Phytochrome and greening in etioplasts

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Stellingen

1. Bij de vorming van de chloroplast uit de etioplast spelen van buiten de etioplast afkomstige factoren een rol.

I. Gollmer en K. Apel, 1983. Eur. J. Biochem. 133, 309-313.
Dit proefschrift.

2. Het fytochroom-transportmodel van Raven en Spruit vormt nog steeds een aantrekkelijke verklaring voor zowel de Zea paradox als voor de zogenaamde "very low fluence responses".

C.W. Raven en C.J.P. Spruit, 1973. Acta Bot. Neerl. 22, 135-143.
Dit proefschrift.

3. De waarneming, dat bicarbonaat de fotosynthese bij waterplanten stimuleert, houdt niet in dat het door de plant wordt opgenomen.

W.J. Lucas, 1983. Annu. Rev. Plant Physiol. 34, 71-104.
R.J. Helder, 1985. Plant, Cell and Environment 8, 399-408.

4. In tegenstelling tot plantesoorten met grote zaden, blijken zich bij plantesoorten met kleine zaden geen "vigour"-problemen bij veldopkomst voor te doen. De aanzienlijke uitlek van (organische) stoffen uit grote zaden vormt een verklaring voor dit verschil.

J. Bekendam, H.L. Kraak en J. Vos, 1986. Acta Horticulturae, in press.

5. Omdat problemen met betrekking tot de kwaliteit en uniformiteit van zaaizaad veelal reeds ontstaan tijdens groei en afrijping van het zaad, dient de zaadteelt meer aandacht binnen het zaadtechnologisch onderzoek te krijgen.

6. De huidige ISTA laboratoriummethode voor meting van hardschaligheid van zaden is aanvechtbaar.

International Seed Testing Association, 1985. Seed Science and Technology 13, 299-355.

7. Het vochtgehalte van een partij zaaizaad dient een belangrijke factor te zijn bij de prijsvaststelling.

8. Het is te verwachten, dat ten gevolge van overproduktie in de landbouw in de EEG, land zal vrijkomen voor andere bestemmingen. Het is noodzakelijk, nu reeds verschillende mogelijkheden voor ander landgebruik te onderzoeken.

9. Bij plantezaden geeft een verjongingskuur betere resultaten dan bij Homo sapiens.

T.A. Villiers en D.J. Edgecombe, 1975. Seed Science and Technology 3, 761-774.
A. Dell' Aquila en G. Taranto, 1986. Seed Science and Technology 14, 333-342.

10. Het verharden van kleiduivenschietbanen is uit het oogpunt van natuurbehoud dringend gewenst en mogelijk rendabel.

11. Paarden worden, zeker in Nederland, te vaak te jong belast met het gewicht van een ruiter en bij de wedstrijdsport te vaak te jong getraind en ingezet.

12. Het feit dat in een land het metrieke stelsel wordt gebruikt, betekent niet dat dan altijd met de juiste maat wordt gemeten.

Proefschrift van H.L. Kraak
Phytochrome and greening in etioplasts
Wageningen, 18 november 1986

Contents

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ABBREVIATIONS

A473, A650, absorbances at 473 and 650 nm, respectively
ACMA, 9-amino-6-chloro-2-methoxyacridine
ALA, 5-aminolevulinic acid
ATP, adenosine triphosphate
BSA, bovine serum albumin
Chl(ide), chlorophyll(ide)
C672, C678, C685, chlorophyll(ide) with absorption maxima at 672, 678 and 685 nm, respectively
CMC, carboxymethylcellulose
CP, chlorophyll-protein complex
D, dark(ness)
D etioplasts, etioplasts isolated from dark-grown seedlings
EDTA, ethylenediamine tetraacetate
FMN, flavin mononucleotide
FR, far-red (light)
high WL, white light of high fluence rate
HIR, high irradiance response
HW, half band-width
K_{av}, the fraction of the gel phase of a gel bed which is available for a substance
kDa, kilodalton
10KP, pellet resulting from centrifugation at 10,000 x g
10KS, supernatant resulting from centrifugation at 10,000 x g
LF, low fluence
LFR, low fluence response
low WL, white light of low fluence rate
MOPS, N-morpholino-3-propane sulfonic acid
mRNA, messenger RNA
NADPH, nicotinamide adenine dinucleotide phosphate
P, phytochrome, or total phytochrome
P628, P636 and P650, protochlorophyll(ide) with absorption maxima at 628, 636 and 650 nm, respectively
PChl(ide), protochlorophyll(ide)
Pfr, far-red absorbing form of phytochrome
PLB, prolamellar body
Pr, red absorbing form of phytochrome
R, red (light)
R etioplasts, etioplasts isolated from red pre-irradiated seedlings
RNA, ribonucleic acid
RNP, ribonucleoprotein
rRNA, ribosomal RNA
TCA, trichloroacetic acid
VLF, very low fluence
VLFR, very low fluence response
V_o, void volume (the volume of the liquid phase of a gel bed)
V_t, total volume of a gel bed
WL, white light

1 GENERAL INTRODUCTION

Light is indispensible for life on earth. Without light, plants would be unable to photosynthesize and so to produce oxygen and biomass on which other living organisms depend. Chlorophyll (Chl) is the principal pigment responsible for light absorption in the process of photosynthesis. This pigment is concentrated, in leaves of light-grown higher plants, in special organelles, the chloroplasts. Apart from enabling plants to photosynthesize, light in various ways influences their growth and development (photomorphogenesis). The principal pigment mediating many photomorphogenetic responses is phytochrome (P). Generally speaking, only very small amounts of light are required for photomorphogenic responses as compared to photosynthesis.

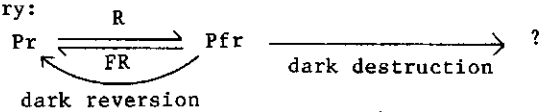
Seeds contain food reserves which enable the seedlings to grow for a period of time without light. Apart from other differences with light-grown seedlings, dark-grown seedlings of angiosperms are unable to form Chl. Instead of chloroplasts, the leaves contain "etioplasts" (Section 1.4.1) which are able, by the action of red light (R), to develop into chloroplasts. At least three photoreceptors control chloroplast development. P controls several processes during Chl accumulation (Schopfer and Apel, 1983). For the expression of these P induced processes, the light dependent reduction of protochlorophyll(ide) (PChl(ide)) into chlorophyll(ide) (Chl(ide)) is required. In this process, PChl(ide) itself is the photoreceptor. Finally, the presence of Chl is required for the formation of the internal chloroplast structure (Mohr and Kasemir, 1975). The present study is mainly concerned with functional and structural aspects of the first stages of the greening process in seedlings and isolated etioplasts after transfer to light and with the role played by P. Some general aspects of the initial photomorphogenic processes in etioplasts and of the pigments involved are discussed in this chapter.

1.1 Phytochrome

P is a photoreversible pigment widely distributed in the plant kingdom. P dependent responses have been observed in angiosperms, gymnosperms, ferns, liverworts, mosses and algae (Borthwick, 1972b). A few examples of such responses are: photoinduction of seed germination, inhibition of hypocotyl growth, induction of leaf expansion and regulation of flowering of photoperiodically sensitive plants (e.g., Shropshire and Mohr, 1983).

P exists in two interconvertible forms characterized by different absorption spectra. In completely dark-grown tissue only the R absorbing form, Pr, is present, with an absorption maximum at 666 nm. When irradiated with light of suitable wavelength, Pr is photoconverted into the far-red light (FR) absorbing form, Pfr, with an absorption maximum at 730 nm (e.g., Pratt, 1979). Due to overlap of the Pr and Pfr absorption spectra, it is impossible to convert all Pr to Pfr. A saturating R irradiation results in a

photoequilibrium between Pr and Pfr with Pfr forming about 86% of total P (Vierstra and Quail, 1983). When irradiated with FR, Pfr is predominantly photoconverted back to Pr. Pfr is considered to be the physiologically active form of P (e.g., Borthwick, 1972a; Pratt, 1979). In dicotyledons, Pfr slowly reverts to Pr in the dark. This "dark reversion" appears to be absent in most monocotyledons (Frankland, 1972). Moreover, Pfr can be irreversibly transformed to a colourless product both in dicotyledons and monocotyledons (Kendrick, 1972; Frankland, 1972). This transformation is called phytochrome destruction. In summary:



The P chromophore is an open-chain tetrapyrrole (e.g., Scheer and Krauss, 1979). The chromophore is covalently attached via a thio-ether linkage to cystein on the apoprotein. The amino acid sequence of the 124 kilodalton P apoprotein of Avena and its major proteolytic cleavage sites are known (Vierstra and Quail, 1986). The exact structure of the chromophore and the changes upon phototransformation are still incompletely understood. Photoconversion of Pr into Pfr involves Z,E- (or cis, trans-) isomerization of the chromophore. However, the observed optical difference of Pr and Pfr is best explained by a combination of changes in both the chromophore and the protein environment (Rüdiger et al., 1985). Several intermediates have been detected in the Pr → Pfr and Pfr → Pr phototransformations (Spruit and Kendrick, 1973; 1977; for reviews see Kendrick and Spruit, 1977; Rüdiger and Scheer, 1983).

R-FR photoreversibility, i.e. the effect of a brief R irradiation being reversed by a subsequent FR irradiation is often used as a criterion for involvement of P in a response of plants to light. However, we may expect from the photochemical properties of the pigment, that responses of completely dark-grown plants to light evoked by extremely small amounts of Pfr should not be photoreversible by FR. In this case, the reversibility criterion breaks down. "High irradiance responses" (HIRs), responses to prolonged FR, have been shown also to be mediated by P (Mohr, 1972; Mancinelli and Rabino, 1978). HIRs are dependent on fluence rate, suggesting that in these responses the rate of interconversion of Pfr and Pr (or cycling) is important.

Although much research has concentrated on P since its detection in 1959 (Butler et al.), still many questions are unanswered, for example, its localization within the cell and the primary mode of action of Pfr.

1.2 Investigations on the localization of phytochrome in plants

1.2.1 Distribution of phytochrome within the plant

Using an antibody-labelling method, Pratt and Coleman (1974) studied the distribution of P in etiolated seedlings. In maize seedlings, a relatively uniform distribution was observed. Seedlings of oat, rye, barley and rice showed high P concentrations in the tip of their coleoptiles and near the

shoot apex. High concentrations of P were observed near the leaf base of rice, barley and rye. In dark-grown roots of oats high P concentrations were found in the root caps. Apparently, P is found primarily in morphogenically active regions of a plant (Pratt et al., 1976). Evidence for inter-organ control of P mediated effects has been obtained by Caubergs (1974); De Greef et al. (1976) and Black and Shuttleworth (1976).

1.2.2 Subcellular distribution

Immunocytological techniques provided evidence that in dark-grown tissue, Pr is distributed throughout the cytoplasm (Coleman and Pratt, 1974; Mackenzie et al., 1974). Electron microscopy indicated that P may also be associated with various membranes. In dark-grown oat and rice seedlings, the diffuse distribution of Pr changed within a minute after R irradiation into an association of P (as Pfr) with discrete but as yet unidentified regions within the cell, about 1 μm in diameter (e.g., Pratt et al., 1976; Epel et al., 1980). Whether this translocation of P represents a binding of Pfr to specific, biologically active receptor sites within the cell, is not known (Pratt, 1983). The original diffuse distribution was slowly resumed over a period of 1-2 h at 25 °C (Mackenzie et al., 1975) after FR irradiation. Although in wheat, barley and rye this redistribution of P was not observed by Mackenzie et al. (1978), different fixing and sectioning techniques have shown its occurrence, at least, in wheat (Epel et al., 1980).

The immunocytochemically observed redistribution of P is possibly related to "pelletability" of Pfr in vitro (Quail, 1983). In several papers (Quail, 1974; Marmé et al., 1974; 1976) specific binding of Pfr to particulate material has been demonstrated. Binding was induced by in vivo R irradiation in zucchini, maize and oats, while in vitro R had a positive effect on pelletability of P only in zucchini (Marmé et al., 1976). Identification of the component(s) with which P becomes associated and the demonstration of a possible biological significance of the binding reaction have not so far been achieved (Quail, 1983).

Several attempts have been made to demonstrate P spectrophotometrically in preparations of cell organelles. Associations of P with the plasma membrane and endoplasmic reticulum (Marmé et al., 1976), mitochondrial and microsomal fractions (Furuya and Manabe, 1976), nuclei (Wagle and Jaffe, 1980) and etioplasts (e.g., Cooke et al., 1975; Evans, 1976) have been claimed. However, a clear picture of the localization of P either in dark-grown, de-etiolated (i.e., briefly irradiated) or green tissue has not yet been obtained.

1.3 Primary effects of phytochrome

The primary mode of action of P is still a matter of discussion. In most reactions, there are probably several steps between the initial action and the final response, which often becomes measurable only after several hours. It has been suggested (Mohr, 1972) that P might act by controlling gene activity.

P mediated changes in levels of rRNA (Thien and Schopfer, 1982) and mRNA (e.g., Gollmer and Apel, 1983) have indeed been observed. However, P responses have been reported on a time scale that seems to exclude gene action (for a review, see Quail, 1983). Membranes have been frequently proposed as sites of action for P (e.g., Hendricks and Borthwick, 1967; Brownlee and Kendrick, 1977). Several of the rapid P mediated events are likely to involve changes in membrane properties. It cannot be excluded, however, that these changes are an indirect consequence of a more rapid primary event elsewhere in the cell (Quail, 1983). The primary action of P may be different in different cells and cell compartments (Mohr, 1972; 1977). Kendrick (1983), on the other hand, proposed a model of primary Pfr action at the membrane level, involving ion transport.

1.4 Greening of dark-grown seedlings and the influence of phytochrome

1.4.1 Etioplasts

Young seedlings contain proplastids: small organelles with a poorly developed thylakoid system. In the light these proplastids directly develop into chloroplasts. In dark-grown seedlings, proplastids develop into etioplasts. Etioplasts are generally slightly smaller than chloroplasts and irregular-ellipsoidal in shape. Like chloroplasts, they have a double membrane envelope. However, instead of grana and stroma thylakoids, they contain one or more so-called prolamellar bodies (PLBs) and some prothylakoids radiating from the PLBs. A PLB is a three-dimensional lattice formed by interconnected membrane tubules that are often arranged in a very regular way, resulting in a "paracrystalline" structure (see e.g. Fig. 2.2 in Chapter 2). The PLB probably mainly functions as a carrier for lipid components necessary for the development of stroma and grana thylakoids (Virgin and Egneus, 1983). Under natural conditions etioplasts are formed in the primary leaves or cotyledons of seedlings germinating in the soil, before they are exposed to light. Plastids containing PLBs are also observed in plants grown in light of low fluence rate (e.g., Weier and Brown, 1970). Etioplast-like organelles are found in some algae, e.g. Euglena gracilis (Klein et al., 1972). In gland cells of tentacle heads of Drosera capensis etioplast-like organelles were observed (Kraak, 1974), even though the plants were light-grown. However, these etioplast-like organelles do not contain regular, para-crystalline PLBs but aggregates of tubules resembling disorganized PLBs instead. The fully-formed etioplasts with large paracrystalline PLBs commonly studied in the laboratory probably rarely occur in nature.

Etioplasts contain substantial levels of many components of mature chloroplasts (Virgin and Egneus, 1983) and in vivo are able to transform rapidly into functional chloroplasts on exposure to light (Wellburn, 1984). Etioplasts in dark-grown angiosperms contain no Chl but only relatively small amounts of PChl(ide) a and PChl(ide) a esters (Section 1.4.3).

1.4.2 Ultrastructural changes in etioplasts during greening

Upon irradiation of dark-grown seedlings, the membranes of the PLBs lose their paracrystalline structure (Gunning, 1965). This process, a rearrangement of the membrane tubules, is called tube transformation or PLB dispersal and can take place in the dark after a brief irradiation. The process is rapid in young seedlings; durations ranging from 1 min to 1 h have been reported (Kirk and Tilney-Bassett, 1978). Following tube transformation, perforated thylakoids (prothylakoids) grow out from the remains of the PLBs ("vesicle dispersal", Gunning, 1965). The perforations in the thylakoids disappear during the first hours of greening (Henningsen and Boynton, 1974). In young dark-grown seedlings PLB dispersal can also take place in the dark following a brief irradiation. The membrane material for the thylakoids at this stage is probably derived from the PLBs (Bradbeer et al., 1974). As irradiation continues, membrane overlaps form, the "stacks" of two thylakoids being the first stage of granum formation. Some phases of the light induced ultrastructural development of etioplasts are P mediated, e.g., the rate of stroma thylakoid and grana formation (Girnth et al., 1979).

1.4.3 Protochlorophyll(ide) species and their photochemistry

PChl(ide) will be used as a term for both unesterified protochlorophyllide and protochlorophyllide esters including protochlorophyll, irrespective of whether they are divinyl or monovinyl species (Cohen and Rebeiz, 1981). At least three spectrally distinct PChl(ide) forms exist: P628, P636 and P650, with absorption maxima around 628, 636 and 650 nm, respectively. On irradiation at 77K, P628 fluoresces at 632 nm, while both P636 and P650 fluoresce at 655 nm (e.g., Kahn et al., 1970). Efficient energy transfer from P636 to P650 accounts for absence of a P636 fluorescence emission band.

General agreement exists on P650 being a phototransformable species (e.g., Shibata, 1957; Kahn et al., 1970) and P628 being nonphototransformable. Whether P636 is directly phototransformable or serves as a precursor of P650 is uncertain (Sundqvist et al., 1980). In older dark-grown seedlings almost certainly only the non-esterified pigment (i.e., PChlide) can be photoreduced (Griffiths, 1974b; Virgin, 1981). However, in young seedlings phototransformation of PChl has been demonstrated (e.g., McCarthy et al., 1982).

The PChl(ide) content and the ratios of the various components vary with the age of the seedlings. In young dark-grown seedlings the amount of PChl(ide) is very low. In dark-grown bean leaves a maximum PChl(ide) content was reached after 10 days (Akoyunoglou and Siegelman, 1968). In 3-day old bean leaves 60% of PChl(ide) present was in the form of non-phototransformable P628 (Klein and Schiff, 1972), most of the phototransformable pigment being in the form P636, with smaller amounts of P650. During subsequent growth (from 3 to 7 days) the percentage of photoconvertible PChl(ide) increased, while P650 increased faster than P628 and P636. At the same time, PLBs were formed and increased in size.

Most PChl(ide) is bound to proteins forming a PChl(ide) holochrome complex (Kasemir, 1983b). The different absorption maxima of the PChl(ide) forms have been proposed to be due to differences of aggregation of the molecules (e.g., Mathis and Sauer, 1972; Virgin, 1981), conformational differences of the PChl(ide) holochrome (Gassman, 1973a), differences in environment (Gassman, 1973b), binding to different kinds of proteins (Guignery et al., 1974) or different modes of binding to proteins (Boardman, 1966). Griffiths (1978; 1980) obtained evidence that photoactive PChl(ide), P636/650, exists as a ternary complex with the enzyme PChl(ide) oxidoreductase and NADPH. The enzyme is probably identical with, or part of, the PChl(ide) holochrome (Apel et al., 1980).

The localization of PChl(ide)-protein complexes at the sub-organelle level is still a matter of debate (Kasemir, 1983a). Fluorescence and phase contrast microscopy indicated PChl(ide) within etioplasts to be located in centres (Boardman and Wildman, 1962). These centres corresponded in number and size to the PLBs observed in electron micrographs of etioplasts and the conclusion has been drawn that PChl(ide) is localized in the PLBs. However, histoautoradiography and biochemical analysis showed that ^{3}H-PChl(ide) synthesized from ^{3}H-5-aminolevulinic acid (ALA) was not only localized in the PLBs but also in the prothylakoids (Laflèche et al., 1972). Lütz and Klein (1979) separated PLBs and prothylakoids of etioplasts of dark-grown oat seedlings and, using saponin markers for the PLBs, concluded that PChl(ide) is mainly localized in the prothylakoids. Ryberg and Sundqvist (1982) showed that the prothylakoids of etioplasts of dark-grown wheat seedlings contain about 60% of the total amount of PChl(ide) present. However, PLBs were found to contain a higher ratio of phototransformable to non-phototransformable PChl(ide) than the prothylakoid fraction.

1.4.4 Biosynthesis of protochlorophyll(ide)

The first stages in (P)Chl(ide) formation are probably the same as for the synthesis of porphyrins in general. The pathway from the earliest Chl precursor that has been unequivocally identified, ALA, to PChl is as follows (Virgin, 1972; Kirk and Tilney-Bassett, 1978; Castelfranco and Beale, 1983):

5-aminolevulinic acid (ALA) $\xrightarrow{\text{ALA-dehydratase}}$ porphobilinogen (PBG) $\longrightarrow$ uroporphyrinogen III $\xrightarrow{-\,CO_2}$ coproporphyrinogen III $\xrightarrow{+\,O_2,\,-\,H_2}$ protoporphyrinogen IX $\xrightarrow{-\,H_2}$ protoporphyrin IX $\xrightarrow{+\,Mg}$ Mg-proto-IX monomethylester $\rightarrow \rightarrow \rightarrow \rightarrow$ Mg-2,4,-divinylpheoporphyrin *a*5 $\longrightarrow$ protochlorophyllide *a*.

Available evidence suggests that formation of ALA in plants follows a pathway different from that in animals and bacteria (Castelfranco and Beale, 1983), the details of which are still uncertain.

Phytochrome (through Pfr) has at least two controlling points in PChl(ide)

biosynthesis. The activity of the enzyme ALA-dehydratase is increased by prolonged exposure to FR, which operates via formation of Pfr (Kasemir and Masoner, 1975; Balangé and Lambert, 1980). However, the Pfr controlled formation of ALA is probably a more important factor (Masoner and Kasemir, 1975; Kasemir, 1983a).

1.4.5 Photoconversion of protochlorophyll(ide) into chlorophyll(ide) and subsequent regeneration of protochlorophyll(ide)

Light absorbed by PChl(ide) itself is effective in its photoconversion (e.g., Koski et al., 1951). A very short light treatment (a few ms) can saturate the conversion (Madsen, 1963). PChl(ide) phototransformation has been observed in vivo, in isolated etioplasts, isolated PLBs and purified PChl(ide) holochrome (e.g., Brodersen, 1976). After (partial) photoconversion of PChl(ide) into Chl(ide), energy transfer from P650 (and P636) to the newly formed Chl(ide) is observed at -196 °C (e.g., Kahn et al., 1970; Sundqvist and Klockare, 1975). The PChl(ide) and Chl(ide) molecules therefore must be located in close proximity. The temperature dependence of PChl(ide) phototransformation has been studied by Smith and Benitez (1954) and Sironval and Brouers (1970). At -196 °C no phototransformation is observed. At -70 °C photoconversion is fairly rapid and extensive. Increase in temperature up to 40 °C increases the rate of phototransformation. Prolonged heating of leaves at 40 °C or short heating at 55 °C destroys the phototransformation capacity (Smith and Benitez, 1954).

After phototransformation of PChl(ide) in dark-grown seedlings by a light-flash, new PChl(ide) is formed in subsequent darkness. The rate of the so-called PChl(ide) regeneration is strongly dependent on the age of the seedlings (Akoyunoglou and Siegelman, 1968). No lag phase in PChl(ide) regeneration was observed in dark-grown bean seedlings younger than 5 days, while in older seedlings the duration of the lag phase increased with increasing age: in 11-day old seedlings it lasted 60 min. Virgin (1955) showed the rate of Chl(ide) formation during a 2 h irradiation at medium fluence rate to equal the rate of PChl(ide) regeneration in darkness.

By examining the effect of FR following a brief R irradiation, a possible influence of Pfr on the PChl(ide) regeneration rate was studied. No significant effect of Pfr was detected in these experiments (Akoyunoglou, 1970; Spruit and Raven, 1970; Jabben et al., 1974), only in older seedlings a small effect of Pfr was observed after a few hours of darkness (Akoyunoglou, 1970; Spruit and Raven, 1970). However, Pfr formed by a R pre-irradiation several hours earlier, appears to stimulate PChl(ide) regeneration (Virgin, 1958; Augustinussen and Madsen, 1965; Jabben et al., 1974; Jabben and Mohr, 1975). A quantitative correlation between the effect of Pfr on the rate of PChl(ide) regeneration and the initial rate of Chl(ide) accumulation in light was shown by Jabben et al. (1974).

1.4.6 Chlorophyll(ide) species and their transformation

When older dark-grown seedlings of angiosperms containing relatively high concentrations of P650 are briefly irradiated, a Chl(ide) *a* species with absorption maximum around 678 nm (C678) is formed. During subsequent darkness the absorption maximum first shifts to 684 nm (Bonner, 1969) and then back to 672 nm (Shibata, 1957). The latter shift has become known as the Shibata shift. Esterification of Chlide *a* with phytol (via geranylgeranyl Chlide, Benz *et al.*, 1980) takes place simultaneously with (Sironval *et al.*, 1965) or after (Akoyunoglou and Michalopoulos, 1971) the Shibata shift. Finally, a stable photosynthetically active form C677 is slowly formed (*e.g.*, Virgin, 1972). Recently, short-lived species intermediate between P650 and C678 have been detected (*e.g.*, Inoue *et al.*, 1981; Belyaeva and Litvin, 1981).

Stimulation of the rate of the Shibata shift by Pfr has been claimed by Jabben and Mohr (1975) and stimulation of the rate of phytylation of Chlide *a* by Liljenberg (1966) and Kasemir and Prehm (1976).

1.4.7 Chlorophyll accumulation

When older dark-grown angiosperm seedlings are continuously irradiated, after the initial photoconversion of PChl(ide) into Chl(ide) a rather slow phase of Chl (including Chlide) accumulation or even a temporary decrease in total Chl is observed (*e.g.*, Raven, 1972): the so-called lag phase in Chl accumulation. Rapid Chl accumulation takes place after the lag phase and proceeds until the pigment content approaches that of the mature, green leaf. The duration of the lag phase is dependent on seedling age (Sisler and Klein, 1963; Akoyunoglou and Argyroudi-Akoyunoglou, 1969) and on the fluence rate. At high fluence rate the lag phase is extended, probably because of photobleaching of Chl (*e.g.*, Raven, 1972; Virgin, 1972).

The lag phase in Chl formation is eliminated or shortened by pre-irradiation of dark-grown seedlings with a low R fluence (working through Pfr), followed by several hours of darkness (Withrow *et al.*, 1957; Mitrakos, 1961; Kasemir *et al.*, 1973; Raven, 1973). Although hardly any reversion of this R effect by FR is obtained in some plant species (Virgin, 1961; Raven and Spruit, 1972) P has been shown to be involved in the response in these species as well (Raven, 1973).

1.5 Transport model for phytochrome

In 1973, Raven and Spruit proposed a transport model for phytochrome accounting for their observations on effects of various pre-irradiation treatments on Chl accumulation in white light (WL). FR reversibility of the effect of R appeared more pronounced in briefly R pre-irradiated ("de-etiolated") seedlings than in seedlings previously grown in complete darkness. Even green "safelight" caused a significant de-etiolation, resulting in an increase of FR reversibility. The increase in R/FR reversibility is accompanied by a marked decrease in sensitivity of the

seedlings to R (Raven and Shropshire, 1975).

In agreement with data of immunocytological studies (Section 1.2) the transport model assumes that initially, Pr is distributed troughout the cytoplasm of plant cells. This soluble P will be called "bulk P". Pfr molecules formed by irradiation of dark-grown seedlings are proposed to migrate to receptors in the cells, thereby eliciting a physiological response. A small fraction of total P is proposed sufficient to saturate the receptor sites, predicting a high sensitivity of dark-grown seedlings to R. The small amount of Pfr formed by FR would be sufficient to saturate a significant proportion of the receptor sites and therefore very limited reversion by FR of the effect of R is anticipated. The model further assumes that binding of Pfr to the receptors is irreversible. Dark reversion of Pfr into Pr was proposed to occur at the receptors, so that after a dark period of sufficient duration, a second R pre-irradiation results in an additional response. However, a considerably higher R fluence would now be needed to give a similar effect as produced by the first R pre-irradiation, as no concentration of Pfr occurs this time. The small percentage of Pfr formed by FR would now have no noticeable effect and therefore this second R effect would be largely reversible by FR.

Interestingly, the P transport model also provides an attractive explanation for the "Zea" paradox (Hillman, 1967). In maize, a small fluence of R insufficient to cause a spectrophotometrically detectable conversion of Pr into Pfr, saturates the R induced enhancement of a phototropic response to blue light (Briggs and Chon, 1966). Paradoxically, the R effect was FR reversible, even though the FR irradiation produced more Pfr than the R irradiation that it reversed. Assuming that Pfr has migrated to the reaction centres before the FR irradiation, these observations are accounted for by the transport model.

1.6 Aim of the investigations

The success of the transport model in explaining a number of otherwise enigmatic physiological responses, asks for a more direct confirmation of its basic assumptions. In particular, the reality of the proposed receptors and their activation by minute fractions of cellular P after its photo-transformation to Pfr has to be demonstrated. A plausible candidate for the receptor sites for P is the etioplast. An increase of the P content of the etioplasts after irradiation of completely dark-grown seedlings, as implied by the model, would provide evidence both in favour of the model and for the etioplasts being a site of action of Pfr. Consequently, etioplasts were isolated from dark-grown and R pre-irradiated seedlings and their P content was measured spectrophotometrically (Chapter 4).

As maize etioplasts were the main object of the present studies, a detailed investigation of the influence of P on the lag phase in Chl accumulation in this species was made (Chapter 3). This appeared to be of even more interest since in monocotyledons probably no Pfr dark reversion occurs (Frankland,

1972). This provided a possibility to test the P transport model, since the model as originally proposed predicts that a second R pre-irradiation will have no additional effect if there is no Pfr dark reversion at the receptors.

The diversity of P controlled processes related to Chl accumulation complicates the analysis of these processes in the intact plant. The isolated etioplast provides an attractive system to investigate the development of the photosynthetic apparatus on a sub-cellular level. In addition to the above investigations, evidence has been sought for a direct influence of P in this respect on the etioplast. Possible P involvement in the following aspects of development of the photosynthetic apparatus was examined in isolated etioplasts: ultrastructural development (Chapter 5), PChl(ide) regeneration (Chapter 6) and changes in the spectral properties of the plastid pigments after a light pulse (Chapter 7).

2 MATERIALS AND METHODS

2.1 Plant material

Seeds of Zea mays L. cv. Capella were obtained from Van der Have B.V. (Kapelle Biezelinge, The Netherlands), seeds of Pisum sativum L. cv. Krombek and Phaseolus vulgaris L. cv. Dubbele Witte z. dr. from Sluis and Groot (Enkhuizen, The Netherlands), seeds of Avena sativa L. cv. Condor from Kweekbedrijf Zelder (Ottersum, The Netherlands) and seeds of Hordeum vulgare L. cv. Julia from Proefbedrijf Nude (Wageningen, The Netherlands). Dr. ir. A.W. de Jong (Kweekbedrijf Zelder) kindly provided seeds of the genetically homogeneous maize varieties Z27 and WJ. Seeds of Zea mays L. cv. Capella were treated with Captan anthra.

2.2 Cultivation of seedlings

In initial experiments, seeds of maize, barley and pea were imbibed for 6 h under dim daylight before sowing. In later experiments all seeds were sown without previous soaking. Seeds were sown in pots filled with sterilized potting compost in dim daylight and the seedlings were grown subsequently in complete darkness as described by Raven (1973) at a temperature of 22 °C. Plants were used at an age of 8 days in most experiments.

2.3 Equipment for irradiations

2.3.1 Equipment for pre-irradiations

In most experiments red (R) pre-irradiation consisted of 5 min R fluorescent light (Philips TL 40, colour 15) at an irradiance of 0.85 W m^{-2} (5 μmol m^{-2} s^{-1}) at the top of the leaves. Maximum emission of this light source was at 658 nm. In the experiments described in Chapter 3, pre-irradiations were given with different fluences of R and far-red light (FR). For various low fluences of R, the R fluorescent light was used for different periods and at different distances of the light source from the plants. Alternatively, a Leitz Prado 500 slide projector equipped with a 667 nm interference filter (Balzers, Liechtenstein, half band-width (HW) 21 nm) was used. A range of fluence rates was obtained by combining this source with neutral glass filters (type NG, Schott und Gen., Mainz, W. Germany). For high fluence rates of R and FR, the "Xenosol V" irradiation equipment described by Spruit et al. (1979) was used. For R irradiation, Baird Atomic interference filters with transmission maxima at 655 nm or 666 nm (HW 15 nm) were used and for FR irradiation Baird Atomic interference filters with transmission maxima at 731 nm (HW 19 nm) or 750 nm (HW 20 nm). Fluence rates were measured with a wavelength corrected photodiode meter (Optometer 80 X, United Detector Technology Inc., Santa Monica, California, USA). The fluence rates at the top of the leaves were 17 W m^{-2} (95 μmol m^{-2} s^{-1}) for the 655 nm filter, 35 W m^{-2} (195 μmol m^{-2} s^{-1}) for the 666 nm filter, 23 W m^{-2} (140 μmol m^{-2} s^{-1}) for the 731 nm filter and 10 W m^{-2} (65 μmol m^{-2} s^{-1}) for the 750 nm filter.

2.3.2 Equipment for continuous irradiation with white light

Greening of plants took place in a controlled climate room at 20 °C under white fluorescent tubes (Philips TL 40 W/33). The light was attenuated by a dense metal wire screen to give a low fluence rate of about 7 W m^{-2} at the top of the leaves. Alternatively, the plants were irradiated in an aluminium cabinet (Joustra, 1970) at 20 °C under similar white fluorescent tubes at a fluence rate of 8.5 W m^{-2} at the top of the leaves (low WL). High fluence rate white light (31 W m^{-2}, high WL) was obtained from white fluorescent tubes (Osram-L, 115 W/20 Sa) placed above and at two sides of the plants.

2.3.3 Safelights

During isolation and purification of etioplasts in the cold room a dim green safelight was sometimes used. This safelight, with maximum emission at about 525 nm, was obtained from a green monophosphor fluorescent tube (Philips TL 40/17), wrapped in 2 layers of no. 62 blue Cinemoid (The Strand Electric Corp.) and 2 layers of no. 46 orange-yellow Cinemoid. The fluence rate of this safelight was 0.3 mW m^{-2} (10^{-3} µmol m^{-2} s^{-1}). Acetone extracts of leaf pigments were prepared under a similar green safelight. However, in this case only one layer of blue Cinemoid and one layer of orange-yellow Cinemoid were used. The fluence rate of this safelight was 0.9 mW m^{-2} (4×10^{-3} µmol m^{-2} s^{-1}).

2.4 Isolation of etioplasts

Leaves were harvested from 8-day old dark-grown or R pre-irradiated seedlings. At this age, leaves of monocotyledons used were usually still completely within the coleoptile. As removal of the coleoptiles took too much time, leaves and coleoptiles were harvested together and subsequently cut in pieces of 5-10 mm. Leaves from pea seedlings were collected together with a small part of the epicotyl hook. Harvesting was done either in complete darkness or under a dim green safelight at room temperature or at 4 °C. All further operations were performed at 4 °C either in darkness or under a dim green safelight.

The leaf material was ground 2 or 3 times during 4 s with isolation medium using an Ultraturrax homogenizer. The medium consisted of 0.15 M Na/K-phosphate buffer, pH 7.3, containing 9% (w/v) sucrose, 0.2% (w/v) bovine serum albumin (BSA) (Sigma, fraction V) and 45 mM 2-mercaptoethanol. A tissue to buffer ratio between 0.5 and 1 was used. The pH of the homogenate was 7.0. In some experiments MOPS buffer, pH 7.5, was used instead of phosphate buffer. This buffer contained 25 mM N-morpholino-3-propane sulphonic acid, 3 mM EDTA (disodium salt), 250 mM sucrose, 0.2% (w/v) BSA and either 14 or 45 mM 2-mercaptoethanol. The leaf homogenate was filtered twice through 4 layers of Nytal gauze (pore diameter about 20 µm). The filtrate was centrifuged for 5 min at 130 x g to remove most of the nuclei and intact cells. The resulting supernatant was centrifuged for 5 min at 1,700 x g. The pelleted etioplasts

were resuspended in isolation medium by dispersing them gently with a fine brush. The etioplast suspension was centrifuged again for 5 min at 1,700 x g, yielding a "crude, washed etioplast pellet".

2.5 Purification of etioplasts by use of a Sephadex G-50 (coarse) column

Wellburn and Wellburn (1971a) developed the Sephadex G-50 (coarse) column method for purification of isolated etioplasts. This method has the advantage over density gradient centrifugation that isotonic media can be used. Intact etioplasts and also mitochondria (Quail, 1977; Hilton and Smith, 1980) readily pass through the column, while nuclei, membranous fragments and broken etioplasts are reported to remain on the top of the column (Wellburn and Wellburn, 1971a).

A column of 40 cm length and 2.6 cm diameter, equipped with a flow adaptor (Pharmacia, Sweden, type K26/40) was used. The Sephadex G-50 (coarse) beads were allowed to expand overnight in excess isolation medium (Section 2.4) at 4 °C, 2-mercaptoethanol being added only shortly before preparation of the column. The Sephadex suspension was stirred before pouring it in the gel reservoir on top of the column. Isolation medium was run immediately through the column at a rate of 3-4 ml min^{-1}. When the column had packed, the gel reservoir was replaced by the flow adaptor and flow of isolation medium at the original rate was brought about by a peristaltic pump. Since the standard 10 µm bed support nets became blocked by the crude etioplast preparations, they were replaced by Nytal nets (Section 2.4).

Etioplasts were isolated as described in Section 2.4. However, the centrifugation step at 130 x g could be omitted since nuclei and cells remained on the top of the column. The crude, washed etioplast pellets were resuspended in 4 ml of isolation medium and loaded on the top of the column. This was done by injecting them carefully by means of a 10 ml plastic syringe with a 0.9 x 50 mm needle in a short tube connected with the column by a LV-4 laboratory valve (Pharmacia). Flow of isolation medium through the column was stopped during loading of the etioplasts on the column and resumed immediately afterwards. A small fraction of the eluate was continuously monitored at 254 nm with a LKB 8300 A Uvicord II to detect fractions containing nucleic acids and their derivatives. In initial experiments the remainder of the eluate was collected by a LKB 7000 Ultrorac fraction collector. In Fig. 2.1 an elution profile of oat etioplasts is shown, being representative also for maize, barley, bean and pea etioplasts. The elution profiles did not show a shoulder on the main peak. In this respect, they resembled those of Evans (1976) and Hilton and Smith (1980) but not those of Wellburn and Wellburn (1971a). Fractions forming the main peak were divided into three portions: fractions collected before maximum absorption at 254 nm was reached, fractions collected around the maximum and those appearing subsequently. The fractions were examined by means of light and electron microscopy. No differences were found other than in number of etioplasts per ml. In fractions forming the low, broad second peak no distinct cell material was detectable by light

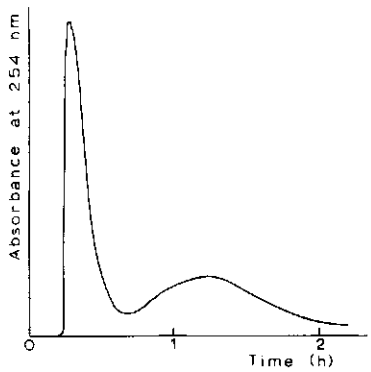


Fig. 2.1 Elution profile obtained by Sephadex G-50 (coarse) column purification of a crude, washed etioplast preparation from 8-day old dark-grown oat seedlings.

microscopy. In later experiments, the fraction collector was no longer used and eluate was collected as soon as the absorbance at 254 nm increased until the main peak had passed.

Phase contrast microscopy indicated that less than 1% of the etioplasts in the eluate was broken. In electron micrographs about 10% of the etioplasts appeared broken, however, during preparation of samples for electron microscopy (Section 2.15) etioplasts may have become damaged. In our experience, the Sephadex G-50 (coarse) column could be used with equal effectiveness for the purification of etioplasts from dicotyledonous and monocotyledonous plants. Fig. 2.2 shows electron micrographs of a crude, washed maize etioplast preparation and a Sephadex G-50 purified preparation.

2.6 Further purification of etioplasts using a discontinuous sucrose gradient

Hilton and Smith (1980) showed that carotenoids (markers for etioplasts) and the mitochondrial membrane marker enzymes cytochrome c oxidase and succinate dehydrogenase elute simultaneously on a Sephadex G-50 (coarse) column. Obviously, it is impossible to separate etioplasts from mitochondria by the Sephadex G-50 technique. A discontinuous sucrose gradient (Hilton and Smith, 1980) was used to obtain etioplast preparations free from mitochondrial contamination. Eluate of the Sephadex G-50 (coarse) column containing etioplasts was centrifuged at 1,700 x g and the pellet was resuspended in 2 ml isolation medium. Such suspensions were layered on to discontinuous sucrose gradients consisting of 7 ml 55 or 65% (w/v) sucrose, 11.2 ml 40% sucrose and 7 ml 25% sucrose. The tubes containing the sucrose gradients were centrifuged in a swing-out rotor in an Omega II ultra centrifuge (Heraeus-Christ) for 20 min at 8,000 x g. According to Hilton and Smith (1980), the fraction banding at the 40/25% interface contains extractable carotenoids and high cytochrome c oxidase activity, while the fraction banding at the 55/40% interface also shows a peak of extractable carotenoids but lacks cytochrome c oxidase

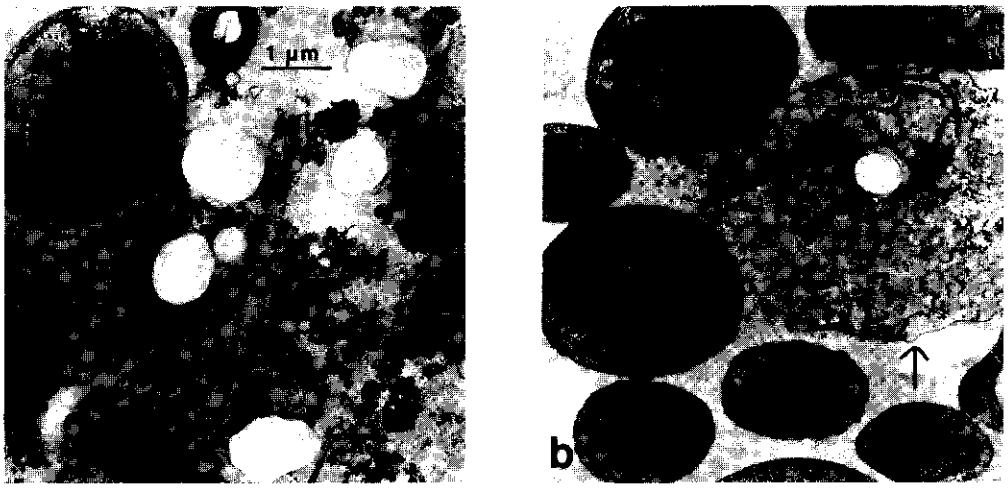


Fig. 2.2 Electron micrographs of etioplast preparations from 8-day old dark-grown maize seedlings, a. a crude, washed etioplast preparation, b. a Sephadex G-50 purified etioplast preparation. The arrow indicates a broken etioplast.

activity. The different fractions were studied by means of light and phase contrast microscopy. The fraction banding at the 40/25% interface consisted of about equal amounts of broken and intact etioplasts. The fraction banding at the 55/40% interface consisted mainly of intact etioplasts with a small percentage of broken etioplasts. Intact etioplasts were also found in the pellet, even if 65% instead of 55% (w/v) sucrose was used.

The 55(or 65)/40% interface fraction was centrifuged at 1,400 x g for 5-10 min either at room temperature or at 4 °C. The 40/25% interface fraction was centrifuged at 16,000 x g for 20 min at 2 °C. The pellets were carefully mixed with 0.5 g $CaCO_3$ for phytochrome (P) measurement.

2.7 Gel filtration on Sepharose CL-2B

Gel filtration on Sepharose CL-2B (Jose, 1977) was used to separate any ribonucleoprotein (RNP)-absorbed P (Quail and Gressel, 1976) from membrane-associated or "true" etioplast P. Etioplasts were purified in the absence of BSA by the Sephadex G-50 method and resuspended in 2.5 or 5 ml 35 mM MOPS buffer, pH 7.0, containing 1 mM EDTA and 14 mM 2-mercaptoethanol (Jose, 1977). Gel filtration using Sepharose CL-2B was carried out at room temperature under a dim green safelight on a K26/40 column (Pharmacia, Sweden) at a flow-rate of 0.5 ml min^{-1}. Fractions (5-6 ml each) were eluted with 25 mM MOPS, pH 7.0, containing 14 mM 2-mercaptoethanol. The void volume (V_o) and total bed volume (V_t) of the column were determined with Blue Dextran 2000 (Pharmacia) and potassium iron(II)cyanide ($K_4[Fe(CN)_6].3H_2O$), respectively, both being eluted with 150 mM NaCl. Blue Dextran was detected spectrophotometrically in fractions eluted from the column at 620 nm. Potassium iron(II)cyanide was

monitored at 750 nm after addition of 0.2 ml of a concentrated ammonium iron sulphate ($NH_4Fe(SO_4)_2.12H_2O$) solution to the fractions.

2.8 Estimation of chlorophyll, protochlorophyll and carotenoid content of leaves and etioplast preparations

Concentrations of chlorophyll(ide) (Chl(ide)), protochlorophyll(ide) (PChl(ide)) and carotenoid pigments in leaves and etioplast preparations were estimated after extraction in acetone/water (final concentration 80% (v/v)). Appropriate samples of whole leaves or segments were weighed on a "Centrogram" balance (Ohaus Scale Corp. model 311) under a green safelight. The samples were ground in acetone in a mortar with a little purified sea sand (Merck, Darmstadt, Germany) and a small amount of $CaCO_3$ was used to minimize pheophytin formation (Bruinsma, 1963). The leaf extract was filtered through a glass filter (Schott und Gen., Mainz, Germany, type 3D3) under suction and the residue on the filter was extracted a second time. Combined extracts were routinely clarified by centrifugation for 30 min at 16,000 x g at 2 °C. In the experiments described in Chapter 3, the opal glass method of Shibata et al. (1954) was used to minimize the effect of light scatter.

For pigment determinations in acetone extracts of etioplast preparations, 25 ml 80% acetone was added to the pelleted etioplasts. The pellet was resuspended and the suspension was centrifuged for 5 min at 1,400 x g at room temperature. The supernatant showed very little light scattering. In the pellet Chl(ide), PChl(ide) and carotenoids were no longer detectable.

The extracts were kept in darkness a few hours until measurement. The absorption of the extracts was measured in cuvettes with either 1 or 5 cm path length (depending on pigment concentration) in a Zeiss model PMQ II spectrophotometer. Any absorption at 800 nm, if present, was subtracted from the absorption at other wavelengths as a correction for remaining light scatter. Pigment concentrations of extracts containing mixtures of PChl(ide) and Chl(ide) were calculated using equations of Anderson and Boardman (1964). For extracts containing negligible amounts of PChl(ide), equations derived from Arnon (1949) and Bruinsma (1963) were used (see also Raven, 1973). Acetone extracts containing water and mixtures of Chlide and its esterified derivatives exhibit slightly different absorption maxima between 663 and 667 nm (Raven, 1973; Scheutjens, 1975). The absorbance at the red maximum was used in the calculations.

As a measure of the concentration of carotenoids, the absorbance at 473 nm in 80% acetone was taken (Bottomley, 1970; Raven, 1973). The contribution of other pigments (mainly Chl b) to the absorption at this wavelength was corrected for when appropriate using data of MacKinney (1941) of specific absorption coefficients of Chl a and Chl b in 80% acetone at 473 nm.

In some experiments (Chapter 3), in vivo measurements were used to estimate the relative Chl content of leaves. Sections of the outer half of the rolled primary maize leaves, at a distance of 2 to 4 cm from the apex, and the outer half of bean leaves were taken for the measurements. For each measurement one

leaf piece was used. A piece of white paper giving about the same scatter was used as a reference. The absorption of the leaves was measured at 672.5 nm in a Cary 14 spectrophotometer. The absorptions at 590 and 730 nm were used to estimate the baseline.

2.9 Estimation of phytochrome content

The P content of samples was estimated spectrophotometrically using the spectrophotometer described by Spruit (1970) in its dual wavelength mode of operation. The measuring beam was at 730 nm and the reference beam at 806 nm. Cuvettes with a volume of about 0.2 ml and a path length of 1.6 mm (Fig. 2.3) were used. $CaCO_3$ was used as a scattering agent (Butler, 1962). For measurements of the P content of a leaf homogenate or supernatant, samples of 0.2 ml were added to cuvettes filled with dry $CaCO_3$. When the P content of an etioplast preparation was measured, 1 g of $CaCO_3$ was carefully mixed with the etioplast pellet and the cuvette was filled with the homogeneous mixture. For a representative example of such a P measurement see Fig. 2.4.

P content is expressed in absorbance units. Differences in absorbance of about $4 \times 10^{-5}\ \Delta\ (\Delta A)730\text{-}806$ as a result of P phototransformation by actinic irradiation were the smallest differences that could be detected.

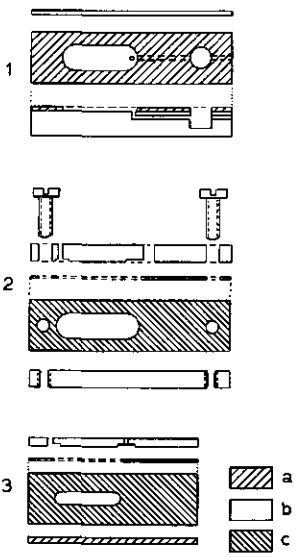


Fig. 2.3 Cuvettes used for measurements of the phytochrome content of etioplast preparations (1) and for absorption measurements (2) and fluorescence measurements (3) of (proto)chlorophyll(ide) in etioplast preparations. a. black "Plexiglass", b. clear "Plexiglass", c. phosphor bronze sheet, 0.2 mm thick. Depth of the cells: (1) 1.6 mm, (2) and (3) 0.2 mm. The front cover was mounted with tape (1), with screws (2) or with clamps (3).

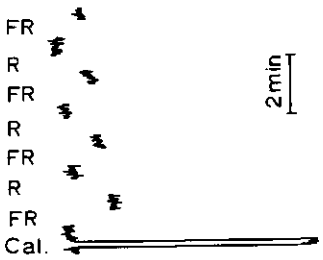


Fig. 2.4 A representative example of a phytochrome measurement of a maize etioplast preparation. Phytochrome measurements were made at room temperature or at 0 °C. Actinic irradiations to interconvert Pr and Pfr were of 1 or 2 min duration and traces were recorded during 1 min after each actinic irradiation. At least three alternating R (653 nm) and FR (732 nm) actinic irradiations were given during each measurement. Cal.: calibration signal, 4 x 10^3 Δ A.

2.10 Estimation of protein content

Protein was assayed by the method of Lowry et al. (1951) using BSA (Sigma, fraction V) as a standard. The Folin-Ciocalteu's phenol reagent used in this method is intensely coloured by 2-mercaptoethanol. Therefore, proteins in preparations containing 2-mercaptoethanol were precipitated by 5% trichloroacetic acid (TCA), washed and dissolved in distilled water before adding the reagent. BSA present in etioplast preparations was removed by washing the etioplasts in isolation medium without BSA.

2.11 Studies of protochlorophyll(ide) regeneration and the Shibata shift using absorption spectrophotometry

PChl(ide) regeneration was studied by absorption spectrophotometry. Absorption spectra of leaves and etioplast preparations were measured in a Cary 14 spectrophotometer. For monocotyledons, leaf pieces 2-4 cm from the apex of the outer half of the rolled leaf were used. One or two layers of leaves or leaf pieces were mounted behind an opening in a piece of brass. A suitable piece of "opaline" perspex sheet or one or two pieces of white paper mounted behind an opening of similar size were placed in the reference beam. For the recording of difference spectra, one or two layers of comparable leaves or leaf pieces were used as a reference. Absorption spectra of etioplasts were measured in dense suspensions contained in 10 µl microcuvettes with a light path of 0.2 mm (Fig. 2.3). After the recording of an absorption spectrum of dark-grown leaves or etioplasts from 400 to 800 nm at room temperature, no photoconversion of PChl(ide) into Chl(ide) by the measuring beam could be detected.

Spectra were measured with the Cary spectrophotometer at different temperatures using an optical cryostat, in which the samples could be

irradiated in situ (Spruit, 1970). Spectra were recorded before and after various dark periods following a R (649 nm) irradiation sufficient to photoconvert all phototransformable PChl(ide) into Chl(ide) (4-12 s, depending on the sample). PChl(ide) regeneration in the dark after R was also measured directly according to the method of Jabben and Mohr (1975). Absorption changes at 650 nm were recorded immediately after the R pulse, using 720 nm as a reference wavelength. The Shibata shift was similarly monitored, by recording absorption changes at 690 nm.

2.12 Fluorescence spectrophotometry

Fluorescence spectra of leaves and etioplast preparations were measured at 77K. The spectrofluorimeter was equipped with an analog differentiator (Fig. 2.5). The excitation source was a 250 W 24 V quartz-halogen incandescent lamp. Excitation light was filtered through 20 mm of saturated copper sulphate solution and an interference filter (Balzers, B 40, 425 nm, HW 12 nm) plus a 2 mm BG 12 filter (Schott und Gen., Mainz). Alternatively, a small high-pressure mercury lamp with a similar copper sulphate filter plus 4 mm BG 12 was used. To separate the fluorescence light from excitation light scattered by the sample, filter F3 was inserted between the sample and monochromator. This filter consisted either of one layer of yellow Cinemoid no. 1 (The Strand Electric Corp.) plus 3 mm GG 495 (Schott und Gen.) or of 3 mm yellow Plexiglas (Röhm und Haas, Darmstadt) plus one layer of yellow Cinemoid.

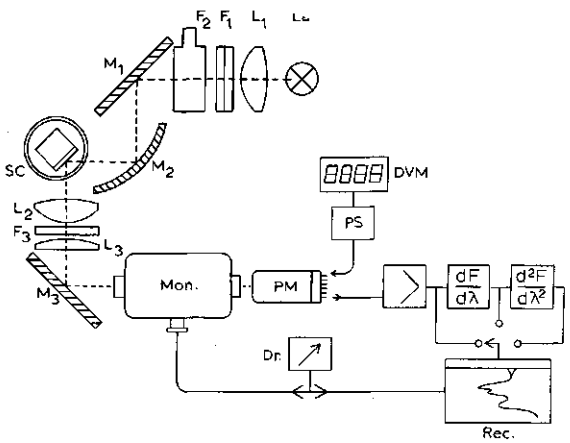


Fig. 2.5 Spectrofluorimeter. La: excitation light source; L_1, L_2, L_3: lenses; F_1, F_3: filters (see text); F_2: 20 mm saturated $CuSO_4$-solution; M_1, M_2, M_3: mirrors; Mon.: grating monochromator; PM: photomultiplier; PS: multiplier power supply; DVM: digital voltmeter; Dr.: multi-speed drive; Rec.: recorder; SC: sample cell (inside cryostat).

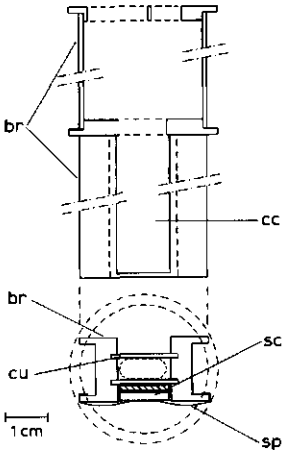


Fig. 2.6 Low temperature sample holder for fluorescence studies. br: brass; cu: copper; cc: sample cell compartment; sc: sample cell (see Fig. 2.3); sp: phosphor bronze springs (2). Liquid nitrogen charge: 110 ml. Cryostat not shown.

Brass sample holders were constructed for leaves and for etioplast preparations (Fig. 2.6). The cuvette used for etioplast samples is shown in Fig. 2.3. Sample holders were placed inside optical cryostats and were filled with liquid nitrogen for measurements at 77K. It took 3 min after addition of liquid nitrogen into the sample holder before the temperature had dropped below 93K as measured with a thermocouple inside the sample holder. If more rapid cooling was desired, the samples in their holders were immersed in liquid nitrogen for 60 s before insertion into the optical cryostat.

Fluorescence probes were added to etioplast preparations to indicate the concentration of etioplasts present in the measuring cuvette. Ideally, a fluorescence probe or indicator should have one or more emission bands located outside the spectrum of the etioplasts. Moreover, the probe should not influence the characteristics of the etioplasts and their spectra (peak positions, peak heights and rate of shifts). Several compounds were tested: fluorescein, acridin yellow, acriflavin, rivanol, rhodamin B, quinacrin, erythrosin, eosin, folic acid, flavin mononucleotide (FMN) and 9-amino-6-chloro-2-methoxyacridine (ACMA). Acriflavin was the only compound acceptably meeting the criteria mentioned above. Its peak position is at 516-520 nm and a minor band is found at 572 nm. At 620 nm all probes, including acriflavin, showed some fluorescence, however at wavelengths above 640 nm fluorescence by acriflavin was negligible.

When etioplast suspensions to which acriflavin was added were washed in isolation medium, the height of the acriflavin fluorescence peak at 518 nm considerably decreased. Apparently, part of acriflavin is not or only weakly bound to the etioplasts. Therefore, carboxymethylcellulose (CMC) was added to

prevent the etioplasts to sink to the bottom of the cuvette. A solution of 5 g CMC and 12.3 mg acriflavin in 200 ml distilled water was dialysed twice during 24 h. 9% (w/v) sucrose was added to the dialysed solution. About 20 drops were added to an etioplast pellet and carefully mixed with a fine brush. Samples from this mixture were taken for recording fluorescence spectra. No influence of CMC and acriflavin on fluorescence characteristics of etioplast preparations were detected.

Since 2-mercaptoethanol has a destructive effect on the phototransformability of PChl(ide) (Schopfer and Siegelman, 1968; Gassman, 1973a), this compound was omitted from the isolation medium for etioplasts after the first centrifugation step at 1,700 x g (Section 2.4).

2.13 Smoothing and differentiation of spectra

The different absorption and fluorescence bands of the PChl(ide) and Chl(ide) species in dark-grown and short-term irradiated leaves and etioplast preparations overlap to some extent. In such cases, maxima in the spectra do not correspond to the true maxima of the components. Moreover, the peak heights observed do not reflect the relative contributions from the components. In the case where one band is small in comparison to other overlapping band(s) and only a shoulder is formed by the small band, it is impossible to accurately determine the position of its maximum and its height by direct inspection of the spectrum. In such cases the determination of derivative spectra can be helpful. First as well as higher derivatives can be used to establish the location of peaks in a spectrum (Vandeginste and De Galan, 1975). If the original spectrum contains only a single band, its maximum corresponds to a passage through zero in derivatives of odd order and to the extreme values (maxima and minima) in even-order derivatives (Fig. 2.7a). This also more or less holds for two or more overlapping bands as illustrated in Fig. 2.7b. With increasing order of the derivative, the sharpness of the bands increases, resulting in improved resolution. On the other hand, the number of extremes increases with increasing order of the derivative. To determine the number of the different components of the fluorescence or absorption spectra and their parameters: peak wavelength, height and HW, second and fourth derivatives of the spectra were used. The second derivatives were obtained either on-line by an analog differentiator or by the use of a suitable computer program. Fourth derivatives were obtained exclusively by computation.

With electronic analog differentiation, the speed of scanning through a spectrum determines the shape of the derivative spectra. Essentially, such spectra are derivatives of a photomultiplier current against time. For this reason, the scanning speed has to be kept constant during a recording. On the other hand, the wavelength dependence of spectrometer transmission and spectral response of the photomultiplier enter into the output and tend to distort the derivative spectra obtained in this way. Fortunately, the response of the RCA 31034 multiplier was remarkably flat over the wavelength

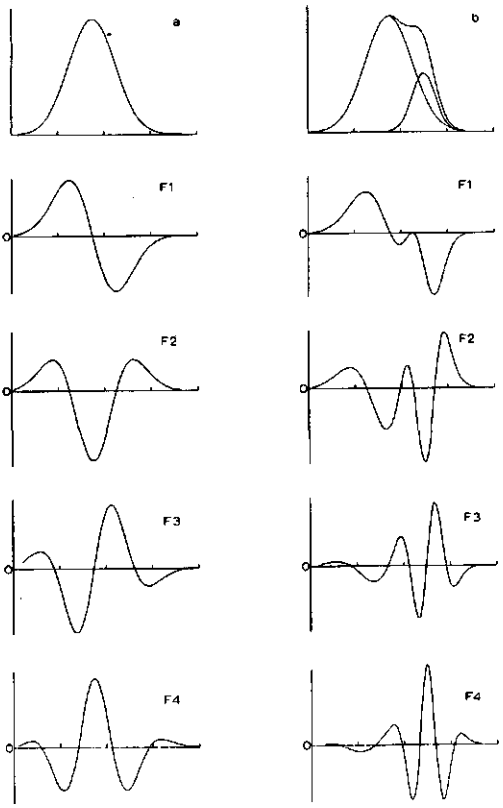


Fig. 2.7 Examples of a Gaussian band (a) and a Gaussian band pair (b) and their first to fourth derivatives.

interval examined and the distortions introduced in this way proved to be small. A second practical problem is that the need for rigorous electrical filtering of the signal before the first and between the first and second differentiation, introduces a time delay which translates into a wavelength shift between the original spectrum and its derivatives. Moreover, the magnitude of the shift depends on the scanning speed. To overcome this problem, the spectra were routinely scanned twice at the same speed, once from low to high, the second time from high to low wavelength values. The average peak position between each pair of runs has been taken as the true wavelength.

For the computer calculations of second and fourth derivatives, the IMSL-routine DCSEVU (IMSL, 1984) was used on a DEC-10 computer. Spectra were digitized by hand at 1 nm intervals, with the result that the spectra are already smoothed to some extent by eye. However, further smoothing was necessary. The original data and, when a fourth derivative was determined,

also the second derivative were smoothed by the IMSL-routine ICSSCU (IMSL, 1984).

2.14 Deconvolution of spectra

The computer program "ACCU" for curve analysis was kindly supplied by Dr. W. Verwer (Laboratory for Biophysics, University of Utrecht). This program was used to improve the band parameters found by derivative spectrophotometry (Section 2.13). The program was originally developed in the Shell Development Laboratory in Emeryville (California) and is described by French et al. (1967) as the RESOL or RESOLV program. The program was modified by Mr. A. Keetman and Mr. G. van Eck (Department of Computer Science, Agricultural University, Wageningen) for use on a DEC-10 computer.

Raw data of spectra were entered in digital form at 1 nm wavelength intervals. Approximate values for maxima, heights and half-widths of bands estimated by derivative spectrophotometry were also entered. The shape of the bands can be specified in the program as Gaussian and/or Lorentzian. The assumption that absorption and fluorescence bands of PChl(ide) and Chl(ide) are symmetric and Gaussian and/or Lorentzian in shape probably is not completely realistic. For absorption bands, the tails of Gaussian bands are slightly too low while the tails of Lorentzian curves are far too high (French et al., 1972; Jabben et al., 1974). However, although an intermediate form might give a better fit, satisfactory results were obtained both for absorption and fluorescence spectra using Gaussian curves alone.

During each iteration all parameters are adjusted within certain specified limits to give a better fit to the original curve. Weighting factors and damping factors can be set to improve the results of each iteration. The program ends after a number of iterations specified by the operator or when the change of standard error is within the limit specified. The final output can be given as plotted curves of the calculated bands, their sum and the original data. The error curve is plotted below the analyzed spectra. Examples of such deconvolutions are shown in Chapters 6 and 7.

2.15 Electron microscopy

For electron microscopical studies of etioplasts "in situ", small (about 4 mm^2) leaf pieces were fixed. The leaf pieces were taken at a distance of about 2 cm from the apex of a primary monocotyledonous leaf or out of the middle of a dicotyledonous leaf. During WL treatment in vitro, etioplast suspensions in the incubation medium described by Wellburn and Wellburn (1973) were rotated at 4 rpm. Fixation of leaf pieces was carried out for about 3 h and fixation of isolated etioplasts for 2 h at room temperature or overnight at 4 ^{o}C in darkness in 5% (v/v) glutaraldehyde in 0.025 M Na/K-phosphate buffer, pH 7.3, containing 5% (w/v) sucrose. Post-fixation was carried out for a similar period in 1% (w/v) OsO_4 in 0.025 M Na/K-phosphate buffer, pH 7.3, containing 5% (w/v) sucrose. Dehydration was carried out at room temperature through a graded ethanol series followed by propylene oxide. All

manipulations were performed under a dim green safelight or in darkness until the material was fixed and dehydrated. The material was embedded in Epon 812 or in Spurr's resin. Electron micrographs were taken of different parts of etioplast pellets. No differences were observed between top, bottom and other parts of the pellet.

In order to quantify the volume of the prolamellar body, a weight method used by Horton and Leech (1975) was adopted. The areas of etioplasts (small sections were disregarded) and their PLBs were cut out of photocopy paper and weighed. Weight was consistently proportional to the area of the paper.

2.16 Statistics

Standard deviation: $S.D.=\sqrt{\sum(x-\bar{x})^2/(n-1)}$, where $\bar{x}$ is the mean value and n is the number of samples.

Standard error of the mean: $S.E._{\bar{x}}=S.D./\sqrt{n}$.

Coefficient of variation: $S.D./\bar{x} \times 100\%$.

Results are expressed as mean ± standard error of the mean. The Student's t-test was used to test whether the difference between two means is statistically significant.

3 THE INFLUENCE OF RED AND FAR-RED PRE-IRRADIATION ON CHLOROPHYLL ACCUMULATION IN CONTINUOUS WHITE LIGHT

3.1 Introduction

Raven and Shropshire (1975) demonstrated that in dark-grown pea seedlings, sensitivity for red light (R) potentiation of rapid chlorophyll (Chl) accumulation in continuous white light (WL) is extremely high (very low fluence response or VLFR). After a previous R pre-irradiation, the sensitivity decreases substantially (low fluence response or LFR). These observations were explained by Raven and Spruit (1973) on the basis of the phytochrome (P) transport model. Dark reversion of receptor-associated Pfr to Pr forms an essential aspect of this model. Frankland (1972) demonstrated that in contrast to dicotyledons, Pfr dark reversion appears to be absent in monocotyledons. The predictions of the model were therefore tested for maize as a member of the latter group.

Since photobleaching of newly formed pigments could be a cause of the lag phase in Chl accumulation, greening in WL of high fluence rate was also examined. Carotenoids probably play a role in protecting Chl against photobleaching (Frosch *et al.*, 1979; Klockare *et al.*, 1981; Malhotra *et al.*, 1982). Therefore, a possible R effect on carotenoid content of maize seedlings was studied.

In this chapter Chl will be used as a collective term for chlorophyllide and its esters, and similarly protochlorophyll (PChl) for protochlorophyllide and its esters.

3.2 Results

3.2.1 Variation in rate of greening

In vivo measurements of the Chl content of individual maize leaves after a 5 h WL period showed considerable variation, even for seedlings grown together in a plant pot. Leaf sections taken at a distance of 2.5 to 3.5 cm from the apex of "long" and "medium long" leaves on average had a higher Chl *a* content than those of "short" leaves (Table 3.1). However, also within classes of seedlings with a certain leaf length the rate of greening was variable. Coefficients of variation (Chapter 2, Section 2.16) were similar for two genetically homogeneous maize varieties Z27 and WJ and for cv. Capella. For the experiments described in this chapter 8-day old seedlings of cv. Capella were used, unless otherwise indicated.

3.2.2 Greening in white light of low fluence rate and the effect of red pre-irradiation

When dark-grown maize seedlings were placed in WL of rather low fluence rate (8.5 W m^{-2}) (low WL), after the first rapid formation of Chl(ide) *a* from endogenous PChl(ide) *a*, further Chl *a* accumulation proceeded slowly during the first 3 h (the "lag phase") (Fig. 3.1). Over the following 3 h the rate of

Table 3.1 Relative chlorophyll concentration in "long", "medium long" and "short" 8-day old maize leaves after 5 h of white fluorescent light (8.5 W m^{-2}) measured as the height of the absorption peak at 672.5 nm (means of 12 experiments each).

Sample	Leaf length (cm)	Relative peak height at 672.5 nm	No. of leaves per experiment
Long leaves	8-9	41 ± 2	6
Medium long leaves	7-8	38 ± 1	8
Short leaves	6-7	32 ± 1	6

Chl a accumulation more than doubled. After 7 h the rate became somewhat slower again, but was still faster than during the lag phase. 8-day old dark-grown seedlings of the maize varieties Z27 and WJ showed lag phases similar to that of cv. Capella.

When a brief standard R pre-irradiation (5 min 658 nm, 0.85 W m^{-2} or 5 µmol m^{-2} s^{-1}) was given 16 h prior to low WL, Chl a accumulation immediately started at an increased rate (Fig. 3.1). After 3-4 h in low WL, the rate of Chl a accumulation increased slightly and after 6-7 h it slowed down to the

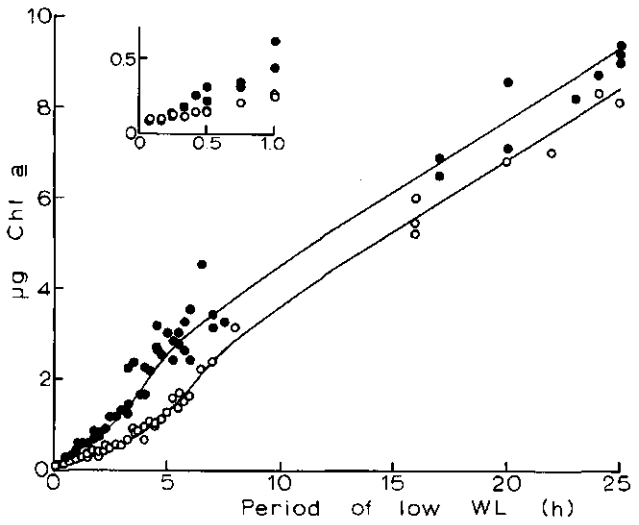


Fig. 3.1 Chlorophyll a (Chl a) accumulation in 1 cm leaf sections (17 mg fresh weight) of 8-day old maize leaves after transfer to continuous white fluorescent light (WL) of low fluence rate (8.5 W m^{-2}) at 20 °C. ○: dark-grown seedlings, ●: seedlings pre-irradiated with a standard fluence of red light (R) 16 h prior to WL. Results have been corrected for the amount of Chl a (0.06 µg per leaf section) formed during R pre-irradiation.

same level as that for dark-grown seedlings. A R pre-irradiation 4 or 40 h prior to low WL had the same potentiating effect as a R pre-irradiation 16 h prior to low WL (see also Section 3.2.4).

3.2.3 Greening in white light of high fluence rate and the effect of red irradiation

When placed in WL of high fluence rate (31 W m^{-2}) (high WL), after 15 min of irradiation a quantity of Chl a was formed similar to that in low WL. During the following 7 h the amount of Chl a hardly increased (Fig. 3.2); after 7 h almost 10 times less Chl a had accumulated than after 7 h low WL (Fig. 3.1). After 20 h high WL, the quantity of Chl a was still approximately 7 times smaller than that formed after 20 h low WL.

Pre-irradiation of dark-grown seedlings with a standard fluence of R 16 h prior to WL strongly stimulated Chl a accumulation in high WL. However, in R pre-irradiated seedlings the Chl a accumulation rate was also lower in high than in low WL: after 7 h high WL about half as much Chl a had accumulated as in low WL.

3.2.4 Effects of various pre-irradiation treatments on greening in white light of low fluence rate

Using the method of Raven (1973), the effects of different pre-irradiation treatments on Chl a accumulation rate in low WL were studied. In these experiments a standard R pre-irradiation consisted of either 5 min R (658 nm) at an irradiance of 0.85 W m^{-2} (5 μmol m^{-2} s^{-1}) or alternatively 20 s R (655

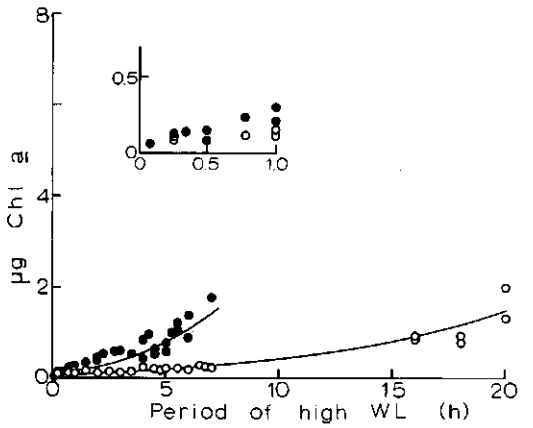


Fig. 3.2 Chlorophyll a (Chl a) accumulation in 1 cm leaf sections (17 mg fresh weight) of 8-day old maize leaves after transfer to continuous white fluorescent light (WL) of high fluence rate (31 W m^{-2}) at 20 °C. ○: dark-grown seedlings, ●: seedlings pre-irradiated with a standard fluence of red light (R) 16 h prior to WL. Results have been corrected for the amount of Chl a (0.06 μg per leaf section) formed during R pre-irradiation.

nm) at an irradiance of 17 W m^{-2} (95 µmol m^{-2} s^{-1}) at the top of the leaves. Both irradiations were considered to be saturating with regard to Pr → Pfr phototransformation.

The amount of Chl a accumulated after 5 h low WL was twice as high for maize seedlings pre-irradiated with a standard fluence of R as for dark-grown seedlings. The potentiating effect of a standard R pre-irradiation on Chl a accumulation in low WL was taken as 100%. Potentiating effects of other pre-irradiation treatments were calculated as follows (see Raven, 1973):

$$\text{potentiation} = \frac{Ct - Cd}{Cr - Cd} \times 100\%$$

where Cd = Chl a content without an inductive light treatment, Cr = Chl a content resulting from a pretreatment with a standard R fluence 16 h before low WL and Ct = Chl a content resulting from a light pretreatment of given wavelength and fluence 16 h before low WL; Chl a was determined after a 5 h period of low WL.

The potentiating effect of a standard R pre-irradiation 16 h prior to low WL was reduced to 48% by 2 or 5 min FR (750 nm, 10 W m^{-2} or 65 µmol m^{-2} s^{-1}) given after R. On the other hand, the FR treatment alone resulted in 20% potentiation (Table 3.2). One min FR was slightly less effective than 2 or 5 min FR, suggesting that with this fluence photostationary equilibrium was not completely attained. Compared with results published for pea (Raven, 1973), the potentiating effect of FR pre-irradiation was only small for maize seedlings and FR gave a better reversion of the effect of R. In this respect, bean is intermediate between pea and maize (Raven, 1973; see Table 3.2).

For pea seedlings, the potentiating effect of R was nearly twice as large when a dark interval of 40 h instead of 16 h was inserted between R and WL (Raven, 1973; see Table 3.2), but for maize seedlings the potentiating effects of R given 16 h and 40 h before low WL were not significantly different (Table 3.2). In maize, the reversion by FR given immediately after R was more pronounced when the pre-irradiations were followed by a dark period of 40 h instead of 16 h. The potentiating effect of FR alone, correspondingly decreased. Similar results were published for bean seedlings (Raven, 1973; see Table 3.2).

3.2.5 Effects of a second pre-irradiation

Calculations of percentage potentiation by two successive pre-irradiations were as follows:

$$\text{potentiation} = \frac{Crlt2 - Cdld2}{Crlr2 - Cdld2} \times 100\%$$

where Cdld2 = Chl a content without any inductive light treatment, Crld2 = Chl a content resulting from a pretreatment with a standard R fluence given 40 h before low WL and Crlt2 = Chl a content resulting from a pretreatment with a standard R fluence 40 h before WL, followed by a second light pretreatment of given wavelength and fluence 16 h before low WL; Chl a was

determined after a 5 h period of low WL.

For pea and bean, the effects of two successive R pre-irradiations have been reported by Raven (1973) to be almost additive. However, for maize seedlings the potentiation resulting from two R pre-irradiation treatments was only about 1.5-fold that resulting from a single R pre-irradiation (Table 3.2).

Table 3.2 Effects of various red (R) and far-red (FR) pre-irradiation treatments on chlorophyll a accumulation in white fluorescent light (WL) (8.5 W m^{-2}) in previously dark grown maize, pea and bean seedlings (data of pea and bean seedlings from Raven, 1973). Note that the pre-irradiation treatments were different for maize. Chlorophyll a was measured after 5 h WL. The effect of a standard R pre-irradiation (given 16 h prior to WL in the case of a single pre-irradiation treatment and 40 h prior to WL in the case of two treatments) was taken as 100% and the effects of all other treatments are presented as a percentage of the effect of this standard pre-irradiation treatment.

Pre-irradiation treatment		Effect (%)		
40 h before WL	16 h before WL	maize[a]	pea[a]	bean[a]
-	R[b]	100	100	100
-	R/FR[d]	48 ± 8 (21)[e]	91	83
-	FR[c]	20 ± 5 (20)	88	48
R	-	102	198	107
R/FR	-	28 ± 5 (11)	-	30
FR	-	12 ± 2 (5)	-	20
R	R	147 (31)	279	181
R	R/FR	98 ± 4 (17)	221	108
R	FR	67 ± 5 (13)	187	-
R/FR	R	122 ± 5 (7)	-	-

[a] the age of the seedlings at the time of WL irradiation was 8 days for maize, 7 days for pea and 10 days for bean. Data for pea from Raven (1973, Fig. 51) and for bean from Raven (1973, Figs. 38, 48 and 52)

[b] R pre-irradiation for maize seedlings: 5 min 658 nm, 0.85 W m^{-2} (5 μmol m^{-2}) or 20 s 655 nm, 17 W m^{-2} (93 μmol m^{-2}), for pea and bean seedlings: 1 min 651 nm, ~3 W m^{-2} (16 μmol m^{-2})

[c] FR pre-irradiation for maize seedlings: 2 or 5 min 750 nm, 10 W m^{-2} (63 μmol m^{-2}), for pea and bean seedlings: 1 min 739 nm, 4.15 W m^{-2} (26 μmol m^{-2})

[d] FR pre-irradiation followed R pre-irradiation immediately

[e] the number of experiments is given within parentheses

Contrary to the effect of a single R pre-irradiation, the effect of a second R pre-irradiation has been claimed to be completely reversible by subsequent FR in bean seedlings. In pea seedlings, FR reversibility of R potentiation also seemed increased for the second R preirradiation (Raven, 1973). However, the present results with maize seedlings show that a FR (750 nm) pre-irradiation 16 h prior to low WL still partially reversed the effect of a R pre-irradiation given 40 h prior to low WL (Table 3.2). For bean seedlings, no comparable data were reported. In pea seedlings (Raven, 1973; Raven and Shropshire, 1975) a FR pre-irradiation 16 h prior to low WL also slightly decreased the effect of R 40 h prior to low WL. Consequently, FR photoreversibility can better be expressed as {(R)-(R+FR)}/{(R)-(FR)}, where (R) is the percentage potentiation by R only, (R+FR) is the percentage potentiation by R followed by FR immediately and (FR) is the percentage potentiation by FR alone. Expressed in this way, in maize and in pea (less evident) the effects of both a first and a second R pre-irradiation were 60-80% reversed by subsequent FR.

3.2.6 Effect of pre-irradiation on carotenoid formation in white light

In 8-day old dark-grown maize seedlings appreciable amounts of carotenoids are present. R pre-irradiation did not significantly influence the amount of carotenoids as measured by the absorption of leaf extracts at 473 nm in 80%

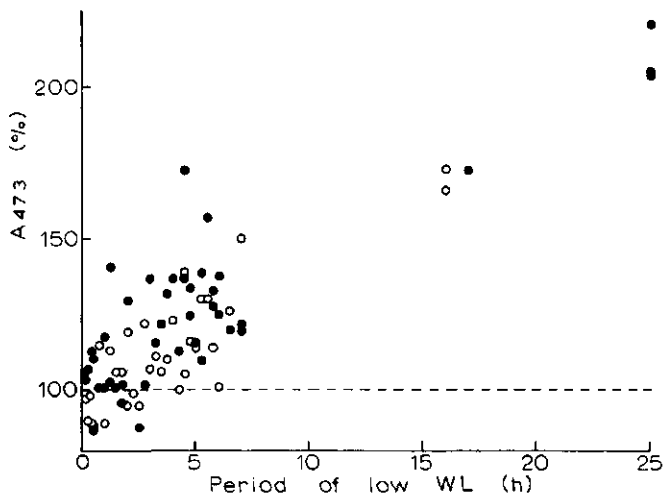


Fig. 3.3 Carotenoid accumulation in 1 cm leaf sections (17 mg fresh weight) of 8-day old maize leaves after transfer to continuous white fluorescent light (WL) of low fluence rate (8.5 W m^{-2}) at 20 °C. O: dark-grown seedlings, ●: seedlings pre-irradiated with a standard fluence of red 16 h prior to WL. Carotenoids were measured by the absorbance at 473 nm of extracts of the leaf sections in 80% aqueous acetone. Results are expressed as percentage of dark controls.

acetone in water. During irradiation of previously dark-grown maize seedlings with low WL, the carotenoid content tends to increase (Fig. 3.3). Although the determination of carotenoids shows considerable variation, the results indicate a slight R induced stimulation of carotenoid synthesis in low WL. This R stimulation was partially reversed by 2 or 5 min FR (750 nm) (Table 3.3).

When 8-day old dark-grown maize seedlings were placed in high WL, the carotenoid content initially showed a slight decrease (Fig. 3.4). However,

Table 3.3 Influence of various red (R) and far-red light (FR) pre-irradiation treatments on carotenoid content of 8-day old maize seedlings after 5 h of white fluorescent light (WL) (8.5 W m^{-2}). Results are expressed as percentage of non-pretreated controls.

Pre-irradiation treatment		Percentage of control without pre-irradiation	No. of experiments
40 h before WL	16 h before WL		
-	R	129 ± 3	44
-	R/FR	111 ± 3	21
-	FR	106 ± 4	20
R	-	141 ± 7	9
R/FR	-	109 ± 7	6
FR	-	105 ± 4	5

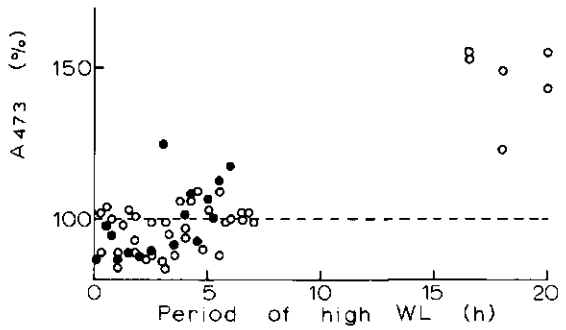


Fig. 3.4 Carotenoid accumulation in 1 cm leaf sections (17 mg fresh weight) of 8-day old maize leaves after transfer to continuous white fluorescent light (WL) of high fluence rate (31 W m^{-2}) at 20 °C. ○: dark-grown seedlings, ●: seedlings pre-irradiated with a standard fluence of red 16 h prior to WL. Carotenoids were measured by the absorbance at 473 nm of extracts of the leaf sections in 80% aqueous acetone. Results are expressed as percentage of dark controls.

after 16-20 h of high WL the carotenoid content had increased. R pre-irradiation did not significantly influence carotenoid formation in high WL.

3.2.7 Fluence-response curves for red potentiation

The logarithmic fluence-response curve for R potentiation of rapid Chl a accumulation in WL has been interpreted to be monophasic (Kraak and Spruit, 1985). However, the data given in Fig. 3.5 can satisfactorily be fitted by a biphasic curve as well. The results in Fig. 3.5 are plotted on the assumption

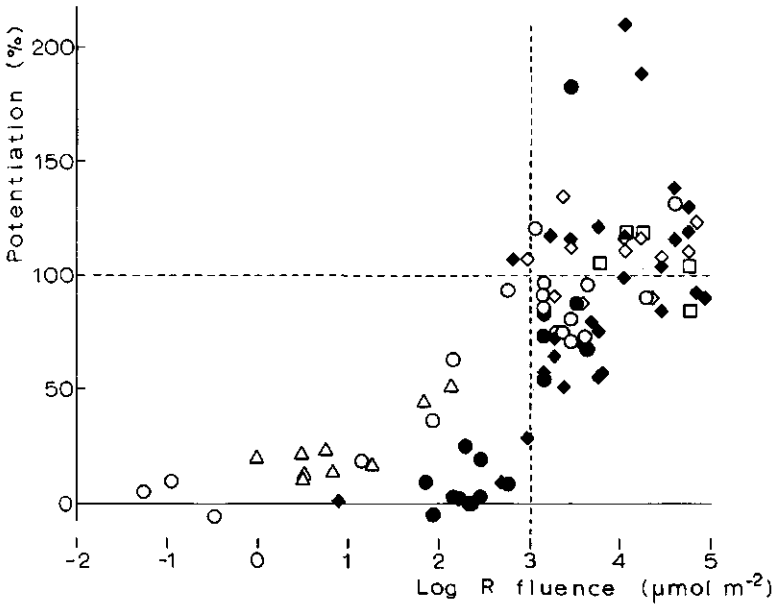


Fig. 3.5 Logarithmic fluence-response curves for R potentiation of rapid chlorophyll a (Chl a) accumulation in white light (WL) of low fluence rate (8.5 W m^{-2}) in 8-day old dark-grown maize seedlings. Open symbols: potentiation of a single red light (R) pre-irradiation 16 h prior to WL. Closed symbols: the additional potentiation of a second R pre-irradiation 16 h prior to WL, subsequent to a standard R fluence 40 h prior to WL. The mean potentiating effect of fluences higher than 1 mmol m^{-2} has been taken as 100% for both R pre-irradiations. Chl a was determined after 5 h WL. The mean amount of Chl a formed after a 5 h WL period was 2.28 times that in the non-pretreated controls after a single R pre-irradiation at fluences higher than 1 mmol m^{-2}; after a second R pre-irradiation at these fluences, the mean Chl a was 2.90 times that in the non-pretreated controls. Exposure times ranged from 4 to 1200 s at the following wavelengths: o : 658 nm, △ : 667 nm, ◇ : 655 nm, □ : 666 nm. Irradiances at the top of the leaves were 5, 95 and 195 μmol m^{-2} s^{-1} for the 658, 655 and 666 nm light, respectively. Irradiances of the 667 nm light were obtained using neutral glass filters (maximum 1 μmol m^{-2} s^{-1}).

that all fluences above 1 mmol m^{-2} (i.e., the fluence required to attain the maximum percentage of Pfr possible) saturate potentiation. The large scatter in the data makes firm conclusions difficult. However, in vivo spectroscopy of maize leaves confirmed that in the VLFR region, 0.05 - 1 μmol m^{-2} R, 10-20% potentiation is obtained. Upon a second R pre-irradiation, no evidence for a VLFR was observed. Both a single and a second R pre-irradiation showed a considerable response in the LFR range. On average, a single R pre-irradiation at fluences higher than 1 mmol m^{-2} yielded 2.28 times more Chl a in a 5 h WL period than the non-pretreated controls, while after two subsequent R pre-irradiations at these fluences 2.90 more Chl a was formed than in non-pretreated controls.

3.3 Discussion

Maize seedlings grown in complete darkness for 8 days show the characteristic lag phase in Chl a accumulation (Virgin, 1978) when placed in low WL (8.5 W m^{-2}). Most of this lag phase is eliminated by a short R pre-irradiation, followed by a dark period (Fig. 3.1). The optimum duration of the dark period between R pre-irradiation and WL is dependent on the plant species and also appears to be age dependent (Virgin, 1956; Akoyunoglou, 1970; Raven, 1973). For 8-day old maize seedlings, the effect of R was the same whether followed by a dark period of 4, 16 or 40 h.

In high WL (31 W m^{-2}) Chl a accumulation was inhibited compared to low WL during the first hours of irradiation (Fig. 3.2). Newly formed Chl is probably photobleached at this high irradiance (e.g., Dorsman et al., 1977; Axelsson and Selstam, 1979; Ryberg, 1980). Although R pre-irradiation did not completely prevent this, its large effect indicates that besides exerting a stimulating effect on the Chl biosynthesis system (Chapters 6 and 7), R also stimulates the formation of a protection mechanism against photobleaching of Chl. A similar conclusion was reached for mustard seedlings by Oelze-Karow et al. (1983). Carotenoids are thought to play an important role in protection of Chl against photobleaching (e.g., Malhotra et al., 1982). In contrast to reports in the literature, under our experimental conditions R pre-irradiation had no effect on total carotenoid content of seedlings in subsequent darkness and little effect on the rate of carotenoid formation in WL (Figs. 3.3 and 3.4). The present results agree with those of Cohen and Goodwin (1962) who found that although R stimulates carotenoid synthesis in 4- or 5-day old maize seedlings, R stimulation is negligible in maize seedlings of more than 6 days old. R dependent formation of specific carotenoids and/or association of Chl molecules with carotenoids may be crucial for the protection of Chl against photobleaching (see Chapter 8).

Logarithmic fluence-response curves for rapid Chl accumulation in WL for pea (Raven and Shropshire, 1975; Spruit et al., 1979), bean (Spruit et al., 1979) and maize (Fig. 3.5) have been interpreted as biphasic. The VLFR and LFR range for maize are very similar to those observed for other biphasic fluence responses in this (e.g., Vanderhoef et al., 1979) and other plant species (e.g., Cone, 1985). Within the error limits of the data, the

fluence-response relationships shown in Fig. 3.5 saturate at 1 mmol m^{-2} R, corresponding to that fluence which attains the maximum percentage of Pfr possible. Previous workers have provided evidence for additional potentiation above this level in pea (Raven and Shropshire, 1975) implicating the involvement of a high irradiance reaction (HIR, Chapter 1, Section 1.1). On the basis of the present data it is impossible to eliminate the involvement of an HIR and obviously more detailed studies at high fluences would be of interest. The present data clearly demonstrate some response in the VLFR range in the case of dark-grown seedlings in agreement with Raven and Shropshire (1975). Additionally, after preliminary irradiation ("de-etiolation") the response is restricted to the LFR range. This decrease in sensitivity for R was explained for pea by the P transport model of Raven and Spruit (1973). The model predicts a second R pre-irradiation to have an additional potentiating effect after dark reversion of receptor-bound Pfr to Pr. Since in maize no Pfr dark reversion has been demonstrated (Frankland, 1972), no effect of a second R pre-irradiation was expected, contrary to observation. However, the possibility has to be considered that in contrast to bulk P (Chapter 1, Section 1.5), Pfr present at the receptors is subject to dark reversion in maize (see Chapter 8 for further discussion).

In maize, a R pre-irradiation 40 h prior to low WL proved still partially reversible by FR given 16 h prior to WL. This implies the presence of Pfr active in potentiation, 24 h after R, despite the fact that Pfr destruction occurs in bulk P during the dark period (Chapter 4, see also Chapter 8). A second pre-irradiation, 16 h prior to low WL, will inevitably influence the Pfr level left after the first R pre-irradiation. Taking this into account, the extent of FR reversibility proved similar for a single and a second R pre-irradiation in maize seedlings. Also, the difference in FR reversibility between completely dark-grown and "de-etiolated" pea and bean seedlings claimed by Raven (1973) should be similarly considered. R potentiation in previously dark-grown seedlings is predicted by the transport model (Raven and Spruit, 1973) to be non-photoreversible by FR during the period of migration of Pfr to the proposed receptors. The claimed lack of photoreversibility by FR of R potentiation in dark-grown pea seedlings was explained in this way (Raven, 1973). However, there are indications that the half-life of migration is only a few seconds at room temperature (e.g., Spruit et al., 1979; Pratt, 1979) implying that migration was complete at the end of a 5 min or even a 20 s R pre-irradiation. In this case, a R pre-irradiation is predicted to be reversible by subsequent FR. In maize, the potentiating effects of both a first and a second R pre-irradiation appeared only partially reversed by FR. Therefore, an alternative explanation for the incomplete photoreversibility has to be found. Most likely, a rapid escape of potentiation from Pfr control takes place. For several P reactions an escape from Pfr control within 5 min has been reported (e.g., Jabben and Mohr, 1975; Girnth et al., 1978; Warner et al., 1981). However, part of the R potentiation in maize seedlings remains under Pfr control even over prolonged dark periods (see above).

4 THE PRESENCE OF PHYTOCHROME IN ETIOPLASTS

4.1 Introduction

Cooke et al. (1975) were the first to report the presence of phytochrome (P) in wheat etioplasts. They detected P spectrophotometrically in preparations purified by the Sephadex G-50 (coarse) column method of Wellburn and Wellburn (1971a). Evans (1976), Evans and Smith (1976a,b) and Hilton and Smith (1980) observed P in barley etioplasts obtained by a similar procedure. However, Quail (1977) claimed that P co-eluting with oat etioplasts on a Sephadex G-50 column was not detectable in the etioplast pellet after centrifugation of the column eluate at 3,000 x g.

Preparations of etioplasts purified by a Sephadex G-50 column are contaminated by mitochondria (Chapter 2, Sections 2.5 and 2.6). Therefore, it remains possible that P detected in these preparations is associated with the contaminating mitochondria. That P is present in or on mitochondria has been reported by Manabe and Furuya (1974) and Billett and Smith (1978). However, Hilton and Smith (1980) showed that a small part of P present in the Sephadex G-50 column eluate was consistently associated with the pure etioplast fraction of the eluate recovered from a discontinuous sucrose gradient.

The authors reporting the presence of P in etioplast preparations have always isolated etioplasts from dark-grown seedlings under a green safelight. Since Raven in 1973 had already shown that even a minimal fluence of green safelight can cause de-etiolation of seedlings, the possibility has to be considered that a minute amount of the far-red light (FR) absorbing form of P, Pfr, formed by the safelight becomes associated with the etioplasts. For this reason etioplasts were isolated in complete darkness. If the etioplasts are the targets for Pfr of the transport model, it is to be expected that they will be enriched in P after red light (R) irradiation.

4.2 Results

4.2.1 Phytochrome assay - general aspects

In liquids with a relatively high P content, for example a filtered leaf homogenate, P was readily detected spectrophotometrically in cuvettes with a 10 mm path length. For measurements of P in e.g. etioplast preparations, a scattering agent such as $CaCO_3$ had to be added to increase the optical path length and hence the detection level. However, P measurements in 10 mm cuvettes with $CaCO_3$ added were not reliable (Table 4.1), probably because the samples are so highly opaque that most of the light reaching the photomultiplier has not traversed the sample, but has e.g. followed a path by multiple reflections along the side walls of the cuvette. Moreover, the low light fluence transmitted by such samples greatly reduces the signal-to-noise ratio. The optimum path length for P measurements in our samples mixed with $CaCO_3$ proved to be 1.6-2.0 mm. Cuvettes with a path length of 1.6 mm were used unless otherwise indicated. The coefficient of variation (Chapter 2,

Table 4.1 Spectrophotometric assay of the phytochrome (P) content ($10^4 \Delta$ (ΔA) 730/806) in a supernatant obtained by centrifugation of a filtered leaf homogenate of dark-grown maize leaves at 1.700 x g, in black-sided cuvettes of different path lengths. The cuvettes with a path length of 10 mm contained 2 ml samples to which 2 g $CaCO_3$ was added. The cuvettes with a path length of 1.6 mm contained approximately 0.2 ml samples plus approximately 0.3 g $CaCO_3$.

Sample	Apparent P content	
	10 mm cuvette	1.6 mm cuvette
Supernatant (undiluted)	10.5	15.9
Duplicate sample	28.3	20.3
Supernatant (diluted 1:1)	38.7	8.4
Duplicate sample	19.9	8.4

Section 2.16) for the measurements was about 15%. Several other scattering and stabilization media for P measurements in etioplast preparations were tried. These included Ficoll, hydroxyethylcellulose and Sephadex G-50 (coarse), but none of these proved satisfactory. The spectrophotometrically detectable P content of samples did not significantly decrease during storage for several days at -18 °C.

4.2.2 Estimation of the phytochrome content of various fractions obtained during etioplast isolation and the Sephadex G-50 procedure

P was observed in most fractions obtained during etioplast isolation and purification (Fig. 4.1). 98-99% of P measured in the leaf homogenate remained in the supernatant upon centrifugation at 1,700 x g. After washing and centrifugation of the first pellet containing the etioplasts, about 65% of P present in the pellet remained in the supernatant. During elution of the resulting pellet (a "crude, washed etioplast preparation") on a Sephadex G-50 column, additional P was lost. This P was possibly associated with (broken) etioplasts and other cell components which remained on the column. After centrifugation of the column eluate at 1,700 x g, the amount of P in the supernatant was near the detection limit. Although the pellet (a "Sephadex G-50 purified etioplast preparation") contained only a small proportion of the P originally present in the leaf homogenate, its P signal usually was far above the detection limit (Chapter 2, Fig. 2.4). The P detectable in Sephadex G-50 purified etioplast pellets was not removed by (repeated) washing of the pellet in isolation buffer (Table 4.2).

P was detected in preparations of purified etioplasts isolated from R pre-irradiated maize seedlings (R etioplasts) as well as from dark-grown

seedlings (D etioplasts), even if the etioplasts were isolated and purified in complete darkness. Addition of Mg^{2+} either as 10 or as 100 mM $MgCl_2$ to the isolation medium had no influence on the amount of etioplast-associated P both in D and in R Sephadex G-50 purified etioplast preparations. Small quantities of P were also detected in Sephadex G-50 purified D and R etioplast preparations from pea, bean and oats.

4.2.3 Phytochrome in etioplast preparations free from mitochondria

The discontinuous sucrose gradient procedure of Hilton and Smith (1980) was used to separate mitochondria from etioplasts. Both D and R maize etioplast preparations purified by the Sephadex G-50 column technique were used. In both cases about 50% of P originally present in the preparations appeared to be present in the purified etioplasts recovered from the fraction banding at the 55/40% or 65/40% sucrose interface as well as from the pellet at the bottom of the sucrose gradient (Chapter 2, Section 2.6).

The pellet obtained by centrifuging the fraction banding at the 40/25% interface at 16,000 x g contained about 15% of the P content of the original etioplast preparation. According to Hilton and Smith (1980) this fraction

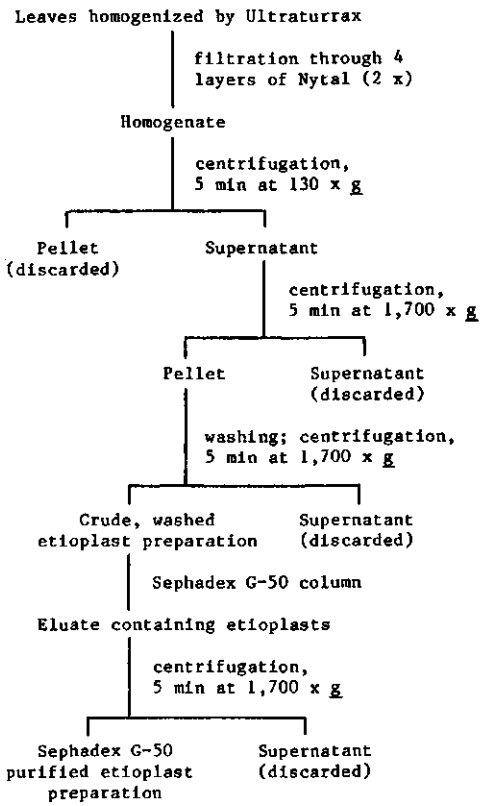


Fig. 4.1 Scheme of etioplast isolation and purification.

Table 4.2 Phytochrome (P) in Sephadex G-50 purified maize etioplast preparations after washing, as a percentage of P in the non-washed preparation.

Pretreatment of intact seedlings	P present after washing (%)	
	1 x washing	2 x washing
Dark grown plants	88	n.d.
5 min R + 25 min D[a]	84	n.d.
5 min R + 2 h D	89	81
5 min R + 4 h D	109	n.d.
5 min R + 5 h D	81	100

[a] D: dark period between red light (R) pre-irradiation of intact seedlings and etioplast isolation
n.d.: not determined

contains both mitochondria and etioplasts. Counts of etioplasts showed that the fraction contained about 10 x less etioplasts than the combined fractions containing pure etioplasts. The etioplasts of the 40/25% interface fraction are predicted to contribute about 5% of the total P content, leaving 10% for possible association with mitochondria. About 35% of P originally present in the preparations was not recovered from the pellet at the bottom of the sucrose gradient plus the 55/40% or 65/40% and 40/25% interfaces.

We can tentatively conclude from our experiments that at least 50% of P measured in Sephadex G-50 purified maize etioplast preparations is present in or on the etioplasts and not in the contaminating mitochondria.

4.2.4 Gel filtration on Sepharose CL-2B

In Cucurbita pepo, two types of association of P with particulate cell material have been demonstrated (Quail, 1975; Jose, 1977). One of them is formed by an association of P with ribonucleoprotein (RNP) and is considered to be an artifact. The other is proposed to be a binding of P to a membrane fraction. Separation of membrane-associated from dissolved and RNP-adsorbed P was obtained by linear sucrose gradients (Quail, 1975) and gel filtration on Sepharose CL-2B (Jose, 1977). The latter technique was used to examine whether P present in the etioplast preparations here was RNP-adsorbed.

After elution of Sephadex G-50 purified etioplast preparations on Sepharose CL-2B, P was detected in two protein bands with K_{av} (see Abbreviations) similar to those in Cucurbita pepo. Apparently some RNP-adsorbed P was present in our etioplast preparations. However, the etioplast band contained at least 50% of P present in the original etioplast preparation.

4.2.5 Suborganelle localization of phytochrome

Etioplasts were isolated from dark-grown and red light (R) pre-irradiated

maize seedlings in MOPS buffer (Chapter 2, Section 2.4) and purified by the Sephadex G-50 column method. The purified preparations were fractionated into envelope, prolamellar body and stroma fractions by the method of Mackender and Leech (1974) as modified by Evans and Smith (1976a), see Fig. 4.2. MOPS buffer without sucrose was used as a lysis medium.

After exposure to the lysis medium for 15 min at 4 °C, many etioplasts appeared still intact when examined by phase contrast microscopy. The possibility exists that under these conditions the envelopes first have become slightly opened and then reconstituted. Therefore, the etioplasts were sonicated in the lysis medium until phase contrast microscopy indicated that all etioplasts were broken.

In agreement with results of Evans and Smith (1976a), P was demonstrated only in the 20 KP fraction (Fig. 4.2) containing the plastid envelopes. In the other fractions P signals were at or below the detection limit.

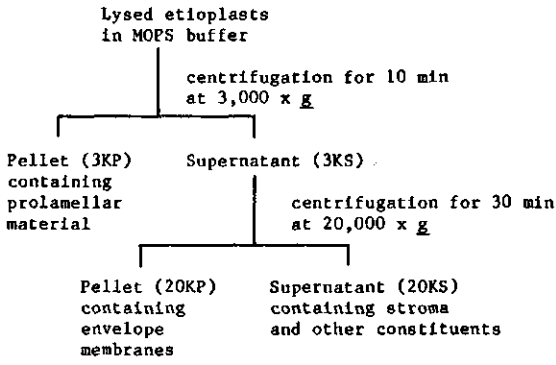


Fig. 4.2 Fractionation of lysed etioplasts.

4.2.6 Phototransformation difference spectrum of phytochrome

To confirm unequivocally the presence of P in the etioplast preparations, a phototransformation difference spectrum was constructed. Measurements of (A) were made at 0 °C at several wavelengths between about 630 to 780 nm, with the reference beam at 806 nm. One etioplast preparation was used for each spectrum. After measurements at three different wavelengths, a measurement at 730 nm was included as a control. These control measurements showed that the P content did not decrease significantly during the measuring period of several hours.

The phototransformation difference spectrum for P in a crude, washed D etioplast preparation from pea leaves is shown in Fig. 4.3. The FR peak (FRmax) was found at about 726 nm and the R peak (Rmax) was between 660 and 670 nm. The isosbestic point in the difference spectrum was located between 680 and 690 nm. The ratio of the absorption peaks at Rmax (A Rmax) and FRmax (A FRmax) was about 1.2 : 1, which falls within the range normally observed *in vivo* (Grill, 1972; Spruit and Kendrick, 1973).

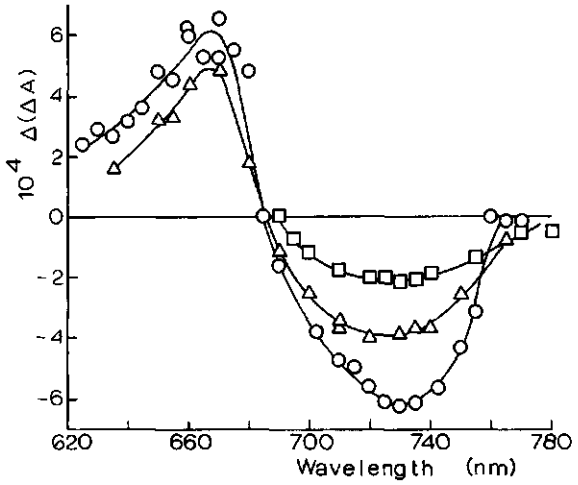


Fig. 4.3 Phototransformation difference spectra (Pfr → Pr) of △ : a crude, washed etioplast preparation from 8-day old dark-grown pea leaves, □: a Sephadex G-50 purified etioplast preparation from 8-day old dark-grown maize leaves (only the far-red part is shown), ○: a homogenate of 8-day old dark-grown maize leaves.

The phototransformation difference spectra for P in Sephadex G-50 purified etioplast preparations from dark-grown or R pre-irradiated maize seedlings had a FRmax between 725 and 730 nm (Fig. 4.3). The 620 - 690 nm region of these spectra could not be measured with precision due to time- and irradiation dependent changes in PChl(ide) and Chl(ide) content. In the pea etioplast preparation used for the measurements shown in Fig. 4.3, (P)Chl(ide) pigments probably did not appreciably interfere with the P measurements for the following reasons. (i) In pea etioplast preparations the ratio P:(P)Chl(ide) appeared to be much higher than in maize etioplast preparations. (ii) The pea etioplast preparation was kept overnight at 4 °C in darkness and probably had lost its ability for PChl(ide) regeneration (see Chapters 6 and 7).

The phototransformation difference spectrum for P in a filtered maize leaf homogenate (Fig. 4.3) appeared "normal" (Quail, 1974). The Rmax was found between 660 and 670 nm, the FRmax at about 730 nm, the isosbestic point at about 685 nm and the A Rmax : A FRmax ratio was about 1 : 1.

4.2.7 Phytochrome content of etioplasts isolated from dark-grown or red pre-irradiated maize leaves

Under our experimental conditions, R pre-irradiation (5 min 658 nm, 0.85 W m^{-2} or 5 μmol m^{-2} s^{-1}) did not significantly influence the fresh weight of maize and pea leaf material over a subsequent 16 h dark period. Also the total amount of carotenoids per gram leaf fresh weight as indicated by the absorbance at 473 nm (A473) was not significantly influenced by R pre-irradiation (Chapter 3). Therefore, the A473 nm in 80% acetone/water (v/v) was

used as an estimate of the number of etioplasts in a maize or pea etioplast preparation.

When Δ (ΔA) 730-806 values were normalized to an A473 of 1.0, the P content of R maize etioplasts slightly exceeded that of D etioplasts (Fig. 4.4). The difference was significant ($p<0.05$) for crude, washed etioplasts and just below significance for Sephadex G-50 purified etioplasts (Student's t-test). The P content of etioplasts isolated in green safelight from dark-grown maize seedlings was slightly higher than that of D etioplasts isolated in complete darkness, but lower than that of R etioplasts for both types of preparations.

4.2.8 Estimation of the percentage of leaf phytochrome present in etioplasts

About 10-15% of leaf P remained in the residue upon filtration of the leaf extract. Therefore, as an estimation of the P content of the leaves 115% of the P content of the leaf homogenate was taken. The proportion of the combined leaf etioplasts present in preparations in which P was measured was estimated assuming a linear relationship between the etioplast content of leaves and etioplast preparations and the carotenoid absorbance at 473 nm in their 80% acetone/water (v/v) extracts. Under the experimental conditions here, addition of $CaCO_3$ resulted in an approximately 14-fold amplification in

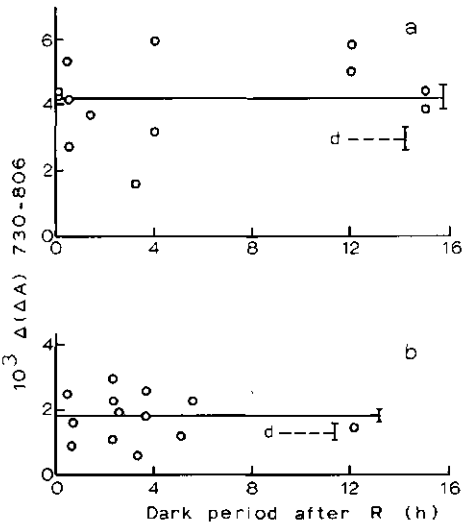


Fig. 4.4 Phytochrome content of etioplasts isolated from 8-day old maize leaves after various dark periods following red (R) pre-irradiation. The phytochrome content is expressed as Δ (ΔA) 730-806 for preparations of A473 = 1.0. a. crude, washed etioplast preparations, b. Sephadex G-50 purified etioplast preparations. The full lines represent the average of the data points. d: average value for etioplasts from 8-day old dark-grown maize leaves. The vertical bars represent the standard error of the mean.

signal both for homogenate and for supernatant samples. Although no exact data are available for etioplast samples, it is estimated that addition of $CaCO_3$ increased their P signal at least 10-fold, making both types of preparations comparable. Assuming the P content of Sephadex G-50 preparations being representative of the P content of etioplasts in vivo, about 1.7% of total maize leaf P appeared associated with D etioplasts. For pea etioplasts, this percentage was about 1.3%.

In R pre-irradiated maize leaves, Pfr dark destruction occurred as indicated by the decrease in P content of the leaf homogenates (Fig. 4.5). Due to the scatter in the values it was not possible to establish the kinetics of the reaction. The half-time agrees with that normally found for Pfr dark destruction (e.g., Stone and Pratt, 1978). After a dark period of 4 h at 22 °C, 25-30% of the amount of P present in homogenates of dark-grown leaves remained. As the P content of R etioplasts remained essentially constant (Fig. 4.4), the percentage of total leaf P associated with the etioplasts increased over this period. A regression line calculated for such data suggests a small but statistically insignificant increase of the P content of etioplasts soon after R pre-irradiation from approximately 1.7% to 2.4% (Kraak and Spruit, 1985).

Although only limited data were obtained for pea etioplasts, they were qualitatively similar to those obtained with maize etioplasts.

4.2.9 Pr and Pfr dark reactions in etioplast preparations

During storage of etioplast preparations in darkness at room temperature, their spectrophotometrically detectable P content decreased. We have studied whether the rate of this decrease was different for Pr and Pfr. After measuring the P content of Sephadex G-50 purified etioplast preparations from dark-grown seedlings, the preparations were irradiated with actinic R or FR

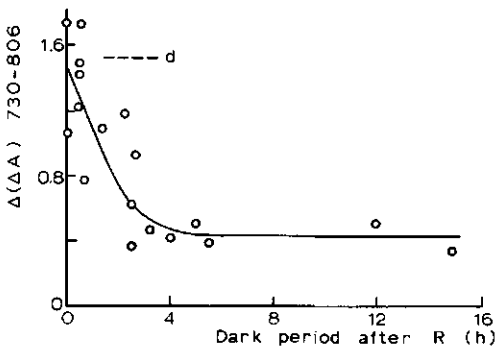


Fig. 4.5 Phytochrome content of 8-day old maize leaves after various dark periods following red light (R) pre-irradiation. Phytochrome was measured in leaf homogenates and is expressed as Δ (ΔA) 730-806 per 100 g of leaf material. d: phytochrome content of 8-day old dark-grown maize leaves.

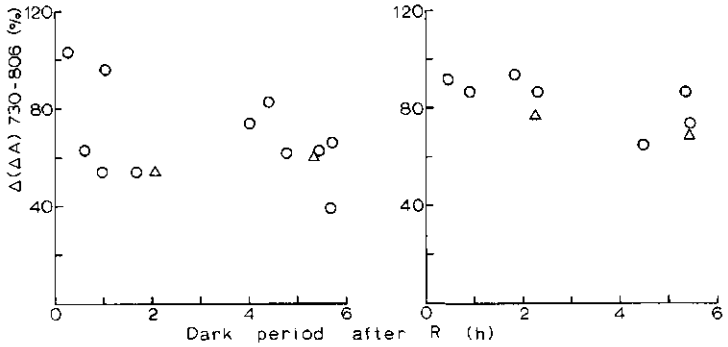


Fig. 4.6 Spectrophotometrically detectable phytochrome in darkness at room temperature after a terminal red (R) (○) or far-red light (Δ) irradiation in a. etioplast preparations from dark-grown maize leaves, b. homogenates of similar leaves. The original phytochrome content of the preparations was taken as 100%.

for 2 min. The cuvettes were subsequently kept in darkness at 22 °C and after a specific time interval their P content was again measured. Before all P measurements, PChl(ide) was photoconverted into Chl(ide) by a 3 min R irradiation. Fig. 4.6 shows a limited loss of both spectrophotometrically detectable Pr and Pfr in maize etioplast preparations as well as in maize leaf homogenates during a storage period of 6 h at 22 °C. The rate was slow compared to Pfr dark destruction in vivo (Fig. 4.5).

Direct spectrophotometric demonstration of Pfr dark reversion in etioplast preparations proved impossible. Even when FR was given as a first actinic irradiation, measurements of Pfr were unreliable because photoconversion of PChl(ide) into Chl(ide) interfered with the measurements.

4.3 Discussion

No completely satisfactory procedure for the isolation of undamaged etioplasts of high purity with a high yield exists. Sephadex G-50 purified etioplast preparations are contaminated by mitochondria (Evans, 1976; Quail, 1977; Hilton and Smith, 1980). In electron micrographs of our Sephadex G-50 purified etioplast preparations, mitochondria were occasionally observed. The activity of triose phosphate isomerase (Quail, 1977) and the presence of cytoplasmic RNA (Evans, 1976) in these preparations have been indications of other contaminants. However, the presence of P remaining after (repeated) washing and further purification of Sephadex G-50 etioplast preparations on a discontinuous sucrose gradient and on Sepharose CL-2B support the conclusion that a substantial fraction of the pigment detectable in these preparations is associated with the etioplasts. Etioplast-associated P appears to be mainly, if not completely, present in or on the etioplast envelope (Section 4.2.5; Evans and Smith, 1976a). Phytochrome phototransformation difference spectra of maize etioplast preparations showed large deviations in the R region due to

interference by PChl(ide) and Chl(ide) pigments. However, the difference spectrum for P in a pea etioplast preparation leaves no doubt that the absorption changes measured at 730/806 nm in etioplast preparations are mainly due to Pr → Pfr interconversions. The absorption maximum of Pfr present in pea and maize etioplast preparations was between 726 and 730 nm, i.e. slightly lower than in vivo (e.g., Quail, 1974). Vierstra and Quail (1982) showed that native 124 kilodalton (kDa) Pfr from Avena has its absorption maximum at 730 nm. On incubation as Pr, this maximum shifts to 722 nm, concomitant with a proteolytic degradation to 118 and 114 kDa P. On the basis of its FRmax, it can be concluded that P in etioplast preparations and in maize leaf homogenates only partially shows this degradation.

It is difficult to exclude the possibility that P becomes attached to the etioplast membranes during isolation of the etioplasts. Bound P, detected in vitro, does not necessarily imply that P is also bound in vivo (e.g., Pratt, 1979). On the other hand, it is possible that P molecules associated with etioplasts in vivo are released during leaf homogenization and subsequent procedures. Crude, washed etioplast preparations contain more P on a carotenoid basis than Sephadex G-50 purified preparations. This may indicate that P is lost from etioplasts during purification, but other possibilities exist (Section 4.2.2). In this connection, the results of Brownlee et al. (1979) are of interest. They found that the large amount of Pr associated with particulate material at low pH could be decreased by washing in media of high pH. It is not known whether a low pH prevents the release of native Pr or causes a non-specific association of Pr. Other factors apart from pH might also influence the amount of P detectable in etioplast preparations. However, it has been shown here that the association of P with etioplasts is not dependent on the presence of divalent cations such as Mg^{2+}, whereas pelletability is enhanced by these ions (Pratt and Marmé, 1976). Also, gel filtration on Sepharose CL-2B showed that most of the P present in etioplast preparations is not bound to ribonucleoproteins. Evidence for P forming an intrinsic component of the etioplast is provided by an in vitro effect of P on the amount of extractable gibberellins by etioplasts (Cooke and Saunders, 1975; Cooke and Kendrick, 1976; Evans, 1976). However, the physiological meaning of this effect is not clear.

If the P content of Sephadex G-50 purified etioplast preparations does indeed reflect the in vivo P content of etioplasts, the results here indicate that etioplasts of dark-grown maize seedlings contain 1.7% of total leaf P (Section 4.2.8). Assuming that the P concentration in cells of dark-grown seedlings is about 10^{-7} M (Kendrick and Frankland, 1983), a plant cell with a volume of about 10^3 μ^3 would contain about 6×10^6 P molecules. At a concentration of about 1.7% of P, about 10^5 P molecules would be present in the combined etioplasts of such a cell. Since there are on an average about 100 etioplasts per cell (Leech, 1984), one etioplast would then contain about 10^3 P molecules. If the etioplasts contain only 25-50% of P present in Sephadex G-50 preparations (Sections 4.2.3 and 4.2.4), still some hundred P

molecules would reside in or on each etioplast.

Results shown in Sections 4.2.7 and 4.2.8 suggest that R pre-irradiation of maize seedlings may result in a small increase of the quantity of P in the etioplasts. Smith *et al.* (1978) reported that when mixtures of the 100,000 x *g* supernatant of the homogenate of etiolated barley leaves (containing soluble P) and Sephadex G-50 purified etioplasts were R irradiated *in vitro*, the amount of pelletable P per mg protein increased two-fold. The authors suggested that Pfr molecules form dimers. If one of the two molecules forming a dimer is a component of the etioplast membrane, then subsequent centrifugation would pellet the dimer. Roth-Bejerano and Kendrick (1979) also found an approximately two-fold increase of pelletable P per mg protein after *in vivo* or *in vitro* R irradiation in 1,000 x *g* and 20,000 x *g* pellets of crude homogenates of etiolated barley leaves. Brownlee *et al.* (1979) similarly speculated that this two-fold increase might result from the formation of Pfr:Pfr dimers after irradiation, one Pfr molecule being a native membrane-associated molecule and one Pfr molecule binding *via* this molecule and to a steroid component of the membrane. However, the results here with maize and pea etioplasts do not support the dimer hypothesis. The increase of the P content of the etioplasts after R irradiation, if any, was less than two-fold.

The P transport model of Raven and Spruit (1973) assumed that the Pfr receptors are devoid of P in dark-grown plants. On the assumption that the etioplasts are the sites of the Pfr receptors, a revised model should incorporate that in dark-grown plants these receptors are already partially occupied by P molecules in the R absorbing form, since P was detected in etioplasts isolated in complete darkness form dark-grown seedlings. In agreement with the original model, we now postulate that unoccupied receptor sites for P molecules are present in etioplasts and that only Pfr molecules are able to bind to these sites. Following R irradiation, Pfr molecules formed in the cytoplasm are proposed to migrate to the etioplasts and become active in potentiation of rapid Chl accumulation in white light (WL). It appeared important to examine whether a relatively small increase in amount of P molecules per etioplast would be sufficient to explain the considerable decrease in sensitivity for R potentiation following R pre-irradiation. Since the quantity of P in the cytoplasm vastly exceeds that in the etioplasts, in the original as well as in the revised model small fluences of R would form enough Pfr molecules outside the etioplasts to saturate the Pfr receptor sites of the etioplasts. However, any P originally associated with the etioplasts would then still be mainly in the Pr form. To give an illustration: results shown in Section 4.2.8 suggest that about 0.7% of total P may migrate to the etioplasts after a standard R pre-irradiation. As a standard R irradiation results in formation of the maximum percentage of Pfr possible, we may assume that all Pfr binding sites then become occupied. A fluence just sufficient to form 0.7% of Pfr in the cytoplasm might be sufficient to saturate the receptor sites in the etioplasts, though higher fluences are required if the etioplasts

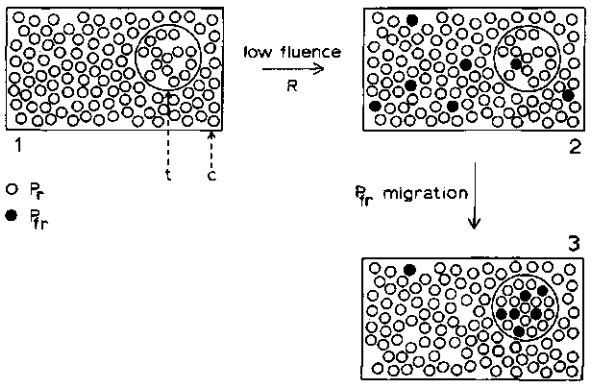


Fig. 4.7 Proposed model of migration of Pfr from the cytoplasm to the etioplasts following a low fluence of red light (R), transforming 0.7% of Pr into Pfr. 1: dark-grown, 100% Pr; 2: low R fluence: 0.7% Pfr formed in cytoplasm and receptor organelle; 3: after migration of Pfr, the receptor organelle has about 30% Pfr, the cytoplasm less than 0.7%. c: cell, t: receptor (or target) organelle.

have to "compete" for Pfr with other organelles and membranes. A R fluence which converts 0.7% of cytoplasmic P into Pfr would also convert 0.7% of Pr originally present in the etioplasts into Pfr. Since we have found that about 1.7% of total leaf P initially resides in the etioplasts, we calculate that after the migration process is complete, 30% of P present in the etioplasts (2.4% of total P) is in the Pfr form (see Fig. 4.7). Obviously, migration to the etioplasts of a fraction of cytoplasmic Pfr, too small to be spectrophotometrically detectable, would meet the requirements of the model.

We may expect that a further potentiation of rapid Chl accumulation occurs when the remainder of Pr in the etioplasts is phototransformed into Pfr. As additional migration of Pfr is impossible, considerably higher R fluences are required to give an appreciable additional potentiating effect by conversion of the organelle Pr. In case a first R pre-irradiation is saturating with regard to Pr → Pfr phototransformation, either occurrence of Pfr dark reversion in the receptor sites or resynthesis of receptor sites has to be postulated for a second R pre-irradiation to give an additional potentiating effect (see Chapters 3 and 8 for further discussion).

The postulated migration of about 0.7% of total P to the etioplasts after R irradiation is statistically not significant. Therefore, no supporting evidence was obtained from our measurements for the P transport model of Raven and Spruit (1973). Although P was consistently found associated with etioplasts, it remains uncertain whether the in vivo P acts directly upon the etioplasts in controlling rapid Chl accumulation in WL. A different approach was therefore followed in an attempt to obtain information on this point (Chapters 5, 6 and 7).

5 ULTRASTRUCTURAL DEVELOPMENT OF ETIOPLASTS *IN SITU* AND *IN VITRO* UPON IRRADIATION

5.1 Introduction

Greening and ultrastructural development of etioplasts into chloroplasts are interconnected. For example, the presence of chlorophyll (Chl) appears to be a prerequisite for the formation of grana (Mohr and Kasemir, 1975). The greening process (*e.g.*, Raven, 1973; Kasemir, 1983b) as well as ultrastructural development of etioplasts (Bradbeer and Montes, 1976; Girnth *et al.*, 1979) is documented to be under the control of phytochrome (P).

Isolated etioplasts display ultrastructural changes upon irradiation (Kohn and Klein, 1976; Wellburn and Wellburn, 1971b,c; 1973a,b). An effect of P upon etioplast development *in vitro* has been concluded by Wellburn and Wellburn (1973b), however according to others (Bradbeer *et al.*, 1974; Bradbeer and Montes, 1976) their experiments are not wholly convincing.

We have examined, whether red light (R) pre-irradiation affects the ultrastructural development of etioplasts in white light (WL) both *in situ* and *in vitro* under conditions similar to those in Chapter 3. However, a WL period of 1 h instead of 5 h was used, since after longer periods in WL little further development of isolated etioplasts is shown and etioplasts begin to deteriorate (Wellburn and Wellburn, 1973b).

Initial experiments were carried out with maize etioplasts. Maize leaves contain two types of plastids: bundle sheath and mesophyll plastids. According to Klein *et al.* (1975), in 10-day old dark-grown maize leaves a circular arrangement of thylakoids, radiating out from the prolamellar body (PLB) is typical of mesophyll etioplasts and a straight arrangement typical of bundle sheath etioplasts. However, in the present electron micrographs the distinction between bundle sheath and mesophyll etioplasts was usually not evident. Since both types of etioplasts might show different developmental changes which would complicate the analysis of effects of a R pretreatment, further experiments were conducted with etioplasts from seedlings of other species.

5.2 Results

5.2.1 The ultrastructure of maize, bean and oat etioplasts after Sephadex G-50 purification

Sephadex G-50 purified maize and oat etioplast preparations contain mainly intact etioplasts with a continuous double outer membrane (envelope) and a dense stroma. The etioplasts are similar in appearance to the corresponding etioplasts in intact leaves, though more rounded. "Broken" or "damaged" etioplasts are swollen and contain only lightly stained stroma (Fig. 2.2, Chapter 2). Although electron micrographs of isolated bean etioplasts usually showed an intact envelope, the internal structure of these etioplasts was unlike that *in situ*. Tubules, crystal-containing bodies (Gunning, 1965) or

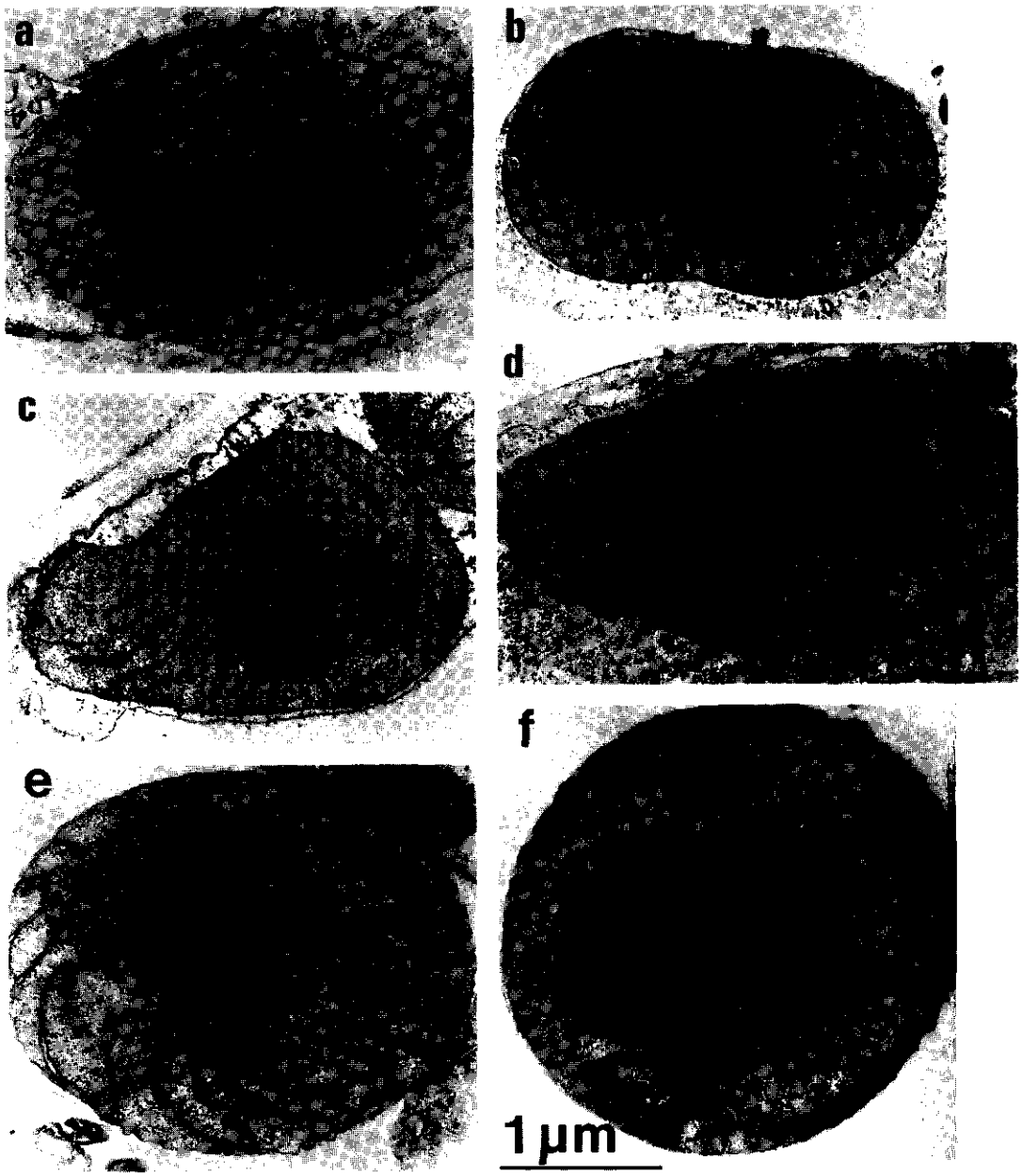


Fig. 5.1 Electron micrographs of etioplasts of 8-day old oat seedlings, a-d *in situ*, e-f *in vitro*, a. in a dark (D)-grown leaf, b. in a leaf pre-irradiated with red light (R) (5 min 658 nm, 5 µmol m^{-2} s^{-1}), 8 h after R pre-irradiation, c. in a leaf after 55 min of white light (WL) (2 W m^{-2}), d. in a leaf after 5 min R followed by 8 h D and 55 min WL, e. etioplast isolated from a R pre-irradiated leaf, f. as e, after 55 min WL *in vitro*.

crystallites (Wellburn and Wellburn, 1971a) and magnograna (e.g., Klein and Bogorad, 1964) were found as isolation artifacts in bean etioplasts. Only remnants of PLBs were observed, while bean etioplasts in situ contained paracrystalline PLBs. Obviously, bean etioplasts are not suitable for studies on effects of light treatments on the ultrastructural development of etioplasts in vitro.

Etioplasts of 8-day old maize and bean seedlings frequently contained one or more starch grains, which were often surrounded by a thylakoid-like membrane. The many free, mostly large, starch grains seen in electron micrographs of purified etioplast pellets suggest that specifically etioplasts containing the larger sized starch grains are susceptible to rupture. In etioplasts of 8-day old oat seedlings starch grains were seen less frequently and were generally small.

The PLBs in etioplasts of dark-grown maize, bean and oat seedlings were mostly paracrystalline (Gunning and Jagoe, 1967) in appearance. An example for oats is shown in Fig. 5.1a. Also (slightly) irregular, but tight, PLBs (Fig. 5.1d), and in oat etioplasts some atypical PLBs (Gunning, 1965) were seen. After 1 h of WL reacted (Weier et al., 1970) or loose (Horton and Leech, 1975) PLBs were present (Fig. 5.1c). Oat etioplasts sometimes contained a stromacentre (Gunning, 1965; Wellburn and Wellburn, 1971a) which is probably composed of molecules of ribulosediphosphate carboxylase (Gunning et al., 1968; Kirk and Tilney-Bassett, 1978). Incipient grana (short thylakoid overlaps) were observed in etioplasts of maize, bean and oat seedlings even when grown in complete darkness (D). Etioplast electron micrographs showed numerous ribosomes. Osmiophilic globuli were observed both inside and outside the PLBs.

Effects of irradiations were studied with respect to four morphological characteristics: PLB structure, PLB volume, the number of thylakoids with a length of at least one third or at least two thirds of the length of the etioplast (medium long and long thylakoids, respectively) and the number of incipient grana with two or more stacks. Maize and bean etioplasts were found unsuitable for these studies for the reasons mentioned above. Therefore the results are mainly restricted to oat etioplasts.

5.2.2 Changes in prolamellar body structure upon irradiation

About 80% of the etioplasts in 8-day old dark-grown oat leaves contained PLBs with a paracrystalline appearance (Fig. 5.1a), the remaining 20% showing irregular but tight PLBs (Table 5.1). After 55 min of WL of low fluence rate (2 W m^{-2}), the percentage of paracrystalline PLBs had considerably decreased and many loose PLBs were observed (Fig. 5.1c).

After R pre-irradiation of the plants (5 min 658 nm, 0.85 W m^{-2} or 5 μmol m^{-2} s^{-1}), followed by 8 h of D, almost all PLBs had a regular, paracrystalline appearance (Fig. 5.1b). After 55 min WL most PLBs in R pre-irradiated oat leaves still had a tight, paracrystalline or slightly irregular appearance (Table 5.1; Fig. 5.1d).

PLBs in etioplasts isolated from R pre-irradiated oat seedlings (R etioplasts) were very similar in structure to those *in situ* (Fig. 5.1e). After 55 min WL *in vitro* the structure of the PLBs had changed (Fig. 5.1f). However, the effect of R pre-irradiation, apparent upon etioplasts in intact oat leaves, was not observed in isolated etioplasts (Table 5.1).

5.2.3 Changes in prolamellar body volume upon irradiation

The area of PLBs compared to total etioplast area, which gives an indication of the PLB volume, was quantified by a weight method described by Horton and Leech (1975) (Section 2.15). It was verified that observed changes in relative PLB area were not due to changes in total etioplast area. Moreover, the number of etioplast micrographs without PLB can be regarded as a criterion for the volume of the PLB (Henningsen and Boynton, 1974).

The average PLB volume in etioplasts *in situ* slightly increased after R pre-irradiation. After 55 min WL the PLB volume in previously dark-grown oat leaves had hardly changed. However, in R pre-irradiated leaves the PLB volume decreased somewhat during 55 min WL. Differences observed were not significant (Student's t-test). However, results obtained by the method of

Table 5.1 Structure of prolamellar bodies (PLBs) of oat etioplasts after different light treatments.

Sample	Light treatment	Percentage of PLBs with the following structure:			No. of PLBs studied
		paracrystalline (including atypical PLBs)	(slightly) irregular, tight	reacted (loose)	
plastids *in situ*	D[a]	80	20	-	20
	5 min R[b] + 8 h D	96	4	-	52
	55 min WL[c]	27	32	41	37
	5 min R + 8 h D + 55 min WL	32	69	-	35
plastids *in vitro*	5 min R + 6 h D[d]	95	5	-	162
	5 min R + 6 h D + 55 min WL[e]	6	47	47	122

[a] D: darkness

[b] R: a standard red light pre-irradiation (5 min 658 nm, 0.85 W m^{-2} or 5 μmol m^{-2} s^{-1})

[c] WL: white light (2 W m^{-2})

[d] treatment *in vivo*

[e] R *in vivo*, WL *in vitro*

Henningsen and Boynton (1974) were consistent with the results of the weight method shown in Fig. 5.2.

A similar small, but significant (Student's t-test) decrease in PLB area to that *in situ* was observed in isolated R etioplasts after 55 min WL *in vitro* (Fig. 5.2).

5.2.4 Growth of thylakoids

In oat etioplasts, the number of medium long and long thylakoids (Section 5.2.1) showed a small increase *in situ* after a brief R irradiation (similar results were obtained with maize). During 55 min WL the length of the thylakoids considerably increased. The increase was similar in dark-grown oat leaves and in R pre-irradiated leaves (Fig. 5.3).

Though significant (Student's t-test), the increase of thylakoid length of R etioplasts during 55 min WL *in vitro* was small compared to that *in situ*.

5.2.5 Formation of incipient grana

In etioplasts of dark-grown oat leaves, incipient grana with 2 stacks were observed. In similar maize etioplasts grana with 3 stacks were also seen. In both oat and maize leaves, R pre-irradiation resulted in a small increase in

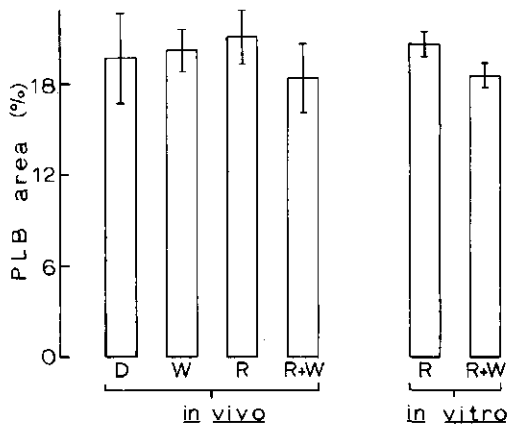


Fig. 5.2 Mean prolamellar body (PLB) area as a percentage of total oat etioplast area (see text) after various light treatments. Treatments of *in situ* plastids: D: darkness, R: 5 min red light (R) (658 nm, 5 μmol m^{-2} s^{-1}) followed by 8 h D, W: 55 min of white light (WL) (2 W m^{-2}), R+W: 5 min R followed by 8 h D and 55 min WL. Treatments of *in vitro* plastids: R: 5 min R followed by 6 h D *in vivo*, no light treatment *in vitro*, R+W: 5 min R followed by 6 h D *in vivo* and 55 min WL *in vitro*. The bars indicate standard errors of the mean. The numbers within parentheses denote the number of plastid electron micrographs studied. Oat seedlings used for the experiments were 8 days old.

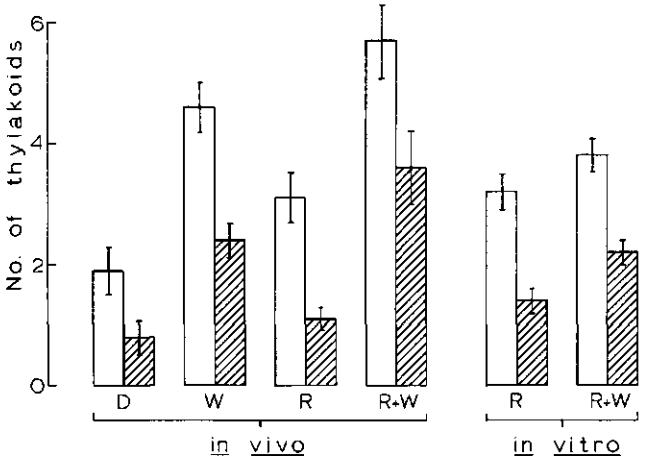


Fig. 5.3 Mean number of stroma thylakoids with a length of at least one third (medium long thylakoids) or two thirds (long thylakoids) of the plastid length after various light treatments (see Fig. 5.2) of oat etioplasts. The bars indicate standard errors of the mean. The numbers within parentheses denote the number of plastid electron micrographs studied. Oat seedlings used for the experiments were 8 days old.

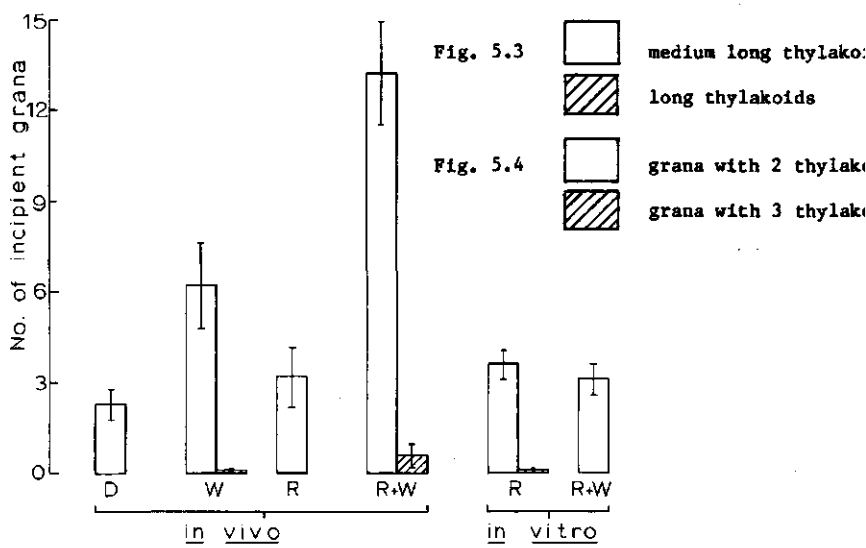


Fig. 5.4 Mean number of incipient grana per plastid electron micrograph after various light treatments (see Fig. 5.2) of oat etioplasts. The bars indicate standard errors of the mean. The numbers within parentheses denote the number of plastid electron micrographs studied. Oat seedlings used for the experiments were 8 days old.

the average number of incipient grana per etioplast. During 55 min WL, formation of incipient grana with 2 (sometimes 3) stacks took place to a greater extent in R pre-irradiated than in dark-grown oat leaves (Fig. 5.4). More than 3 stacks were not observed in oat etioplasts in the present experiments. Bean etioplasts *in situ* contained grana with up to 5 stacks after R pre-irradiation and even 6 stacks following a subsequent WL period of 75 min.

The mean number of incipient grana did not increase during 55 min WL in isolated R oat etioplasts. After 55 min WL, some isolated etioplasts differed from those *in situ* in possessing some (long) thylakoids running partially adjacent to each other, resembling the magnograna observed in isolated bean etioplasts (Section 5.2.1).

5.3 Discussion

In oat seedlings, R pre-irradiation appeared to retard PLB transformation from a tight (mostly paracrystalline) into a loose form. Ryberg and Virgin (1978) suggested that the disruption of PLBs in WL is faster with PLBs in a paracrystalline form than with those which are loose. The present results, although sometimes not significant, support this suggestion: after 55 min WL the PLB volume had not changed or even slightly increased in previously dark-grown oat leaves, but decreased in R pre-irradiated leaves. Irradiation can have two effects on the size of the PLB. R pulses or continuous far-red (FR) irradiation cause the formation of large, paracrystalline PLBs in mustard seedling etioplasts (Kasemir *et al.*, 1975). WL irradiation can induce an immediate decrease of the PLB volume (Henningsen, 1970; Girnth *et al.*, 1979). Results shown by Bradbeer *et al.* (1974) indicate an increase in PLB volume after about 10 min of R and a decrease after prolonged R irradiation. In the experiments here, first an increase in PLB volume may have been induced by WL and a subsequent decrease may have been accelerated by R pre-irradiation. However, changes in PLB volume were small in these experiments.

An effect of R pre-irradiation on the WL mediated PLB transformation as observed in our experiments was also reported by Girnth *et al.* (1979) for mustard seedlings. Four R pulses were given in their experiments, the first three of which were followed by 4 h of D and the last by 12 h D. Their characterization of PLBs was based both on the arrangement of the PLB tubules and on the location of plastoglobuli. However, in our experiments the location of plastoglobuli did not change in WL. Girnth *et al.* (1979) reported that the effect of R pulses could be reversed by 756 nm FR (reversibility being lost after 5 min) and concluded that P was involved.

Girnth *et al.* (1978) found that in mustard seedlings, R pulses increased the number of stroma thylakoids having a length of at least two thirds of the length of the etioplast section after 1 h WL. FR reversibility was demonstrated and again escape from reversibility occurred within 5 min. The R pulses themselves did not increase the number of long thylakoids in their experiments as observed here in oat seedlings (Section 5.2.4). On the other

hand, in oat seedlings R pre-irradiation hardly influenced the rate of growth of thylakoids in WL.

The present results on the effect of R on the rate of formation of incipient grana in WL agree with those of Girnth *et al.* (1978). Although FR reversibility was not examined, it appears likely that in oat seedlings R pre-irradiation is also working *via* P.

Changes in ultrastructure occurring in isolated etioplasts have been studied by several authors. Light induced changes of PLB transformation have been reported by Wrischer (1973a,b), Horton and Leech (1975) and Kohn and Klein (1976) for maize etioplasts and by Wellburn and Wellburn (1971b) for oat etioplasts. Light induced changes in PLB volume were reported by Kohn and Klein (1976) and light induced stroma thylakoid formation and incipient grana formation by Wellburn and Wellburn (1971b) and Kohn and Klein (1976). In the experiments here, WL induced PLB transformation was observed together with a decrease in PLB volume in etioplasts isolated from R pre-irradiated oat seedlings. Some thylakoid growth was also observed but no formation of incipient grana. With regard to the PLB transformation, an effect of *in vivo* R pre-irradiation was only apparent in etioplasts in leaves and not in isolated etioplasts. Apparently some factor from the cytoplasm is required for the expression of the R effect (see Chapter 8). The decrease of the PLB volume in WL, which appeared controlled by R, occurred similarly in both isolated etioplasts and *in situ*. However, changes in PLB volume were small. Consequently, in order to obtain statistically significant results much larger numbers of electron micrographs should be examined. In addition, since the ultrastructure of isolated etioplasts after only 1 h of low WL showed some deviations from that observed *in situ*, priority was given to a study of spectral changes in isolated etioplasts which can be observed on a shorter time scale (Chapters 6 and 7).

6 PROTOCHLOROPHYLL(IDE) REGENERATION IN MAIZE LEAVES AND ISOLATED ETIOPLASTS - ABSORPTION STUDIES

6.1 Introduction

Isolated etioplasts contain phototransformable protochlorophyll(ide) (PChl(ide)). After photoconversion to chlorophyll(ide) (Chl(ide)) by a red light (R) pulse, subsequent regeneration of photoactive PChl(ide) can be observed (Griffiths, 1974a; 1975). In intact leaves, the rate of PChl(ide) regeneration is controlled by the far-red light (FR) absorbing form of phytochrome, Pfr (Virgin, 1958; Augustinussen and Madsen, 1965; Jabben et al., 1974; Jabben and Mohr, 1975). This chapter reports on experiments aimed at studying the effect of phytochrome (P) on PChl(ide) regeneration in isolated etioplasts from 8-day old maize leaves. A possible effect of P on the Shibata shift (Chapter 1, Section 1.4.6) was also studied. A stimulation of the rate of the Shibata shift by Pfr was reported by Jabben and Mohr (1975) for mustard seedlings and by De Greef (1978) for bean seedlings. However, Klockare (1979; 1980) found no stimulation of the Shibata shift in wheat seedlings by continuous long wavelength FR, which phototransformed part of P into Pfr and stimulated Chl biosynthesis. A very slow "Shibata-like" shift has been observed in etioplasts isolated from 8-day old dark-grown maize seedlings by Horton and Leech (1975). Spectra published by Griffiths (1974a; 1975) show a more rapid dark shift of the absorption maximum of newly formed Chl(ide) in etioplasts isolated from 7-day old barley seedlings.

6.2 Results

6.2.1 Absorption spectra - general aspects

The absorption spectra were recorded with a Cary 14 spectrophotometer at high sensitivity and had a low signal-to-noise ratio. Also, unavoidable small differences in light scattering between sample and reference depended on wavelength. Therefore, the absorption bands were superimposed upon a sloping, curved baseline, precluding a direct determination of peak positions. For a number of absorption spectra, curve analysis was applied using the computer program ACCU (Chapter 2, Section 2.14). Estimations of peak positions, peaks heights and half band-widths (HWs) have to be entered into the program. The noise in the original spectra made it impossible to use derivative spectroscopy (Chapter 2, Section 2.13) in order to obtain values for these parameters. Therefore we used data of Jabben et al. (1974) for the PChl(ide) components and data of Virgin and French (1973) for Chl(ide) components.

6.2.2 Protochlorophyll(ide) species in 8-day old dark-grown maize leaves and isolated etioplasts

Curve analysis indicated three PChl(ide) bands with peak positions at 629, 638 and 652 nm and HWs of about 25, 17 and 16 nm, respectively, in maize leaves and isolated etioplasts. Values for both peak positions and HWs agree

satisfactorily with those determined by Jabben et al. (1974). To conform to the literature the bands will be referred to as P628, P636 and P650. The ratio of the peak heights of the three bands differed considerably in vivo and in vitro. Compared to the band of P628, the band of P636 was lower and the band of P650 considerably higher in intact leaves (Fig. 6.1a) than in

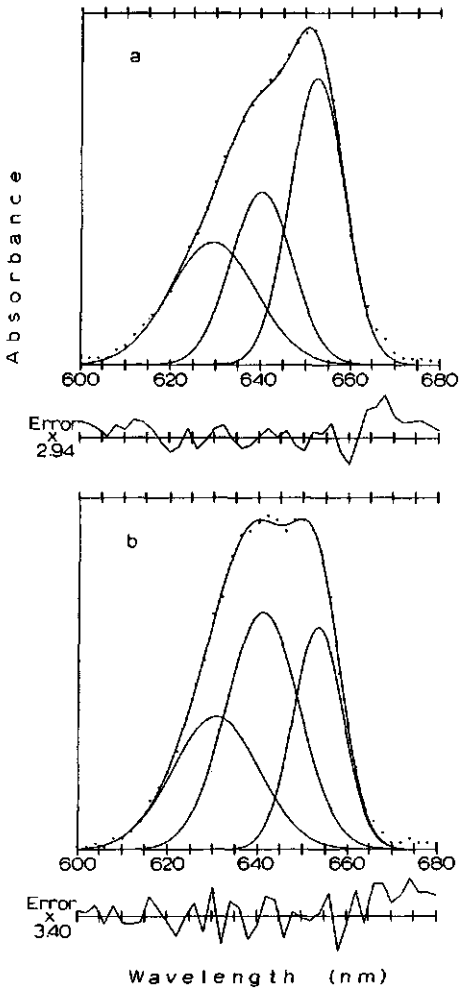


Fig. 6.1 Absorption spectra and their components of two superimposed 8-day old dark-grown maize leaves (a) and a crude, washed etioplast preparation from similar leaves (b). Data points were read at 2 nm intervals from the original spectra and are plotted. The line through the points represents the sum of the component curves. The error of fit at each point is shown, with its magnification factor, below the spectra.

isolated etioplasts (Fig. 6.1b). During storage at room temperature, 4 or 0 °C, the P650 content of etioplasts decreased further.

6.2.3 Protochlorophyll(ide) photoconversion

When samples of dark-grown maize leaves or isolated etioplasts were briefly irradiated at room temperature or at 0 °C with saturating R (649 nm), P650 almost completely disappeared (Figs. 6.2 and 6.3). The absorption increase due to newly formed Chl(ide) was about 2.5 times as large as the absorption decrease at 650 nm. Curve analysis showed that a small band at about 650 nm remained after R irradiation, irrespective of its duration (8 s to 1 min). Curve analysis also showed that part (about 50%) of P636 disappeared upon R irradiation, which was directly evident on examination of several difference spectra of leaves and isolated etioplasts (Fig. 6.4). Photoconversion of P628 was not observed.

6.2.4 Protochlorophyll(ide) regeneration

In the dark following R irradiation, both in intact leaves and isolated etioplasts photoactive PChl(ide) (mainly P650) reappeared. As was the case for leaves, P650 regeneration could be observed repeatedly in etioplast preparations, however in contrast to leaves the amount of regenerated P650 in isolated etioplasts gradually decreased.

The rate of P650 regeneration was examined by monitoring absorbance changes at 650 nm, using 720 nm as a reference to correct for wavelength-independent baseline changes (method of Jabben et al., 1974). Initially it was verified

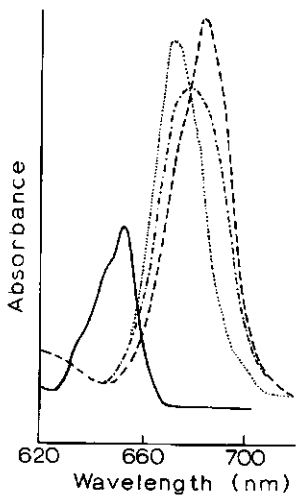


Fig. 6.2 Absorption spectra of 2 superimposed 8-day old maize leaves, recorded at a fast speed at room temperature. Non-irradiated (———), after 12 s red light (R) (649 nm) followed by 2 min (- - -), 5 min (-.-.-) and 11 min (.....) of darkness.

that the shift of the peak position of Chl(ide) observed in maize leaves (Section 6.2.5) had no significant influence on the measurements at 650 nm. Assuming that the absorption bands are Gaussian in shape (Chapter 2, Section 2.14) they can be described (e.g., Talsky et al., 1978) as

$$A_\lambda = A_{max}\, e^{(-2/s^2)(\lambda-\lambda_{max})^2}$$

where A is the absorbance at wavelength λ, A_{max} is the maximum absorbance (or peak height), 2s is the distance between the 2 inflection points of the band and λ_{max} is the peak position. The value for s is related to HW as 2s = 1.67 HW. The Chl(ide) band in maize leaves before the Shibata shift has its maximum at about 685 nm (C685) and a HW of about 17.5 nm (Section 6.2.5). During the Shibata shift the peak position of the band shifts to 672 nm (C672) and its HW increases to 19 nm. We calculate that at 650 nm, C685 and C672 contribute 0.001% and 2.1%, respectively, of their maximum absorbance. The final absorbance increase at 650 nm during P650 regeneration was 100-150% of

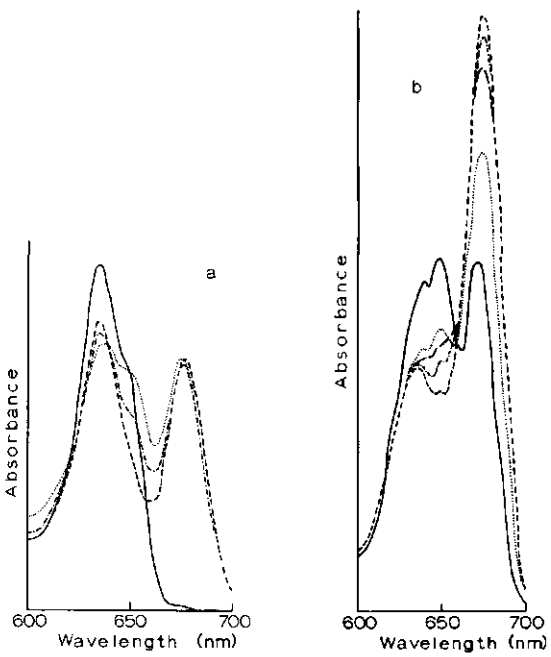


Fig. 6.3 Absorption spectra of etioplast preparations, a. a crude, washed preparation from 8-day old dark-grown maize leaves, non-irradiated (———) and after 12 s red light (649 nm) followed by 2 min (- - -), 11 min (-.-.-) and 35 min (.....) of darkness, b. a crude, washed preparation from 8-day old maize leaves red pre-irradiated (5 min 658 nm, 5 μmol $m^{-2}s^{-1}$) 17 h prior to etioplast isolation, non-irradiated (———) and after 12 s red light (649 nm) followed by 1 min (- - -), 3 min (-.-.-), 4.5 min (— —) and 21 min (.....) of darkness.

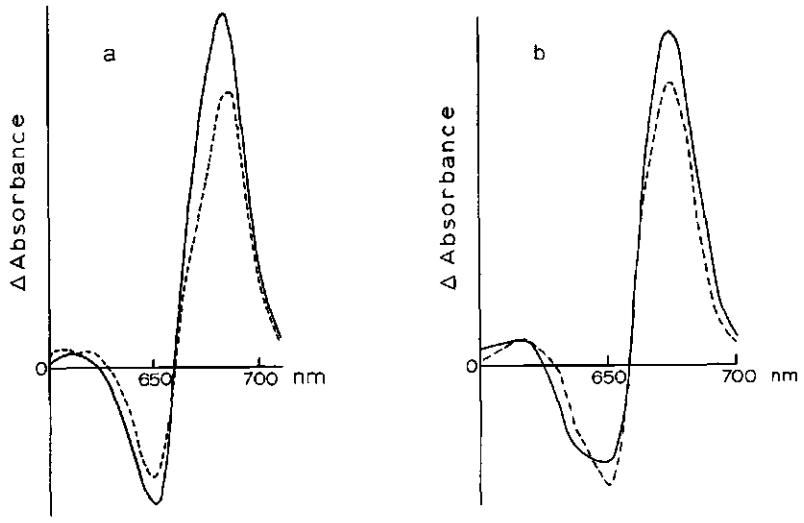


Fig. 6.4 Absorption difference spectra (red minus dark) of a. two superimposed 8-day old maize leaves irradiated with saturating red light (R) (649 nm), ——— previously dark-grown leaves, - - - leaves pre-irradiated with R (5 min 658 nm, 5 μmol m^{-2} s^{-1}) 7 h before recording of the spectrum, b. etioplasts isolated from 8-day old dark-grown maize leaves, ——— a crude, washed preparation, R irradiated at 22 °C, - - - a Sephadex G-50 purified preparation, R irradiated at 0 °C.

the initial absorbance decrease as a result of P650 photoconversion, i.e. 40-60% of the absorbance increase at 685 nm (Fig. 6.4; Section 6.2.3). The contribution of C685 to the absorbance at 650 nm calculated from these data is insignificant, however 3.5-5% of the absorbance increase at 650 nm in leaves can be attributed to the absorbance of C672. Since the beginning of P650 regeneration coincides with the end of the Shibata shift (see below), only the initial absorbance increase at 650 nm could be due to the Shibata shift (Fig. 6.5). Similarly, in isolated etioplasts absorption measurements at 650 nm are probably not significantly influenced by the wavelength shift of Chl(ide).

The absorbance of P636 at 650 nm is about 14% of the value at its peak wavelength. Therefore, absorbance changes at 650 nm represent photoconversion and subsequent regeneration of the total photoconvertible PChl(ide) pool rather than of P650 alone. However, as the major part of these changes is due to P650, they are referred to here as P650 photoconversion and regeneration.

In 8-day old dark-grown maize leaves, P650 regeneration in the dark following P650 photoconversion showed a lag phase of several minutes (Fig. 6.5). The duration of the lag phase is dependent on temperature (Fig. 6.6). At 0 °C, even after 1 h of darkness (D) no P650 regeneration was observed. A standard R pre-irradiation (Chapter 3, Section 3.2.2) given

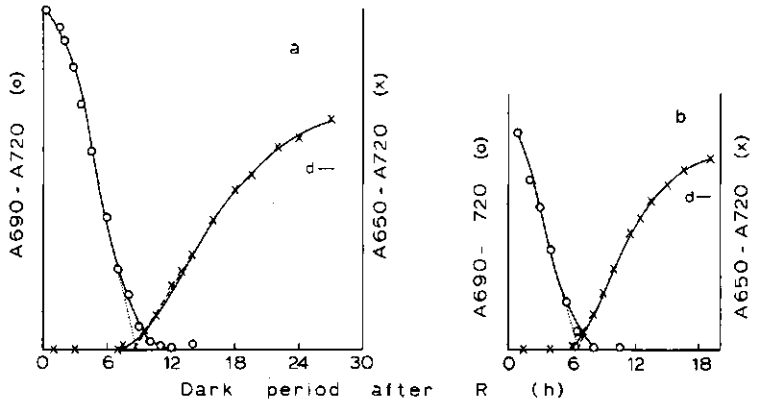


Fig. 6.5 Absorption changes at 650 nm and 690 nm, using 720 nm as a reference, in darkness at 22 °C following irradiation with a saturating 10 s red light (R) (649 nm). The durations of the lag phase of P650 regeneration and the Shibata shift are defined by the crossing of the extrapolated dashed and dotted lines, respectively, with the base line. 8-day old dark (D)-grown maize leaves without (a) and with (b) R pre-irradiation (5 min 658 nm, 5 μmol m^{-2} s^{-1}) 17 h prior to measurement.

several hours before PChl(ide) photoconversion shortened the lag phase (Fig. 6.6) in 8-day old seedlings. This effect was even more pronounced in 9-day old seedlings (Table 6.1). The effect of R was partially reversible by FR (Table 6.1) indicating involvement of P, which is in agreement with the

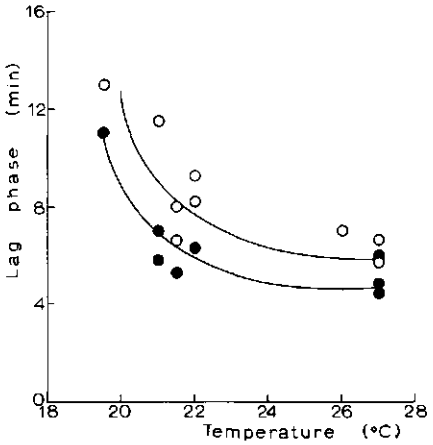


Fig. 6.6 Duration of the lag phase in P650 regeneration (for its definition see Fig. 6.5) in 8-day old dark-grown maize leaves in the dark following protochlorophyll(ide) photoconversion as a function of temperature and red (R) pre-irradiation. Open symbols: dark-grown leaves, closed symbols: leaves pre-irradiated with R (5 min 658 nm, 5 μmol m^{-2} s^{-1}) 5 to 22 h prior to the measurements.

literature (Section 6.1). Contrary to intact leaves, P650 regeneration in isolated etioplasts showed no lag phase (Fig. 6.7).

After the lag phase, the absorbance at 650 nm in intact leaves increased approximately linearly with time for several minutes (Fig. 6.5), after which the rate slowed down. In some experiments, P650 seemed to reach a steady state level after about 30-40 min, corresponding with 100-150% of the initial dark level. However, in other experiments the absorbance at 650 nm continued to increase slowly. This appeared independent of temperature or R pre-irradiation. The rate of P650 regeneration after the lag phase was higher in R pre-irradiated intact leaves than in dark controls (e.g., Fig. 6.5 and Table 6.1). Again, FR gave a partial reversion of the R effect (Table 6.1). Results of similar experiments with etioplasts are summarized in Table 6.2. In isolated etioplasts, unlike leaves, P650 regeneration was observed at 0 °C. Even at this temperature, the absorbance at 650 nm increased at the beginning of measurement (15-30 s after R irradiation) without a lag phase. In order to estimate the absorbance at 650 nm immediately after PChl(ide) photoconversion,

Table 6.1 The effect of various pre-irradiation treatments on P650 regeneration and the chlorophyll(ide) Shibata shift at 22 °C in darkness following photoconversion of P650. Data are given for two superimposed 8- or 9-day old dark-grown maize leaves. P650 photoconversion was brought about by 17 s red light (R) (649 nm). Data are results of two independent experiments for 8-day old leaves and of one experiment for 9-day old leaves.

Sample	Pre-irradiation treatment	P650 regeneration		Shibata shift (min)
		lag phase (min)	half time[a] (min)	
8-day old leaves	D[b]	8.8	8.9	9.1
	R[c] + ~1 h D	6.3	4.5	6.8
	R/FR[e] + ~19 h D	8.0	5.5	7.5
	FR[d] + ~19 h D	9.0	7.4	9.4
9-day old leaves	D	13.5	12.0	10.8
	R + 22 h D	6.4	4.5	6.6
	R/FR + 22 h D	7.6	6.5	9.1

[a] time after the lag phase until 50% of the amount of photoconverted P650 was regenerated

[b] D: darkness

[c] R: 655 nm (1.9 mmol m^{-2} given in 20 s)

[d] FR: far-red light of 750 nm (20 mmol m^{-2} given in 5 min)

[e] R/FR: FR followed R immediately

The data in the table are accurate to about 1%.

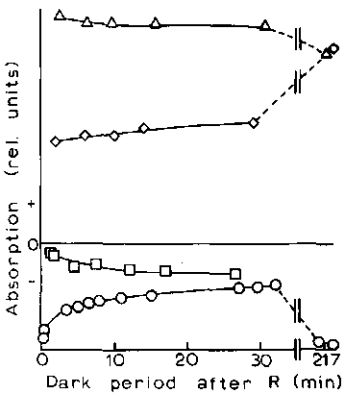


Fig. 6.7 Absorption changes in darkness at 22 °C following irradiation with 8 s saturating R (649 nm) in a crude, washed etioplast preparation from 8-day old dark-grown maize seedlings, measured at 630 nm (□), 650 nm (○), 670 nm (◇) and 680 nm (△), using 730 nm as a reference.

differences with the final level were plotted logarithmically against time and the resulting straight line was extrapolated to time zero. Irrespective of R pre-irradiation in vivo, the final level of regenerated P650 was between 20% and 70% of the initial amount of P650 in crude, washed etioplast preparations (13 experiments) and about 100% in Sephadex G-50 purified preparations (2 experiments). A final level was reached after about 30 min at room temperature. At 0 °C, regeneration was much slower (Table 6.2) and continued for several hours. After prolonged dark periods at room temperature (1 h or longer), the absorbance at 650 nm decreased again, probably as a result of

Table 6.2 P650 regeneration in etioplasts isolated from 8-day old dark-grown or red pre-irradiated maize leaves (D and R etioplasts, respectively).

Sample	Temperature (°C)	Half time[a] of P650 regeneration (s)	Fraction of P650 regenerated (%)	No. of experiments
D etioplasts	0	725	24	2
	23 ± 2	104 ± 19	52 ± 10	6
R etioplasts	23 ± 2	179 ± 37	55 ± 12	7

[a] time for 50% of the final absorption increase at 650 nm to be obtained

etioplast deterioration. There was no indication in isolated etioplasts of the enhancement of the P650 regeneration rate by R pre-irradiation that was observed in leaves.

6.2.5 Wavelength shifts of chlorophyll(ide) bands

Immediately after a brief R irradiation, maize leaves showed an absorption maximum at 685 nm (Fig. 6.2). The initial rapid shift from about 678 to 685 nm reported in the literature (e.g., Bonner, 1969) was not observed at room temperature. However, at 77K the absorption maximum of newly formed Chl(ide) was at 679 nm when leaves were frozen in liquid nitrogen immediately upon R irradiation and at 683 nm when a dark period of 1-2 min was inserted between R and cooling.

Within several minutes after R irradiation at room temperature, the absorption maximum shifted from 685 (HW 17.5 nm) to 672 nm (HW 19 nm) (Fig. 6.2). This shift is referred to as the Shibata shift. A small band at 672 nm appeared to be already present in spectra recorded within 1-2 min after R irradiation. In such spectra, the ratio C672 : C685 was about 1 : 5. During the Shibata shift the ratio increased to about 4 : 1. The method of Jabben and Mohr (1975) was adopted to study the duration of the Shibata shift (for its definition see Fig. 6.5). After irradiation of the leaves in the sample compartment of the spectrophotometer, the absorption change at 690 nm was measured, using 720 nm as a reference. The first reliable measurements were made about 30 s after a 4-12 s R irradiation. In several experiments a

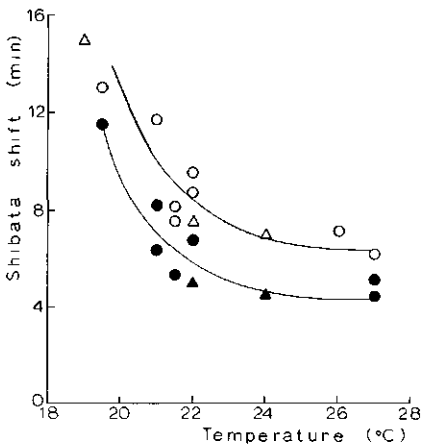


Fig. 6.8 Duration of the Shibata shift (for its definition see Fig. 6.5) in 8-day old dark-grown maize leaves as influenced by temperature and red light (R) pre-irradiation. Open symbols: dark-grown leaves, closed symbols: leaves pre-irradiated with R (5 min 658 nm, 5 μmol m^{-2} s^{-1}) 5 to 22 h prior to the measurements. O: data from absorption measurements, Δ : data from fluorescence measurements (Chapter 7).

small initial increase in the 690 nm absorption was observed, probably caused by the initial fast shift of the Chl(ide) peak from 678 to 685 nm. Kinetics of the changes at 690 nm have already been shown in Fig. 6.5.

A strong temperature dependence of the duration of the Shibata shift in leaves was observed (Fig. 6.8). At 0 °C no absorption decrease at 690 nm or absorbance increase at 672 nm occurred for at least 1 h. A standard R pre-irradiation shortened the duration of the Shibata shift (Fig. 6.8). The R effect appeared to be partially reversed by FR (Table 6.1) implicating the involvement of P. In all experiments, P650 regeneration in maize leaves did not start until the Shibata shift was (almost) complete.

Although in fluorescence emission spectra of etioplast preparations shifts of the peak position of newly formed Chl(ide) were clearly observed (Kraak and Spruit, 1980; Chapter 7), similar shifts were not obvious in absorption spectra. Curve analysis showed that the 676 nm Chl(ide) absorption band of etioplasts after irradiation could be fitted with considerably smaller error using 2 bands with peak positions at 672 and 685 nm than with a single band at 676 nm. The HWs of the 2 bands were similar to those in intact leaves. The ratio C672 : C685 immediately after R irradiation was much higher (about 1 : 1) in isolated etioplasts than in intact leaves (about 1 : 5). The slow absorption decrease at 680 nm and absorption increase at 670 nm (Fig. 6.7) indicate a slight increase of the ratio in darkness following R irradiation.

6.2.6 Stability of newly formed chlorophyll(ide)

Newly formed Chl(ide) appeared less stable in isolated etioplasts than in intact leaves. During continuous recording or after a storage period in darkness, a marked absorption decrease attributed to Chl(ide) destruction was observed in etioplast preparations. Instability of newly formed Chl(ide) was also observed in isolated barley etioplasts (Griffiths, 1975).

6.3 Discussion

Photoconversion and subsequent dark regeneration of P650 was demonstrated in 8-day old dark-grown leaves and isolated etioplasts from maize by absorption (difference) spectrophotometry. The kinetics of P650 regeneration in isolated etioplasts differed considerably from those in leaves. At room temperature, P650 regeneration in isolated etioplasts started at a high rate immediately after a brief R treatment, whereas a lag phase of several minutes was observed in intact leaves. At 0 °C, P650 regeneration occurred in isolated maize etioplasts (Section 6.2.4) confirming the observations in barley etioplasts (Griffiths, 1975). However, in intact maize leaves no P650 regeneration was observed during 1 h at 0 °C. Virgin (1955) found no evidence for P650 regeneration at 0 °C in barley leaves after a dark period of 8 h.

In maize leaves the amount of regenerated P650 was 100-150% of the amount before photoconversion and in etioplast preparations only 20-100%. The low regeneration in isolated etioplasts may be due to simultaneous degradation of P650. A decrease of P650 absorption during storage of maize etioplasts was

observed, confirming the observations of Horton and Leech (1972; 1975). Griffiths (1974b; 1975) reported a substantial decrease in photoconvertible PChl(ide) when barley etioplasts were lysed. Disappearance of the absorption band of P650 can be due to denaturation of the holochrome protein (Gassman, 1973; Dujardin, 1976) or to a (reversible) alteration of the PChl(ide)-lipoprotein complex to a non-photoconvertible form (Horton and Leech, 1972; Gassman, 1973a). Alternatively, disaggregation of dimeric P650 into a monomeric form (e.g., Butler and Briggs, 1966) might be the cause of the disappearance of the P650 absorption band. According to Henningsen (1970), physical treatments inducing a derangement of the tubular membranes of the prolamellar body (PLB) result in formation of photoactive P636 from P650 and complete denaturation of the PLB membranes results in formation of photoinactive P628. It is possible to speculate that during storage of isolated etioplasts P650 decreases as a result of alteration of the PLB structure after disruption of the plastid envelope. However, electron micrographs of broken etioplasts show a PLB structure similar to that of intact etioplasts (see Chapter 2, Fig. 2.2). In vivo transformation of P650 by H_2S into P636 and/or P628 has been observed to be reversible (Gassman, 1973a). Griffiths (1975) showed that addition of NADPH to an etioplast membrane preparation with maximum absorption around 630 nm restored their P650 (and P636) content. This observation provides an alternative explanation for the decrease of the P650 content during storage of etioplasts. Leakage of NADPH from the etioplasts upon deterioration of the etioplast envelope may cause transformation of P650 to photoinactive PChl(ide).

In maize leaves, P650 photoconversion gave rise to C685 and some C672. The presence of a Chl(ide) absorbing at 668-672 nm immediately after P650 photoconversion has been reported by several authors, e.g. Klockare and Virgin (1983). In the experiments here with maize leaves, the ratio C672 : C685 after P650 photoconversion was low and increased considerably during the Shibata shift. In isolated etioplasts, about 50% of newly formed Chl(ide) was already in the C672 form at the time of the first measurements (1-2 min after photoconversion of P650). Hardly any increase of C672 was observed in subsequent darkness.

It has been observed (e.g., Goedheer, 1961; Brodersen, 1976) that no P650 regeneration occurs as long as all Chl(ide) is still in the C685 form. The measurements here with intact leaves confirm this. Under all conditions, the completion of the Shibata shift was observed to coincide with the start of P650 regeneration. Also the data for isolated etioplasts, where fast P650 regeneration occurs, agree with these observations since a large proportion of Chl(ide) was already present as C672 soon after R irradiation. The above observation is commonly explained by assuming that C685 formed by photoreduction of P650 is still attached to the holochrome protein (e.g., Henningsen et al., 1974). Only after removal of Chl(ide) can new PChl(ide) become attached to the protein. Dissociation of Chl(ide) from the holochrome

is assumed to take place during or after the Shibata shift. Binding of free, photoinactive PChl(ide) to the holochrome protein could then result in regeneration of P650. Indeed, during regeneration of P650 a concomitant decrease of P628 was observed (Fig. 6.7). On the other hand, Oliver and Griffiths (1982) proposed that in leaves C685 is stable until a significant amount of PChl(ide) has accumulated by de novo synthesis. Dissociation of Chl(ide) from the holochrome, accompanied by the absorption shift of 685 to 672 nm, is then induced by PChl(ide).

The aim of the present experiments was, to examine the effect of Pfr on P650 regeneration in isolated etioplasts. In maize leaves, formation of Pfr shortens the duration of the Shibata shift as well as the lag phase of P650 regeneration. Moreover, the rate of P650 regeneration after the lag phase is higher in the presence of Pfr. In isolated etioplasts from dark-grown and R pre-irradiated plants there was no lag phase in P650 regeneration. The rate of P650 regeneration in isolated etioplasts appeared not to be increased when the leaves had received R pre-irradiation. In the following chapter (7), a possible influence of Pfr on the rate of the dark shifts of the fluorescence emission maxima of newly formed Chl(ide), both in intact leaves and isolated etioplasts is described.

7 THE SHIBATA SHIFT IN MAIZE LEAVES AND ISOLATED ETIOPLASTS - FLUORESCENCE STUDIES

7.1 Introduction

Absorption studies (Chapter 6) indicate that the rate of the Shibata shift in maize leaves is stimulated by the far-red light (FR) absorbing form of phytochrome, Pfr. In isolated maize etioplasts, the 77K fluorescence emission maximum of newly formed chlorophyll(ide) (Chl(ide)) was observed to shift to shorter wavelengths (blue shift) at room temperature (Kraak and Spruit, 1980). In this chapter, the effect of red light (R) pre-irradiation on the rate of this in vitro "Shibata-like" fluorescence shift is examined. Data on P650 regeneration were obtained concomitantly and are compared with those obtained from absorption studies. A few experiments with bean leaves and bean etioplast preparations, containing only mesophyll cell plastids, were included to examine whether their fluorescence characteristics differed from those of maize, containing both mesophyll cell and bundle sheath plastids. Protochlorophyll(ide) (PChl(ide)) photoconversion and dark shifts of the 77K fluorescence emission maxima of newly formed Chl(ide) were also examined in a holochrome preparation from bean leaves.

7.2 Results

7.2.1 Fluorescence spectra - general aspects

In this chapter, fluorescence emission spectra measured at 77K will be considered, unless otherwise indicated. Spectra were recorded from 500 to 800 nm. Curve analysis was applied to the 620-710 nm region. Fluorescence studies of changes in pigment composition meet with some complications that are not observed in absorption spectroscopy. Energy transfer from P636 to P650 and from P650 (and P636?) to Chl(ide) occurs and, moreover, during or following shifts of fluorescence maxima of newly formed Chl(ide), changes in fluorescence yield are to be expected (Thorne, 1971a,b; Van der Cammen, 1982). Fortunately, changes in fluorescence yield of Chl(ide) observed at room temperature (Section 7.2.10) appeared not to occur at 77K. Therefore, the sum of the Chl(ide) peaks of spectra of maize leaves could be used as an internal standard. For etioplast preparations containing carboxymethyl cellulose (CMC) and acriflavin (Chapter 2, Section 2.12), acriflavin fluorescence was used as a standard. Comparing this with the method used for leaves confirmed the validity of the above procedure.

7.2.2 Derivative spectra

The use of derivative spectroscopy in the resolution of composite bands has been outlined in Chapter 2, Section 2.13. The various Chl(ide) and PChl(ide) bands were not completely resolved in second derivative spectra of leaves and etioplast preparations. On the other hand, in fourth derivatives the noise was often too large to allow an accurate estimation of the band parameters.

Considerable trial and error was therefore involved before the most suitable parameters could be introduced in the "ACCU" program for curve analysis, the criteria being a small standard error and a minimum number of bands.

7.2.3 Fluorescence spectra of dark grown maize leaves and isolated etioplasts

The fluorescence emission spectra of an 8-day old maize leaf and isolated etioplasts are shown in Fig. 7.1. The main band at 656 nm (F656) originates from P650 and P636 (Kahn et al., 1970) and a weaker band at 630 nm (F630) can be ascribed to P628. A very weak band around 643 nm was sometimes visible in the zero order spectra. Derivative spectra revealed this band in all spectra and it is proposed that it is due to emission from P636 (e.g., Kahn et al., 1970). Calculated half band widths (HWs) are 16.5 nm for F630, 15 nm for F643 and 10 nm for F656, both for leaves and etioplasts. Vibrational bands, i.e. emission bands originating from vibrational energy levels of PChl(ide), are responsible for the long-wavelength tails of the spectra.

In isolated etioplasts, the relative height of F656 appeared considerably lower than in leaves (Table 7.1), in agreement with results of absorption studies (Chapter 6). Data from spectra of etioplast preparations containing CMC and acriflavin showed that during storage of etioplasts at 4 °C in darkness, the height of F656 further decreased. After storage for 20 h, only about half of F656 remained. Concomitantly, F630 and F643 increased to about 150% and 130% of their original magnitudes, respectively. Decreased energy transfer from P636 to P650 may be the reason for the increase of F643, rather than an increase in the absolute amount of P636. However, P628 is probably formed from P650 during storage of etioplast preparations.

7.2.4 Photoconversion and subsequent regeneration of protochlorophyll(ide)

When dark-grown leaves or etioplast preparations were irradiated at room temperature for 4 s with 649 nm (25 W m^{-2} or 135 μmol m^{-2} s^{-1}), saturating PChl(ide) photoconversion, 77K fluorescence emission at 656 nm was considerably reduced and emission bands of Chl(ide) were observed (see Figs. 7.2 and 7.3 and Section 7.2.5). Experiments with freshly isolated etioplasts containing CMC and acriflavin showed that on an average F630/F518 (the height of F630, divided by the height of the acriflavin emission band at 518 nm) and F643/F518 in spectra of preparations frozen to 77K immediately after R were 97% and 107%, respectively, of the values in non-irradiated preparations. This indicates that P628 (F630) is not photoconverted by R, in agreement with absorption studies. The latter indicated that part of P636 is photoconverted, whereas its fluorescence (F643) slightly increases. This apparent discrepancy may be explained by a simultaneous decrease of energy transfer from P636 to P650. Energy transfer from P636 (via P650?) to Chl(ide) may also occur (Thorne, 1971a) but with lower efficiency than energy transfer from P636 to P650.

Peak positions and HWs of F630 and F643 in leaves and etioplasts after R irradiation were similar to those in non-irradiated samples. However, both

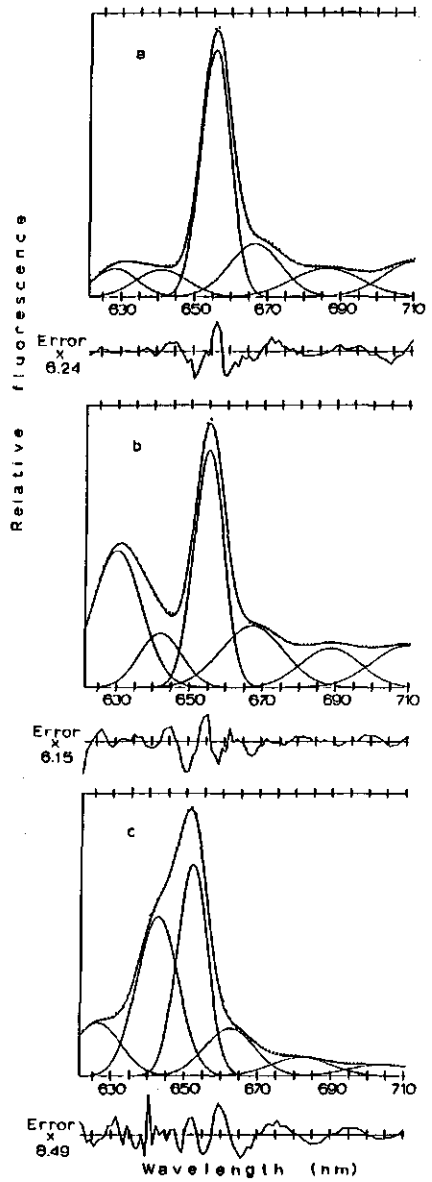


Fig. 7.1 77K fluorescence emission spectra of 8-day old dark-grown maize leaves (a), a crude, washed etioplast preparation isolated from similar leaves (b) and a protochlorophyll(ide) holochrome preparation from 10-day old dark-grown bean leaves (c) fitted by the sums of Gaussian components by the "ACCU" computer program. Data points were read at 1 nm intervals from the original spectra and are plotted. The line through the points represents the sum of the component curves. The difference between observed and computed fluorescence at each point is shown, with its magnification factor, below the spectra. Excitation was at 425 nm. The spectra have been corrected for instrument response.

derivative spectra and curve analysis consistently showed fluorescence emission by the small amount of P650 remaining after R irradiation (Section 6.2.3) to have its peak at somewhat shorter wavelength (654.5 nm) than in non-irradiated samples (656 nm). The HWs of F656 and F654.5 were similar.

In leaves, the low level of this F654.5 remained essentially constant during the first few minutes after R (Figs. 7.2 and 7.4), which is in agreement with results of absorption studies. After this lag phase, fluorescence emission by P650 both increased and shifted from 654.5 to 656 nm. Energy transfer from excited PChl(ide) to Chl(ide) slows down the increase of F656, whereas energy transfer from P636 to P650 also influences the magnitude of F656. Therefore, determinations of the duration of the lag phase of F656 re-appearance as shown in Table 7.2 can only be an approximation. Nevertheless, as was the case in absorption studies, a strong temperature

Table 7.1 Relative heights of the protochlorophyll(ide) fluorescence emission bands at 77K in various samples as computed by the ACCU computer program. For etioplast preparations, only data of spectra recorded shortly after etioplast isolation are used.

Sample	Relative height[a] of			No. of spectra examined
	F630	F643	F655	
D maize leaves[b]	10	7	83	3
R maize leaves[c]	15	7	78	3
D bean leaves[d]	10	7	83	1
D maize etioplasts[e]	30	13	57	6
R maize etioplasts[f]	33	12	55	6
D bean etioplasts[g]	35	13	52	1
D bean PChl(ide) holochrome[h]	8	28	64	2

[a] Percentage of the sum of the heights of the three protochlorophyll(ide) bands

[b] 8-day old dark-grown maize leaves

[c] 8-day old maize leaves pre-irradiated for 2 or 5 min with 658 nm light (5 μmol m^{-2} s^{-1}) (R) about 16 h prior to recording of the spectra

[d] 11-day old D bean leaves

[e] Crude, washed etioplast preparations from 8-day old D maize leaves

[f] Crude, washed etioplast preparations from 8-day old maize leaves pre-irradiated for 0.5, 2 or 5 min with R about 16 h prior to etioplast isolation

[g] Crude, washed etioplast preparation from 10-day old D bean leaves

[h] Protochlorophyll(ide) holochrome from 10-day old D bean leaves in glycerol-tricine buffer 3:1, pH 8.0

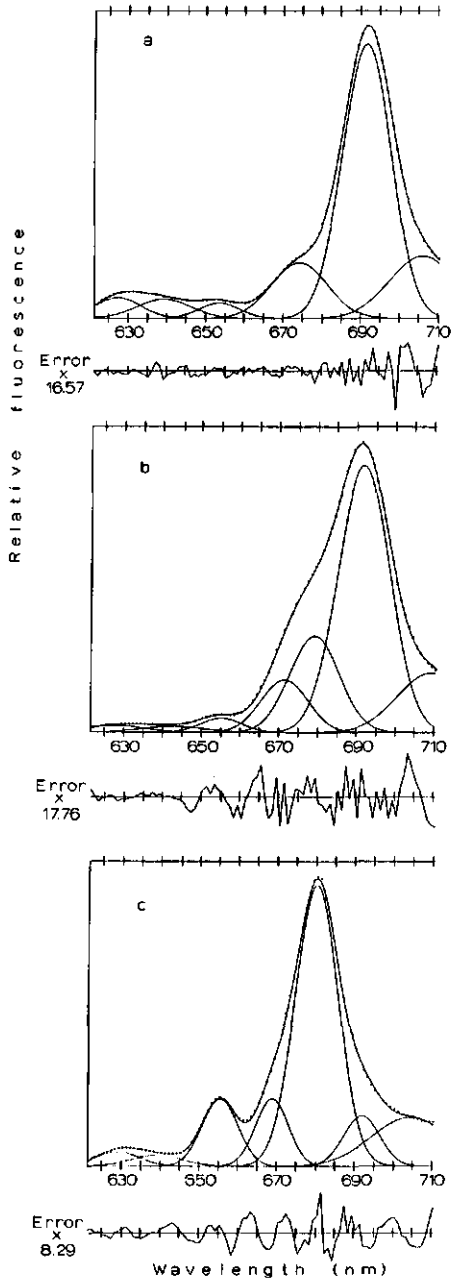


Fig. 7.2 77K fluorescence emission spectra of 8-day old previously dark-grown maize leaves, cooled immediately (a) and after a dark period of 4 min (b) or 20 min (c) at 22 °C following irradiation with saturating red light (4 s 649 nm, 134 μmol m^{-2} s^{-1}). Excitation was at 425 nm. See also Fig. 7.1.

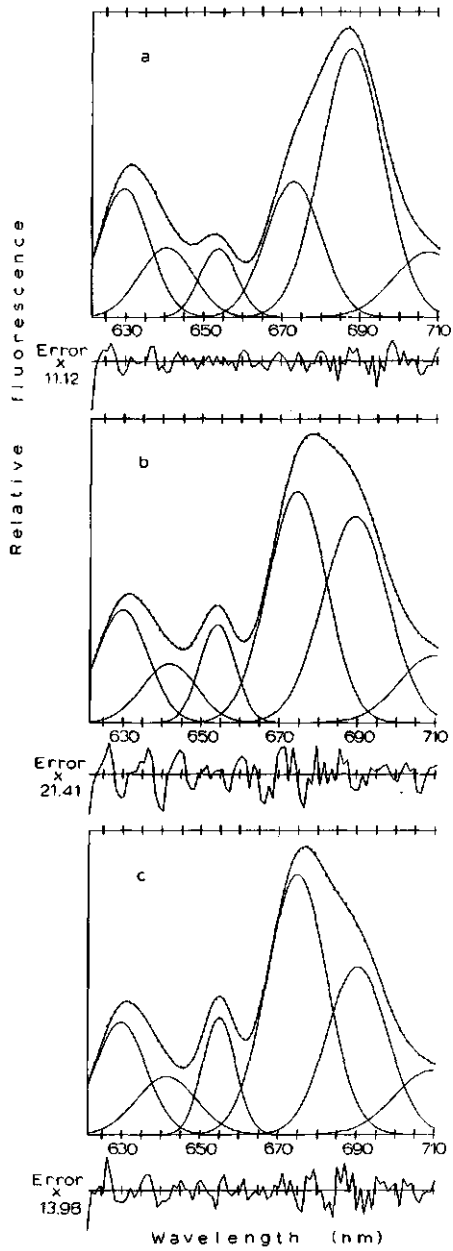


Fig. 7.3 77K fluorescence emission spectra of a crude, washed etioplast preparation from 8-day old dark-grown maize leaves, cooled immediately (a) and after a dark period of 1.5 min (b) or 10 min (c) at 22 °C following irradiation with saturating red light (4 s 649 nm, 134 μmol m^{-2} s^{-1}). Excitation was at 425 nm. See also Fig. 7.1.

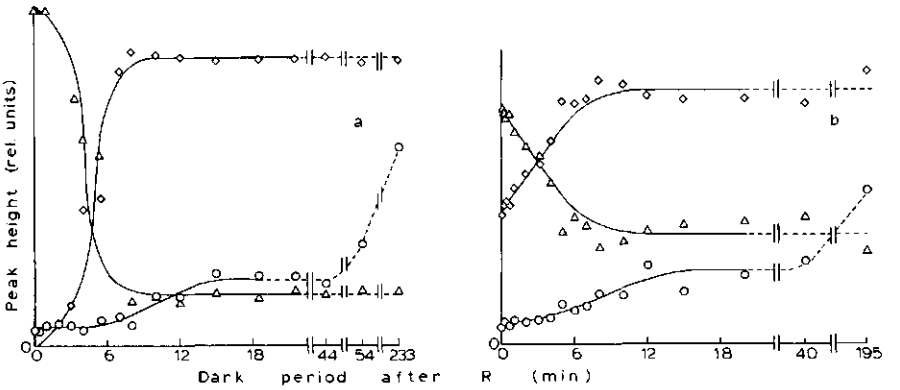


Fig. 7.4 Relative heights of the 77K fluorescence emission bands of the various protochlorophyll(ide) and chlorophyll(ide) pigments in spectra of 8-day old maize leaves recorded after various dark (D) periods at 22 °C following a saturating red irradiation (4 s 649 nm, 134 μmol m^{-2} s^{-1}) (R). All fluorescence spectra were normalized for the sum of the bands at 680 and 692 nm. ○ : F655, ◇ : F680, △ : F692. a. dark-grown maize leaves, b. maize leaves red pre-irradiated (5 min 658 nm, 5 μmol m^{-2} s-1) about 16 h prior to recording of the spectra.

effect was observed in maize leaves. In agreement with absorption studies, a short (2 or 5 min) R pre-irradiation (658 nm, 0.6 or 1.5 mmol m^{-2}) appeared to decrease the duration of the lag phase.

Table 7.2 Estimated duration of the lag phase of F655 re-appearance in 8-day old dark-grown (D) and red pre-irradiated (R) maize leaves (see Table 7.1 for details) at various temperatures.

Temperature (°C)	Estimated lag phase (min)	
	D leaves	R leaves
13	10-30[a]	n.d.
19	9	8
20	7	n.d.
22	5.5	4
24	4	3

[a] after 30 min a substantial amount of F655 had re-appeared

n.d., not determined

Spectra of etioplasts were made with samples from a stock stored at 4 °C in darkness. The period between recording of the first and last spectrum of a series was usually in excess of 10 h. It was observed that within a given time interval, less F656 re-appeared after photoconversion of P650 in "aged etioplasts" than in fresh samples. Taking this into account, F656 re-appearance in isolated etioplasts was observed without a lag phase (Figs. 7.3 and 7.5). After a prolonged dark period following R irradiation, the F656 level decreased. Table 7.3 shows data on the duration of the dark periods after which half the final level of F656 was reached at various temperatures. Although the data are only provisional, they indicate that R pre-irradiation in vivo has no significant effect on the rate of F656 re-appearance in isolated etioplasts. These data agree with data from absorption studies (Chapter 6).

7.2.5 The initial red shift of the main chlorophyll(ide) band in maize leaves and isolated etioplasts

Isolated etioplasts contain relatively small amounts of photoconvertible PChl(ide) compared with intact leaves (Chapter 6; Section 7.2.3). Consequently, only small amounts of Chl(ide) are formed by in vitro R irradiation. In etioplasts isolated from R pre-irradiated maize leaves (R etioplasts), shifts of the emission maxima of in vitro formed Chl(ide) are partially obscured by the relatively large Chl(ide) band resulting from the R

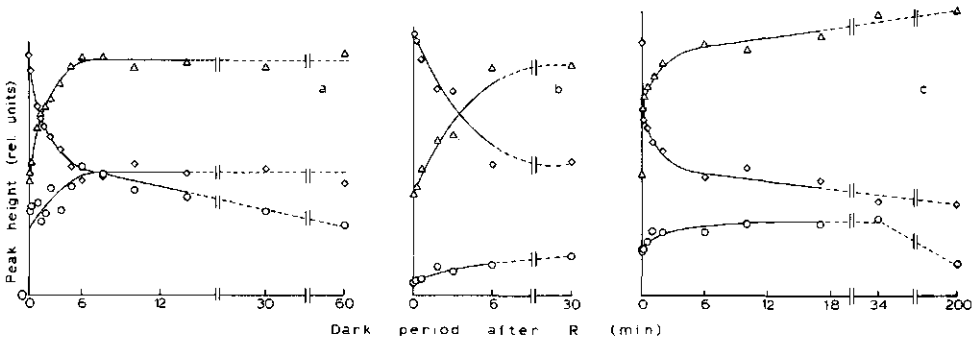


Fig. 7.5 Relative heights of the fluorescence emission bands at 77K of the various protochlorophyll(ide) and chlorophyll(ide) pigments in spectra of etioplasts isolated from 8-day old maize leaves, recorded after various dark periods following a saturating red irradiation (4 s 649 nm, 134 μmol m^{-2} s^{-1}) (R) in vitro. Spectra were normalized for the sum of the bands at 674-677 nm and 692 nm (a, b), or acriflavin fluorescence was used as an internal standard (c). ○ : F655, △ : F674-677, ◇ : F692. a. a crude, washed etioplast preparation from dark grown leaves, at 22 °C, b. a similar etioplast preparation as in a, at 0 °C, c. a crude, washed etioplast preparation from leaves pre-irradiated for 30 s with 658 nm red light (0.85 W m^{-2} or μmol m^{-2} s^{-1}) 17 h prior to etioplast isolation, at 22 °C.

Table 7.3 Half-time of F655 re-appearance in etioplasts isolated from 8-day old dark-grown or red pre-irradiated maize seedlings (D and R etioplasts, respectively) (see Table 7.1 for details) at various temperatures.

Temperature (°C)	Half time of P655 re-appearance (min)	
	D etioplasts	R etioplasts
19	1	n.d.
20	n.d.	2
21	2	1
23	0.5	1
24	0.5	n.d.

n.d., not determined

pre-irradiation. Therefore, in later experiments an *in vivo* pre-irradiation of 0.5 or 2 min instead of 5 min 658 nm (0.85 W m^{-2} or 5 µmol m^{-2} s^{-1}) was given. Although such treatments photoconverted only part of PChl(ide) into Chl(ide), they resulted in 65 and 85%, respectively, of the potentiation of rapid Chl *a* accumulation in white light by a standard R pre-irradiation, respectively (Chapter 3, Fig. 3.5).

In spectra of leaves frozen to 77K immediately after a saturating 4 s irradiation with R of 649 nm, two Chl(ide) fluorescence bands are present (Fig. 7.2a): a main band at 692 nm which originates from C685 and a weaker band at 674 nm visible as a shoulder in the spectra, which can be ascribed to C672. The corresponding bands in isolated etioplasts were observed at 688 and 674 nm (Fig. 7.3a). In maize leaves, only the last part of the shift of the main band from 690 to 694-696 nm (*e.g.*, Gassman *et al.*, 1968) could be observed: within a dark period of 10 s at room temperature the peak position shifted from 691.8 to 692.8 nm. These bands will be referred to collectively as F692. In isolated etioplasts, a red shift of the main fluorescence peak was more pronounced. The peak shifted from 688 to 692 nm within 1 min at room temperature (Fig. 7.3). At 0 °C, the duration of this shift was about 3 min (Table 7.4). To conform to the situation in leaves, the initial main Chl(ide) band in isolated etioplasts will be referred to as F692. No effect of *in vivo* R pre-irradiation on the rate of the initial red shift in isolated etioplasts was detected (Table 7.4).

7.2.6 The Shibata shift in maize leaves and isolated etioplasts and the effect of red pre-irradiation

In intact leaves, F692 began to decrease and a band at 680 nm (F680) was formed after a dark period of 1 - 2 minutes following R irradiation (Figs.

7.2 and 7.4) (Shibata shift). In isolated etioplasts, unlike in leaves, F692 decreased and F674 increased immediately in the dark following R irradiation (Figs. 7.3 and 7.5). After its initial fast increase, the ratio F674 : F692 continued to increase slowly in several experiments.

For reasons dicussed below (Section 7.2.7), the decrease of the F692 band was used as a measure for the Shibata shift rather than the change of the ratio F692 : F680. Such data fit in well with those derived from absorption measurements (Chapter 6, Fig. 6.8). Fluorescence studies support the conclusion of absorption studies that R pre-irradiation appears to decrease the duration of the Shibata shift in maize leaves.

In etioplasts stored for several hours at 4 °C in darkness ("aged etioplasts"), the amount of F674 formed upon photoconversion of PChl(ide) was as high as, or even higher than in fresh etioplasts. However, the amount of F692 formed was considerably lower. Therefore, the storage period before PChl(ide) photoconversion has to be taken into account. In one experiment the etioplast samples were irradiated in rapid succession, which appeared the best procedure. In this experiment the duration of the "Shibata-like" shift was relatively long (Table 7.5), suggesting that "ageing" of etioplasts increases the rate of the "Shibata-like" shift. It is therefore conceivable that in "ideal", completely native etioplasts, rates approaching those in intact leaves could be observed. The data shown in Table 7.5 suggest a small increase in the rate of the "Shibata-like" shift in isolated etioplasts induced by R pre-irradiation *in vivo*. However, results were variable and further experiments with simultaneously irradiated etioplasts would be needed

Table 7.4 Estimated duration of the initial red shift of the fluorescence maximum of newly formed chlorophyll(ide) from 688 to 693 nm in etioplasts isolated from 8-day old dark-grown or red pre-irradiated maize leaves (D and R etioplasts, respectively) (see Table 7.1 for details) at various temperatures.

Temperature (°C)	Duration of the red shift (s)	
	D etioplasts	R etioplasts
0	165	n.d.
19	55	n.d.
20	n.d.	60
21	n.d.	50
22	50	n.d.
23	40	45
25	30	n.d.

n.d., not determined

to examine whether the difference between etioplasts isolated from dark-grown and R pre-irradiated plants is statistically significant.

7.2.7 Other shifts in maize leaves and isolated etioplasts

Immediately after R irradiation, the fluorescence spectra of maize leaves showed a weak band at 674 nm in addition to the main band at 692 nm (Fig. 7.2). The ratio of the peak heights of the bands was 1 : 5, i.e. similar to the ratio of the peak heights of the corresponding absorption bands. After a 1-2 min dark period, the peak height of "F674" increased and a red shift of the peak was obvious in derivative spectra and curve analysis. Near completion of this wavelength shift, F680 had appeared and a weak band at about 669 nm was observed (Fig. 7.2c). The HWs of the "intermediate forms" between F674 and F680 exceeded those of F674 and F680 by several nm. It is now proposed that the intermediate forms are in fact composed of both F674 and F680, the latter being formed in increasing amounts from F692 by the Shibata shift. Indeed, curves could be fitted equally well with one intermediate form and with two bands, with peaks at 674 and 680 nm (Fig. 7.2b). The band at 669 nm may then be formed by a blue shift from F674 (see Section 7.3). Since P650 was present in insignificant amounts at the time F669 was first detected (Fig. 7.4), F669 can not be attributed to a vibrational band of P650.

In isolated etioplasts, a red shift of F674 to 677 nm was observed which

Table 7.5 Estimated half time of the "Shibata-like" fluorescence shift in etioplasts isolated from 8-day old dark-grown or red pre-irradiated maize seedlings (D and R etioplasts, respectively) (see Table 7.1 for details) at various temperatures.

Temperature (°C)	Half time of the "Shibata-like" shift (s)	
	D etioplasts	R etioplasts
0	180	n.d.
19	40	n.d.
20	-	20
21	45	35
22	100[a]	n.d.
23	30	25
24	20	n.d.

[a] The etioplast samples of this series were irradiated in rapid succession soon after isolation and after an appropriate dark period at 22 °C were cooled and stored at 77K until measurement

n.d., not determined

was faster but less complete than the similar one in intact leaves: its duration was less than 1 min at room temperature and about 3 min at 0 °C. No lag phase was observed for this shift in isolated etioplasts. As in the case of leaves, F677 in isolated etioplasts is probably composed of F674 or F669 and F681. The complicated behaviour of bands in the 669-680 nm region led to the decision to use the decrease of F692 rather than the ratio F692 : F680 as a measure for the Shibata(-like) shift (Section 7.2.6).

7.2.8 Fluorescence characteristics of bean leaves, etioplasts and protochlorophyll(ide) holochrome

The fluorescence spectra of primary leaves from 11-day old dark-grown bean seedlings and etioplasts isolated from 10-day old bean seedlings, resembled spectra of maize leaves and etioplasts with regard to peak positions, HWs and ratio of the heights of the PChl(ide) bands. Following R irradiation, similar shifts of Chl(ide) emission peaks in bean leaves and etioplasts were observed as in the case of maize.

A PChl(ide) holochrome preparation in glycerol-tricine buffer 3:1, pH 8.0, was obtained from 10-day old dark-grown bean leaves. The fluorescence spectrum of non-irradiated PChl(ide) holochrome showed a main peak at 653 nm with distinct shoulders at about 630 and 644 nm (Fig. 7.1). Maximum emission

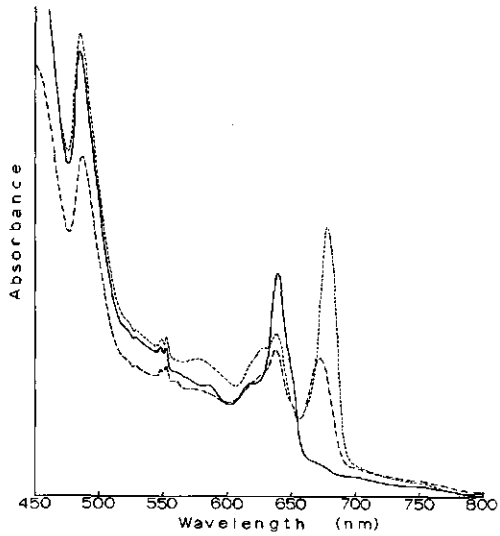


Fig. 7.6 77K absorption spectra of a protochlorophyll(ide) holochrome preparation from 10-day old dark-grown bean leaves in glycerol-tricine buffer 3:1, pH 8.0. ——— : non-irradiated; — — : warmed to 5 °C, irradiated for 3 min with red light (649 nm, 134 μmol m^{-2} s^{-1}) (R) at 5 °C and subsequently recooled to 77K; - - - : warmed to 5 °C, R irradiated for 3 min at 5 °C, kept in darkness for 210 min at 19.5 °C and subsequentely recooled to 77K.

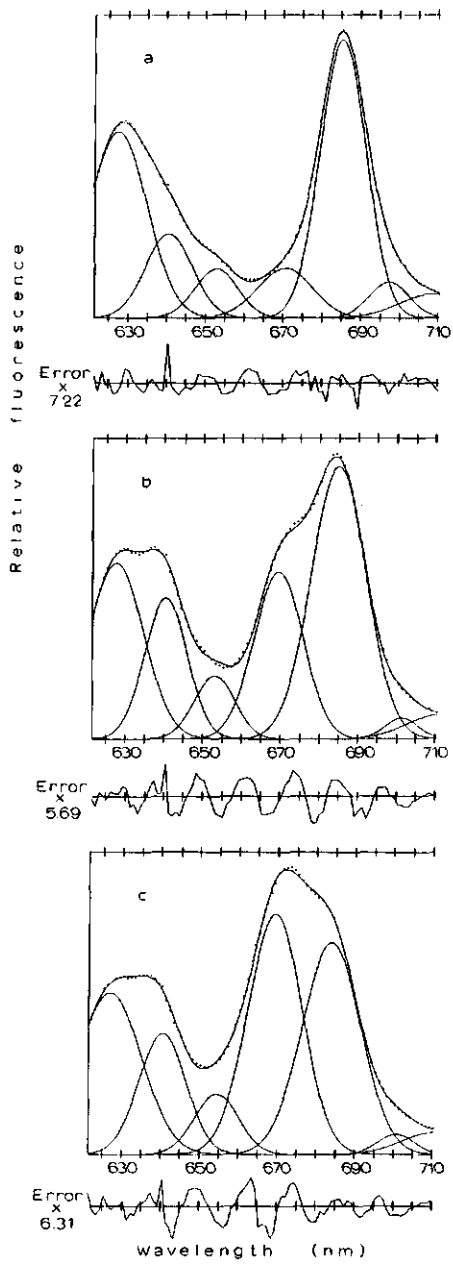


Fig. 7.7 77K fluorescence emission spectra of the bean protochlorophyll(ide) holochrome preparation of Fig. 7.6, cooled to 77K immediately after irradiation for 6 s with red light (649 nm, 134 μmol m^{-2} s^{-1}) (R) (a) or after a dark period of 30 min (b) or 126 min (c) following R irradiation at 19.5 °C. Excitation wavelength: 425 nm. See also Fig. 7.1.

by P650 at 653 nm as compared to 656 nm for leaves was also observed by Kahn et al. (1970). Whereas in intact leaves a fluorescence band at F654.5 remains after R irradiation, F653 in the holochrome preparation proved largely photoconvertible. The band with maximum emission at 642 nm was relatively high in bean PChl(ide) holochrome as compared to F643 in bean and maize leaves and etioplasts (Table 7.1). The 77K absorption spectrum of bean PChl(ide) holochrome showed a peak at 639 nm and only a shoulder at 652 nm (Fig. 7.6), in agreement with data of Kahn et al. (1970). Upon cooling to 77K, the absorption maximum of P636 shifts slightly to the red and the P650 absorption is increased more than the P636 absorption (Kahn et al., 1970). Apparently, the ratio of P650 : P636 in bean PChl(ide) holochrome is even lower than in etioplasts. Consequently, relatively less energy can be transferred from P636 to P650 and emission by P636 is observed at 643 nm (Fig. 7.1).

Immediately after photoconversion of PChl(ide) holochrome, the 77K fluorescence emission maximum of the main Chl(ide) band was at 686 nm (Figs. 7.7 and 7.8). Second and fourth derivative spectra and curve analysis revealed a second Chl(ide) band at 671 nm. During a dark period at room temperature following photoconversion, the low temperature ratio F671 : F686 increased slowly (Figs. 7.7 and 7.8). A similar "Shibata-like" shift was also observed in holochrome absorption spectra (Fig. 7.6).

7.2.9 77K fluorescence emission maxima of chlorophyll(ide) formed by in vivo red pre-irradiation of high and low fluence

77K fluorescence emission spectra of maize leaves R pre-irradiated during 2 or 5 min (658 nm, 0.6 and 1.5 mmol m^{-2}, respectively) about 17 h prior to cooling and of etioplasts isolated from similar leaves showed a main band at 680 nm and weaker bands at 669 and 692 nm. However, spectra of etioplasts isolated from leaves pre-irradiated with a subsaturating fluence of R (150

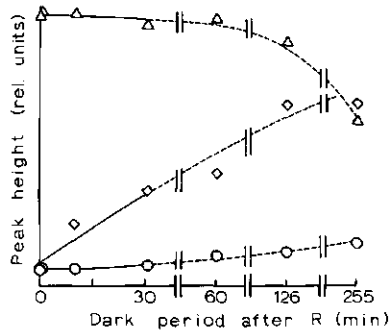


Fig. 7.8 Relative heights of the 77K fluorescence emission bands of the various protochlorophyll(ide) and chlorophyll(ide) pigments in spectra of the bean protochlorophyll(ide) holochrome preparation of Fig. 7.6. The spectra were recorded after various dark periods at 19.5 °C following irradiation for 6 s with red light (649 nm, 134 μmol m^{-2} s^{-1}) (R). ○: F652, ◇: F671, △ : F686.

μmol m^{-2}) showed a major band at 674 nm and weaker bands at 680 and 692 nm. Also, in a sample of etioplasts isolated from dark-grown leaves, in which PChl(ide) was only partially photoconverted in vitro, the ratio F674 : F692 at 77K was much higher than in similar samples following saturating irradiation.

7.2.10 Changes in fluorescence yield of chlorophyll(ide) in maize leaves and isolated etioplasts at room temperature

Maize leaves and isolated etioplasts were examined at room temperature for possible changes in Chl(ide) fluorescence yield as observed in bean leaves (Thorne, 1971a,b; Van der Cammen, 1982). Samples were irradiated within the fluorimeter with blue excitation light (see Section 2.12). P650 was rapidly photoconverted at this temperature. As soon as the light was turned on, the fluorescence emission spectrum was repeatedly scanned from 660 to 700 nm (Figs. 7.9 and 7.10). The first reliable measurements were about 25 s after the onset of the irradiation. In maize leaves, a slight initial decrease of the fluorescence yield of Chl(ide) formed by photoconversion of P650 was observed. The emission maximum shifted from 685.5 nm to 678 nm. Before this shift was completed, the fluorescence yield started to increase until after about 8 min at room temperature a final level was attained, which was almost 250% of the original level.

In etioplasts, the shift of the emission maximum of Chl(ide) was from about 684 nm to 680 nm. This shift took place immediately after the onset of irradiation and was accompanied by a decrease rather than an increase of the Chl(ide) fluorescence yield (Figs. 7.9 and 7.10).

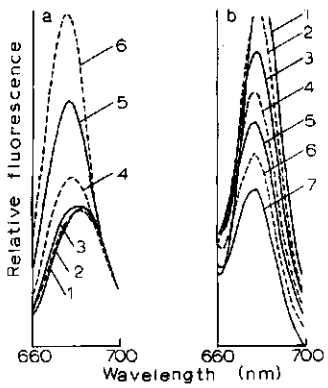


Fig. 7.9 Fluorescence emission spectra at room temperature during continuous irradiation with blue excitation light (425 nm). a. spectra of an 8-day old dark-grown maize leaf, recorded at 21 °C. Peaks reached after about 25 s (1), 55 s (2), 2 min (3), 3 min (4), 5 min (5) and 8 min (6), b. spectra of a crude, washed maize etioplast preparation from dark grown leaves, recorded at 22 °C. Peaks reached after about 20 s (1), 40 s (2), 1.5 min (3), 3 min (4), 4.5 min (5), 8 min (6) and 11 min (7).

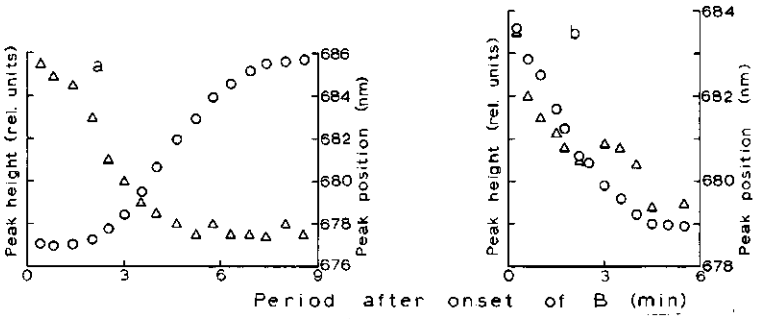


Fig. 7.10 Changes in peak height (O) and peak position (Δ) of chlorophyll(ide) fluorescence emission bands at room temperature during continuous irradiation with blue excitation light (425 nm) (B), a. an 8-day old dark (D)-grown maize leaf, at 21 °C, b. a crude, washed etioplast preparation from similar leaves, at 22 °C.

7.3 Discussion

Visual inspection of 77K fluorescence emission spectra of maize leaves and isolated etioplasts reveals a shift of the peak position of newly formed Chl(ide) in darkness at room temperature. However, the existence of overlapping bands and the occurrence of simultaneous shifts complicated kinetic studies. Particularly in the case of R pre-irradiated samples Chl(ide) resulting from the pre-irradiation masks wavelength shifts of newly formed Chl(ide). In such cases, curve fitting was indispensible. However, derivative spectroscopy as well as curve fitting have their pitfalls (Shrager, 1983; Vandeginste and De Galan, 1975). Application of these methods to fluorescence spectra of maize leaves yielded results, essentially in agreement with data in the literature, both with respect to the various PChl(ide) and Chl(ide) components and to wavelength shifts, therefore indicating their usefulness. Gaussian curves provided a satisfactory fit for the bands in the 620-710 nm region of the spectra.

In 77K spectra of leaves and etioplasts from maize, four PChl(ide) bands were detected. In addition to the main bands at 630 nm and 656 nm, a fluorescence emission band at 643 nm was observed. This band can be attributed to P636, since a fluorescence emission peak at 643 nm was observed in spectra of leaves containing high levels of P636 after treatment with 5-aminolevulinic acid (ALA) (e.g., Sundqvist and Klockare, 1975). Also in spectra of bean PChl(ide) holochrome with a high P636 : P650 ratio (Kahn et al., 1970; Section 7.2.8) had a pronounced band at 643 nm. A 77K fluorescence band of P636 has not been previously reported in etiolated leaves older than 6 days without ALA treatment. Following saturating photoconversion of PChl(ide) by R, a small band remained around 654.5 nm in leaves and etioplasts, i.e. at slightly shorter wavelength than photoconvertible P656. In absorption spectra a corresponding persistent band around 650 nm was observed, in agreement with data of Virgin and French (1973). A non-photoconvertible PChl(ide) species with maximum 77K emission at 649 nm was detected by El Hamouri and Sironval (1980) after incubation of isolated

cucumber etioplasts. The F654.5 : F630 and F654.5 : F643 ratios in isolated etioplasts were similar to those in intact leaves and did not increase during storage at 4 °C in darkness. In 77K fluorescence spectra of bean PChl(ide) holochrome, a phototransformable band was observed at 653 instead of 656 nm. Apparently, in this sample the configuration of the holochrome protein is altered somewhat by the isolation procedure resulting in a change of the fluorescence emission maximum of P650, without affecting its capacity for phototransformation.

In agreement with results of Horton and Leech (1972; 1975) for maize etioplasts and of El Hamouri and Sironval (1980) for cucumber etioplasts, a decrease of the etioplast P650 content was observed during storage. Concomitantly, P628 was observed to increase, suggesting that P628 is formed from P650. Whether P636 changed during storage can not be concluded from these fluorescence studies (Section 7.2.3). However, absorption measurements showed the ratio P636 : P628 in isolated etioplasts to be higher than in leaves, so that apparently part of P650 is also transformed to P636 during isolation.

Absorption spectroscopy proved more suitable than fluorescence emission spectroscopy for studies on P650 regeneration in darkness. However, fluorescence studies confirmed results of absorption measurements in that R pre-irradiation shortens the duration of the lag phase in P650 regeneration in maize leaves. In absorption as well as fluorescence studies P650 regeneration without a lag phase was observed in isolated etioplasts. No stimulation by in vivo R pre-irradiation on the rate of P650 regeneration in isolated etioplasts could be observed.

Both in leaves and in isolated etioplasts, photoconversion of PChl(ide) resulted in "immediate" formation of some F674. At the same time, F692 (leaves) and F688 (etioplasts) were formed. In etioplasts isolated from seedlings pre-irradiated with a low R fluence, F674 was the dominant Chl(ide) band. This band probably corresponds to F675 observed by Litvin and Belyaeva (1971) in bean leaves after low irradiance or short-time irradiation. Láng and Sárvári (1974) have reported the presence of F674 in maize leaves and suggested that it represents a PChl(ide)-Chl(ide) dimer or aggregate. According to Litvin and Belyaeva (1971) this form is rather stable, however Láng and Sárvári (1974) reported a blue shift from 675 to 670-672 nm of the fluorescence maximum when partially phototransformed leaves were kept in the dark for 5-10 min. After complete photoconversion of PChl(ide) in maize leaves a similar shift of the fluorescence maximum from 674 to 669 nm is likely from the present experiments (Section 7.2.7). However, in maize seedlings which were pre-irradiated with a non-saturating R fluence, F674 was apparently stable.

The initial 77K fluorescence emission maximum of the main Chl(ide) band formed in isolated maize etioplasts by a brief saturating R irradiation, shifted from 688 to 693 nm during a dark period of about 1 min at room temperature. In maize leaves, this initial red shift appeared to be more

rapid and only the last part was observed. According to Bonner (1969), in 6-day old maize leaves the shift is complete after 20-30 s at 25 °C. In bean PChl(ide) holochrome such a red shift was not detected, in agreement with Schultz and Sauer (1972). Oliver and Griffiths (1982) obtained evidence that the Chl(ide) forms before and after the initial red shift, C678 and C685, respectively, represent ternary complexes of the enzyme protochlorophyllide reductase with Chl(ide) and either $NADP^+$ (C678) or NADPH (C685). The NADPH required for the shift can be supplied by the high level of endogenous NADPH found in etioplasts in dark-grown plants (Mapleston and Griffiths, 1978). Lack of NADPH in holochrome preparations is held responsible for the absence of this shift (Oliver and Griffiths, 1982). A low supply of NADPH in isolated etioplasts may then explain the relatively slow rate of the shift in this object. El Hamouri and Sironval (1980) reported that after irradiation of isolated cucumber etioplasts, the 77K fluorescence emission maximum of Chl(ide) was at 688 nm. However, when a NADPH regenerating enzyme system was added before irradiation, the maximum was found at 696 nm. In addition to the effect of NADPH, the red shift has been ascribed to a re-arrangement of newly formed Chl(ide) (Mathis and Sauer, 1973), which is consistent with the change in fluorescence lifetime observed by Van der Cammen and Goedheer (1980). The shorter lifetime of the species after the shift indicates a greater interaction with its molecular environment. The corresponding decrease in fluorescence yield at room temperature observed by Thorne (1971a) in bean leaves, was hardly detectable in the present exeriments with maize leaves (Section 7.2.10), probably because of the rapidity of the shift. In isolated maize etioplasts, a decrease in fluorescence yield did occur, however no red shift of the Chl(ide) fluorescence emission peak was observed at room temperature. The decrease in fluorescence in isolated etioplasts is most likely caused by photobleaching of Chl(ide).

A Shibata(-like) blue shift of the Chl(ide) emission maximum from 692 to 680 nm was observed in maize and bean leaves, isolated etioplasts and bean PChl(ide) holochrome. Analysis of a number of spectra taken in the course of the shift consistently pointed to the presence of only the two components F692 and F680 in this wavelength interval, no indications of intermediate peak position being observed. This suggests interconversion of two discrete pigment forms, rather than a continuous wavelength shift, as *e.g.* caused by a gradual change in the physical environment. That no isosbestic point was seen, may be explained by simultaneous changes in the level of a number of other pigments having appreciable fluorescence in the same region. The 77K emission maxima of the two Chl(ide) species involved in the Shibata(-like) fluorescence shift were at shorter wavelengths in the holochrome preparation than in leaves and isolated etioplasts, which may be attributed to a different conformation or some denaturation of the holochrome protein during isolation. The duration of the shift differed in the various preparations. The high glycerol concentration of the medium is probably the cause of the slow rate of

the shift in the PChl(ide) holochrome preparation (cf. e.g., Brodersen, 1976). In contrast to the initial red shift, which was slower in isolated etioplasts than in leaves, the Shibata(-like) fluorescence shift appeared considerably more rapid in isolated etioplasts. In experiments with bean leaves, Thorne (1971a) also observed that the rates of the initial red shift and the Shibata shift are not necessarily proportional. Interestingly, ALA treated leaves having a high P636/P650 ratio typical of isolated etioplasts, similarly show a rapid Shibata-like shift (Klockare and Sundqvist, 1977; Oliver and Griffiths, 1982). In very young dark-grown leaves, as well as in the alga Euglena, where P636 is the only photoconvertible PChl(ide) species, Chl(ide) has its absorption maximum at 672 nm immediately after R irradiation and there is no Shibata shift (Klein and Schiff, 1972). Similar results were obtained with frozen and thawed leaves (Butler and Briggs, 1966). Nielsen (1975) observed that in a barley mutant producing only photoconvertible PChl(ide), the Chl(ide) absorption maximum was at 684 nm and no Shibata shift to 672 nm occurred. However, C672 was formed in this mutant after ALA feeding which results in formation of PChl(ide) (probably both P628 and P636) with absorption maximum around 633 nm. These observations suggest that the presence of initially photo-inactive PChl(ide) is a prerequisite for the Shibata shift. The inactive PChl(ide) possibly causes Chl(ide) to dissociate from the holochrome protein. This suggestion is supported by the observation of Oliver and Griffiths (1982), that the absorption maximum of Chl(ide) formed by irradiation of etioplast membranes enriched in photo-active PChl(ide), remains at 684 nm. Only upon addition of exogenous PChl(ide), the maximum shifts to 672 nm. It remains unclear whether the C672 which is formed either by a rapid Shibata shift or directly without a Shibata shift and the C672 formed from C684 by a slow Shibata shift, are identical. Van der Cammen and Goedheer (1980) showed that C672 in bean leaves after freezing and thawing has a longer fluorescence lifetime than C672 after the Shibata shift, indicating that the interaction of C672 with its surroundings is less for the form after freezing and thawing. Possibly, inability of isolated etioplasts to incorporate Chl(ide) at specific sites of the membranes protecting them against photobleaching and denaturation, is the reason for the instability of newly formed Chl(ide) in the isolated organelles.

Both absorption and fluorescence measurements indicate that R pre-irradiation increases the rate of the Shibata shift in 8-day old maize leaves. Data obtained with isolated etioplasts were too variable to demonstrate a stimulation of the in vitro shift by in vivo R pre-irradiation. The results presented in Chapter 6 and in this chapter show that isolation of etioplasts and storage at 4 ^{o}C in darkness drastically influence the kinetics of both P650 regeneration and wavelength shifts of newly formed Chl(ide). Even if better techniques become available for the isolation of etioplasts, it appears doubtful whether an effect of an in vitro R pre-irradiation can be established. Only a positive effect of R could provide evidence for a direct action of Pfr upon the etioplasts with regard to Chl accumulation.

8 GENERAL DISCUSSION

8.1 Sensitivity of plants to R potentiation of rapid chlorophyll accumulation

The action of phytochrome (P) in the development of etioplasts to chloroplasts has been studied in seedlings of maize. This plant was chosen in preference to pea and bean (Raven, 1973), since etioplasts, isolated from the latter proved insufficiently stable for in vitro studies (e.g., Chapter 5). Maize seedlings proved less sensitive to red light (R) potentiation of rapid chlorophyll (Chl) accumulation in white light (WL) than pea. However, dark-grown maize seedlings clearly show a small response in the very low fluence response (VLFR) region. Sensitivity of R pre-irradiated maize seedlings to a second R irradiation was considerably lower and such plants only showed a low fluence response (LFR). This decrease in sensitivity upon de-etiolation is common in photomorphogenic reactions of plants, although the opposite effect has also been observed (Beggs et al., 1980). A decrease in sensitivity was observed for photocontrol of hook opening in bean seedlings (Klein et al., 1967), of elongation of pea segments (Fox and Hillman, 1968), of hypocotyl elongation of Cucumis seedlings (Black and Shuttleworth, 1976) and of wheat seedling growth (Smith et al., 1985). Probably related to this phenomenon are the biphasic fluence-response curves observed for inhibition of maize mesocotyl elongation (Vanderhoef et al., 1979), for stimulation of oat coleoptile growth and for mesocotyl growth inhibition (Mandoli and Briggs, 1981), for geotropic reactions of oat coleoptiles (Blaauw-Jansen and Post, 1985) and for seed germination (e.g., Blaauw-Jansen and Blaauw, 1975; Small et al., 1979; Cone, 1985; VanDerWoude, 1985). In most of these cases, a VLFR is observed at fluences of 10^{-4} to 10^{-1} μmol m^{-2} R, which produce 0.1% Pfr or less. The LFR on the other hand is observed at fluences of about 1 to 10^{3} μmol m^{-2}, producing from 1 to 86% Pfr (Cone, 1985; Vierstra and Quail, 1983). VLF and LF responses have also been observed for the phytochrome (P) induced increase in transcript abundance of mRNA of the light-harvesting Chl a/b binding protein (Kaufman et al., 1985). Fluence-response curves for R induction of rapid Chl accumulation for pea (Raven and Shropshire, 1975; Spruit et al., 1979), bean (Spruit et al., 1979) and maize (Chapter 3) are more or less biphasic, however for bean a "VLFR" and a "LFR" were observed at fluences higher than in most other cases. In maize seedlings, the VLFR is saturated at about 1 μmol m^{-2} R, resulting in about 0.1-1% Pfr, and within the error limits of the data the LFR appears to saturate at about 1 mmol m^{-2} R which attains the maximum % Pfr possible.

Several hypotheses have been put forward to explain biphasic responses to R, some of which may also provide an explanation for the decrease in sensitivity of pea and maize seedlings to R, or loss of the VLFR, upon de-etiolation. Small et al. (1979) proposed that induction of germination in lettuce seed is under the control of two processes acting in series, the first being extremely sensitive to Pfr, the second much less so. A similar

mechanism might also be involved in R potentiation of rapid Chl accumulation. Several P controlled processes related to Chl accumulation in WL have been reported (Schopfer and Apel, 1983). An indication for at least two different initial actions of Pfr is provided by the observation of Jabben and Mohr (1975) that the effect of a R pulse on the rate of protochlorophyll(ide) (PChl(ide)) regeneration in mustard seedlings remains fully reversible by a far-red light (FR) pulse for more than 5 min, while reversibility of the effect of a R pulse on the duration of the Shibata shift is lost within 2 min. Effects of P on processes both inside the etioplasts (e.g., synthesis of 5-aminolevulinic acid (Masoner and Kasemir, 1975)) and outside the organelles (e.g., induction of the appearance of mRNA activity for the nuclear coded apoprotein of the light-harvesting Chl a/b protein complex (Apel, 1979)) give additional support for the idea that more than one P controlled process is involved in induction of rapid Chl accumulation in WL. It would be interesting to determine the sensitivity of these processes to R in the light of Small's hypothesis. Blaauw-Jansen (1983) proposed the existence of a Pfr destroying enzyme which was assumed to become active at a certain critical level of Pfr, accounting for a plateau or even for a decrease in response, the latter increasing again when the enzyme becomes saturated upon increasing Pfr formation. In this model, the decrease in sensitivity to R upon de-etiolation in maize and pea seedlings requires the assumption that the enzyme remains active throughout the dark period of 24 h between both R pre-irradiations. Cone et al. (1985) attempted to explain similar complex fluence responses on the basis of a Pfr dependent VLFR changing the response to the LFR range. A mechanism of P action based on interaction of dichromophoric P with receptors was proposed by VanDerWoude (1985). He assumed that P exists in three interconvertible species, viz. Pr:Pr, Pr:Pfr and Pfr:Pfr and that a VLFR involves interaction of Pr:Pfr with a specific receptor X. Photoconversion of at least one of the two chromophores of a dimer into Pfr would induce binding of P. The VLFR to Pr:Pfr is thought to be dependent on the state of the membrane. For an explanation of the decrease in sensitivity to R induced by de-etiolation, it is essential that P remains bound to X after dark reversion of Pr:Pfr or Pfr:Pfr to Pr:Pr. A decrease in sensitivity to R, or loss of VLFR, upon de-etiolation is also readily explained by the P transport model proposed by Raven and Spruit (1973) (Chapter 1, Section 1.5). This model, which will be discussed in more detail in Section 8.4, is similar in some aspects to that of VanDerWoude (1985) but does not require the existence of P dimers.

8.2 Phytochrome in etioplasts

With regard to potentiation of rapid Chl accumulation, etioplasts are candidates for the sites of action of P. Cooke et al. (1975) and Smith and coworkers (e.g., Evans and Smith, 1976a,b) had already demonstrated the presence of P in etioplasts isolated under a dim green safelight. Spectrophotometric measurements of etioplast preparations obtained in complete darkness from completely dark-grown maize, pea and bean leaves (D etioplasts)

showed that P was already associated with the organelles in darkness (Chapter 4). The P content of etioplasts isolated from leaves of R pre-irradiated maize seedlings (R etioplasts) marginally exceeded that of D maize etioplasts.

It can not be excluded that P measured in etioplast preparations is an isolation artifact. However, some evidence for etioplast-associated P being involved in potentiation of rapid Chl accumulation in WL is provided by the observation that the potentiating effect of a R pre-irradiation of maize seedlings is still partially reversible by FR even after a dark period of 24 h (Chapter 3, Table 3.2). This implies the presence of Pfr molecules at that moment, whereas P measurements of leaf homogenates show disappearance of Pfr as a result of in vivo dark destruction in bulk P within 4 h of darkness after R irradiation. The amount of P associated with etioplasts did not decrease during the first 12 h of darkness (Chapter 4, Fig. 4.4), indicating that no dark destruction occurs in etioplast-associated Pfr. Different rates of Pfr dark destruction in a single plant species have been reported: in Amaranthus, Brassica and Pharbitis seedlings, the initial fast Pfr destruction is followed by a slow Pfr disappearance after a low Pfr/P ratio has been reached (Heim et al., 1981; Brockmann and Schäfer, 1982). Slow destruction rates or even complete stability of Pfr have been observed in tissues grown for a long period in light (Jabben and Holmes, 1983). However, evidence exists that P in light-grown tissue is a different species from that in etiolated tissue (Tokuhisa et al., 1985). It is attractive to attribute at least that part of R potentiation which shows a long-term reversibility by FR, to stable etioplast-associated Pfr.

8.3 Phytochrome and developmental processes in isolated etioplasts

Direct evidence for involvement of etioplast-associated Pfr in the potentiation reaction was sought by studying several light-induced developmental processes related to Chl accumulation and possible P involvement in isolated etioplasts. For comparison, these processes were also studied in vivo.

Some limited ultrastructural development in WL was shown by isolated R oat etioplasts. However, this did not parallel development in vivo. Preliminary experiments described in Chapter 5 show that prospects of detecting in vitro effects of Pfr on ultrastructural development of etioplasts are not very promising.

When isolated maize etioplasts are briefly irradiated, chlorophyll(ide) (Chl(ide)) is formed from phototransformable PChl(ide). In subsequent darkness, regeneration of phototransformable PChl(ide) (mainly P650) occurs (Chapter 6). The lag phase of several minutes in P650 regeneration as observed in vivo was absent in isolated etioplasts. Also, contrary to the situation in vivo, no effect of Pfr on the rate of P650 regeneration was observed. Moreover, the proportion of PChl(ide) that was photoconvertible in vitro appeared to be lower than in vivo. Curve fitting of 77K fluorescence emission spectra with Gaussian components was applied to study the kinetics of

wavelength shifts of the fluorescence maxima of newly formed Chl(ide) (Chapter 7). Although the results have to be interpreted with caution, similar shifts, except for differences in their duration, appear to occur *in vivo* and *in vitro*. *In vivo*, the duration of the Shibata shift was shortened by Pfr, but this was less evident in isolated etioplasts.

Observations on regeneration of photoconvertible PChl(ide) *in vivo* are in agreement with reports in the literature (*e.g.*, Kasemir, 1983b) in that Pfr appears to increase the biosynthetic capacity of seedlings to form Chl(ide) *via* a stimulation of PChl(ide) formation. However, the lag phase in Chl accumulation is more pronounced when greening occurs in WL of high irradiance (Virgin, 1972; Raven, 1973; Chapter 3). Raven (1973) also observed that at 0 °C, the initial photoconversion of PChl(ide) yielded significantly more (about 50%) Chl(ide) in green (529 nm) than in R (650 nm). This indicates that photodestruction of Chl(ide) also plays a role in the lag phase and indeed Pfr has been shown to protect Chl against photodestruction (*e.g.*, Oelze-Karow *et al.*, 1983). Singlet oxygen formed by energy transfer from Chl(ide) triplet states is probably responsible for Chl(ide) photodestruction and may ultimately lead to damage of other systems, *e.g.*, prolamellar body (PLB) structure and enzymes involved in PChl(ide) synthesis (Dorsman *et al.*, 1977; Ryberg *et al.*, 1981). A stimulation of the rate of the Shibata shift (Jabben and Mohr, 1975; Chapters 6 and 7) may be part of the protection mechanism by Pfr, since Axelsson (1976) has shown that C685, the Chl(ide) form preceding the Shibata shift, is 4-5 times less photostable than the C672 form present afterwards. Carotenoids play an important role in protection against singlet oxygen (Krinsky, 1978). Although the bulk carotenoid content of maize seedlings was not increased by R pre-irradiation, R induced a slight stimulation of carotenoid formation in subsequent WL (Chapter 3). Association of Chl and carotenoids in pigment-protein complexes within the thylakoid membranes is probably required for protection of Chl. A relationship may exist between the rate of the Shibata shift and the rate at which Chl and carotenoids become incorporated into such complexes. The apoprotein of a major complex, the light-harvesting Chl *a*/*b* protein complex, is synthesized on cytoplasmic ribosomes (Apel and Kloppstech, 1978). Both the amount of mRNA activity (Apel, 1979) and the steady-state level of mRNA sequences encoding the apoprotein (Gollmer and Apel, 1983) are controlled by Pfr. Probably, the two constituent polypeptides of the apoprotein are synthesized as soluble precursors which are transported across the plastid envelope by a post-translational mechanism, converted there to their smaller mature form and inserted into the thylakoids (Schmidt *et al.*, 1980). Although the apoproteins of some Chl-protein complexes (CPs) appear to be synthesized within the plastids, that of at least one other CP, the minor Chl *a*/*b* complex CP29, is under nuclear control (Green, 1982). It is not yet known whether newly synthesized Chl(ide) molecules in previously dark-grown or R pre-irradiated seedlings are bound immediately to the final protein complexes. With the present techniques, the proteins of these complexes are detectable only after

about 1 h or more irradiation. More early labile CPs have been detected (Tanaka and Tsuji, 1985), the synthesis sites of which are not known. However, the apoprotein of the ternary NADPH-PChl(ide) oxidoreductase complex is also synthesized in the cytoplasm and the role of the cytoplasm in providing plastids with structural proteins and other compounds which are essential for their development into chloroplasts becomes more and more appreciated.

Changes of both ultrastructure and spectral characteristics of etioplasts which occur upon isolation may at least partially be attributed to disturbance of pigment-protein complexes. For example, isolation of etioplasts results in a decrease of phototransformable PChl(ide) which forms part of the ternary NADPH-PChl(ide) oxidoreductase complex (Griffiths, 1978), a major constituent of the PLB. Dehesh and Ryberg (1985) argue that the paracrystalline structure of the PLB is dependent on the presence of this complex. An alteration of the complex, which may be caused either by insufficient NADPH (Chapter 7) or by a lack of supply of the apoprotein from the cytoplasm, may account for both a decrease of photoconvertible PChl(ide) (Chapters 6 and 7) and the modified behaviour of isolated etioplasts with regard to PLB transformation (Chapter 5). Failure of isolated etioplasts to successfully incorporate newly formed Chl(ide) within Chl-protein complexes may account for the lability of newly formed Chl(ide) (Chapter 6) and its sensitivity to photodestruction (Chapter 7). Furthermore, it is conceivable that isolated etioplasts are unable to form incipient grana (Chapter 5) because they lack essential polypeptides synthesized in the cytoplasm.

Although the above findings suggest that P control of ultrastructural development, P650 regeneration and Shibata shift is mainly exerted from the cytoplasm, they do not necessarily imply that etioplast-associated P is not involved in Chl accumulation in WL. Isolated etioplasts may lack one or more factors originating in the cytoplasm, essential for the expression of the effect of etioplast-associated P on the above mentioned processes. Fractionation studies (Evans and Smith, 1976b; Chapter 4) have shown that P may be localized exclusively in, or attached to, the plastid envelope. This may point to a control by etioplast-associated P of the permeability of the plastid envelope for e.g. apoproteins of Chl-protein complexes as well as other essential compounds, such as metabolites from mitochondria (Wellburn, 1984). Indeed, evidence for Pfr mediated changes in envelope permeability of both etioplasts and mitochondria has been presented by Hampp and Schmidt (1977).

8.4 The locus of phytochrome action

Chloroplast development as well as numerous other processes under P control are often characterized by biphasic fluence-response relationships which are difficult to interpret. It is indeed remarkable that the VLFR occurs upon transformation to Pfr of only one in a hundred thousand Pr molecules, or even less. Previous irradiation with R or even with green

"safelight" (Raven, 1973) results in loss of the VLFR. In such cases sensitivity to further activation decreases by several orders of magnitude. This behaviour is very clearly shown by the greening process in pea seedlings (Raven and Shropshire, 1975) and in maize seedlings (Chapter 3). An attractive explanation for these effects appears to be offered by the phytochrome transport model, originally proposed by Raven and Spruit (1973) and discussed in Section 1.5 and Chapter 4. The present experiments suggest that, upon an initial irradiation, migration of some 1% of total cytoplasmic P to maize etioplasts as Pfr is possible, leading to a local Pfr/P ratio of about 30%. This amount of migration satisfies the requirements of the transport model. Although it is notable that a pre-irradiation with a R fluence producing not more than 1% Pfr saturates the VLFR in maize seedlings (see Section 8.1) as well as in pea seedlings (Spruit et al., 1979), this does not prove that the etioplast is the only, or even the main target of Pfr migration in terms of the model. However, even if a (high) concentration of Pfr in other organelles is demonstrated, this would not necessarily invalidate the model. Nevertheless, some minor modifications of the original scheme seem to be necessary to adapt it to the present observations.

Raven and Spruit (1973) observed that in seedlings of pea, bean and maize, the potentiating effect of R is not or only partially reversible by FR. However, if the R irradiation was followed, after an appropriate dark interval, by a second R irradiation, the additional potentiation by this second R in pea and bean was largely or completely FR reversible. These findings were successfully explained by their model assuming that the migration of Pfr is slow: FR being ineffective if applied during migration. In Chapter 3 it is shown that, if the effect of FR as such is accounted for, in maize both a first and a second R pre-irradiation are 60-80% reversible by FR. This may also apply to pea where it is more difficult to demonstrate, since the potentiating effect of a first FR pre-irradiation is about equal to that of R (e.g., Raven and Spruit, 1973) due to the large VLFR. On the basis of the model, an initial R pre-irradiation is predicted to be reversible by FR when Pfr migration is complete before FR irradiation starts (20 s in the present experiments). FR given after R will then revert receptor-bound Pfr to Pr and further binding by Pfr will be blocked since all receptor sites are now occupied. Interestingly, the Zea paradox (Hillman, 1967; Chapter 1, Section 1.5) can be explained in a similar way. Indeed, a study on the time course of reversibility of the R effect by FR in bean provided evidence for a half life of Pfr migration of only about 3 s at room temperature (Spruit et al., 1979; Spruit, 1980). Such a rapid migration is in agreement with data from immunological studies (Pratt and Marmé, 1976) and pelletability studies (Quail and Briggs, 1978). The occurrence of a second reaction which rapidly escapes reversibility by FR may account for the observed incomplete reversibility of the effect of R (see also Spruit et al., 1979). On the other hand, in maize seedlings the VLFR induced by a first R pre-irradiation is small in comparison to its effect in the LFR range, so that a possible difference in reversibility

of a first and a second R pre-irradiation would hardly be observable.

The potentiating effect of a second R pre-irradiation given 24 h after a standard R pre-irradiation of maize seedlings is compatible with the P transport model when after the initial R irradiation Pfr dark reversion to Pr takes place at the receptors. In view of the small fraction of total cellular P bound to the receptors, this is not necessarily in disagreement with the established fact that in maize no dark reversion can be observed spectrophotometrically. In this respect, observations of Cordonnier et al. (1985) are of interest. Addition of a monoclonal antibody to a preparation of 124-kilodalton oat P, which in the absence of antibody did not exhibit Pfr reversion, induced reversion of Pfr to Pr. Binding of Pfr to receptor sites in the cell might, as well as binding to antibody, result in Pfr reversion to Pr. The observation that the LFR upon a second R pre-irradiation is smaller than after a first R (Table 3.2), suggests that Pfr dark reversion may not be complete at the time of a second R pre-irradiation. This is compatible with the long-term reversibility by FR of the effect of a first R, discussed above. However, the interpretation of the additional potentiation of Chl accumulation by a second R pre-irradiation must be cautious, since 24 h is a sufficient period for many changes to occur during etioplast development, including the possible synthesis of new receptor sites.

Although the results of the present studies can be explained without the assumption of P transport, they are equally consistent with the transport model as proposed by Raven and Spruit (1973) and do not contradict the assumption that the postulated receptor sites are located in or on the etioplasts. It appears attractive to attibute the VLFR to migration of Pfr to the etioplasts and/or other organelles or membranes. Part of the LFR may be attributed to photoconversion into Pfr of Pr already residing in the etioplasts in darkness.

However, though there is now evidence that P is associated with etioplasts, no appreciable P effect on developmental processes could be observed once these organelles were removed from their natural environment. The effect of P on the level of gibberellins extractable from isolated etioplasts found by Cooke et al. (1975) and Evans and Smith (1976a) suggests that etioplast-associated P may be physiologically active. In the present study, indirect indications for involvement of etioplast-associated P in potentiation of rapid Chl accumulation in WL were obtained. It appears highly likely that for the development of etioplasts into chloroplasts, P processes associated with the etioplasts as well as with other cell components are important.

9 SUMMARY

This thesis is concerned with the role played by phytochrome (P) in the development of etioplasts into chloroplasts.

Previously dark-grown maize seedlings are not as sensitive as pea seedlings to very low fluences of red light (R) with regard to induction of rapid chlorophyll (Chl) accumulation in white light (WL), but a very low fluence response (VLFR) has been established in this plant species as well. Much higher fluences of a second R pre-irradiation are required to give an additional effect (low fluence response or LFR). When the effect of far-red light (FR) as such is accounted for, the effects of both a first and a second R pre-irradiation are 60-80% reversible by FR in maize seedlings. In high irradiance WL, the lag phase of Chl accumulation is of considerably longer duration. This indicates that photodestruction of Chl plays a role in the occurrence of a lag phase in Chl accumulation. R has a relatively large effect in high irradiance WL (Chapter 3).

Phytochrome (P) was measured spectrophotometrically for the first time in purified etioplast preparations obtained in complete darkness from dark-grown seedlings (D etioplasts) (Chapter 4). The P content of etioplast preparations from R pre-irradiated seedlings marginally exceeded that of D etioplasts. While the total P content of maize leaves, as measured in homogenates, decreased after R irradiation as a result of Pfr dark destruction, the P content of etioplasts from similar seedlings remained constant.

Attempts to demonstrate a physiological effect of etioplast-associated P were not successful. Preliminary studies on ultrastructural development of etioplasts (Chapter 5) showed that the *in vitro* development during 1 h WL did not completely parallel development *in situ*. An effect of *in vivo* R pre-irradiation on prolamellar body transformation, which was evident *in situ*, was not observed *in vitro*. *In situ*, formation of incipient grana in WL was stimulated by R pre-irradiation, however, isolated etioplasts proved incapable of forming incipient grana.

In the dark, following a short irradiation, regeneration of phototransformable protochlorophyll(ide) (PChl(ide)) was observed in isolated etioplasts (Chapters 6 and 7). However, regeneration kinetics differed from those *in vivo* and no effect of *in vivo* R pre-irradiation could be demonstrated. *In vivo*, the rate of PChl(ide) regeneration was increased by Pfr (Chapter 6).

Wavelength shifts of the 77K fluorescence emission maxima of newly formed chlorophyll(ide) (Chl(ide)) after a short irradiation were studied in leaves and isolated etioplasts. Derivative spectroscopy and curve fitting were applied to study kinetics of these shifts (Chapter 7). The first shift, a red shift, was slower in isolated etioplasts than in leaves. No effect of R pre-irradiation was observed on the rate of this shift. The subsequent blue shift, the so-called Shibata shift, was more rapid, but less complete in isolated etioplasts than in leaves. Whereas in leaves the rate of the Shibata

shift was increased by Pfr, this was hardly, if at all, detectable in isolated etioplasts. The amount of phototransformable PChl(ide) decreased and the rate of the Shibata shift increased during storage of isolated etioplasts at 4 °C in darkness. Newly formed Chl(ide) proved unstable in isolated etioplasts.

The above results point to a decisive influence of the cytoplasm on the development of etioplasts in WL. In this respect, polypeptides of Chl-protein complexes synthesized in the cytoplasm may play an important role. However, a direct influence of etioplast-associated P in the development of etioplasts into chloroplasts, e.g. on permeability of the etioplast envelope, can not be excluded. Evidence for such an effect is found in the observation that the potentiating effect of a R pre-irradiation with regard to rapid Chl accumulation in WL is still partially reversible by FR after a dark period of 24 h. While Pfr in bulk P had already disappeared due to dark destruction after 4 h of darkness, the amount of P associated with etioplasts appeared not to decrease (see above). It is attractive to attribute at least that part of R potentiation which shows a long-term reversibility by FR, to apparently relatively stable etioplast-associated Pfr.

The results are discussed in relation to the phytochrome transport model of Raven and Spruit (Chapter 8). It is concluded that, though they do not provide a direct support for the model, they are not in disagreement with it. The transport model still appears to give an attractive explanation for a number of P responses, such as the VLFR and the Zea P paradox.

10 SAMENVATTING

De invloed van fytochroom op de ontwikkeling van etioplasten tot chloroplasten vormt het centrale thema van dit proefschrift. Het onderzoek was vooral gericht op de z.g. aanloopfase bij chlorofylvorming in wit licht.

Door een korte voorbelichting van 8 dagen oude kiemplanten van maïs met rood licht wordt inactief fytochroom, Pr, omgezet in de actieve vorm Pfr. De aanloopfase bij chlorofyl-accumulatie in wit licht is dan vrijwel afwezig. Volledig in het donker opgegroeide kiemplanten zijn gevoelig voor zeer lage doses rood licht, maar bij een tweede rood-belichting zijn veel hogere doses vereist om een effect te verkrijgen (Hoofdstuk 3). Belichting met ver-rood licht, onmiddellijk na belichting met rood licht, doet het effect van zowel een eerste als een tweede roodbelichting voor een groot deel teniet. In wit licht van hoge intensiteit is de aanloopfase bij chlorofylaccumulatie langer dan in wit licht van lage intensiteit. Dit wijst erop, dat afbraak van chlorofyl onder invloed van licht een oorzaak is van deze aanloopfase. Voorbelichting met rood licht heeft een relatief grote invloed op chlorofylaccumulatie in wit licht van hoge intensiteit.

Voor het eerst werd fytochroom spectrofotometrisch gemeten in preparaten van gezuiverde etioplasten, die in volledig donker waren geïsoleerd uit kiemplanten die in volledig donker waren opgekweekt ("D etioplasten") (Hoofdstuk 4). De hoeveelheid fytochroom in preparaten van etioplasten uit rood-voorbelichte planten was iets groter dan in D etioplasten, echter het verschil was nauwelijks significant. De totale hoeveelheid fytochroom in maïskiemplanten nam ten gevolge van donkerafbraak van Pfr sterk af na een voorbelichting met rood licht, in tegenstelling tot de hoeveelheid in etioplasten uit rood-voorbelichte planten.

De afname van gevoeligheid voor rood licht na voorafgaande roodbelichting kan verklaard worden met een transportmodel voor fytochroom, dat in 1973 werd gepubliceerd door Raven en Spruit. De bovengenoemde resultaten kunnen met dit model in overeenstemming worden gebracht, als het model enigszins wordt gewijzigd. Hierbij wordt aangenomen, dat de receptor-plaatsen, waar de door een roodbelichting in het cytoplasma gevormde Pfr-moleculen zich volgens het model zouden concentreren, zich in of op de etioplasten bevinden. In tegenstelling tot wat in het oorspronkelijke model verondersteld werd, blijken de receptor-organellen dan reeds in kiemplanten, die in het donker zijn opgegroeid, fytochroom (in de Pr-vorm) te bevatten. De aanwezigheid van Pr-moleculen in de etioplasten van in het donker opgegroeide kiemplanten biedt een goede verklaring voor het tweede deel van de dosis-effect curve voor inductie van snelle chlorofylaccumulatie in wit licht ("low fluence response" of LFR). Activering van de etioplasten door Pfr, dat zich na verhuizing vanuit het cytoplasma in deze organellen concentreert, zou juist het eerste deel ("very low fluence response" of VLFR) van deze curve verklaren. Verhuizing van slechts een klein, spectrofotometrisch nauwelijks meetbaar, deel van de totale hoeveelheid fytochroom uit het cytoplasma naar de

receptoren kan kwantitatief de grote gevoeligheid voor Pfr van kiemplanten die volledig in het donker gegroeid zijn en de afname in gevoeligheid na een eerste belichting, goed verklaren. Dat zowel het effect van een eerste, als dat van een tweede roodbelichting grotendeels door ver-rood licht omkeerbaar zijn in maïsplanten, maakt een tweede wijziging van het model noodzakelijk. In het oorspronkelijke model werd verondersteld, dat Pfr na een roodbelichting langzaam verhuist, zodat de ver-rood-belichting tijdens de verhuizing plaatsvindt. In plaats daarvan wordt nu verondersteld, dat de verhuizing plaatsvindt voor het begin van de ver-rood-belichting. Een snelle "ontsnappingsreactie" zou dan verklaren dat het effect van rood licht niet geheel door ver-rood licht teniet wordt gedaan. Evenals in het oorspronkelijke model wordt aangenomen dat Pfr, dat zich in de receptoren heeft geconcentreerd, daar aanwezig blijft. Bij een tweede roodbelichting kan dan niet opnieuw een concentratie van Pfr in de receptoren plaatsvinden. Om het effect van een tweede roodbelichting in maïskiemplanten in overeenstemming met het model te kunnen verklaren, wordt aangenomen dat donker-omzetting van Pfr in Pr in de receptoren plaatsvindt.

Pogingen om een fysiologisch effect van het in de etioplasten aanwezige fytochroom rechtstreeks aan te tonen, door processen die in vivo door fytochroom beïnvloed worden in geïsoleerde etioplasten te bestuderen, hebben geen positief resultaat opgeleverd.

De ontwikkeling van de ultrastructuur van geïsoleerde etioplasten verliep niet geheel parallel aan de ontwikkeling van etioplasten in bladeren (Hoofdstuk 5). In bladeren werd een invloed van rood-voorbelichting op de verandering van de prolamellaire lichamen geconstateerd, maar niet in geïsoleerde etioplasten. Het begin van de vorming van grana in etioplasten in bladeren werd gestimuleerd door rood-voorbelichting, maar geïsoleerde etioplasten bleken niet in staat grana te vormen.

In het donker na een korte belichting vond, zowel in bladeren als in geïsoleerde etioplasten, opnieuw vorming van licht-omzetbaar protochlorofyl(lide) plaats (Hoofdstuk 6). In bladeren versnelde rood-voorbelichting de vorming van deze voorloper van chlorofyl, maar in etioplasten uit rood-voorbelichte kiemplanten was de snelheid van vorming niet hoger dan in etioplasten uit in volledig donker opgegroeide planten. De kinetiek van de regeneratie van protochlorofyl(lide) in geïsoleerde etioplasten verschilde van die in bladeren.

Het fluorescentie-maximum bij 77K van chlorofyl(lide), dat door een lichtflits uit protochlorofyl(lide) wordt gevormd, verschuift in het donker bij kamertemperatuur. De kinetiek van de optredende verschuivingen en de invloed van rood-voorbelichting daarop, werden bestudeerd bij bladeren en geïsoleerde etioplasten (Hoofdstuk 7). Hierbij werd gebruik gemaakt van afgeleide spectra en het ontleden van spectra in Gauss-krommes. De eerste waarneembare verschuiving van het maximum, naar langere golflengtes, bleek langzamer plaats te vinden in geïsoleerde etioplasten dan in bladeren.

Rood-voorbelichting had geen invloed op de snelheid van deze verschuiving. De daaropvolgende verschuiving, naar kortere golflengtes (de Shibata shift), vond in geïsoleerde etioplasten sneller, maar minder volledig, plaats dan in bladeren. In bladeren nam de snelheid van de Shibata shift toe na rood-voorbelichting, maar eenzelfde effect van in vivo voorbelichting werd bij geïsoleerde etioplasten niet of nauwelijks waargenomen. In geïsoleerde etioplasten nam de hoeveelheid licht-omzetbaar protochlorofyl(lide) af, en de snelheid van de Shibata shift toe, naarmate de etioplasten langer bewaard werden bij 4 °C in het donker. Pasgevormd chlorofyl(lide) bleek in geïsoleerde etioplasten instabiel te zijn.

Deze resultaten wijzen op een grote invloed van het cytoplasma op het vermogen van etioplasten om zich te ontwikkelen onder invloed van wit licht. Hierbij zouden polypeptiden van chlorofyl-eiwit complexen, die in het cytoplasma gevormd worden, een belangrijke rol kunnen spelen. Toch sluiten de resultaten een invloed van in de etioplasten aanwezig fytochroom op de ontwikkeling van etioplasten tot chloroplasten niet uit. Het in etioplasten aanwezig fytochroom zou bijvoorbeeld de permeabiliteit van de dubbele membraan, die de organellen omgeeft, kunnen beinvloeden. Door de volgende waarnemingen wordt een invloed van in etioplasten aanwezig fytochroom aannemelijk gemaakt. Het effect van een rood-voorbelichting op chlorofylaccumulatie in wit licht bleek na een periode van 24 uur nog gedeeltelijk omkeerbaar te zijn door ver-rood licht. Reeds 4 uur na een roodbelichting leek Pfr door donkerafbraak verdwenen te zijn, alleen de hoeveelheid fytochroom in etioplasten bleek constant te blijven (zie boven). Het is aantrekkelijk om tenminste dat deel van het effect van rood licht, dat na lange tijd nog omkeerbaar is door ver-rood, toe te schrijven aan Pfr, dat kennelijk in de etioplasten betrekkelijk stabiel is.

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Curriculum vitae

Henriëtte Louise Kraak werd geboren op 5 juni 1950 te Djakarta, Indonesië. Zij behaalde in 1968 het diploma gymnasium β aan het Gemeentelijk Lyceum te Kampen. In hetzelfde jaar begon zij met de studie biologie aan de Rijksuniversiteit te Groningen. In 1972 werd het kandidaatsexamen B1 (cum laude) behaald. Het doctoraalexamen, met onderwijsbevoegdheid, werd (cum laude) behaald in 1975. Hoofdvak: plantenfysiologie, bijvakken: fytopathologie en elektronenmicroscopie. Van 1 september 1972 tot 1 september 1974 was zij daarbij werkzaam als studentassistente bij het 1e jaars-praktikum plantenfysiologie. In het schooljaar 1975/1976 gaf zij les in biologie aan het Jeroen Bosch College te 's-Hertogenbosch. Van 1 juni 1976 tot 1 juni 1979 was zij als wetenschappelijk ambtenaar in dienst van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek in het kader van de Stichting voor Biologisch Onderzoek (BION). In deze functie werd zij in de gelegenheid gesteld om onder leiding van dr. C.J.P. Spruit bij de Vakgroep Plantenfysiologisch Onderzoek van de Landbouwuniversiteit (voorheen Landbouwhogeschool) te Wageningen het onderzoek, dat in dit proefschrift beschreven is, te verrichten. Van 1 november 1979 tot 1 november 1980 werd in dienst van de Landbouwhogeschool verder gewerkt aan het onderzoek. Sinds 1 augustus 1981 werkt zij als wetenschappelijk ambtenaar bij het Rijksproefstation voor Zaadonderzoek te Wageningen.