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Protochlorophyllide Spectral Forms

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Abstract: This study summarized recent results on POR and plastid development in order to find an explanation for the existence of Pchlide spectral forms. This review has summarized many researches about the Pchlide spectral forms and their phototransformability in different higher plants which have been published. Chlorophyll (Chl) is the most important pigment on the Earth. Each spring millions of tons Chl are formed during bud break and leaf development. The sun light needed for photosynthesis is captured by Chl and transformed to chemical energy. In the Biosynthesis of chlorophyll (Chl) begins with the synthesis of δ-aminolevulinic acid (ALA) from glutamic acid. Chl biosynthetic pathway, a light dependent enzyme protochlorophyllide oxidoreductase (POR) catalyses a key light-driven reaction, trans addition of hydrogen across the C-17-C-18 double bond of the Chl precursor, protochlorophyllide (Pchlide), that triggers a profound transformation in plant development. Pchlide is spectrally heterogeneous and exist in different spectral forms having slightly different absorption and fluorescence peaks. The identified Pchlide spectral forms can be sorted into three groups. The first group is designated as short-wavelength forms. This group with fluorescence in the 625-646 nm spectral region has a heterogeneous nature and is made by four components. The second group includes the long-wavelength Pchlide forms with emission maxima between 652 and 657 nm. The third group is found in the extreme red region (670-730 nm) of the fluorescence emission spectra and includes a number of pigment forms with spectral bands of low intensity. This region is also influenced by vibrational bands of the short-and long-wavelength Pchlide forms.

Key words: Chlorophyll, fluorescence excitation, fluorescence emission, fluorescence lifetime, NADPH-protochlorophyllide oxidoreductase, protochlorophyllide

INTRODUCTION

Biosynthesis of chlorophyll (Chl) begins with the synthesis of δ-aminolevulinic acid (ALA) from glutamic acid. Two molecules of ALA react to yield prophobilinogen. Four molecules of porphobilinogen form the ring structure of protoporphyrin IX (proto). To make Chl, a magnesium chealatase inserts Mg into proto ring. Chl synthesis involves further modifications of the ring, including attachment of the phytol chain. In angiosperms, the synthesis of Chl requires a light-dependent Pchlide reductase enzyme (Buchanan *et al*., 2000).

In the dark grown angiosperms, the light-dependent synthesis of Chl is arrested, which in turn, blocks the thylakoid formation and results in the formation of etioplasts (Staehelin, 2003). Etioplasts represent an arrested stage in the normal development of proplastids into chloroplasts. The most notable feature of the etioplasts is the presence of one or more prolamellar bodies, PLBs. The lack of functional thylakoids, the absence of Chl, the accumulation of monovinyl (MV) and divinyl (DV) derivatives of Pchlide and the presence of POR are some other features of etioplasts (Whatley *et al*., 1982; Ryberg and Sundqvist, 1991; Ryberg *et al*., 1993).

A complex signalling network that combines environmental and genetic information coordinates the activities of the nucleus and the chloroplast. Necessary for this is a transduction of information from plastids to the nucleus as well as the flux of information from the nucleus to the plastids (Strand *et al*., 2003). Such a signal is required continuously for the expression of nuclear photosynthetic genes (Sullivan and Gray, 1999). It has been demonstrated that the accumulation of Mg-proto IX is necessary and sufficient to regulate the expression of many nuclear encoded chloroplastic proteins (Strand *et al*., 2003). The transmission of a plastid signal does not obligatorily require light but can occur in the dark and both in leaves and roots (Sullivan and Gray, 1999). A large number of proteins localised to the chloroplast are actually encoded in the nucleus.

Chl biosynthesis starts from the formation of ALA a common tetrapyrrole precursor. The ALA biosynthesis is regulated by the levels of POR and Pchlide or by heme

(Ilag *et al*., 1994). The subsequent energy-independent reactions of ALA lead to proto. Energy-dependent incorporation of magnesium into Proto gives rise to the specific Chl series (Beale, 1999). A light governed reduction of Pchlide results in formation of chlorophyllide (Chlide). Pchlide reduction in the dark and under irradiation revealed two different types of enzymes to which can be referred to as a light-independent Pchlide reductase (DPOR) and a light-dependent Pchlide reductase (LPOR), which have been reviewed by Timko (1998) and Schoefs (2005). Angiosperms are missing the light-independent enzymes. LPOR in Arabidopsis thaliana has three isoforms, PORA, PORB and PORC. However, some angiosperms such as cucumber have only one isoform of POR (Kuroda *et al*., 1995).

Conformational change of POR within sub-nano seconds switches the enzyme into an active state. Sytina *et al*. (2008) reported that prior to excitation of the enzyme-substrate complex with a laser pulse induces a more favourable conformation of the active site than non-active state. This effect is triggered during the Pchlide excited state lifetime and persists on a long timescale (Sytina *et al*., 2008).

Chl is associated with proteins localized to the thylakoids in the chloroplast. The Chls are present in different spectral forms in the Chl-protein complexes. They are organized in light-trapping antennas in such a way that the pigments absorbing light of relatively short wavelengths are found at the periphery of the antenna whereas those absorbing at longer wavelength are generally found closer to the reaction centre (Grondelle *et al*., 1994). Light energy is thus transferred from the antenna and trapped in the reaction centre.

Spectral forms of Pchlide, as a pigment, characterized by its absorbance and excitation and emission fluorescence. Initially two forms, one phototransformable form absorbing around 650 nm and one non-transformable form absorbing around 635 nm were identified. Pchlide is bound to the specific site of the POR protein and might also be present in a free form but the reason for the presence of different forms are still obscure. This review summarizez the knowledge about different spectral Pchlide forms and attempt to find an explanation for their existence.

The prolamellar body: The etioplast inner membranes are organized into two structurally different systems. One is the PLBs, with tubular membranes connected into a highly regular three-dimensional lattice of which the membranes are physically continuous with the other system the prothylakoids, PTs, flat perforated membranes that extend from the surface of the PLBs. The PLBs are composed of regular tetrapodal units made up from a continuous bilayer assembled to form a diamond cubic lattice. The POR enzyme is the main protein in the PLBs and constitutes nearly 90% of its protein content. In the PLBs, POR in ternary complex with Pchlide and NADPH forms a large aggregate of photoactive Pchlide form (Böddi *et al*., 1990; Schulz and Senger, 1993). At the onset of greening the PLBs can be of importance for efficient capture of light energy for optimal photoconversion of Pchlide to Chlide (Masuda *et al*., 2003). The PLBs are also considered as a storage room for membrane lipids and in fact it was shown that many photosynthetic proteins are present already in the dark in the PLBs. The flat perforated PT membrane stretching out into the stroma may act as a precursor of the thylakoids.

The PLB formation is correlated to the presence of POR. The overexpression of POR allowed the creation of large PLB membranes indicating that the formation of PLBs is correlated to the aggregation of ternary complexes of NADPH-POR-Pchlide. Also Masuda showed that POR functions in PLB assembly (Masuda *et al*., 2003). However, an intact PLB structure is not a prerequisite for the preservation of the spectral properties of the ternary complex of POR. High salt concentrations caused a disintegration of the structure of isolated PLBs, but had no effect on the spectral properties of the ternary complex of POR, especially in the presence of NADPH (Selstam *et al*., 2007).

Irradiation induces a series of changes in chemichal, ultra structural and spectral properties of etioplasts. Pchlide is photoconverted to Chlide and then esterified to Chl. After irradiation the PLBs lose their regular structures and the perforated PTs increase in length. The PLBs are then dispersed and disappear completely within a few hours and are replaced by newly formed thylakoids (Henningsen, 1970). The photoreduction of Pchlide to Chlide not only triggers the transformation of the PLBs but the formation of the first Chl molecules initiate the synthesis of some and stabilize the accumulation of other chloroplast proteins.

POR: POR catalyzes trans addition of hydrogen across the C-17-C-18 double bond of Pchlide. Pchlide reduction occurs by dynamically coupled nuclear quantum tunneling of a hydride anion followed by a proton on the microsecond time scale in the Pchlide excited and ground states, respectively (Heyes *et al*., 2009). POR is a nucleus-encoded enzyme, which has been cloned and sequenced from different plants has been identified in Arabidopsis and its gene is cloned and sequenced (Oosawa *et al*., 2000; Su *et al*., 2001). Different POR genes have been reported in various angiosperms. *Arabidopsis* has a small gene family consisting of PorA, PorB and PorC. PORA and PORB, two structurally similar but differentially regulated isoforms of POR were first identified in barley (Holtorf *et al*., 1995) and *Arabidopsis* (Armstrong *et al*., 1995) but recently also in wheat. The third isozyme, PORC, characterized in *Arabidopis*, is not present in darkness but is induced by high light irradiation and was suggested to also have a photoprotective role during greening (Oosawa *et al*., 2000; Masuda *et al*., 2003). PORA is known to be down-regulated by phytochrome (Apel, 1981). Far-red light working through phytochrome A can thus cause a down-regulation of PORA and an inhibition in greening. However, far-red stimulated Pchlide and Chl formation has also been known for a long time (Klockare, 1980). Sineshchekov found that the phytochrome regulation of the Pchlide level was dependent on plant species and development in a complex pattern (Sineshchekov *et al*., 2006).

The chloroplast DNA of *Arabidopsis thaliana* is a circular molecule of 153 kb. However, estimations show that the chloroplast hosts more than 2000 (*Arabidopsis* 2100, rice 4500) different proteins (Lopez-Juez and Pyke, 2005). More than 90% of them are encoded by nuclear genes. Import of nucleus-encoded proteins into the plastid has been shown to occur mainly by a general import pathway (Chen and Schnell, 1999). The precursor form of the nuclear encoded POR protein, pPOR, consists of approximately 400 amino acids and has a molecular weight of about 41 to 44 kDa. The mature form has a molecular weight of about 33 to 38 kDa. The pPOR follows the general import pathway (Aronsson *et al*., 2001). Pea pPOR could be cross-linked to pea Toc75, a major protein in the translocation complex indicating that POR uses the general import pathway (Aronsson *et al*., 2000). Teakle and Griffiths (1993) showed that the pPOR of 41 kDa was imported into isolated wheat chloroplasts and that stromal-processing proteases cleaved off the transit peptide, giving POR its mature size of approximately 36 kDa (Teakle and Griffiths, 1993).

The amino acid sequences of POR from barley (PORA), oat, *Arabidopsis* (PORA) and pea have large similarities (Schulz and Senger, 1993). The monocotyledons, oat and barley, displayed 96% similarity and compared to the dicotyledons, *Arabidopsis* and pea, the barley sequence revealed similarities of 81 and 83%, respectively. The sequences are characterised by high contents of basic and hydrophobic amino acids and contain highly conserved regions, for instance in the parts where the cysteines are located (Schulz and Senger, 1993).

In Arabidopsis, the sequence similarities for the precursor POR protein were 87% between PORA and PORB, 74% between PORC and PORA and 76% between PORC and PORB. In the mature form the sequences have a higher similarity 83% between PORC and PORA or PORB leaving the main sequence diversity to be in the transit peptide (Oosawa *et al*., 2000). In barley, the precursors of PORA and PORB share 75% and the mature forms 81.5% sequence similarities, whereas the transit peptides have only 46% sequence similarity (Holtorf *et al*., 1995).

A study of *Synechocystis* POR showed that the ternary complex initially is formed by the binding of NADPH to POR and thereafter Pchlide (Heyes *et al*., 2000). The presence or absence of Pchlide did not influence the binding parameters of NADPH to POR. This indicated either independent binding sites for NADPH and Pchlide or an regulated binding of the co-factor and the substrate. Pchlide could bind to POR in the absence of NADPH. When NADPH binds to POR-Pchlide it is suggested to induce a conformational change of the complex leading to protection of sensitive cysteine residues (Heyes *et al*., 2000).

The ternary complex is present as a dimer but can also form larger complexes. The dimer interface was predicted to correspond to residues 198-208 and 271-292, based on results from X-ray structural analysis of other members of the reductases/epimerases/dehydrogenases, RED family, (Jörnvall *et al*., 1995). Analysis of POR from the etioplast inner membranes of wheat by two-dimensional electrophoresis showed five different isoforms of PORA. Four with a pI around 8-9 and one with an pI more close to five (Blomqvist *et al*., 2008) indicating a certain degree of posttranslational modification.

Pchlide and Chl formation: Pchlide as an intermediate plays an important role in the regulation of Chl biosynthesis. Pchlide accumulates in dark-grown angiosperms and is mainly localized to the PLBs (Sundqvist and Dahlin, 1997; Beale, 1999). The Pchlide content of isolated PLBs are more than ten times of that of PTs. The protochlorophyll (Pchl), which esterified form of Pchlide and Pchlide content of leaves increases rapidly up to 7 to 9 days and then the rate of accumulation slows down (Klein and Schiff 1972). Cotiledons contain Pchl and as leaf development proceeds in the dark, Pchlide accumulation increases while the formation of Pchl is attenuated and eventually ceases before the maximum in total pigment accumulation is achieved (Lancer *et al*., 1976).

In vivo, Pchlide exists in four closely related species: MV-and DV-Pchlide or MV-and DV-Pchl, Pchl. However, Pchlide can also exist in two other types with different chemical structure, i.e., Pchlide a and b. (Reinbothe *et al*., 1999). The occurrence of Pchlide b, however, has been proven not to be present (Kolossov and Rebeiz, 2003).

The proportion of DV-Pchlide in the non-photoactive and photoactive Pchlide pools increases in correlation to the POR content. The MV and DV Pchlide function equally well as POR substrates, although minor spectroscopic differences can be traced in the intermediates (Heyes *et al*., 2006).

Photoreduction of Pchlide is a major regulatory step in the Chl biosynthesis. Pchlide reduction catalyzed by POR is an ultra fast enzymatic reactionoccuring within femto seconds. In the reaction the photoactive Pchlide is transformed to Chlide via a light driven step and two dark reactions (Wilks and Timko, 1995). However, Heyes and Hunter using a thermophilic form of POR identified two additional dark steps (Heyes and Hunter, 2004). Photochemistry and the first dark step result in formation of the POR-Chlide-$NADP^+$ complex. The second dark reaction and two additional dark steps identified by Heyes and Hunter have been shown to represent a series of ordered product-release and cofactor-binding events: $NADP^+$ is released from the enzyme and then replaced by NADPH, before release of the Chlide product and subsequent binding of the Pchlide substrate to enable the next catalytic cycle to proceed (Heyes and Hunter, 2004, 2005). During this process two hydrogen atoms which are derived from the NADPH and Tyr 275 of the POR protein, are added at carbons 17 and 18 of Pchlide, respectively (Wilks and Timko, 1995).

In the photoreduction reaction the proper substrate for POR is the excited Pchlide not the pigment in the ground state (Fujita, 1996). The catalytic mechanism of POR involves two additional steps, which do not require light. The first involves the conversion of the product of the initial light-driven reaction, a non-fluorescent radical species, into a new intermediate that has an absorbance maximum at 681 nm and a fluorescence peak at 684 nm. During the second dark step the absorption and the emission bands gradually blue shift to yield the product, Chlide. Another new pathway of Chl biosynthesis from long-wavelength Pchlide fluorescing at 686 nm is also suggested in which a photo transformation of Pchlide fluorescing at 686 nm into Pchlide fluorescing at 653 nm, which is then photo transformed to Chlide (Ignatov and Litvin, 2002).

The first recognizable product of photoreduction at room temperature is a transient Chlide with absorption and emission maxima at 678 and 690 nm, respectively, which then converts to a Chlide form absorbing at 684 nm. This form in turn, has a blue shift, called Shibata shift, to a 672 nm absorbing form. These spectral shifts occur also during Chl accumulation in plants grown in photoperiodic light (Schoefs and Franck, 2008). The shifts seems to disappear during maturation of leaves and might be coupled to the regulation of development (Lee *et al*., 2007). The shift has been interpreted as a liberation of the pigment from the enzyme and the Chlide absorbing at 672 nm has been regarded as a free pigment. Several other proposals have been suggested as explanations for the different Chlide spectral forms and their interconversions such as pigment aggregation, esterification, association or disassociation with a specific type of membrane or enzyme (Franck *et al*., 1997). The spectral shifts coincide with the transformation of the PLB structure and a re-localization of POR from the PLBs to the developing thylakoids. Most of the newly formed Chlide is esterified to Chl a in parallel to or slightly slower than the Shibata shift. A Pchll esterified with phytol already in dark-grown plants was found to be important as a structural component substituting the Chl in the cytochrome b_6f complex. In this case the phytol moity of the pigment was the important part (Reisinger *et al*., 2008).

Spectral properties of Pchlide in dark-grown plants: Pchlide is spectrally heterogeneous. Several spectral forms of Pchlide are present in dark-grown plants (Böddi *et al*., 1992; Stadnichuk *et al*., 2005). The proportions of spectral Pchlide forms varies depending on plant species (Fig. 1A, B), plant tissue (Fig. 2) and

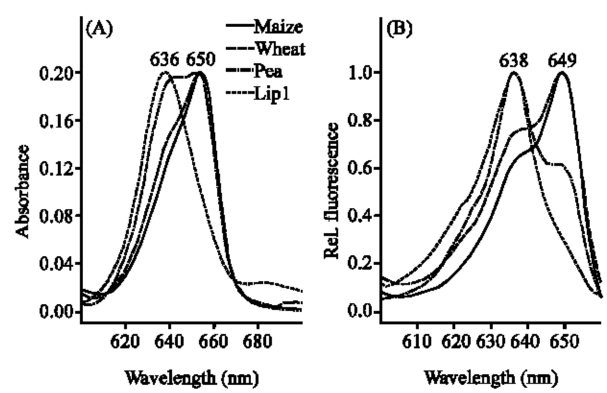

Fig. 1: Room temperature absorption (A) and low temperature (77 K) excitation (B) spectra of 7-day-old dark-grown maize, wheat, pea and lip1 showing the species dependency of spectral forms. Excitation spectra were measured by the emission set at 705 nm. The spectra were normalized at the highest peak

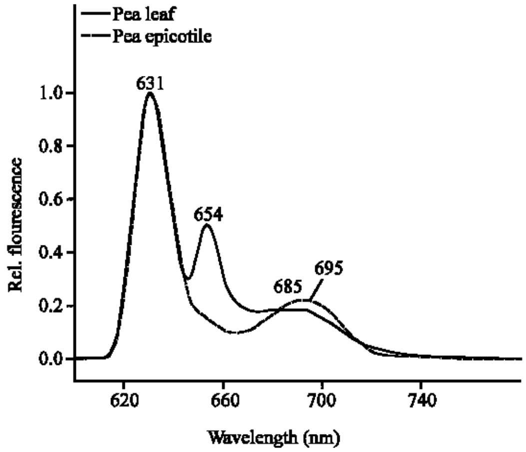

Fig. 2: Low temperature (77 K) fluorescence emission spectra of dark-grown pea leaves and epicotyls. The spectra are normalized at the highest peak. Excitation wavelength was 440 nm

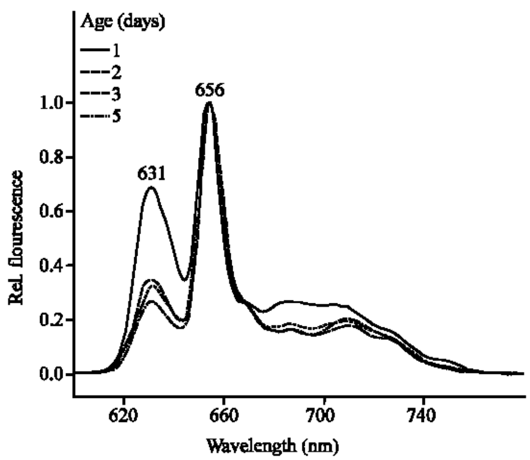

Fig. 3: Low temperature (77 K) fluorescence emission spectra of dark-grown wheat at various ages. The spectra are normalized at the highest peak. The plant age, calculated from the start of imbibition, is indicated. Excitation wavelength was 440 nm

developmental age of the plant (Fig. 3) (Mysliwa-Kurdziel *et al*., 2003; Amirjani *et al*., 2006). Marchand found that plastids in the bundle sheath cells of maize accumulated preferentially the short-wavelength form of Pchlide (Marchand *et al*., 2004). In green and greening leaves of maize Pchlide accumulated after a period of dark treatment also participates in the formation of different spectral forms (Amirjani and Sundqvist, 2004). The heterogeneous spectroscopic properties of the various Pchlide forms reflect differences in their environments. Some contributing factors are the association of Pchlide to the POR protein and NADPH to make a ternary complex, a possible POR phosphorylation and an aggregation of ternary complexes. Short-wavelength form of Pchlide is presumably localized in lamellar membranes of PTs and/or small and loose PLBs unlike the longer wavelength Pchlide complexes (Böddi *et al*., 1994). Such changes in localization can be of basic importance. Short-wavelength forms of Pchlide can come together, thus the ð-electron systems of the Pchlide molecules in their active sites interact and the exciton interaction causes the red shift in their emission maxima to 644 or 655 nm (Kosa *et al*., 2006). The appearance of different Pchlide forms can also be due to a change in the oxidative state of the cofactor, NADP (Franck *et al*., 1999). The lipid composition surrounding the enzyme is also of importance for the spectral properties of POR bound Pchlide (Klement *et al*., 2000). Recent measurements on the chromophores Pchl and Pchlide in different solvents indicate that spectral properties such as the Stokes shifts and the fluorescence lifetimes are affected phytol chain (Mysliwa-Kurdziel *et al*., 2008). To characterize the spectral forms of Pchlide the absorption, fluorescence excitation and emission spectra have been used.

Absorption and fluorescence excitation: Absorption spectrum of Pchlide in organic solutions shows two strong bands, a Soret band located around 440 nm and a long-wavelength band located around 625 nm. The peak positions, however, vary in different solution as the latter band has a peak position at 623 or 626 nm in 80% acetone or in methanol, respectively (Hendrich and Bereza, 1993). The *in vivo* absorption spectrum of Pchlide at both room and low temperature (77 K) has a peak at 650 nm and a shoulder at 636 nm, the proportion of which depends on the plant species. For example the 636-nm shoulder of the 7-day-old maize is slightly smaller than that of 7-day-old wheat and for pea the short-and long-wavelength peaks have approximately the same height. In contrast, in lip1 mutant of pea, which shows the morphology of light grown plants when grown in dark, the 636-nm band is dominating (Fig. 1). The ratio of the mentioned absorption peaks changes during growth of the plants due to an initial increase of the 650 nm peak (Amirjani *et al*., 2006) followed by a decrease during senescence.

The 77 K fluorescence excitation spectra show similar differences as absorption spectra which varies with plant species and different developmental stages. For instance it has been reported that excitation spectra of wheat have a dominant peak at 649 nm when the emission wavelength was set at 710 nm (Fig. 4). The variations are not restricted to plant species but are found also between different parts of a given plant, e.g., epicotyls and leaves.

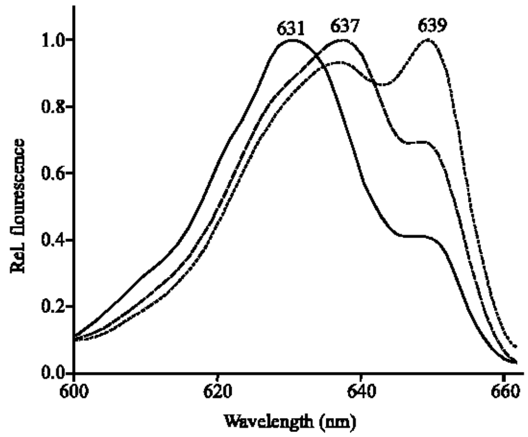

Fig. 4: Low temperature (77 K) excitation spectra of ALA treated maize leaves. The spectra are recorded at 695 (—), 710 (– –) and 725 (---) nm. The spectrum of control (–—) is recorded at 710 nm. The spectra were normalized at the highest peak

In the excitation spectra of pea epicotyls the 672 nm peak is most pronounced (Stadnichuk *et al*., 2005). Furthermore, at an early developmental age the excitation spectra also changed in the Pchlide region. In 1-day-old pea, only a small band is seen at 649 nm but one day later a 636-nm shoulder appeared which became the dominant peak when the plants were 5 days old (Amirjani *et al*., 2006).

The heterogeneous nature of Pchlide may best be shown by a series of excitation spectra recorded at an increasing emission wavelength from ALA treated dark-grown maize leaves (Fig. 4). The excitation spectra recorded with the emission set at 695 nm contained contributions from four Pchlide forms emitting at 628, 635, 644 and 656 nm (Stadnichuk *et al*., 2005). With increasing emission wavelength the dominant band gradually shifted to the red side of the spectra. When measured at 710 nm the vibrational band of the 656 nm Pchlide form was dominating and the excitation peak at 649 nm was evident. With a stepwise increase in the emission wavelength the peak position remained at 649 nm. But the short-wavelength bands were also found in the excitation spectra. This fact can be ascribed to an energy transfer from short-to long-wavelength Pchlide forms.

Fluorescence emission: During fluorescence emission the electronic transition occurs from the lowest energy level, Qy (0,0) and from its vibrational sublevel, Qy (0,1) (Gouterman, 1978).

Böddi *et al.* (1992) reported four spectral forms of Pchlide with emission maxima at 633, 645, 657 and 670 nm. Later experiments with bean leaves indicate that the 633 nm band of non-photoactive Pchlide is made of four bands at 625, 631, 637 and 643 nm. Thus at least seven Pchlide species are present in bean leaves (Schoefs *et al*., 2000).

The identified Pchlide spectral forms can be sorted into three groups. The first group is designated as short-wavelength forms with fluorescence in the 625-646 nm spectral region with corresponding excitation bands in the 620-640 nm region. This group has a heterogeneous nature and is made by four components fluorescing at around 628, 635, 642 and 644 nm (Table 1) (Stadnichuk *et al*., 2005). The 628 and 635 nm forms are both monomeric and the 628 nm form is suggested to be unbound Pchlide while the 635 nm form represents a Pchlide bound to the POR protein. The second group includes the long-wavelength Pchlide forms with emission maxima between 652 and 657 nm. A Pchlide form belonging to this group with peak position at 653 nm was found shortly after flash irradiation of dark-grown leaves. The presence of $NADP^+$ can contribute to the formation of this form. The third group is found in the extreme red region (670-730 nm) (Table 1) of the fluorescence emission spectra and includes a number of pigment forms with spectral bands of low intensity. This region is also influenced by vibrational bands of the short- and long-wavelength Pchlide forms. Sironval has claimed that the region of 660-725 in addition to the vibronic bands also contains bands coming from electronic transitions (Sironval *et al*., 1967). The main short-wavelength bands between 628-635 nm have vibronic bands in the region 685-693 nm. The long-wavelength bands have vibronic satellites above this value. The long-wavelength 710 nm emission band of Pchlide fulfils the conditions of a vibronic satellite of the intensive main 656-nm emission peak. With regard to the distance of the Qy (0,0) and Qy (0, 1) bands, the band-widths and the presence of the 728 nm band even after excitation with long-wavelength (677 nm) light, the 710 nm band is the most probable vibrational band for 656 nm Pchlide form. Analyses of low-temperature fluorescence emission and excitation spectra suggest that there are Pchlide spectral forms fluorescing at 666, 680, 690, 698 and 728 nm together with vibrational (0,1) bands positioned at 675, 687, 697 and 710, which then corresponds to the 628, 635, 644 and 656 nm band, respectively (Table 1). Suppression of vibrational Qy (0,1) bands of the short-wavelength forms of Pchlide can verify the presence of far-red Qy(0,0) components. This could be achieved by using actinic light of wavelengths longer than the absorption peak positions of the short-wavelength. Subtraction of the emission spectrum of heat-denatured leaves excited with the same excitation light enhances the fluorescence emission

Table 1: Spectral parameters of Pchlide bands. Spectral parameters of Pchlide bands obtained after curve resolution of the 77 K excitation spectrum (emission 740 nm) and two types of emission spectra (excitation 440 and 460 nm) of etiolated leaves of wheat. The peak positions can vary slightly when different plant varieties are used for measurements

Parameter	(Spectral values)									
Excitation (Em: 740)	620	628	635	641	649	658	668	677	686	69
Emission (Ex: 440)	628	635	(642)	644	656	666				
Emission (Ex: 460)				646	656	668	680	690	698	728

spectrum as the heat-denatured leaves contained no long-wavelength or far-red Pchlide forms. Subtracting the spectrum obtained (Stadnichuk *et al*., 2005). The peak positions and relative ratio between the fluorescence emission peak heights depend on plant (Fig. 1) species and plant tissues (Fig. 2) (Stadnichuk *et al*., 2005) as well as external factors such as salt stress (Abdelkader *et al*., 2007).

The peak position and relative ratio between intensity of the different peaks vary also with the age of tissues. To examine the effect of developmental age on peak positions and intensities the tissues from 1-to 12-day-old dark-grown plants have been used. The 631 nm band is the first Pchlide form, which appeared in dark-grown tissues (Amirjani *et al*., 2006). In the very young stage the short-wavelength Pchlide forms are dominating with about 80% of the total Pchlide fluorescence for pea and maize. In a later stage of development the Pchlide emission spectra was dominated of the two well-known peaks, 631 and 654-656 nm. The ratio of fluorescence from the short-wavelength Pchlide to that of total Pchlide fluorescence decreased to a minimum of 0.22 in the 12-day-old pea and to a minimum of 0.1 for 6-day-old maize leaves. In older leaves the ratio slightly increased (Fig. 5A, B) (Amirjani *et al*., 2006).

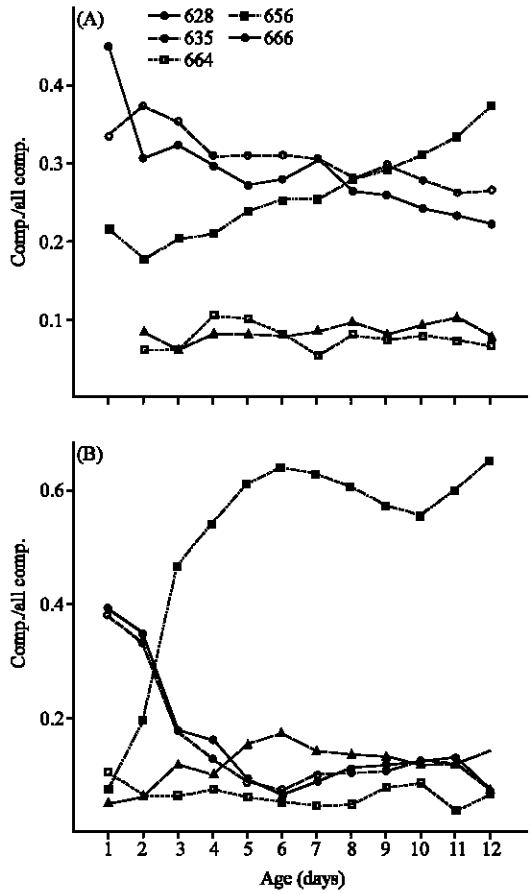

Fig. 5: The ratio of single Gaussian components to the sum of the components during development in darkness. The components were obtained from Gaussian deconvolution of low temperature (77 K) fluorescence emission spectra of dark-grown pea (A) and maize (B) at various ages. The excitation was 440 nm.

Phototransformability of Pchlide forms: Pchlide forms are divided into flash-photoactive and non-flash-photoactive on the basis of their ability to transform to Chlide. Those Pchlide molecules sitting in the active site of the POR-NADPH complex are flash-photoactive, those which are not or have $NADP^+$, are not flash-photoactive (Franck *et al*., 1999). However, this characterization of the different forms is not crystal clear, as depending on irradiation time, different Pchlide forms can be photo transformed or not. The non-flash-photoactive Pchlide forms have fluorescence excitation peaks at 620-640 nm and emission peaks at 627-646 nm (Stadnichuk *et al*., 2005). During a prolonged irradiation, short-wavelength Pchlide can also be photo transformed to Chlide, but this process is probably dependent on a conversion of non-flash-photoactive short-wavelength Pchlide into long-wavelength flash-photoactive Pchlide. The ratio of non-flash-photoactive Pchlide forms to the sum of Pchlide forms in the emission fluorescence spectra decreased while the total amount of Pchl(ide) increased during development (Fig. 5) (Amirjani *et al*., 2006). It has been shown that a treatment with ALA results in an increase of the non-flash-photoactive Pchlide forms (Amirjani and Sundqvist 2006). The non-flash-photoactive Pchlide has been considered to represent a pool of free pigment (Kahn *et al*., 1970) since, its band position is similar to Pchlide in solution. Bystrova reported, however, that a 635-nm Pchlide form is bound to a protein and transmit the excitation energy to the long-wavelength pigment forms

Table 2: Fluorescence lifetime (ns) for pea (wild type), pea (lip1), wheat and maize using excitation light of 440 or 460 nm. For each lifetime corresponding fractions are given in brackets

		Species			
Em (Ex) (nm)		Pea	Lip1	Wheat	Maize
633 (440)	$\tau_1(f_1)$	0.4 (0.18)		0.6 (0.11)	
	$\tau_2(f_2)$	6.7 (0.82)	7.1 (0.99)	6.2 (0.89)	
637 (440)	$\tau_1(f_1)$	0.6 (0.12)			
	$\tau_2(f_2)$	7.0 (0.88)	6.8 (0.99)		
642 (440)	$\tau_1(f_1)$	0.4 (0.12)	7.1 (0.99)		
	$\tau_2(f_2)$	6.2 (0.88)			
642 (460)	$\tau_1(f_1)$		0.6 (0.16)		
	$\tau_2(f_2)$	4.0 (1.00)	7.0 (0.84)		
656 (440)	$\tau_1(f_1)$	0.5 (0.20)	0.7 (0.16)	0.6 (0.09)	0.7 (0.04)
	$\tau_2(f_2)$	6.4 (0.77)	6.5 (0.84)	5.1 (0.91)	6.7 (0.96)
656 (460)	$\tau_1(f_1)$	0.6 (0.11)	0.3 (0.36)	0.8 (0.27)	0.4 (0.28)
	$\tau_2(f_2)$	5.4 (0.89)	7.0 (0.63)	5.6 (0.79)	6.3 (0.72)
670 (440)	$\tau_1(f_1)$	0.3 (0.24)			
	$\tau_2(f_2)$	5.7 (0.67)	5.6 (0.99)		
670 (460)	$\tau_1(f_1)$				
	$\tau_2(f_2)$	2.0 (1.00)	2.7 (0.99)		

(Bystrova *et al*., 1988). Furthermore, lifetime measurements indicated that the short-wavelength Pchlide forms are not free pigments but probably combined with proteins or lipids since their lifetime are shorter than that of Pchlide in diethyl ether, τ = 9.3 ns, or acetone, τ = 8.8 ns measured at 77 K (Table 2).

The main pigment form of mature etiolated leaves in many plant species is the flash-photoactive Pchlide form, depending on the age species, etc as discussed above. This form can be photo transformed by a short (1 m sec) light flash. The main flash-photoactive Pchlide form has a long-wavelength fluorescence emission band located around 656 nm, with slight variations depending on species. In some plants, such as pea and its lip1 mutant, this form is, however, a minor form (Stadnichuk *et al*., 2005; Amirjani and Sundqvist, 2006). The main flash-photoactive Pchlide is regarded to be an aggregate of ternary complexes, composed of the photoenzyme, POR, its Pchlide substrate and its reduced NADPH cofactor. The photoactive Pchlide pool is spectrally and chemically heterogeneous (Stadnichuk *et al*., 2005). A minor form of photoactive Pchlide absorbing around 638 nm and fluorescing at 643-644 nm has been characterized both *in vivo* and *in vitro*, which seems to be the first transformed Pchlide form into Chlide when etiolated leaves are illuminated with light of low pfd weak irradiation (Böddi *et al*., 1991). This is interesting as there seems to be a formation of an excited state with fluorescence around 644 nm (Dietzek *et al*., 2006). Schoefs reported the photoactive Pchlide form to be composed of three components fluorescing at 644, 652 and 657 nm (Schoefs *et al*., 2000).

Photoactive Pchlide exists not only in the leaves of etiolated plants but also in greening and green leaves after a period of darkness (Amirjani and Sundqvist, 2004).

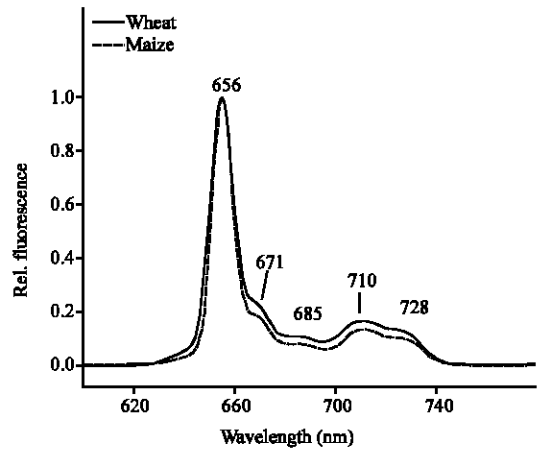

Fig. 6: Low temperature (77 K) fluorescence emission spectra of dark-grown plants. Maize and wheat leaves excited by 460-nm light, respectively. The spectra were normalized to the highest peak

Ignatov demonstrated the occurrence of photoactive Pchlide in green leaves under light conditions (Ignatov and Litvin, 2002). We showed that in angiosperms the pathway of Chl biosynthesis in greening and green leaves proceeds through the 655-nm Pchlide form, similar to the pathway in dark-grown leaves (Amirjani and Sundqvist, 2004).

Pchlide spectral forms in different plant species: *In vivo* the Pchlide peak positions and relative intensity ratio vary with different plant species. The variation is evident in absorption,fluorescence emission and excitation spectra. The absorption spectrum of maize, barley and wheat has in the red region a peak at 650 and a 636 nm band appears as a shoulder. For pea the 636 nm band is an evident peak and in lip1 the 636-nm absorption band is dominant (Fig. 1) (Amirjani *et al*., 2006).

In the fluorescence emission spectra the differences between the species are conspicuous. The fluorescence spectra of wheat and maize have a dominant peak located at 656 nm and a small peak at 631 nm. The latter peak is for maize slightly smaller than for wheat (Fig. 6) (Mysliwa-Kurdziel *et al*., 2003).

As an extreme the emission spectrum of the *aurea* mutant of tomato (*Solanum lycopersicum* L.), which is unable to synthesize the linear tetrapyrrole chromophore of phytochrome and is one of the most exhaustively characterized photomorphogenic mutants (Terry, 1997; Terry and Kendrick, 1999; Terry *et al*., 2001), does not have a short-wavelength Pchlide form in darkness and during an early stage of greening. In contrast in pea and its lip1 mutant the 631 nm emission band is the dominant band with 440 nm excitation. Pea has a small peak at 654 nm whereas lip1 mutant does not show any evident peak in this region. The epicotyl of dark-grown pea plants also has a dominant short-wavelength Pchlide peak (Fig. 2). The peak is, however, slightly asymmetric around 654 nm indicating the presence of the long-wavelength form. The same is true for maize mesocotyles and coleoptiles of triticale (Savchenko *et al*., 2005).

Dark-forced wine (*Vitis vinifera*) leaves and stems were dominated by the short-wavelength Pchlide form. Differences are also seen in the wavelength region between 660 and 730 nm. The 666, 680 and 728 nm wavelengths Pchlide forms are more pronounced in wheat and maize (cf. (Fig. 6). Using a statistical method calculating the average of the absolute deviation (AVEDEV) of datapoints in a spectrum from their mean function and plotting the AVEDEV values against the wavelength clearly showed the bands at 626, 636 and 656nm for pea epicotyl (Szenzenstein *et al*., 2008).

The cause for the species dependent spectral differences can be sought on different levels. As mentioned the fluorescence spectra from epicotyls of pea are quite different from those of the leaf tissues. In maize and wheat the spectra from different part of leaves are different. Therefore the presence of immature palisade parenchyma or spongy parenchyma, or like in wheat and maize, immature mesophyll cells might be important for the spectral forms. The occurrence of different types of PLBs can also be of importance. The correlation between $Pchlide_{650\text{-}656}$ and the presence of PLBs has been shown earlier (Ryberg and Sundqvist, 1982). Differences in the interaction between Pchlide, POR and the PLB/PT membranes depending on a variation in the lipid composition of the different membranes can also be possible. Finally, when it comes to POR itself there might be differences in the interaction between the Pchlide pigment and the POR protein.

Reaccumulation of Pchlide during greening: When illuminated plants return to darkness they re-accumulate photoactive Pchlide. The re-accumulation proceeds in different ways. Some Pchlide, which are bound to an $NADP^+$ in a ternary complex, can transform into photoactive Pchlide within seconds when the $NADP^+$ is reduced. The 633 nm fluorescing form can be transformed into photoactive Pchlide within minutes, when newly formed Chlide is removed from the active site of POR and replaced by new Pchlide from the precursor pool. During a prolonged dark-period (h), newly synthesized Pchlide also contribute to the re-accumulation. Duration and intensity of the illumination light determine the ratio between re-accumulated Pchlide fluorescing at 633 and 653 nm. The spectral properties of Pchlide re-accumulated in plants illuminated for a short time (1 h) were specific for the plant species and resembled those of the Pchlide accumulated in the dark-grown plants (Amirjani and Sundqvist, 2004). The re-accumulation of long-wavelength Pchlide probably reflects the re-formation of the PLBs since the plastids still have a capacity to develop etioplast features when the plants are returned to darkness after illumination (Schoefs and Franck, 2008). With prolonged illumination (24 h) the re-accumulation of the long-wavelength Pchlide form apparently decreased. The decrease might be due to a decrease in POR as the PORA gene is known to be down-regulated after irradiation (Apel, 1981). However, the excitation spectra showed that there could be more Pchlide present absorbing around 650 nm than revealed by the fluorescence emission spectra. There seems to be an energy transfer from the long-wavelength Pchlide to the Chl (Brouers and Sironval, 1974) and this energy transfer diminish the contribution of the long-wavelength Pchlide form to the fluorescence emission at 656 nm (Amirjani and Sundqvist, 2004). Energy may transfer from a donor chromophore, initially in its electronic excited state, to an acceptor chromophore (in proximity, typically less than 10 nm) through nonradiative dipole-dipole coupling. This mechanism is termed Förster resonance energy transfer and is named after the German scientist Theodor Förster. When both chromophores are fluorescent, the term fluorescence resonance energy transfer is often used instead, although the energy is not actually transferred by fluorescence (Lakowicz, 1999). Thus judging from both fluorescence emission spectra and fluorescence excitation spectra the spectral forms of the re-accumulated Pchlide also in green leaves seemed to be dependent on plant species and present in proportions similar to those in the corresponding dark-grown material.

Fluorescence lifetime of Pchlide forms: The fluorescence lifetime is one of the most important characteristics of a fluorophore. When a fluorophore absorbs the energy of a photon the molecule become excited and can be in the excited state during a time period corresponding to its fluorescence lifetime. The lifetime determines the time available for the excited fluorophore to diffuse and interact with its environment before it returns to its ground state. During the fluorescence lifetime, energy migration can occur.

Earlier lifetime measurements on Pchlide indicate that photoactive and non-photoactive Pchlide have different lifetimes. Two lifetime components were found in time-resolved fluorescence measurements on etioplast membranes. The low temperature *in vivo* fluorescence lifetime was found to be shorter (5.1-7.1 ns) than the fluorescence lifetime of Pchlide in organic solvents measured under the same experimental conditions (around 9 ns) (Mysliwa-Kurdziel *et al*., 2003). However, similar to the absorption, excitation and emission fluorescence spectra, the lifetimes of the Pchlide forms differed between the plants (Table 2).

The lip1 mutant excited at 440 nm, has one Pchlide fluorescence lifetime of approximately 7 ns with emission set at 633, 637 or 642 nm. This fluorescence lifetime can be related to both the 635 and 642 nm forms of Pchlide since both forms are present, although in different proportions, in the steady-state fluorescence spectra. When the emission wavelength was set at 670 nm, only one component, now with a lifetime of 5.6 ns can be found. For the 656 nm emission two components of the fluorescence lifetime were observed. When the sample was excited at 460 nm two lifetime components are observed for the emission at 642 and 656 nm. For this excitation wavelength, the fast component has a lifetime value about two times longer (0.6 ns) for the emission at 642 nm than at 656 nm. However, its fractional intensity was lower (Table 2). The fast component decreases concomitant with the increase of the fractional intensity, when comparing the results obtained for emission at 656 nm with the two excitations of 440 and 460 nm. Wild-type pea leaves excited at 440 nm have two fluorescence lifetime components for different emission wavelengths. For the excitation at 440 nm and the emission at 633 nm, the main contribution to total fluorescence is from the $Pchlide_{628\text{-}635}$ form (0.78) and the rest (0.22) from $Pchlide_{635\text{-}642}$. Thus, in wild-type pea it seems the fast component came from the $Pchlide_{635\text{-}642}$ form. For the emission wavelength set at 637 and 642 nm, the contribution of $Pchlide_{628\text{-}633}$ in the steady-state spectra decrease and that of $Pchlide_{635\text{-}642}$ increases and finally reach 0.18 and 0.77, respectively. However, the fractions of the fluorescence lifetimes stay more or less at the same levels, which do not reflect the changes in the steady-state fluorescence of the different Pchlide forms. The slow component has a lifetime between 5.7 and 7.0 ns. For the emission wavelength of 642 nm, the slow component for wild-type pea has a lower value (6.2 ns) than the slow component for lip1 (7.1 ns). For the excitation at 460 nm two lifetime components are found for the emission at 656 nm, whereas only one component was determined for the 642 and 670 nm emission (Table 2). Decay Association Spectra (DAS) resembled the fluorescence spectrum of wild-type pea leaves for both the slow and fast lifetime components (Mysliwa-Kurdziel *et al*., 2003). Thus, in the case of wild-type pea, not only 654-nm Pchlide but also 635 and 642 nm Pchlide forms show a complex character of the fluorescence decay that can be described only using two fluorescence lifetime components.

Organization of pigment-protein complexes: The Pchlide molecule suits a pocket on the POR protein with the active site at the bottom (Townley *et al*., 2001). During phototransformation the hydride transfer occurs from NADPH, which is located at the bottom of the substrate-binding pocket. Conserved Tyr and Lys residues in POR supply the protons for the reaction (Wilks and Timko, 1995). Three sites on the Pchlide molecule are of special importance for POR activity. The central metal atom should preferably be a magnesium atom. The propionic side chain on the porphyrin ring D should be whole and the complete structure of ring E should be present. The penta-coordination of the magnesium has contributions from the four tetrapyrrole nitrogens and one ligand from the POR. The POR enzyme allows a certain degree of modifica-tion of the side chains on ring A and B even if these modifications give spectral shifts. Thus Pchlide a and b with different electronic spectra have almost identical reaction rates *in vitro*.

The short-wavelength Pchlide forms are monomeric and probably bound to a protein. It might be that the monomeric Pchlide forms are not phototransformable but a dimeric form is necessary. Then the 635 nm Pchlide form may be bound to the POR and even in the active site. The upper part of the Pchlide molecule containing ring A and ring B protrudes from the POR protein (Townley *et al*., 2001). This creates a possibility for an interaction between different Pchlide molecules. A formation of dimers can be caused by an interaction between two POR-bound Pchlide molecules with overlapping A and B rings (Fig. 7). The 642-644 nm fluorescing Pchlide form can be regarded as a dimer of the ternary complex of NADPH, POR and Pchlide. This form is flash-photoactive and the phototransformation is faster than for the main photoactive Pchlide form. This form is mostly present in small amounts suggesting that it easily form larger aggregates when it becomes more abundant.

If the interaction between the Pchlide molecules is limited to only a part of the free end of the Pchlide molecule there can be room for an interaction also for a third POR-bound Pchlide molecule. In this way large aggregates with interacting Pchlide molecules can be formed. The aggregates are then associated with the PLB membrane and this interaction also seems to influence the spectral properties of the Pchlide. Far-red forms of Pchlide could be regarded as representing a higher degree of aggregation (Fig. 7). Another possibility is that the dimers formed via the pigments interact through the POR molecules and creates the larger aggregates. The Pchlide

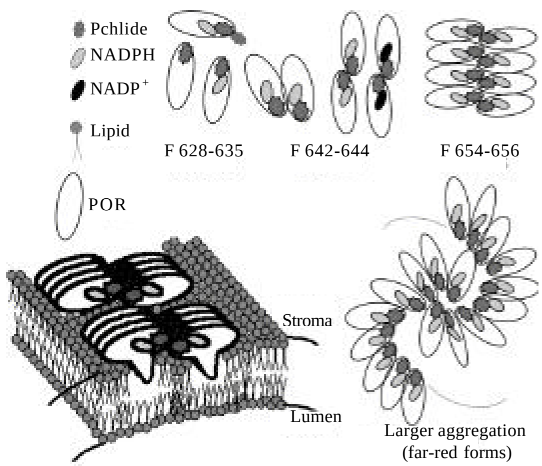

Fig. 7: Schematic diagram illustrating the aggregation state of ternary complexes of Pchlide, POR and NADPH

molecules are in good contact for the interaction of the π electron system. A size of the aggregates of 8 interacting POR molecules (Fig. 7) has been suggested as energy transfer units. A possibility is also that a free (not POR-bound) Pchlide molecule can be attached to a POR-bound Pchlide. At a flash irradiation such a Pchlide molecule would not be phototransformed as it is not at the active site. After phototransformation it can rapidly replace the newly formed Chlide and contribute to a rapid regeneration of phototransformable Pchlide. If such non-POR bound Pchlide molecules could substitute for a POR-bound Pchlide during formation of the large aggregates a considerable variation in the spectral properties and the degree of phototransformability could be expected.

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I would like to dedicate this paper to Professor Christer Sundqvist.

REFERENCES

Abdelkader, A.F., H. Aronsson, K. Solymosi, B. Boddi and C. Sundqvist, 2007. High salt stress induces swollen prothylakoids in dark-grown wheat and alters both prolamellar body transformation and reformation after irradiation. J. Exp. Bot., 58: 2553-2564.

Amirjani, M.R. and C. Sundqvist, 2004. Regeneration of protochlorophyllide in green and greening leaves of plants with varying proportions of protochlorophyllide forms in darkness. Physiol. Plantarum, 121: 377-390.

Amirjani, M.R. and C. Sundqvist, 2006. Red region excitation spectra of protochlorophyllide in dark-grown leaves from plant species with different proportions of its spectral forms. Photosynthetica, 44: 83-92.

Amirjani, M.R., K. Sundqvist and C. Sundqvist, 2006. Protochlorophyllide and POR development in dark-grown plants with different proportions of short-wave length and long-wavelength protochlorophyllide spectral forms. Physiol. Plantarum, 128: 751-762.

Apel, K., 1981. The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Phytochrome-induced decrease of translatable mRNA coding for the NADPH: Protochlorophyllide oxidoreductase. Eur. J. Biochem., 120: 89-93.

Armstrong, G.A., S. Runge, G. Frick, U. Sperling and K. Apel, 1995. Identification of NADPH: Protochlorophyllide oxidoreductases A and B: A branched pathway for light-dependent chlorophyll biosynthesis in *Arabidopsis thaliana*. Plant Physiol., 108: 1505-1517.

Aronsson, H., K. Sohrt and J. Soll, 2000. NADPH:Protochlorophyllide oxidoreductase uses the general import route into chloroplasts. Biol. Chem., 381: 1263-1267.

Aronsson, H., C. Sundqvist, M.P. Timko and C. Dahlin, 2001. The importance of the C-terminal region and Cys residues for the membrane association of the NADPH: Protochlorophyllide oxidoreductase in pea. FEBS Lett., 502: 11-15.

Beale, S.I., 1999. Enzymes of chlorophyll biosynthesis. Photosynth. Res., 60: 43-73.

Blomqvist, L.A., M. Ryberg and C. Sundqvist, 2008. Proteomic analysis of highly purified prolamellar bodies reveals their significance in chloroplast development. Photosynth. Res., 96: 37-50.

Brouers, M. and C. Sironval, 1974. Evidence for energy transfer from protochlorophyllide to chlorophyllide in leaves treated with ä-amino-levulinic acid. Plant Sci. Lett., 2: 67-72.

Buchanan, B.B., W. Gruissem and R.L. Jones, 2000. Biochemistry and Molecular Biology of Plants. Courier Companies Inc., Rockville, Maryland.

Bystrova, M.I., I.G. Nazarova and A.A. Krasnovskii, 1988. The state and functional activity of protochlorophyllide formed from exogenous 5-aminolevulinic acid in green leaves. Biokhim, 53: 1950-1961.

Böddi, B., A. Lindsten, M. Ryberg and C. Sundqvist, 1990. Phototransformation of aggregated forms of protochlorophyllide in isolated etioplast inner membranes. Photochem. Photobiol., 52: 83-87.

Böddi, B., B. McEwen, M. Ryberg and C. Sundqvist, 1994. Protochlorophyllide forms in non-greening epicotyls of dark-grown pea (*Pisum sativum*). Physiol. Plantarum, 92: 160-170.

Böddi, B., M. Ryberg and C. Sundqvist, 1991. The formation of a short-wavelength chlorophyllide form at partial phototransformation of protochlorophyllide in etioplast inner membranes. Photochem. Photobiol., 53: 667-673.

Böddi, B., M. Ryberg and C. Sundqvist, 1992. Identification of four universal protochlorophyllide forms in dark grown leaves by analysis of the 77 K fluorescence emission spectra. J. Photochem. Photobiol. B: Biol., 12: 389-401.

Chen, X. and D.J. Schnell, 1999. Protein import into chloroplasts. Trends Cell Biol., 9: 222-227.

Dietzek, B., W. Kiefer, A. Yartsev, V. Sundstrom and P. Schellenberg *et al*., 2006. The excited-state chemistry of protochlorophyllide a: A time-resolved fluorescence study. Chemphyschem, 7: 1727-1733.

Franck, F., B. Bereza and B. Böddi, 1999. Protochlorophyllide-$NADP^+$ and protochlorophyllide-NADPH complexes and their regeneration after flash illumination in leaves and etioplast membranes of dark-grown wheat. Photosynth. Res., 59: 53-61.

Franck, F., P. Eullaffroy and R. Popovic, 1997. Formation of long-wavelength chlorophyllide (Chlide695) is required for the assembly of photosystem II in etiolated barley leaves. Photosynth. Res., 51: 107-118.

Fujita, Y., 1996. Protochlorophyllide reduction: A key step in the greening of plants. Plant Cell Physiol., 37: 411-421.

Gouterman, M., 1978. Optical Spectra and Electronic Structure of Porphyrins and Related Rings. Academic Press Inc., New York, USA.

Grondelle, R., J.P. Dekker, T. Gillbro and V. Sundström, 1994. Energy transfer and trapping in photosynthesis. Biochim. Biophys. Acta, 1187: 1-65.

Hendrich, W. and B. Bereza, 1993. Spectroscopic characterization of protochlorophyllide and its transformation. Photsynthetica, 28: 1-16.

Henningsen, K.W., 1970. Macromolecular physiology of plastids. VI. Changes in membrane structure associated with shifts in the absorption maxima of the chlorophyllous pigments. J. Cell Sci., 7: 587-621.

Heyes, D.J., G.E. Martin, R.J. Reid, C.N. Hunter and H.M. Wilks, 2000. NADPH: Protochlorophyllide oxidoreductase from Synechocystis: Overexpression, purification and preliminary characterisation. FEBS Lett., 483: 47-51.

Heyes, D.J. and C.N. Hunter, 2004. Identification and characterization of the product release steps within the catalytic cycle of protochlorophyllide oxidoreductase. Biochemistry, 43: 8265-8271.

Heyes, D.J. and C.N. Hunter, 2005. Making light work of enzyme catalysis: Protochlorophyllide oxidoreductase. Trends Biochem. Sci., 30: 642-649.

Heyes, D.J., P. Heathcote, S.E. Rigby, M.A. Palacios, R. van Grondelle and C.N. Hunter, 2006. The first catalytic step of the light-driven enzyme protochlorophyllide oxidoreductase proceeds via a charge transfer complex. J. Biol. Chem., 281: 26847-26853.

Heyes, D.J., M. Sakuma, S.P. de Visser and N.S. Scrutton, 2009. Nuclear quantum tunneling in the light-activated enzyme protochlorophyllide oxidoreductase. J. Biol. Chem., 284: 3762-3767.

Holtorf, H., S. Reinbothe, C. Reinbothe, B. Bereza and K. Apel, 1995. Two routes of chlorophyllide synthesis that are differentially regulated by light in barley (*Hordeum vulgare* L.). Proc. Nat. Acad. Sci. USA., 92: 3254-3258.

Ignatov, N.V. and F.F. Litvin, 2002. A new pathway of chlorophyll biosynthesis from long-wavelength protochlorophyllide Pchlide 686/676 in juvenile etiolated plants. Photosynth. Res., 71: 195-207.

Ilag, L.L., A.M. Kumar and D. Soll, 1994. Light regulation of chlorophyll biosynthesis at the level of 5-aminolevulinate formation in arabidopsis. Plant Cell., 6: 265-275.

Jörnvall, H., B. Persson, M. Krook, S. Atrian, R. González-Duarte, J. Jeffery and D. Ghosh, 1995. Short-chain dehydrogenases/reductases (SDR). Biochem. J., 34: 6003-6013.

Kahn, A., N.K. Boardman and S.W. Thorne, 1970. Energy transfer between protochlorophyllide molecules: Evidence for multiple chromophores in the photoactive protochlorophyllide-protein complex *vivo* and *in vitro*. J. Mol. Biol., 48: 85-101.

Klement, H., U. Oster and W. Rudiger, 2000. The influence of glycerol and chloroplast lipids on the spectral shifts of pigments associated with NADPH: Protochlorophyllide oxidoreductase from *Avena sativa* L. FEBS Lett., 480: 306-310.

Klein, S. and J.A. Schiff, 1972. The correlated appearance of prolamellar bodies, protochlorophyll(ide) species and the shibata shift during development of bean etioplasts in the dark. Plant Physiol., 49: 619-626.

Klockare, B., 1980. Far-red induced changes of the protochlorophyllide component in wheat leaves. Physiol. Plantarum, 48: 104-110.

Kolossov, V.L. and C.A. Rebeiz, 2003. Chloroplast biogenesis 88. Protochlorophyllide b occurs in green but not in etiolated plants. J. Biol. Chem., 278: 49675-49678.

Kosa, A., Z. Marton, K. Solymosi, K. Boka and B. Boddi, 2006. Aggregation of the 636 nm emitting monomeric protochlorophyllide form into flash-photoactive, oligomeric 644 and 655 nm emitting forms *in vitro*. Biochim. Biophys. Acta, 1757: 811-820.

Kuroda, H., T. Masuda, H. Ohta, Y. Shioi and K. Takamiya, 1995. Light-enhanced gene expression of NADPH-protochlorophyllide oxidoreductase in cucumber. Biochem. Biophys. Res. Commun., 210: 310-316.

Lakowicz, J.R., 1999. Principles of Fluorescence Spectroscopy. Kluwer Academic, New York.

Lancer, H.A., C.E. Cohen and J.A. Schiff, 1976. Changing ratios of phototransformable protochlorophyll and protochlorophyllide of bean seedlings developing in the dark. Plant Physiol., 57: 369-374.

Lee, K.P., C. Kim, F. Landgraf and K. Apel, 2007. EXECUTER1-and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. PNAS., 104: 10270-10275.

Lopez-Juez, E. and K.A. Pyke, 2005. Plastids unleashed: their development and their integration in plant development. Int. J. Dev. Biol., 49: 557-577.

Marchand, M., D Dewez , F. Franck and R. Popovic, 2004. Protochlorophyllide phototransformation in the bundle sheat cells of *Zea Mays*. J. Photochem. Photobiol. B: Biol., 75: 73-80.

Masuda, T., N. Fusada, N. Oosawa, K. Takamatsu and Y.Y. Yamamoto *et al*., 2003. Functional analysis of isoforms of NADPH: Protochlorophyllide Oxidoreductase (POR), PORB and PORC, in *Arabidopsis thaliana*. Plant Cell Physiol., 44: 963-974.

Mysliwa-Kurdziel, B., M.R. Amirjani, K. Strzalka and C. Sundqvist, 2003. Fluorescence lifetimes of protochlorophyllide in plants with different proportions of short-wavelength and long-wavelength protochlorophyllide spectral forms. Photochem. Photobiol., 78: 205-212.

Mysliwa-Kurdziel, B., K. Solymosi, J. Kruk, B. Boddi and K. Strzalka, 2008. Solvent effects on fluorescence properties of protochlorophyll and its derivatives with various porphyrin side chains. Eur. Biophys. J., 37: 1185-1193.

Oosawa, N., T. Masuda, K. Awai, N. Fusada, H. Shimada, H. Ohta and K. Takamiya, 2000. Identification and light-induced expression of a novel gene of NADPH-protochlorophyllide oxidoreductase isoform in *Arabidopsis thaliana*. FEBS Lett., 474: 133-136.

Reinbothe, C., F. Buhr, S. Pollmann and S. Reinbothe, 2003. *In vitro* reconstitution of light-harvesting POR-protochlorophyllide complex with protochlorophyllides a and b. J. Biol. Chem., 278: 807-815.

Reinbothe, C., N. Lebedev and S. Reinbothe, 1999. A protochlorophyllide light-harvesting complex involved in de-etiolation of higher plants. Nature, 397: 80-84.

Reisinger, V., A.P. Hertle, M. Plöscher and L.A. Eichacker, 2008. Cytochrome b6f is a dimeric protochlorophyll a binding complex in etioplasts. FEBS J., 275: 1018-1024.

Ryberg, M. and C. Sundqvist, 1982. Spectral forms of protochlorophyllide in prolamellar bodies and prothylakoids fractionated from wheat etioplasts. Physiol. Plantarum, 56: 133-138.

Ryberg, M. and C. Sundqvist, 1991. Structural and Functional Significance of Pigment-Protein Complexes of Chlorophyll Precursors. CRC Press Inc., New York, pp: 587-592.

Ryberg, H., M. Ryberg and C. Sundqvist, 1993. Plastid Ultrastructure and Development. In: Pigment-Protein Complexes in Plastids: Synthesis and Assembly, Sundqvist, C. and M. Ryberg (Eds.). Academic Press, San Diego, ISBN: 0-12-676960-5, pp: 25-62.

Savchenko, G.E., E.A. Klyuchareva and L.F. Kabashnikova, 2005. Tissue-specific features of pigment biogenesis in coleoptiles of greening etiolated seedlings of cereals. Russian J. Plant Physiol., 52: 591-595.

Schoefs, B., M. Bertrand and F. Franck, 2000. Spectroscopic properties of protochlorophyllide analyzed in situ in the course of etiolation and in illuminated leaves. Photochem. Photobiol., 72: 85-93.

Schoefs, B., 2005. Protochlorophyllide reduction-what is new in 2005?. Photosynthetica, 43: 329-343.

Schoefs, B. and F. Franck, 2008. The photoenzymatic cycle of NADPH: Protochlorophyllide oxidoreductase in primary bean leaves (*Phaseolus vulgaris*) during the first days of photoperiodic growth. Photosynth. Res., 96: 15-26.

Schulz, R. and H. Senger, 1993. Protochlorophyllide Reductase: A Key Enzyme in the Greening Process. In: Pigment-Protein Complexes in Plastids: Synthesis and Assembly, Sundqvist, C. and M. Ryberg (Eds.). Academic Press Inc., New York, ISBN 0-12-676960-5 pp: 25-62.

Selstam, E., J. Schelin, W.P. Williams and A.P. Brain, 2007. Structural organisation of prolamellar bodies (PLB) isolated from *Zea mays*. Parallel TEM, SAXS and absorption spectra measurements on samples subjected to freeze-thaw, reduced pH and high-salt perturbation. Biochim. Biophys. Acta, 1768: 2235-2245.

Sineshchekov, V., A. Loskovich, N. Inagaki and M. Takano, 2006. Two native pools of phytochrome A in monocots: Evidence from fluorescence investigations of phytochrome mutants of rice. Photochem. Photobiol., 82: 1116-1122.

Sironval, C., Y. Kuyper, J.M. Michel and M. Brouers, 1967. On the primary photoact in the conversion of protochlorophyllide into chlorophyllide. Stud. Biophys., 5: 43-50.

Stadnichuk, I.N., M.R. Amirjani and C. Sundqvist, 2005. Identification of spectral forms of protochlorophyllide in the region 670-730 nm. Photochem. Photobiol. Sci., 4: 230-238.

Staehelin, L.A., 2003. Chloroplast structure: From chlorophyll granules to supra-molecular architecture of thylakoid membranes. Photosynth. Res., 76: 185-196.

Strand, A., T. Asami, J. Alonso, J.R. Ecker and J. Chory, 2003. Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. Nature, 421: 79-83.

Su, Q., G. Frick, G. Armstrong and K. Apel, 2001. POR C of Arabidopsis thaliana: a third light-and NADPH-dependent protochlorophyllide oxidoreductase that is differentially regulated by light. Plant Mol. Biol., 47: 805-813.

Sullivan, J.A. and J.C. Gray, 1999. Plastid translation is required for the expression of nuclear photosynthesis genes in the dark and in roots of the pea lip1 mutant. Plant Cell, 11: 901-910.

Sundqvist, C. and C. Dahlin, 1997. With chlorophyll pigment from prolamellar bodies to light harvesting complexes. Physiol. Plantarum, 100: 748-759.

Sytina, O., D.J. Heyes, C. Neil Hunter, M.T. Alexandre, I.H.M. van Stokkum, R. van Grondelle and M.L. Groot, 2008. Conformational changes in an ultrafast light-driven enzyme determine catalytic activity. Nature, 456: 1001-1004.

Szenzenstein, A., A. Kosa and B. Böddi, 2008. Biological variability in the ratios of protochlorophyllide forms in leaves and epicotyls of dark-grown pea (*Pisum sativum* L.) seedlings (A statistical method to resolve complex spectra). J. Photochem. Photobiol. B: Biol., 90: 88-94.

Teakle, G.R. and W.T. Griffiths, 1993. Cloning, characterization and import studies on protochlorophyllide reductase from wheat (*Triticum aestivum*). Biochem. J., 296: 225-230.

Terry, M.J., 1997. Phytochrome chromophore deficient mutants. Plant Cell Environ., 20: 740-745.

Terry, M.J. and R.E. Kendrick, 1999. Feedback inhibition of chlorophyll synthesis in the phytochrome chromophore-deficient aurea and yellow-green-2 mutants of tomato. Plant Physiol., 119: 143-152.

Terry, M.J., M. Ryberg, C.E. Raitt and A.M. Page, 2001. Altered etioplast development in phytochrome chromophore-deficient mutants. Planta, 214: 314-325.

Timko, M.P., 1998. Pigment Biosynthesis: Chlorophylls, Heme and Carotenoids. In: The Molecular Biology of Chlamydomonas: Chloroplasts and Mitochondria, Rochaix, J.D., M. Goldschmidt-Clermont and S. Merchant (Eds.). Kluwer Academic Publishers, Dordrecht, ISBN: 0-7923-5174-6.

Townley, H.E., R.B. Sessions, A.R. Clarke, T.R. Dafforn and W.T. Griffiths, 2001. Protochlorophyllide oxidoreductase: A homology model examined by site-directed mutagenesis. Proteins, 44: 329-335.

Whatley, J.M., G.R. Hawes, J.C. Horne and J.D.A. Kerr, 1982. The establishment of the plastid thylakoid system. New Phytol., 90: 619-629.

Wilks, H.M. and M.P. Timko, 1995. A light-dependent complementation system for analysis of NADPH: Protochlorophyllide oxidoreductase: Identification and mutagenesis of two conserved residues that are essential for enzyme activity. Proc. Nat. Acad. Sci. USA., 92: 724-728.