Photoreduction of protochlorophyllide *a* to chlorophyllide *a* during the biogenesis of the photosynthetic apparatus in higher plants

by

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Habilitation thesis

Dr Benoît SCHOEFS

1999

To my wife

CONTENT

ABBREVIATIONS

Norm
A: value of the normalized absorbance intensity at the time t
t

c: the proportion of chlorophyllide *a* formed during the photoreduction
CD: circular dichroism
Chlide: chlorophyllide
Chl: chlorophyll
CX-Y: means chlorophyllide *a* absorbing at X nm and emitting fluorescence at Y nm
DHGG: dihydrogeranylgeraniol

Norm
F: value of the normalized fluorescence intensity at the time t
t

F_0: Level of the fluorescence mainly originating from the antenna chlorophylls of photosystem II before the excitons are transferred to the reaction center

GG: geranylgeraniol
i: the fluorescence intensity emitted at 690 nm by protochlorophyllide *a* and chlorophyllide *a*
k_F: rate constant of the 690 nm fluorescence kinetic
k_A: rate constant of the 440 nm absorbance kinetic
LPOR: light-dependent NADPH:protochlorophyllide *a* oxidoreductase (E.C. 1.3.1.33)
Pchl: protochlorophyll *a*. Except when specified, this term designates a mixture of protochlorophyllide *a* esterified with either geranylgeraniol or tetrahydrogeranyl-geraniol, dihydrogeranylgeraniol, phytol
Pchlide: protochlorophyllide *a*

Ph: phytol
Pheo: pheophytin
PLB: prolamellar body
pLPOR: unprocessed LPOR
PT: prothylakoid
PX-Y: means protochlorophyllide *a* absorbing at X nm and emitting fluorescence at Y nm
t: time
THGG: tetrahydrogeranylgeraniol

Foreword

The development and the maintenance of life on earth is predominantly dependent on photosynthesis which transforms the radiant energy, coming from the sun, into the chemical energy stored in various molecules.

In photosynthetic eukaryotic organisms, this process takes place in a specialized organelle called chloroplast (Engelmann 1881, Arnon et al. 1954). At the heart of the photosynthetic process are chlorophyll (Chl) and carotenoid pigments which are principally associated with proteins (reviewed by Green & Durnford 1996). These pigment-protein complexes are organized in units called photosystems (Emerson et al. 1957, Kok 1957). Each photosystem consists of a reaction center that performs the charge separation and of a set of light-harvesting complexes that collect the radiant energy and deliver it to the reaction center (Emerson & Arnold 1932). The photosystems are located in the chloroplast internal membranes called the thylakoids (Schimper 1885, Heitz 1932).

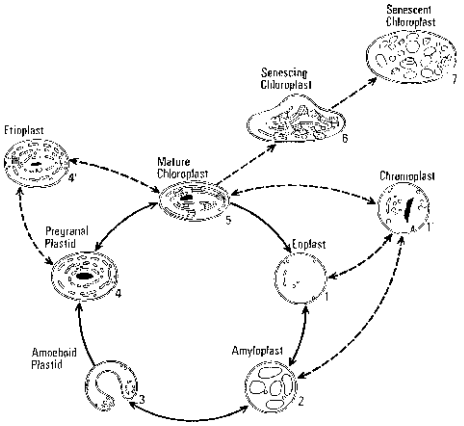

Figure 1. General scheme of the chloroplast development. From Thomson &Whatley. Reprinted with permission, from The Annual Review of Plant Physiology, Vol. 41 copyright 1980, by Annual Reviews http:\\www.Annual Reviews.org

In angiosperms, chloroplast formation is a light-dependent process (Morren 1858) which starts from the proplastid stage, a mixture of eoplasts, amyloplasts and amoeboid plastids (Figure 1). This stage is characterized by the presence of few internal membranes and of starch. In further development of the proplastid, two pathways are possible: in the light, proplastids directly differentiate into chloroplasts whereas in the dark, proplastids develop into etioplasts, as reviewed by Thomson & Whatley (1980), Kutík (1985) and Hudák (1997) (Figure 1).

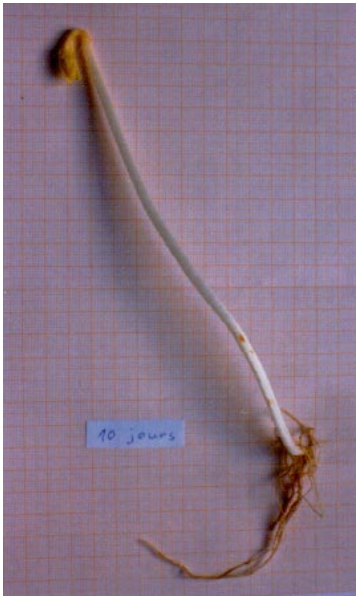

Figure 2. ***Left***: Seeds of *Acer* sp. germinating at the soil surface. Only the radicle is out of the seed (Picture taken the 14th of March 1999 in the park of the Hluboká castle, Hluboká nad Vltavou, Czech Republic).
Right: 10-d-old etiolated bean seedling.

Because etiolated[1] material - leaf or cotyledon - has larger leaves (compare figure 2 right with figure 1 in **II**) and contain more pigments per surface area than the embryonic tissues, chlorophyll synthesis, and more generally, chloroplast development are studied on this material. Although the etiolated plants cannot be taken as a model for plants developing in nature, they can probably be used to study the chloroplast development in plants cultivated in the field. In fact, the modern agricultural methods bury the seeds deep into the soil and therefore the leaves start to grow almost in the absence of light. *In situ* measurements demonstrate that in these conditions, the leaves perceive light when they reach the level approximately 2 mm below the soil surface (Woolley & Stoller 1978; reviewed by Smith 1994). It is very probable that at this moment, the proplastids are already developed into etioplasts (reviewed by Schnepf 1980). Even if in the natural environment, the seeds fall on the ground the embryonic leaves can hardly see the light before germination. When the appropriate conditions are combined, the seed germinates *i.e.* the radicle emerges from the seed (Côme 1968; Figure 2 left panel). This event modifies the light environment of the embryonic leaves since the radicle can conduct light to them as a optic guide would do (Mandoli & Briggs 1984). In literature, we can often find a confusion in the terms designating the material used for greening experiments. Therefore throughout this thesis, the term old and young leaves were used to designate 10 to 15-d-old (usually designated as etiolated leaves with etioplasts) and 2 to 3-d-old leaves (usually called embryonnic leaves with proplastids), respectively.

In angiosperms, chloroplast biogenesis invariably begins with the photoreduction of photoactive

[1] In the text, this word is synonymous of old plants (*e.g.*, for bean 10 to 15-d-old) grown in complete darkness

protochlorophyllide (Pchlide *a*) to chlorophyllide (Chlide *a*) since the formation of the first Chlide *a* molecules initiates the synthesis of the chloroplast-encoded proteins which will further bind the pigments (Eichacker et al. 1990). The latter synthesis appears to be coordinated with that of the nucleus-encoded polypeptides during the development of the photosynthetic apparatus (reviewed by Mullet 1988).
To summarize, one can say that the direct development of proplastids to chloroplasts occurs during the natural development of seeds whereas that involving the etioplast stage is met in field cultures.

Most of the data about the development of the photosynthetic apparatus including pigment biogenesis, have been obtained using etiolated plants (reviewed by Šesták 1984, 1985). In addition and despite that the alternation of the day-light and night-dark periods has been recognized as an essential environmental factor, most of the studies dealing with plant greening are still performed using continuous illumination period(s) exceeding 24 h ! (*e.g.,* Babani et al. 1996, Bette & Kutschera 1996). Therefore, we find necessary to regularly check whether the conclusions formulated on the basis of such measurements also apply to plants growing in a natural environment. The almost complete absence of data on pigment biosynthesis and on the biogenesis of the photosynthetic apparatus in plants cultivated under conditions mimicing natural ones (reviewed by Šesták 1984, 1985) make these investigations particularly interesting and needed.

Throughout our work, we have cultivated the plants in conditions close to the natural environment, including the intactness of the seedlings during the illumination period. This gave us the guarantee that the observed events really occur and have a significant importance in the life of the plants.

This thesis is divided into 4 chapters summarizing my contribution on selected topics related to the chlorophyll biosynthesis pathway and the biogenesis of the photosynthetic apparatus.

The **first chapter** deals with the state of Pchlide *a* in nonilluminated leaves. The comparison of the photoactive Pchlide *a* absorbance spectra of nonilluminated leaves, recorded throughout the development in the dark, reveals that the proportion of the spectral forms, P638-645[2] and P650-657, is not much modified during this period. The Gaussian deconvolutions of 77 K fluorescence spectra of nonphotoactive and photoactive Pchlide *a* indicated that the photoactive and nonphotoactive Pchlide *a* are composed by three and 5 spectral forms, respectively. The study of the modification in their proportion indicated that none of the nonphotoactive Pchlide *a* spectral forms is especially accumulated during the growth in the dark. In constrast, the photoactive Pchlide P650-657 is particularly accumulated. Fluorescence measurements also indicate that only 40 % of the nonphotoactive Pchlide *a* is transferring energy to the photoactive one.

In the **second chapter**, we describe the photoreduction of photoactive Pchlide *a* to Chlide *a* upon an illumination. The kinetic of the transformation at room temperature can be fitted with a monoexponential curve which rate constant does not depend on the leaf developmental stage. This indicates that the mechanism of the photoreduction is also independent on the leaf developmental stage. Using 77 K fluorescence spectroscopy,

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[2] PX-Y means Pchlide *a* absorbing at X nm and emitting fluorescence at Y nm. The position of the maxima can slightly vary from one study to another and also as a function of the temperature at which the measurements have been performed (Buschman & Sironval 1984).

the Chlide *a* spectral forms corresponding to the three photoactive Pchlide *a* have been identified. The proportion of the different first products of the photoreduction is greatly influenced by the leaf developmental stage. In young leaves (*i.e.*, 2- to 4-d-old leaves), C670-675[3] is mainly formed whereas in old ones (*i.e.*, 10- to 15-d-old leaves), another Chlide *a* spectral form, C684-696 is produced.

During the 1st hour of greening, the newly formed Chlide *a* molecules are not protected by carotenoids. The evidence for the involvement of a photocycle between two different spectral forms of Chlide *a* in the photoprotection of the newly formed Chlide *a* is also presented.

In the **third chapter**, a new method for the isolation of photoactive Pchlide *a* which yields complexes characterized by spectral properties similar to those found in intact leaves is described. Such a procedure is the first step towards establishing the structure of photoactive Pchlide *a*. In addition with the help of the data, the structural modifications and the mechanism of the photoreduction can be better understood.

In the **fourth chapter**, it is explained that the different fates of the first products of the photoreduction, described in the 2nd chapter, strongly influence the greening process. In fact, the Chlide *a* spectral form, C684-696, which is required for the assembly of the PSII, is only produced in small amounts in young plants (see chapter II). Therefore the increase of the photosynthetic activity of these leaves is delayed. Nevertheless, during the first 8 h of greening, a weak photosynthetic activity is detected in the young leaves.

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[3] CX-Y means Chlide *a* absorbing at X nm and emitting fluorescence at Y nm. The position of the maxima can slightly vary from one study to another and also as a function of the temperature at which the measurements have been performed (Buschman & Sironval 1984).

HPLC measurements indicate that this weak activity is due to a low level of photosynthetic pigments during this period. In addition, fluorescence measurements reveal that a large part of the Chl remains nonintegrated to the photosystems. In young leaves, the rapid Chl integration into the photosynthetic units occurs when both carotenoids and Chl are massively synthesized.

Most of the data presented in this thesis have been published since 1990 in international journals after peer-review. Some of them appeared as part of conference proceedings or have been included in chapters in monographs. I also included some unpublished data. They are chronologically listed in the chapter entitled «Personnal literature». References corresponding to the papers in which my contribution was the major one are referred to in the text by Roman numbers whereas the papers in which I was involved as a collaborator, Arabian figures are used.

Most of the results have been obtained during my postdoctoral stays at the Laboratory of Photobiology at the University of Liège in Belgium and during several research stays at the Laboratory of Biochemistry at the University of Stockholm in Sweden and at the Laboratory of Plant Cytophysiology and Phycology at the University of Lille 1 in France. The most recent data have been collected at the Laboratory of Biomembranes at the University of South Bohemia in Czech Republic.

I want to express here my gratitude to the organizations which financially supported my scientific activities. These are the following: the "Commissariat Général aux Relations Internationales», the Ministry of Education, the Belgian Funds for Scientific Research, the Royal Academy of Belgium, the University of Liège, the Hela Foundation and the Rasquinet Foundation (**Belgium**); the Ministry of Education, Youth and Sports (VS 96085), the Grant Agency (204/98/P120) (**Czech Republic)**; the Foreign

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I also would like to give my thanks to all of those, collegues, researchers, technicians and friends who collaborated with me or/and encouraged me during all these years. Many thanks to all of you.

Chapter I

Formation and organization of the pools of photoactive and nonphotoactive protochlorophyllide *a* in nonilluminated leaves

1.1. Introduction.

Chlorophyll synthesis in angiosperms is a strictly light-dependent process[4]. The light-dependent step is the photoreduction of protochlorophyllide. The reaction is catalyzed by the light-dependent NADPH:Pchlide *a* oxidoreductase (LPOR; 1.3.1.33; (reviewed in **X**, **XV**[5])).

In vivo, two kinds of Pchlide *a* can be distinguished on the basis of their ability to be transformed to Chlide *a* during a short illumination (< 5 ms). Pchlide *a* which can be photoreduced to Chlide *a* by such a short light pulse is called photoactive Pchlide *a* (reviewed by Virgin 1981, **X**, **XV**). When Pchlide *a* cannot be transformed to Chlide *a* upon such a light pulse, it is called nonphotoactive Pchlide *a*. Nonphotoactive and photoactive Pchlide *a* differ in spectral properties. On the basis of low temperature absorbance and fluorescence spectra, it is usually assumed that an etiolated leaf contains two spectral forms of photoactive Pchlide *a*, denoted P638-645 and P650-657 and one single form of nonphotoactive Pchlide *a*, denoted P628-633 (reviewed by Virgin 1981, **X**, **XV**).

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[4] Dark-grown tissues can contain some traces of chlorophyll (*e.g.,* Durchan et al. 1992) which appears to be deposited into the embryo during its formation rather than synthesized *in vivo* during dark-growth (Böddi et al. 1999). For a full discussion on the possibility that Chl synthesis occurs in the dark in angiosperms tissue, see Adamson et al. (1997).

[5] The Roman numbers and Arabian figures indicated in bold in the text refer to the publications of the author. They are listed in the first appendix (see also p 11).

1.2. *On the degree of functional connectivity between the nonphotoactive and photoactive protochlorophyllide a pools.*

The first study on the localisation of Pchlide *a* in isolated etioplasts has been performed using fluorescence microscopy. Boardman & Anderson (1964) observed that the fluorescence is emitted from a restricted area and not from the whole surface of the organelle. The fluorescing area was called the stroma center. When the electronic microscopy technique became available, several authors observed that etioplasts contain two types of internal membranes: the prolamellar body (PLB), a huge paracrystalline lipidic network connected to some non-appressed membranes, called the prothylakoids (PTs) (reviewed by Kutík 1985, Hudák 1997). A picture of an etioplast is displayed in Figure 3.

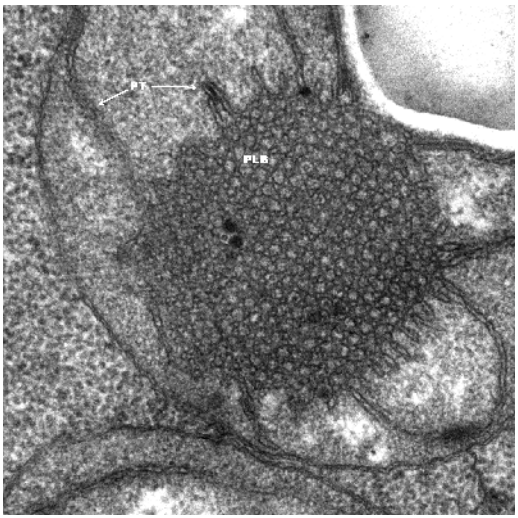

Figure 3. Picture of an etioplast from a 10-d-old bean leaf (*Phaseolus vulgaris* var Red Kidney). (60000 X). PLB: prolamellar body; PT: prothylakoid.

Using X-ray diffraction, Williams et al. (1998) definitely confirmed that PLB is composed of regular units *i.e.*, tetrapodal units made up from a continuous lipid bilayer assembled to form a diamond cubic lattice. A similar conclusion was drawn from a mathematical analysis of PLB sections observed by electronic microscopy (Schoefs B, Hucek S, Hušák M, Štys D, 1999, to be published). In contrast, PTs are flat and perforated membranes (Figure 3). It was therefore concluded that PLB contains Pchlide *a* and corresponds to the stroma center observed by fluorescence microscopy.

A detailed study of the Pchlide *a* and LPOR locations in internal membranes of etioplasts had to await the elaboration of methods for PLB and PT purification (reviewed by Sundqvist & Ryberg 1989). From spectroscopic measurements, pigment analysis and immunocytochemical studies, the general concept emerged that most of the photoactive Pchlide *a* and LPOR is concentrated in the PLB whereas minor amounts of Pchlide *a* and LPOR have been also detected in the PTs, in the organelle envelope, in the plasmalemma and even in the cytoplasm (reviewed by Sundqvist & Ryberg 1989). The view, that the pools of photoactive and nonphotoactive Pchlide *a* are physically separated, emerged from these studies. This conclusion seems in contradiction with the observation of a band corresponding to nonphotoactive Pchlide *a* in the low temperature excitation spectra of photoactive Pchlide *a* which proves that excitation energy can be transfered from nonphotoactive to photoactive Pchlide *a* (Ignatov et al. 1983). On the other hand, studies on pigment-pigment interactions by circular dichroism (Böddi et al. 1989) have confirmed that photoactive Pchlide *a* is an aggregate of Pchlide *a* within which energy migration can occur (Thorne 1971, Brouers et al.1972). Therefore two cases should be

considered: 1) the transfer of energy between photoactive Pchlide *a* and 2) a transfer from nonphotoactive Pchlide *a* to photoactive Pchlide *a*.

1.2.1. The energy transfer between photoactive Pchlide *a*.

Brouers & Sironval (1978) developed a method which allows the determination of the number of the Pchlide *a* transferring the excitation energy *per* Chlide *a*. This method is based on the variations of the absorbance (at 678 nm) and fluorescence (at 688 nm) of the Chlide *a* formed during the photoreduction of photoactive Pchlide *a*. They defined «c» as the proportion of the Chlide *a* formed at the time t whereas «i» is defined as the proportion of the pigments, *i.e.* Pchlide *a* and Chlide *a*, which are responsible for the emission at 688 nm at the same time. It has been shown that «c» and «i» are equivalent to the absorbance at 678 nm and the fluorescence intensity at 688 nm, respectively (Sironval 1972). The Sironval & Brouers's theory established that «c» and «i» are linked by the equation 1

$$\frac{i}{c} = \frac{A}{K} - \frac{i}{K} \qquad \text{(Equation 1)}$$

where A and K are two constants linked by the relation A - K = 1. The validity of equation 1 has been verified over a large range of temperature *i.e.* from 77 to 263 K (Sironval & Kuyper 1972, Franck 1982). According to the theory, the ratio A/K measures the number of pigments which transfer the energy *per* Chlide *a*. When this method is applied to measurements made at 77 K, one finds that the mean number of pigment transferring the excitation energy *per* Chlide *a* is approximately 12 Pchlide *a* (Sironval & Kuyper 1972). Similar values (15-20) have been obtained by other authors

(Thorne 1971, Litvin et al. 1976). Such a group of pigment exchanging energy has been called a transfer unit and A/K is a tool to estimate its size.

On the basis of the existence of energy transfer units at 77 K, it was proposed that these units are functioning like photosystems within the role of the accessory pigments is accomplished by either nonphotoactive and photoactive Pchlide *a* (Sironval & Brouers 1980) or Pchlide b (Reinbothe et al. 1999)[6]. We demonstrated previously that the nonphotoactive Pchlide *a* can hardly play the role of accessory pigment inside the transfer unit (see p. 18) and Scheumann et al. (1999) have demonstrated that etiolated leaves do not contain Pchlide b. Therefore, only an excitation energy transfer between photoactive Pchlide *a* inside the unit transfer can be considered. This idea is however in complete contradiction with the definition of the photoactive Pchlide *a* *i.e.* a complex between LPOR-Pchlide *a*-NADPH organized in such a way that when Pchlide a is excited, it is transformed to Chlide *a*.

In order to clarify this point, we modified the method of measuring «i» and «c», initially planned for low temperature (see p. 20), to the room temperature conditions (**IV, V**). The new method is based on the simultaneous recording of the absorbance and fluorescence variations at 440 nm and 690 nm, respectively. The mathematical derivations (see appendix II for details) indicate that the size of the transfer unit - *i.e.* A/K - is equal to the ratio of the rate constant of the fluorescence (k_F) and absorbance (k_A) kinetics determined from the measurements of the photoreduction of photoactive Pchlide *a* (Equation 2)

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[6] This recent work is only cited to be comprehensive. In this paper, the authors claimed that Pchlide b is abundant in etiolated leaves and plays the role of accessory pigments, transferring the excitation energy to photoactive Pchlide *a*.

$$\frac{A}{K} = \frac{k_F}{k_A} \quad \text{(Equation 2)}$$

Using the data published by Schoefs et al. (see Figure 6 in **V**), we calculated that at room temperature, the mean number of Pchlide *a* per transfer unit is between 1 and 2! This clearly indicates that at physiological temperature each photoactive Pchlide *a* is absorbing photons for its own photoreduction. The weak efficiency of the fluorescence and the high quantum yield of the photoreduction observed at similar temperature (Dobek et al. 1981, Böddi & Franck 1997) agree with this result. We conclude that the transfer units are not playing a crucial role during the photoreduction. As we will see later, they are involved in the photoprotection of the newly formed Chlide *a* (see chapter II, p. 52).

1.2.2. The energy transfer between nonphotoactive Pchlide *a* and photoactive Pchlide *a*.

Litvin et al. (1976) estimated that each transfer unit contains approximately 2 nonphotoactive Pchlide *a* *per* 18 photoactive Pchlide *a*. We reached a similar conclusion by measuring the variations of the ratio of the *in situ* 77 K fluorescence intensities at 657 nm (photoactive Pchlide *a*) and 632 nm (nonphotoactive Pchlide *a*) as a function of the ratio between the amounts of photoactive and nonphotoactive Pchlide *a* as obtained after pigment extraction. A linear relationship between the two ratios was found (see Figure 5 in **XVI**). This can be only explained if we assume that the fraction of transferring pigments is constant. The ordinate value at the origin gives the proportion of nonphotoactive Pchlide *a* in the transfer unit (see the appendix in **XVI**). This value is 0.13 *i.e.* 1 molecule of nonphotoactive Pchlide *a* are

transferring the excitation energy to approximately 8 molecules of photoactive ones. The mean photoactive and nonphotoactive Pchlide *a* amounts in one 12-d-old etiolated leaf pair are approximately 0.76 and 0.24 nmoles, respectively (**XVI**). Therefore we calculated[7] that 60 % of nonphotoactive Pchlide *a* pool is not transferring the excitation energy to the photoactive Pchlide *a*. This suggests, the existence of two pools of nonphotoactive Pchlide *a*: the first one transfers the excitation energy to photoactive

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[7] The mean number of photoactive Pchlide *a* molecules (Nb_{PA}) per 12-d-old leaf pair is

$$Nb_{PA} = N_{AVOG} \times [\text{photoactive Pchlide } a] = 6.02\ 10^{23} \times 0.76\ 10^{-9} = 4.58\ 10^{14} \text{ molecules}$$

Since we calculated that the mean number of nonphotoactive and photoactive Pchlide *a* molecules in each transfer unit is 2 and 16, respectively, the total number of nonphotoactive ($Nb_{PI,MAX}$) Pchlide *a* molecules transferring the energy is

$$Nb_{PI,MAX} = \frac{Nb_{PA} \times 2}{16} = 0.46\ 10^{14} \text{ molecules}$$

We measured that the mean total amount of nonphotoactive Pchlide *a* (Nb_{PI}) *per* leaf is

$$Nb_{PI} = N_{AVOG} \times [\text{nonphotoactive Pchlide } a] = 6.02\ 10^{23} \times 0.24\ 10^{-9} = 1.44\ 10^{14} \text{ molecules.}$$

From these figures, we can estimate that approximately 40 % of the nonphotoactive Pchlide *a* are transferring the excitation energy to the photoactive Pchlide *a*.