductase (POR) polypeptides. Representative sequences from dicotyledonous (*Arabidopsis thaliana*, pea) and monocotyledonous (barley) angiosperms and from a gymnosperm (pine) have been aligned: AtPOR A, *A. thaliana* POR A (Armstrong *et al.* 1995); AtPOR B, *A. thaliana* POR B (Benli *et al.* 1991, Armstrong *et al.* 1995); PsPOR, pea (*Pisum sativum*; Spano *et al.* 1992a); PmPOR 1, mountain pine (*Pinus mugo*; Forreiter and Apel 1993); HvPOR, barley (*Hordeum vulgare*; Schulz *et al.* 1989). POR sequences from wheat (Teakle and Griffiths 1993) and oat (Darroh *et al.* 1990) are *ca.* 95 % identical with the barley HvPOR, whereas a loblolly pine POR polypeptide (Spano *et al.* 1992b) is 99 % identical with the mountain pine PmPOR 1. Residue numbers for the POR polypeptides are given on the right side. Putative cleavage sites for the plastid transit peptides are indicated by a triangle. Hyphens represent gaps inserted to optimize the sequence alignments, asterisks highlight residues conserved between AtPOR A and AtPOR B, and plus signs indicate residues identical with those found in the NADPH-dependent human carbonyl reductase (Wermuth *et al.* 1988). Cysteine residues located within the mature POR polypeptides are indicated. The consensus sequence represents those residues conserved in at least four of the five higher plant POR proteins, including underlined residues that form a putative βαβ-ADP-binding fold involved in NADPH binding. Reprinted with permission from Armstrong *et al.* (1995) (copyright by the American Society of Plant Physiologists).

The NADPH:Pchlide oxidoreductases

The POR apparent molecular mass of *ca.* 36 kDa has been estimated by SDS-polyacrylamide gel electrophoresis (Ikeuchi and Murakami 1983). It was estimated that each POR molecule can bind 2 Pchlide molecules (Apel *et al.* 1980) but recent results indicate that only one of them is bound to the active site (Wilks and Timko 1995, Rüdiger *et al.* 1998). Recently, cDNAs coding for POR have been isolated from several angiosperms and gymnosperms (reviewed by Schulz and Senger 1993). A POR homologue cDNA was also isolated from cyanobacteria (Suzuki and Bauer 1995). All POR proteins have a high degree of homology (Suzuki and Bauer 1995; Fig. 4). Evidences were presented that two very closely related genes encode POR proteins. The products of these genes, termed PORA and PORB, are present in photosynthetic cells (Armstrong *et al.* 1995, Holtorf *et al.* 1995) except in cyanobacteria (Suzuki and Bauer 1995), *Chlamydomonas reinhardtii* (Li and Timko 1996), and pea (Spano *et al.* 1992a) which contain only one POR gene. Both PORs are able to form $P_{650\text{-}657}$ and also induce the formation of a prolamellar body (Sperling *et al.* 1998).

In dark-grown leaves, PORA is more abundant than PORB (Holtorf and Apel 1996). Although exceptions have been found (Kuroda *et al.* 1995), the experimental results generally agree that during the first hours of irradiation, PORA and its mRNA concentration decrease (reviewed by Schulz and Senger 1993). In contrast, PORB, which is constitutively expressed, ensures Chl formation during rapid Chl accumulation (Holtorf *et al.* 1995).

The POR substrate specificity has been partially studied. 13^2(R) Pchlide can only be used (Helfrich *et al.* 1996). POR can utilize either 3-vinyl,8-ethyl-Pchlide or 3,8-divinyl-Pchlide (Bertrand *et al.* 1990, reviewed by Rebeiz *et al.* 1994; Fig. 1). Usually etioplasts contain both Pchlide types whose relative abundances depend on the taxa, the developmental stage, and the environmental conditions (reviewed by Ioannides *et al.* 1994). All plants accumulate 3-vinyl,8-ethyl-Chl, except some marine prochlorophytes which contain 3,8-divinyl-Chls or both pigment Chl types in mixture (reviewed by Jeffrey and Vesk 1997). To be photoreduced, the 17^3-position of Pchlide should be a free carboxylic acid (Griffiths 1980, Fig. 1). POR is able to convert Zn-protopheophorbide *a* and Zn-protopheophorbide *b* to their respective Zn-pheophorbides (Bombart and Dujardin 1984, Schoch *et al.* 1995). Finally, POR is an absolutely NADPH-dependent enzyme (Griffiths 1974).

On the basis of the similarity of POR with short-chain alcohol dehydrogenase, it was proposed that the proton added to the C_{18} is transferred from Y_{275} (numbering in pea POR; Fig. 4) which might bind NADPH. K_{279} would lower the pKa of the Y residue in order to facilitate deprotonation. The replacement of one of these two amino acids impairs Pchlide reduction (Wilks and Timko 1995). Comparison of the different POR (deduced) sequences indicates the conservation of two C residues, which may be involved either in Pchlide binding or in the photoreduction itself (Teakle and Griffiths 1993). Cysteine residues might be involved in Pchlide and/or NADPH binding (Oliver and Griffiths 1981). The deletion of the last 54 amino acid residues (homozygous mutant of *Synechocystis* carrying a POR interruption between M_{263} and V_{264}; V_{264} seems not to be conserved in higher plants) does not impair

Pchlide photoreduction but affects the enzyme affinity for Pchlide. Another mutant [photosystem-1-less/chlL$^-$/*por* (del)], having all the amino acids beyond residue 111 deleted, was unable to photoreduce Pchlide (He *et al.* 1998).

To obtain more insight about the mechanism of photoreduction as well as on the organization of the complexes and the aggregates, several attempts to isolate and purify or even reconstitute photoactive complexes have been reported. Since POR is a membrane-associated protein, the use of detergent is necessary to solubilize prolamellar bodies. Thus 1 % digitonin and 10 mM *Triton X-100* modified significantly the absorption properties of the aggregates (shift from 650 to 640 nm). The removal of the detergent by centrifugation restores the original absorption spectra only in the case of the *Triton X-100*-treated sample (Ikeuchi *et al.* 1984). When larger amounts of detergent are added (Birve *et al.* 1996) or when exogenous Pchlide *a* is added to an extract containing the product of the POR-gene expressed in *Escherichia coli*, only one fluorescence peak is found around 633 nm (Schulz *et al.* 1989). When such complexes are irradiated in the presence of NADPH, the formed Chlide emits fluorescence at 675 nm (Schulz *et al.* 1989).

Using a new solubilization procedure, Ouazzani Chahdi *et al.* (1998) isolated two different photoactive POR-Pchlide complexes having spectral properties very similar to $P_{638\text{-}645}$ and $P_{650\text{-}657}$. The structure of the enzyme and the binding sites of both NADPH and Pchlide remains, however, largely unknown. Solubilized PORs from wheat (with 1 mM *Genapol X-080*) have been studied by circular dichroism spectroscopy (185-250 nm) to obtain information about the enzyme secondary structure (Brive *et al.* 1996). The analysis revealed that POR is composed of around 33 % α-helix, 19 % β-sheet, 20 % turn, and 28 % random coil. These proportions are similar to that found with other oxidoreductase (see Table 1 in Birve *et al.* 1996).

The light-independent Chl synthesis

As mentioned in the Introduction, Chl can be synthesized in absence of light in most photosynthetic organisms. Using plasma membranes from *Synechococcus* sp. PCC6301, Peschek *et al.* (1989) demonstrated that the dark-Pchlide reduction was NADPH-dependent. The reaction can be reverted if $NADP^+$ is added.

Three genetic loci designated *bchB*, *bchL*, and *bchN* were identified as necessary for the light-independent Pchlide reduction in *Eubacteria* (anoxygenic photosynthetic bacteria; reviewed by Suzuki *et al.* 1997). This suggests that the light-independent POR is a multimeric enzyme composed of at least three subunits. Later, similar genes were found in eukaryotic organisms synthesizing Chl in the dark (see Table 1 in Armstrong 1998). Consequently, ChlB, ChlL, and ChlN proteins are believed to compose the light-independent POR in eukaryotic organisms. None of the three polypeptides have significant similarities with that of POR (Suzuki and Bauer 1992). In contrast, the ChlL proteins presents homologies with the NifH polypeptide of nitrogenase of *Eubacteria* (Fujita *et al.* 1989) whereas ChlN has only limited similarities with the other polypeptides of eubacterial nitrogenase (*i.e.*, NifD and NifK; Fujita *et al.* 1992, 1993). Therefore, it was hypothesized that light-independent POR

is structurally similar to the oxygen-sensitive nitrogenase (Peters *et al.* 1995). BchlL has a 32 % homology with BchX (Burke *et al.* 1993a), a polypeptide of the Chlide oxidoreductase, the enzyme which reduces the C_7-C_8 double bond of ring B in the Bchl biosynthetic pathway (reviewed by Suzuki *et al.* 1997). The other polypeptides of the Chlide oxidoreductase, *i.e.*, BchY and BchZ are also similar to BchN/ChlN and BchB/ChlB, respectively (Burke *et al.* 1993a,b). This indicates that the dark-reduction of the C_{17}-C_{18} double bond, leading to the formation of Chlide, and that of the C_7-C_8 double bond, leading to the formation of Bchlide, are performed by two structurally related enzymes.

Antibodies against ChlL and ChlB react with a polypeptide of 31 kDa present in the soluble extracts of *Marchantia polymorpha* (Fujita *et al.* 1989) and with a polypeptide of 58 kDa of a membrane fraction of *Plectonema boryanum* (Liu *et al.* 1993), respectively. It was predicted from the deduced amino acid sequence of the *chlN* gene that ChlN which has no membrane spanning region has a molecular mass of 61 kDa (Choquet *et al.* 1992). Hence the information indicates that the light-independent POR is probably membrane-bound with a structural domain facing the chloroplastic stroma. This could account for the difficulties met during the isolation and purification of the light-independent POR in an active state.

Pchlide photoreduction during greening, in green plants and in plants where the light-independent Pchlide reduction also occurs: After the initial Pchlide reduction, Chl synthesis shows a lag phase whose length is dependent on the developmental stage (Sisler and Klein 1963, Akoyunoglou and Argyroudi-Akoyunoglou 1969, Henningsen and Boynton 1974, Schoefs *et al.* 1998). After this delay, Chl is rapidly accumulated (Liro 1908, Akoyunoglou and Argyroudi-Akoyunoglou 1969, Henningsen and Boynton 1974, Schoefs *et al.* 1998). If during this process, Chl formation also involves photoactive Pchlide, a steady-state amount of this photoactive complex should be detected in the light. That this is the case was shown by *in situ* fluorescence (Schoefs and Franck 1991, Franck *et al.* 1993) and by absorbance measurements (Franck and Strzalka 1992, Schoefs and Bertrand 1997). Precise measurements indicate that the amount of photoactive Pchlide observed during greening is directly related to the irradiance used for cultivation (Franck and Strzalka 1992). This photoactive Pchlide has a 77 K emission maximum slightly shifted to the blue (653 nm) which could reflect a small but significant modification(s) of the organization of the aggregates. Under a saturating irradiance, the remaining photoactive Pchlide, *i.e.*, that not photoreduced by the radiant energy used to drive greening, is transformed to $C_{678\text{-}688}$ which is in turn transformed to $C_{684\text{-}696}$ and subsequently to $C_{672\text{-}682}$. The duration of these shifts, similar in nature to those observed when an etiolated leaf is irradiated for the first time, is dramatically accelerated when compared with the etiolated material (Franck *et al.* 1993). In plants cultivated under a light/dark regime, the PORB concentration shows diurnal variations (Holtorf and Apel 1996) which might be correlated with variations of the Chl amount in leaves from plants cultivated in similar conditions (Sironval and Michel-Wolwertz 1963). PORA and Pchlide are reaccumulated at the end of the dark phase in plants cultivated in light/dark regime (Holtorf and Apel 1996, Schoefs *et al.* 1998). Although both PORA and PORB are able to induce prolamellar body formation

(Sperling *et al.* 1998), this reaccumulation of PORA is probably correlated with the formation of small prolamellar bodies during the dark-phase following an irradiation (Rebeiz and Rebeiz 1986). *In vitro* experiments have indicated that POR is a peripheral protein attached on the stromal side of the membranes (Dahlin *et al.* 1995).

The light-dependent Pchlide reduction in dark-grown gymnosperm cotyledons and primary needles involves similar photoactive Pchlide forms as those found in non-irradiated leaves (Michel-Wolwertz 1977, Selstam *et al.* 1987, Schoefs *et al.* 1995, Raskin and Marder 1997, Schoefs and Franck 1998). In addition, the same spectral and chemical heterogeneities of nonphotoactive Pchlide were found in pine tissues (cotyledons and primary needles) and in dark-grown higher plants (Schoefs *et al.* 1995, 1997, Schoefs and Franck 1998).

Plastids from dark-grown pine cotyledons are differentiated into grana and thylakoids and, in addition, contain prolamellar bodies. Both membranes contain POR (Selstam and Widell 1986). Two POR enzymes with different localisations have been found in those plastids (Forreiter and Apel 1993). The first one, PORA (36 kDa), is associated with the prolamellar body while another one (38 kDa) is found in the thylakoids.

In organisms where both light-dependent and light-independent pathways coexist, it seems that regulation of Chl synthesis is organism-specific. For instance, in *Plectonema boryanum*, *chlL* gene is expressed only in inhibiting nitrogen fixation (Fujita *et al.* 1991). In the green alga *Chlamydomonas moewusii* cultivated in light-dark cycles, ChlB mRNA starts to accumulate at the beginning of each dark phase (Richard *et al.* 1994). At the biochemical level, the regulation of the Pchlide pool utilisation for the dark-Chl synthesis and the formation of photoactive Pchlide in the dark has not yet been studied. It is only known that decreasing the temperature induces the preferential accumulation of photoactive Pchlide (Michel-Wolwertz 1977).

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