Photocontrol of seed germination of wildtype and long-hypocotyl mutants of *Arabidopsis thaliana*.

Lichtregulatie van de zaadkieming van wildtype en lange-hypocotyl mutanten van *Arabidopsis thaliana*.

Promotor: dr. W.J. Vredenberg,
hoogleraar in de plantenfysiologie, met
bijzondere aandacht voor de fysische
aspekten

Co-promotor: dr. R.E. Kendrick,
wetenschappelijk hoofdmedewerker

Stellingen

1. Binnen een zaadpopulatie bestaat een normaal verdeling voor de logarithme van de Pfr behoefte voor de kieming, en niet, zoals vaak abusievelijk is verondersteld, een normaal verdeling voor de logarithme van de lichtbehoefte.

   Dit proefschrift.

2. Het verschil dat Taylorson vindt in de "ontsnappingstijden" van de kiemingsremming na verrood en ethanol behandeling kan niet verklaard worden door aan te nemen dat ethanol tijd nodig heeft om het reactiecentrum te bereiken.

   Taylorson, R.B. (1984) Prevention of action of far-red-absorbing phytochrome in *Rumex crispus* L. seeds by ethanol. Plant Physiol. 74, 223-226.

3. Daar het hypothetisch model van Blaauw-Jansen voor de verklaring van twee-fasen dosis-effect curven inhoudt dat zaden onder bepaalde omstandigheden energie verbruiken zonder dat ze kiemen, is het model niet erg voor de hand liggend.

   Blaauw-Jansen, G. (1983) Thoughts on the possible role of phytochrome destruction in phytochrome-controlled responses. Plant Cell and Environ. 6, 173-179.

4. Men dient bij de interpretatie van aktiespectra zeer voorzichtig te zijn met conclusies ten aanzien van het voorkomen van verschillende pigmenten of verschillende vormen van een pigment.

   Dit proefschrift.

5. Het feit dat zaden extreem gevoelig kunnen zijn voor licht duidt op de aanwezigheid van een systeem waarmee zij ook onder de grond kunnen reageren op licht.

6. Fysiologisch onderzoek aan hormoon- en fotoreceptormutanten is onmisbaar om een goed inzicht te krijgen in de processen die een rol spelen tijdens de groei en ontwikkeling van een plant.

7. Hoewel lignine de verteerbaarheid van ruwvoeders beïnvloedt, lijkt het onwaarschijnlijk dat alleen lignine de mate van verteerbaarheid bepaalt.

8. Het uitdrukken van de hoeveelheid lipide per gram drooggewicht bij de bestudering van de invloed van NaCl op het lipidegehalte van *Plantago sp.* kan tot verkeerde interpretaties leiden.

   Erdei, L., Stuiver, C.E.E., Kuiper, P.J.C. (1980) The effect of salinity on lipid composition and on activity of Ca^{2+}- and Mg^{2+}-stimulated ATPases in salt-sensitive and salt-tolerant Plantago species.

9. Daar steeds meer jongeren pas een vaste baan kunnen krijgen nadat zij enige tijd vrijwillig, met behoud van een uitkering, ervaring hebben opgedaan, is de term "vrijwillig" misplaatst.

10. Wil de kleuring van de Hilversumse radiozender goed tot zijn recht komen, dan dient men bij de indeling van de zendtijd alleen naar de aard van het programma te kijken en niet naar de omroep die het uitzendt.

11. De belastingwetten in Nederland dienen zodanig te veranderen, dat de belastingbetaler niet langer afhankelijk is van de interpretatie van de wet door de belastinginspecteur.

12. Het gezegde "Early to bed and early to rise, makes a man healthy, wealthy and wise" blijkt onjuist, daar sinds de invoering van de zomertijd de gezondheid en wijsheid niet merkbaar zijn toegenomen en de rijkdom alleen maar is afgenomen.

J.W. Cone

Photocontrol of seed germination of wildtype and long-hypocotyl mutants of *Arabidopsis thaliana.*

Wageningen, 6 september 1985.

J.W. Cone

Photocontrol of seed germination of wildtype and long-hypocotyl mutants of *Arabidopsis thaliana*.

Proefschrift
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in het openbaar te verdedigen
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Voorwoord

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Contents

List of abbreviations and symbols

B	blue light
D	dark
FR	far-red light
G	green light
HIR	high irradiance reaction
Hy	long hypocotyl mutant
K	differential screening relative to 660 nm
kD	kilodalton
LER	low energy reaction
LFR	low fluence response
N	fluence rate
P	phytochrome
Pfr	far-red absorbing form of phytochrome
Pr	red absorbing form of phytochrome
Ptot	total amount of phytochrome
R	red light
SD	standard deviation of ln Pfr around μ
t	exposure time
ttg	transparent, testa, glabra mutant
UV	ultra violet
VLFR	very low fluence response
WT	wildtype
X	the unknown reaction partner of Pfr
y	percentage germination
z	proportion of a population responding to an overriding factor
$\Delta\Delta A$	difference in absorbance difference
ϕ	the maximum Pfr/Ptot established by a given wavelength
ϕ_0	initial Pfr
σ_1	apparant molar conversion cross section for the transition of Pr → Pfr
σ_2	apparent molar conversion cross section for the transition of Pfr → Pr
μ	ln Pfr required for 50% germination

CHAPTER 1, INTRODUCTION

Life on earth would not be possible without the existence of light. Green plants need light for photosynthesis to synthesize biomass from CO_2 and H_2O. All other forms of life depend directly or indirectly on the biomass produced by plants. However plants do not only need light for photosynthesis but also utilize light for the regulation of growth and development (photomorphogenesis). The amount of light needed for photomorphogenesis is very small in comparison with that needed for photosynthesis. Plants must possess some kind of sensor with which they can measure the light quality of their environment, such as the spectral quality, irradiance and daylength. Unlike animals, plants do not have a complex light sensitive organ, but they have a more homogeneously distributed receptor-molecule, called phytochrome.

Phytochrome is a red/far-red (R/FR) reversible pigment which exists in two forms: a physiologically inactive R absorbing form Pr, which can be transformed by R in the physiological active FR absorbing form Pfr, which in turn can be transformed by FR to Pr. Many processes in plant growth and development are controlled by phytochrome, such as seed germination, hypocotyl growth, flowering, leaf fall, anthocyanin synthesis, the activity of many enzymes and phototropism. In most cases phytochrome does not act on its own but in combination with environmental factors such as temperature, humidity and the availability of nutrients and oxygen. Phytochrome action varies from one species to another and with the genetic constitution of the plant.

History of photomorphogenetic research

Early photomorphogenetic research was done by Bonnet (1752) studying the etiolation of plants and by Darwin (1880) studying phototropism. They found that plant development and growth is regulated by light, although they were not aware of the mechanisms involved. In the early 20th century Kinzel (1908, 1909) and Heinricher (1899, 1908) investigated the influence of light on seed germination of many species. They found that germination of some species was inhibited by light, while in others it was promoted. Flint and McAlister (1935, 1937) were the first to recognize the promotive effect of R and the inhibitory effect of FR on germination of lettuce seed. They pre-treated their seed samples by giving them an initial promotive irradiation sufficient to induce 50% germination. They

then irradiated the samples for 24 h and noted promotory and inhibitory deviations from 50% germination. Actually they were the first to determine the effectiveness of different wavelengths of light for the promotion and inhibition of germination ("action spectrum"). However they were not aware of the importance of the R/FR reversibility of the pigment system. Their papers were neglected until the 1940's, when Borthwick, Hendricks, Parker and Siegelman studied the influence of light on flowering in the U.S.D.A. at Beltsville. By that time it was known that flowering of plants could be inhibited by a short light pulse in the middle of the dark period. The first action spectra of this flower inhibition by a night-break were made for soybean (Parker et al. 1945) and cocklebur (Parker et al. 1946). It was shown that the effect of light to prevent flowering was high in the R spectral region 600 - 680 nm with its limit at about 700 nm. Minimum effectiveness was at about 480 nm with an increase again at shorter wavelengths. The most obvious conclusion from these first experiments was that the photo-active pigment was blue or green since action was predominantly in the R. Also for other processes such as: promotion of flowering of long day plants (Borthwick et al. 1948, Parker et al. 1950), the inhibition of stem growth in barley (Borthwick et al. 1951), promotion of leaf growth of dark grown pea seedlings (Parker et al. 1949) and promotion of germination of lettuce seeds (Borthwick et al. 1952a, Borthwick et al. 1954) similar action spectra were found. The action spectra for induction and inhibition of seed germination were crucial in the series leading to the discovery of the photoreversibility of phytochrome. Lettuce seed germination was shown to be inhibited by light of 700 - 800 nm. This phenomenom led the workers at Beltsville to the idea of the existence of a R/FR reversible pigment with absorption maxima responsible for promotion and inhibition at 660 and 730 nm respectively (Borthwick et al. 1954). This R/FR reversibility was also shown for other processes; e.g. inhibition of flowering (Borthwick et al. 1952b) and elongation growth of internodes of bean (Downs et al. 1957).

Detection of phytochrome was made possible in plant tissue by the use of dual wavelength spectrophotometry, following alternate irradiation of plant material with R and FR (Butler et al. 1959, Norris 1968, Spruit 1970, 1972).

In the late 1950's, the name phytochrome was introduced by Butler. A few years later Siegelman and Firer (1964) developed a method to isolate and purify the protein phytochrome from oat seedlings. Pratt and Coleman (1971) developed an immunocytochemical method for the localization of phytochrome. Flash exitation experiments (Linschitz et al. 1966) showed that the transition of Pr to Pfr and of Pfr to Pr are stepwise and the nature of these intermediate steps have been

extensively studied (Kendrick and Spruit 1977, Rüdiger and Scheer 1983).

Properties of phytochrome

Phytochrome exists in two forms, one R absorbing (Pr) and the other FR absorbing (Pfr), which are repeatedly interconvertible by light. Pfr is the physiologically active but unstable form, while Pr is stable but physiologically inactive. In darkness Pfr can revert to Pr, the so-called dark reversion, or it may undergo an irreversible transformation, called destruction. Since the discovery of phytochrome, the majority of research has focussed on the study of phytochrome mediated responses rather than on the molecule itself (Borthwick 1972). One of the reasons for this relative lack of attention to the molecular properties of phytochrome derives from difficulties associated with obtaining the photoreceptor in the highly purified undenaturated and undegraded form necessary for its physiochemical characterization. The degradation of Pfr was minimized by working under a green safelight (Smith 1975, Pratt 1984a). Conventional approaches to the purification of phytochrome derive from the original procedure developed by Siegelman and Firer (1964). These approaches involve various combinations of ammonium sulphate fractionation, brushite and/or hydroxyapatite chromatography, ion exchange chromatography and gel exclusion chromatography. In the early 1970's it was found that phytochrome was highly susceptible to proteolysis (Gardner et al. 1971), which is minimized by maintaining phytochrome in vitro as close as possible to 0 ^{o}C and by completing its purification rapidly. Since the conventional purification procedures were time consuming, they often yielded phytochrome with a molecular weight of 60 kD (small phytochrome). Recent efforts have focussed on the development of rapid affinity purification procedures that provide both high yields and uncontaminated preparations of large (120 kD) phytochrome. The first affinity purification procedure for phytochrome utilized the availability of specific antiphytochrome immunoglobulins (Pratt 1984b). A second affinityprocedure has been developed by Smith and Daniels (1981). Their method depends upon an interaction between phytochrome and agarose-immobilized Cibacron Blue F3GA. A third method developed by Yamamoto also utilized preliminary column chromatography to enrich for phytochrome. The final affinity step then involves irradiation of the phytochrome preparation with R followed by absorption to an ω-aminooctyl agarose column (Yamamoto and Smith 1981). After washing the affinity column with buffer, phytochrome is eluted by its conversion back to Pr by FR.

Initial work was carried out with small phytochrome and is reviewed by Briggs, Gardner and Hopkins (1972). However small phytochrome (60 kD) probably does not exist in vivo (Grombein and Rüdiger 1976, Pratt et al. 1974, Pratt 1982) and is only a degradation product of large phytochrome (120 kD) which is produced by proteolytic degradation during the isolation procedure. Large phytochrome (120 kD) behaves as a dimer under nondissociating conditions (Pratt 1982). Boeshore and Pratt (1980, 1981) and Bolton and Quail (1981) have found that phytochrome from oat is 5 kD larger than 120 kD. More recently Vierstra and Quail (1982) and Kerscher and Nowitzki (1982) found that inhibition of proteolysis of Pr by phenylmethylsulfonyl fluoride yields a homogeneous monomer of *Avena* phytochrome of 124 kD, which has molecular properties that differ significantly from those of partially degraded preparations. They called the 124 kD monomer native phytochrome. Proteolysis obviously removes a small proportion of the protein that is critical for spectral and structural integrity.

The phytochrome molecule consists of a protein and a chromophore. As a consequence of the spectral similarities of Pr and phycocyanin, a bilitriene pigment, having an open chain tetrapyrrole structure was suggested for the phytochrome chromophore (Parker et al. 1950) and was later confirmed (Rüdiger and Correll 1969, Klein et al. 1977). The visible absorption spectra of chromoproteins are due to the chromophore being held by the apoprotein in a specific conformation and environment. The bilitriene chromophore of the Pr form of phytochrome is indeed very similar to that of the algal antenna pigment c-phycocyanin (Lagarias and Rapoport 1980, Rüdiger 1980). Phototransformations of phytochrome could arise from rotation around a single methine bond or by cis-trans isomerization reaction of the double methine bonds (Kendrick and de Kok 1983, Rüdiger 1983). However the exact structure of the chromophore of phytochrome and the changes in its configuration upon phototransformation are still not completely known.

Phototransformations of phytochrome from Pr to Pfr and from Pfr to Pr conform to simple first order photoreactions. However the phototransformations are not single step processes but involve initial photoreactions followed by dark relaxations. Several intermediates have been detected. The subject has been reviewed by Rüdiger and Scheer (1983) and Kendrick and Spruit (1977). The latter suggested a nomenclature similar to the one used for the rhodopsin transformations. Each type of study have resulted model pathways for the phototransformation of Pr → Pfr and Pfr → Pr (e.g. Fig. 1).

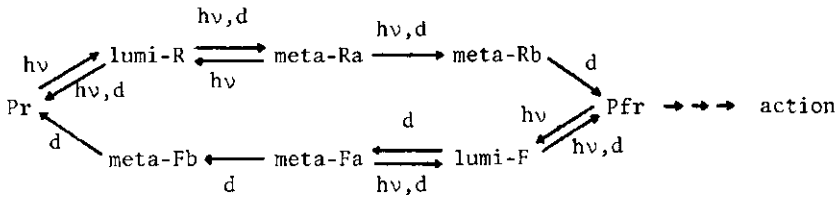

Fig. 1. Intermediates of phytochrome transformations.

Recently experiments were carried out with large and native phytochrome (Pratt et al. 1982, Furuya 1983, Braslavsky 1984), suggesting that there are different parallel ways for the transition of Pr to Pfr and of Pfr to Pr.

Phytochrome action

The distinction is made between two types of action, the low energy reaction (LER) and the high irradiance reaction (HIR). For the LER only a low fluence is needed to produce a physiologically measurable effect. Induction of a response by short R can be reversed by short FR. In the LER, the response is constant for a given fluence irrespective of the exposure time. Processes regulated by the LER are for instance: seed germination (Toole 1973, Frankland and Taylorson 1983), flower induction or inhibition by a night break (Borthwick et al. 1948, Vince-Prue 1983), rapid inhibition of extension growth (Vanderhoef et al. 1979) and hook opening of etiolated bean seedlings (Withrow et al. 1957). The action spectrum (Shropshire 1972, Schäfer et al. 1983) of a LER shows a maximum at 660 nm for the induction and at 730 nm for the inhibition of the induction. Action spectra of LER responses show similarities to the absorption spectrum of purified phytochrome (Rice et al. 1973). The fact that the absorption spectra of Pr and Pfr overlap means that R is capable to some extent of converting Pfr back to Pr. This means that a saturating irradiation with R produces a mixture of Pfr and Pr. The maximum level of Pfr that can be produced is still a point of discussion, but it is believed to be between 75 and 86% (Butler et al. 1964, Butler 1972, Bartley 1982, Vierstra and Quail 1982, Smith 1983). Similarly saturating FR (730 nm) produces approximately 2.5% Pfr.

Plant photomorphogenesis in the natural environment occurs with irradiations over long periods. Response to longterm irradiation is thought to involve the high irradiance reaction (HIR) (Borthwick et al. 1969, Hartmann 1966, 1967,

Mancinelli and Rabino 1978). In contrast to a LER response, HIR responses are not photoreversible and the response is dependent on fluence rate and exposure time. Processes regulated by the HIR are for instance: suppression of hypocotyl lengthening (Hartmann 1966, Evans et al. 1965), inhibition of seed germination by prolonged irradiation (Gwynn and Scheibe 1972), promotion of anthocyanin synthesis (Mohr 1957, 1983, Mancinelli 1983) and chlorophyll accumulation (Mohr 1983, Kasemir 1983). Action spectra of the HIR show different peaks and depend on the pretreatment of the plant material. While dark grown plants show peaks in the FR and B for the induction of a response, for deetiolated plants the long-wavelength effectiveness shifts to the R (Beggs et al. 1980). In some etiolated plants an action peak for the induction of a response is found at c. 716 nm. Hartmann (1966) found that an equal effectiveness could be attained by simultaneous irradiation with 658 and 768 nm bands, both of which are ineffective when used alone. It is therefore believed that although the action spectrum of the HIR does not conform with the absorption spectrum of either Pr or Pfr, phytochrome is responsible for the HIR action. By irradiation with 658 and 768 nm both Pr and Pfr are excited, resulting in a cycling of phytochrome (Kendrick and Frankland 1983, Frankland and Taylorson 1983). The HIR does not only have an action peak in the 716 nm region but also in the 340 to 500 nm region. The question as to whether phytochrome is acting alone or together with a B receptor is in most cases unanswered. The comparatively high responsiveness of several HIR responses in the B compared to the R and FR regions argues against phytochrome exclusively involved. Several reports support the idea of a separate B receptor pigment and possibly also of a separate UV-receptor pigment (Meyer 1968, Gaba and Black 1979, Beggs et al. 1980, Koornneef et al. 1980).

It has been suggested that phytochrome functions by interaction with cellular membranes (Hendricks and Borthwick 1967, Marmé 1977). Action of phytochrome through the control of membrane functions implies that Pfr might associate with a specific receptor on the membrane. The possible molecular nature of the binding of phytochrome to cellular membranes was examined by Gressel and Quail (1976) and they concluded that phytochrome may be associated with membrane proteins. Studies by Yamamoto and Furuya (1979) of the in vitro binding of Pfr to pea shoot membrane fractions treated with phospholipase-C or trypsin supported this conclusion. The findings of Roth-Bejerano and Kendrick (1979) suggest that steroids as membrane compounds might also be involved in phytochrome binding. Closer studies are needed to draw conclusions on the nature of the phytochrome-membrane interactions.

Ultimately phytochrome action results in modified gene expression (Schöpfer 1977, Lamb and Newton 1983). Although for many enzymes the nature of the photoregulation of the synthesis have not been elucidated (Newsbury 1983), the regulation of chloroplast development is reasonably well understood (Jenkins et al. 1983, Mohr 1980). It has been shown that phytochrome can regulate synthesis of the mRNA for synthesis of the light harvesting complex protein (Apel and Kloppstech 1978). Phytochrome is also known to be involved in the control of chlorophyll biosynthesis.

Since the exact nature of the primary reaction partner of phytochrome is not known, it is called the factor X. Schäfer (1975) attempted to explain the differences between the HIR and the LER in terms of changes in the PfrX complex, in his so-called open phytochrome receptor model (fig. 2).

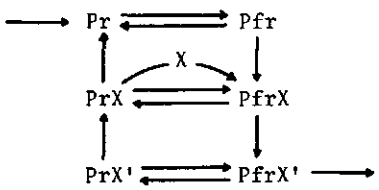

Fig. 2. Open phytochrome receptor model (after Schäfer 1975).

The model considers de novo synthesis of Pr as well as destrution of Pfr and includes an interaction of Pr and Pfr with a receptor X or X'. PfrX' is the state present in the dark following a brief irradiation, whereas the HIR could be mediated by the state PfrX. As the reaction PfrX → PfrX' is slower than the reaction Pfr + X → PfrX, it is the latter state which will tend to accumulate under continuous, high irradiance. This model can be used to explain why the HIR peak of action is in the FR region of the spectrum. The possibility that the actual conversion of Pr to Pfr could drive some reaction has been considered for a long time. Johnson and Tasker (1979) suggested that under high irradiance conditions the continuous interconversion of Pr to Pfr could be coupled to the movement of some metabolite across a membrane. Kendrick and Frankland (1983) also proposed a model with which they can explain the difference between LER and HIR. However they started from the principle that seed germination and seedling photomorphogenesis have different initial states and that phytochrome is membrane

bound. The association between phytochrome and a membrane provides a structural basis for the orientation and also for possible co-operative interaction between phytochrome molecules. A light induced change in the phytochrome chromophore leads to a change in the conformation of the phytochrome protein. This in turn could lead to changed membrane conformation and hence to changed membrane function, making the membrane more permeable, allowing passive diffusion of some essential ion or other metabolite along a concentration or electrochemical gradient. Phytochrome cycling could be coupled to the active pumping of some molecule across a membrane. This model explains how passive and active transport across a membrane could act synergistically in seedling photomorphogenesis but antagonistically in seed germination.

Light control of seed germination

Non-dormant seeds germinate after imbibition of water at an appropriate temperature and in the presence of oxygen. Germination begins with enlargement of the embryonic axis and ends with protrusion through the seed coat. In many seeds germination occurs by cell enlargement and not by cell division, which begins after germination. Naked embryos from dormant seeds often germinate (Kugler 1951), but may be prevented by doing so by replacing the restricting effect of the seed coat by an osmotic stress (Scheibe and Lang 1965). When a viable seed fails to germinate under conditions favourable for seedling growth, it is said to be dormant. The dormancy may be referred to as innate or primary in freshly matured seeds, or as induced or secondary in imbibed seeds exposed to unfavourable germination conditions (this thesis). Duke et al. (1977) argued that failure of germination upon a light stimulus after induction of secondary dormancy is related to declining levels of the Pfr reaction partner X, because levels of total phytochrome and rate of Pfr dark reversion to Pr remained constant. Similar conclusions were reached by Karssen (1970) and Taylorson and Hendricks (1973).

Germination of an individual seed is an all-or-nothing event. Germination of a population of seeds may be quantified by the proportion which germinate under a given set of conditions. Germination data are usually presented as final percentage germination. There is usually a sigmoid relationship between percentage germination and logarithm of light fluence which is thought to reflect a normal distribution among individual seeds in their Pfr requirements for germination (Frankland 1976, Duke 1978, this thesis). Probit of germination (Finney 1952)

plotted against log fluence consequently gives a linear relationship (Borthwick et al. 1954, Toole et al. 1955, Taylorson and Hendricks 1973, this thesis). Biphasic fluence-response curves were observed by Blaauw-Jansen and Blaauw (1975), Small et al. (1979a) and VanDerWoude and Toole (1980) for R germination induction of dormant lettuce seeds. There appeared to be at least two types of response, distinguished in fluence requirement by four orders of magnitude.

To investigate the absorption characteristics of an unknown photoreceptor, action spectra must be determined. The first true action spectra of photostimulation and subsequent inhibition of seed germination were determined by Borthwick et al. (1952a, 1954). Two action peaks were observed at c. 660 for stimulation and c. 730 nm for inhibition, corresponding to the absorption peaks of Pr and Pfr respectively. Similar spectra have been obtained for seeds of *Arabidopsis thaliana* (Shropshire et al. 1961). Although few detailed action spectra have been published, R/FR reversibility has been widely demonstrated (Toole 1973). Action spectra cannot be identical with the absorption spectra of Pr and Pfr, because of the overlapping nature of the absorption spectrum (Small et al. 1979a).

A short irradiation with FR reverses the R inductive effect by photoconversion of Pfr to Pr. Short FR can reduce germination below the dark control in some light requiring species (Borthwick et al. 1954) and in a few dark germinating species (Mancinelli et al. 1966). This is evidence that endogenous Pfr in seeds sown in darkness can cause germination. Failure of FR to inhibit germination could have several reasons: germination may not be under phytochrome control or may have progressed beyond phytochrome control; the low Pfr/Ptot ratio established by far-red light may be sufficient to initiate germination of sensitive seeds; or that more Pfr appears from intermediates following FR, that were trapped during dehydration (Kendrick and Russel 1975).

Many effects of B on plants may involve a photoreceptor pigment, other than phytochrome, possibly a flavoprotein (Senger 1980). However conclusive evidence for a photomorphogenetic photoreceptor other than phytochrome in seed germination is lacking. Small et al. (1979b) found that thermodormant lettuce seeds would germinate after B, giving an action spectrum with peaks at 422 and 446 nm. However they were not able to discriminate between a B receptor or energy transfer to phytochrome. Note also green light, often used as safe light, may induce germination (Baskin and Baskin 1979, Blom 1978).

After a R inductive irradiation, germination can be inhibited by subsequent FR. However if the period between R and FR increases, seeds escape from photo-

control and germinate. This indicates that Pfr action has been completed in seeds so that reversion to Pr will not affect subsequent germination. Escape may be defined as the time of Pfr action required in half the population. The rate of escape may be dependent to the pre-treatment prior to irradiation and to the dormancy of the seeds (Duke et al. 1977, this thesis). After seeds have escaped from inhibition by short FR, germination may still be inhibited by prolonged irradiation (Mohr and Appuhn 1963, Rollin 1963, Hartmann 1966, Karssen 1970, Gwynn and Scheibe 1972). Inhibition is greatest by FR (710 - 720 nm) and B (c. 470 nm). However in some species R can also be inhibitory (Schulz and Klein 1963, Rollin and Maignan 1967). Photoinhibition is dependent on fluence rate, suggesting that the rate of interconversion of Pr and Pfr is important and that photoinhibition is similar to the HIR. The Pfr/Ptot ratio is little affected by fluence rate, whereas the photostationary flux is directly proportional to the fluence rate (Kendrick and Frankland 1983).

The effect of light on seed germination is strongly dependent on the temperature (Toole 1973). Temperature effects result from changes in germination potential or degree of dormancy of the seeds that reflect an altered Pfr requirement for germination. In *Amaranthus retroflexus*, germination in prolonged white light is decreased at 20 ^{o}C but increased at 30 ^{o}C (Kadman-Zahavi 1960). Short irradiations increase germination more at the higher temperature. VanDerWoude and Toole (1980) studied the effects of chilling on lettuce seeds in which Pfr was reduced to very low levels by FR and imbibition at 20 ^{o}C for 24 h. Chilling did not increase dark germination, confirming Pfr dependence, but rendered the seeds responsive to low Pfr levels. It was suggested that the chilling effect arose from altered membrane properties. Interaction between chilling and phytochrome in *Betula papyrifera* seeds has been interpreted as membrane changes affecting availability or sensitivity of Pfr receptor sites (Bevington and Hoyle 1981). It was suggested that a single short high temperature treatment acts in the same way. In *Rumex obtusifolius* seeds studied by Takaki et al. (1981) only a few minutes of high temperature stimulated germination and there was close interaction between temperature and Pfr.

Ecological significance of phytochrome

Phytochrome operates in nature as a signaltransducing photoreceptor enabling the plant to acquire information on the light environment which may be applied

to the modulation of cellular processes. A fundamental function of phytochrome is to detect the R/FR ratio of natural radiation. This is very important since growing conditions are less favourable when shaded by a leaf canopy. Phytochrome can detect the shading through its R/FR reversible properties. Since chlorophyll absorbs R light more effectively than FR, shade light will have a relative low ratio of R/FR; about 0.1 compared to 1.2 for sunlight (Holmes and Smith 1975, Frankland and Letendre 1978, Frankland and Poo 1980).

It is found that germination of many species is inhibited by shade light (Gorski et al. 1977, 1978, Silvertown 1980). Germination of light requiring seeds sown on the soil surface under various degrees of plant shade decreased as R/FR ratio of incident light decreased, reflecting progressive decreases of phytochrome as Pfr (Frankland and Poo 1980, Frankland 1981). In seeds, phytochrome is basically a light detector enabling germination when seeds are near the soil surface. It provides a mechanism for maintaining a supply of dormant weed seeds within the soil which may germinate only after light exposure accompanying disturbance of the surface soil (Taylorson 1970, 1972, Roberts 1972). If seeds are sown at different depths and subsequently exposed to light, germination declines progressively with depth reaching the dark control level at 6 to 9 mm (Frankland and Poo 1980, Frankland 1981). In well aerated soil, the decline in germination correlates with the progressive decrease in fluence rate reaching the seeds. Highly dormant freshly shed seeds may be buried before developing an ability to respond to light. Conversely, seeds initially capable of dark germination may develop a light requirement during burial in soil (Wesson and Wareing 1969) and exhibit a seasonal sensitivity to light (Karssen 1980/81). Seeds may be inhibited to germinate in continuous light through the cycling of phytochrome, while the promotion of germination depends on the proportion of phytochrome in the Pfr form (Bartley and Frankland 1982).

Phytochrome appears to be distributed throughout the seedling, but shows peaks of concentration in meristematic tissue, coleoptile tips, leaf bases and nodes in monocots and the apical regions (hypocotyl hook, leaves, first node) in dicots. In roots of monocotyledons phytochrome is concentrated almost exclusively in the root tip (Pratt and Coleman 1971). It is probably no coincidence that the phytochrome is associated with the apical regions of the seedling, since these are the organs which will normally be exposed first to light. For large seeded species and for renewed sprouting from underground organs, the phenomena of etiolation in the dark, and de-etiolation following exposure to light are obviously

of ecological significance (Mohr 1972). Etiolation may be considered an adaption for growth under the soil. Expansion and greening of the leaf laminae and inhibition of stem extension are the most striking of the variety of changes which occur in white light (Mohr 1972, Smith 1975). When shade-intolerant plant species are found growing in vegetational shade they characteristically have longer internodes and fewer branches than shade-tolerant species (Grime 1966). This systematic difference can be explained ecologically as a shade-avoiding response in the sun-adapted species, and a shade acclimative response in the shade adapted species. There is a great deal of evidence to suggest that stem elongation, apical dominance and many more subtle responses are a result of the perception of vegetational shadelight by phytochrome.

Since flowering and dormancy induction are seasonal in the natural environment, it seems self-evident that photoperiodism is a critically important factor in plant strategy. It seems possible that plants can measure the length of the days and/or nights and consequently the time in the season. Phytochrome may also have a key-function in photoperiodism, although the mechanisms are not clear at the moment. The influence of B and the involvement of a B receptor in plant strategy is not very well documented.

Scope and outline of the present study

Mutants of *Arabidopsis thaliana* which showed reduced photoinhibition, compared to wildtype, of hypocotyl growth in white light (Koornneef et al. 1980), have been used in this study. Some of the mutants showed no inhibition of hypocotyl growth by R and/or FR, while others showed no inhibition in B and UV. It was suggested that these mutants might either be mutated with respect to phytochrome, another R absorbing photoreceptor or a B/UV absorbing receptor. Especially the absence of responses attributed to a R/FR reversible photosystem in some mutants, while B and UV light apparently still were active, provided good evidence of the existence of different pigment systems. If different pigment systems are active, the mutants would be excellent tools to investigate the involvement of them in the control of seed germination and seedling development.

The involvement of the different receptor pigments in seed germination was investigated by determining action spectra for the induction and inhibition of germination of the different long-hypocotyl mutants and wildtype (chapter

7). Construction of action spectra requires the determination of detailed fluence--response curves. Since the differences in the fluence-response curves could not be explained with conventional models, a new model was designed (chapter 3). This model also enables theoretical action spectra to be calculated (chapter 4). Preliminary studies were carried out to investigate the optimal imbibition conditions in order to obtain maximal germination (chapter 5). Chapter 6 reports on the time course of phytochrome action by investigating the rate of escape and germination. Biphasic fluence-response curves are interpreted on the basis of a hypothetical model (chapter 8).

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CHAPTER 2, MATERIALS AND METHODS

Plant material

The crucifer *Arabidopsis thaliana* (L.) Heynh. is widely used in plant research, because it has a very short life cycle, less than 8 weeks, the plants require little space but still produce up to 10,000 seeds per plant and the chromosome number is low (2n = 10). Moreover it is selffertile which facilitates mutant detection and the isolation of genetically homozygous lines.

The seeds of *Arabidopsis thaliana*, used in this research were supplied by Dr. M. Koornneef (Dept. of Genetics, Agricultural University, Wageningen, The Netherlands) and were derived from the ecotype "Landsberg erecta" (Redei 1962). The batches were harvested at different times of the year. Dormancy is greatly influenced by the light conditions during seed maturation, seeds maturing under long-day conditions being more heavily dormant than those maturing under short-day conditions (Baskin and Baskin 1972). Two batches of wildtype were used, one harvested in the summer of 1979, called WT79 and the other in the winter of 1981, called WT81.

For the production of seed, seeds were sown in 9 cm petri dishes on perlite with a standard mineral solution (Oostindier-Braaksma and Feenstra 1973). To break dormancy, the petri dishes were kept at 2 - 4 ^{o}C for 5 days after sowing. They were subsequently placed in a climate room at 24 ^{o}C under continuous illumination with fluorescent light of c. 8 Wm^{-2} irradiance. After 8 days the seedlings were transplanted into soil in an air-conditioned greenhouse where additional continuous fluorescent light was given from October to April. Despite these long-day conditions throughout the year, WT79, harvested in the summer, proved to be more dormant than WT81, harvested in the winter. Presumably, the supplementary light did not simulate completely natural long-days. The seeds were harvested and dried at room temperature and stored in the refrigerator at 4 - 6 ^{o}C.

Long-hypocotyl mutants (Hy)

The long-hypocotyl mutants (Hy-mutants) were isolated by Dr. M. Koornneef (Koornneef et al. 1980). To induce mutation, *Arabidopsis* seeds were either treated with ethylmethanesulfonate (EMS, 10 mM, 24 h, 24 ^{o}C) or irradiated

with X-rays or fast neutrons. Hy-mutants were recognized by having a significantly longer hypocotyl after 8 days growth at 24 °C. The mutations causing an elongated hypocotyl are located at 5 different loci (Koornneef et al. 1980). Some of the Hy-mutants have reduced phytochrome content (Table 1).

mutant	locus	phytochrome content 10^3 x ΔΔ A	
		seed	hypocotyl
wildtype	+	0.19	1.15
21.84	Hy-1	< 0.04	< 0.05
TO76	Hy-2	< 0.04	< 0.05
Bo64	Hy-3	0.07	1.35
2.23N	Hy-4	0.22	0.97
Ci88	Hy-5	0.13	1.03

Table 1. Spectrophotometrically detectable phytochrome content of seed and darkgrown, one week old seedlings of wildtype *Arabidopsis* and 5 long-hypocotyl mutants (after Koornneef et al. 1980 and Koornneef, unpublished data).

Although Hy-1 and Hy-2 appear to have no phytochrome, the possibility cannot be excluded that they still contain some phytochrome below the detection limit of the spectrophotometer (c. 0.05 x 10^{-3} ΔΔA).

Koornneef et al. (1980) tested the spectral dependence of the hypocotyl growth inhibition (Fig. 1). In wildtype, all wavelength regions tested display inhibiting effects, green light being less effective. However, in the Hy-mutants, some of the wavelength regions proved to be ineffective. In Hy-1 and Hy-2 mutants, the inhibition by red (R) and far-red (FR) is almost completely absent. In Hy-3 no inhibition takes place by R but does so by FR. Blue light (B) is less effective in Hy-4, and to some extent in Hy-5 mutants. Koornneef et al. (1980) conclude that the results in Fig. 1 cannot be explained from the action of a single light receptor pigment. In Hy-1 and Hy-2, the phytochrome poor mutants, R and FR are not active, while B and UV are active, suggesting

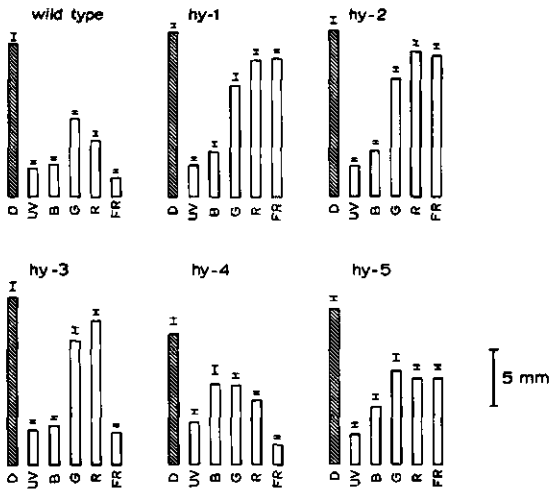

Fig. 1. Hypocotyl lengths of wildtype and long-hypocotyl mutants of *Arabidopsis*, grown under continuous irradiation with various spectral regions. D = dark control, UV = ultra violet, B = blue, G = green, R = red, FR = far-red (After Koornneef et al. 1980).

that there is a B-UV absorbing receptor pigment working in the HIR, apart from phytochrome. The fact that UV is more effective than B in Hy-4 and Hy-5 may suggest that there is more than one pigment absorbing in the B and UV. They conclude that the Hy-1 and Hy-2 genes regulate the synthesis of phytochrome, at least in the hypocotyl and that high activity of the short wavelength regions under continuous irradiation is not related to phytochrome. This appears to exclude the possibility of UV- and B-absorbing pigments, acting by transferring their excitation energy to phytochrome. The fact that mutant Hy-3, which has a normal phytochrome content, has normal sensitivity to FR but a strongly reduced sensitivity to R, may suggest the possibility that a second R-absorbing pigment, apart from phytochrome, contributes to the hypocotyl inhibition under continuous irradiation. Mutant Hy-5 is different to the other mutants. Whereas its measured phytochrome content is fairly high, it is only moderately sensitive to FR. Apparently the reactivity to light in this genotype is blocked by some other factor than its capacity for phytochrome synthesis.

In the present study seeds of the mutants 21.84 (Hy-1), TO76 (Hy-2),Bo64 (Hy-3), 2.23N (Hy-4) and Ci88 (Hy-5), harvested in the winter of 1981, were used.

Transparent, Testa, Glabra mutants (ttg)

The ttg mutants used in this study were also obtained from Dr. M. Koornneef (Koornneef 1981). The gene symbol ttg stands for transparent, testa, glabra. The gene ttg is located on chromosome 5. The ttg plants do not have trichenes on the leaf surface and the stem base and they have no anthocyanins. The seed coats are transparent, giving the seeds a yellow appearance and there is no mucilage on the surface of the seeds. Normally *Arabidopsis* seeds develop a mucilage layer very rapidly upon imbibition. For phytochrome measurements, absence of a mucilage layer is of great advantage, because the light of the measuring beam now passes preferentially through the seeds and not through the mucilage layer. The seed dormancy of ttg seeds is reduced as compared to wildtype (Koornneef 1981). The seeds used in the present study were harvested in late summer 1981.

General germination procedure

Seeds were imbibed in lots of 50 to 100 in 5 cm petri dishes on 5 layers of 4.5 cm diameter filter paper discs (Schleicher and Schüll nr 589[2]). The filter paper was moistened with 1.8 ml solution, containing 0.01 M Na,K-phosphate buffer (pH 7.5) and 0.001 M KNO_3. The dishes were kept in a dark room in plastic containers in light tight wooden boxes during the imbibition period. Different imbibition periods and conditions were used (for details see each chapter). At the end of the dark imbibition period the dishes were transferred in darkness to 20 ^{o}C for 1 h, before irradiation with a modified projector lamp assembly equiped with a 250 W quartz iodine lamp (Philips, The Netherlands). Narrow wavelength bands were obtained by interference filters (Balzers, B40, Liechtenstein), with c. 10 nm bandwidth at 50% of the transmission maximum. As a precaution against any possible traces of long-wavelength irradiation in the optical system, a 3 cm saturated $CuSO_4$-filter was placed in the light beam when irradiation was with wavelengths shorter than 550 nm. The fluence rate was varied by inserting neutral glass filters (NG, Schott u Gen., Mainz, West Germany) in the light beam or by varying lamp voltage. The fluence rate was measured with an Optometer, type 80X (United Detector Technology Inc., Santa Maria, Ca, USA) or calculated on the basis of measured characteristics of neutral filters. The calibration of the optometer was checked

by comparison with a calibrated thermopile (Kipp en Zonen, Delft, The Netherlands). During the irradiation of the seeds, lids were removed from the petri dishes (for irradiation time see each chapter). After the irradiation, the seeds were incubated in the dark for 4 days at 20 ^{o}C before germination was monitored. For each experiment at least 3 - 4 dishes were used and most experiments were repeated at least once with qualitatively similar results. For details and deviations from this general procedure see each chapter.

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CHAPTER 3, FLUENCE-RESPONSE CURVES FOR INDUCTION AND INHIBITION OF SEED GERMINATION IN *Arabidopsis thaliana*.

The fluence-response curves of wildtype and long-hypocotyl mutants of *Arabidopsis thaliana* for induction and inhibition of seed germination, expressed as percentage germination on a probit scale against logarithm (ln) of fluence are different in shape. In this chapter we discuss parameters which can influence the shape of fluence-response curves for phytochrome controlled seed germination. Calculations of the amount of the far-red absorbing form of phytochrome (Pfr) produced at a given fluence have been made taking into account pre-existing Pfr in the seeds. This pre-existing Pfr can dramatically change the slope of a fluence-response curve. Other factors or interactions of them are shown to influence the form and/or position of a fluence-response curve. Examples of these are an overriding factor, stimulating germination by a non-phytochrome related process, the total amount of phytochrome, the range of Pfr requirement in the seed population and differential screening. A normal distribution of ln Pfr requirement in the population may therefore not be reflected in the ln fluence requirement for induction of germination.

Introduction

Light has a promotory and/or inhibitory effect on seed germination of many plant species (Toole 1973). The red/far-red (R/FR) reversible pigment phytochrome (P) in its FR absorbing form (Pfr) has been shown to promote germination (Rollin 1972, Kendrick 1976) with the exception of *Bromus sterilis* where it inhibits (Hilton 1982). Photoinhibition of germination is more complex and can be brought about by photoconversion of pre-existing Pfr to the R absorbing form (Pr) or by the fluence rate dependent process of P cycling (Frankland 1981, Bartley and Frankland 1982).

The photoconversion of P from Pr → Pfr and from Pfr → Pr are first order reactions (Butler et al. 1964). Since the absorption spectra of Pr and Pfr overlap, a wavelength dependent photostationary state ($Pfr/(Pr + Pfr) = \phi$) is established upon irradiation. While ϕ is theoretically irradiance independent, the time required for this state to be achieved will clearly be irradiance dependent. The requirement of Pfr to promote germination can be quantified by determination of the fluence-response relationship. In the case of seed ger-

mination (Duke 1978) and some other photomorphogenetic responses controlled by P, e.g. inhibition of mesocotyl growth in oat (Loercher 1966), there is a good correlation between logarithm of Pfr and response. The fluence-response relationship for seed germination plotted as percentage germination on a linear scale against logarithm of fluence (ln fluence) is sigmoid (Shropshire 1972, Frankland 1976). This indicates a normal distribution of ln fluence requirement in the seed population. We feel this reflects a normal distribution in ln Pfr requirement. Alternatively the probit of percentage germination against ln fluence can be plotted (Hartmann 1966, Frankland 1976). Probit transformation is a mathematical method to linearize a normal sigmoid curve (see Finney 1952 for details and appendix II). This plot enables a more precise determination of the fluence at each wavelength that gives a standard response. The fluences determined in this way are utilized in constructing action spectra. Obviously the line will curve at low fluences if there is any dark germination and at high fluences if not all the seeds germinate in the light. This means that in practice even on a probit plot, fluence-response curves are somewhat S-shaped. The practice of subtracting the dark germination and/or seeds failing to germinate in the light and plotting results as probit of percentage response often makes the fluence-response relationship linear. In calculating theoretical fluence-response curves many assumptions have to be made. One factor which has not been taken into account previously in calculating fluence-response curves is the influence of pre-existing Pfr in the seeds. Duke (1978) analysed the influence of such factors as: range of ln fluence requirement, absolute ln fluence range, as well as the amount of total P (Ptot) on fluence-response curves for the promotion of seed germination. He also introduced the term overriding factor for a process independent of the P system that influenced germination. An overriding factor that reduces the R-induced percentage germination, reduces the apparent slope of the fluence-response curve for the smaller proportion of the population that responds to light. He further proposed that the only other cause of a change in slope of a fluence-response curve for promotion of seed germination could arise from a broadening of the range of the ln fluence requirement of the population.

In this chapter the factors which influence the shape and absolute ln fluence range of fluence-response curves for promotion and inhibition of seed germination were investigated, utilizing wildtype and mutants of *Arabidopsis thaliana* (Koornneef et al. 1980).

Materials and methods

See also chapter 2. Two wildtype batches, one harvested in winter 1981 (WT81) and the other harvested in summer 1979 (WT79) and the mutants 21.84 (Hy-1) and TO76 (Hy-2) were used. The wildtype batches were imbibed for 7 days at 7 ^{o}C, whereas Hy-1 and Hy-2 were imbibed for 2 days at 7 ^{o}C. All irradiations were done to individual dishes in 1 min and at least 3 dishes were used for each treatment. SE's always less than 5% of the mean, were omitted from the figures for clarity.

Results and discussion

Figure 1 shows fluence-response curves for the induction of germination in two batches of wildtype (WT79, WT81) and two long-hypocotyl mutants (Hy-1, Hy-2). In an attempt to make a meaningful comparison, a pre-irradiation with a saturating fluence of FR (730 nm) was given to establish the same Pfr/Ptot in all seed batches. The fluence-response curves of the wildtype batches are parallel but shifted along the ln fluence axis, since they have different sensitivities to light. The fluence-response curves of the mutants are also parallel to each other, showing different sensitivities to light but they are not parallel with the wildtype curves.

Duke (1978) came to the conclusion that differences in the slopes of fluence-response curves can only be explained by changes in the range of Pfr requirement of the seed population. In calculating fluence-response curves, we assume that there is a normal distribution in ln Pfr requirement in the seed population around a mean level of Pfr required for 50% germination (Fig. 2). The relationship between percentage germination and Pfr in such a seed population can be described as:

$$y = \int_{-\infty}^{\ln Pfr} \frac{1}{SD\sqrt{2\pi}} \cdot e^{-\frac{(\ln Pfr - \mu)^2}{2\,SD^2}} \, d\ln Pfr \cdot 100\ \% \qquad (1)$$

where y = percentage germination, μ = ln Pfr required for 50% germination and SD = standard deviation of ln Pfr around μ.

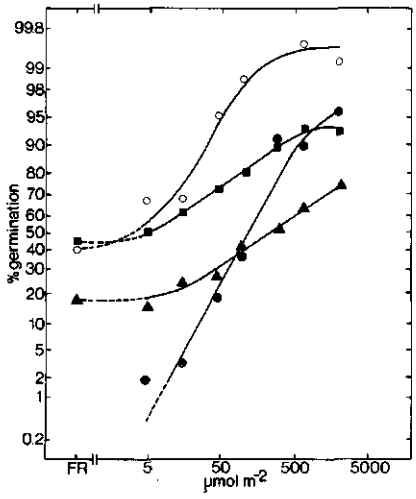

Fig. 1. Fluence-response curves for the induction of germination with red light (660 nm) plotted as % germination on a probit scale. All batches were pre-irradiated with 5.5 x 10^{-3} mol m^{-2} far-red light (730 nm). The batches WT79 (●) and WT81 (o) were imbibed for 7 days at 7 °C and Hy-1 (■) and Hy-2 (▲) for 2 days at 7 °C, irradiated and incubated for 4 days at 20 °C in darkness.

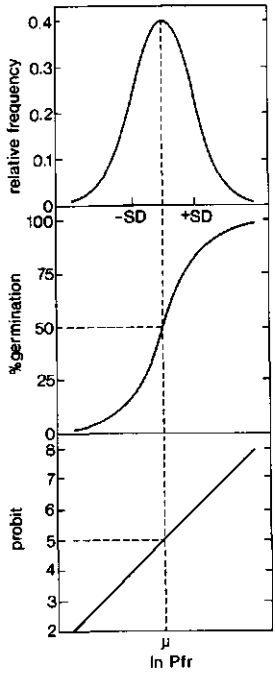

Fig. 2. Relationship between the logarithm of Pfr and the germination. The upper figure shows the normal distribution curve around µ with standard deviation SD. The middle figure shows the % germination for a population with normal distribution for ln Pfr requirement. The lower figure is the linearized form of the middle figure where % germination is plotted on a probit scale (see also appendix II).

Equation (1) assumes all seeds are viable and the whole population is capable of germinating, provided enough Pfr can be produced. The linearized form of equation (1) in terms of probits (Finney 1952) is:

$$\text{probit } y = \frac{\ln Pfr - \mu}{SD} + 5 \qquad (2)$$

Probit values for different values of y are given in appendix II.

Whereas equation (1) gives a sigmoid curve for % germination against ln Pfr, equation (2) gives a straight line for probit % germination against ln Pfr (Fig. 2). From equation (2) we can see that a change in μ, which is a change in sensitivity of the seed population will give a parallel shift in the fluence-response curve. A change in SD, which represents the range of ln Pfr requirement of the seed population, will change the slope of the fluence-response curve. In Fig. 3 fluence-response curves were calculated for populations with different μ and SD in their normal distribution. In these calculations Ptot is taken to be 100. The results are plotted as percentage germination on a probit scale against ln fluence. The amount of Pfr established by different fluences of 660 nm was calculated according to the modified version (Hartmann and Cohnen-Unser 1972) of Butler's (1972) formula:

$$Pfr = \left(1 - e^{-(\sigma_1 + \sigma_2) Nt}\right) \phi \, . \, Ptot \qquad (3)$$

where Nt = fluence in mol m^{-2}, σ_1 and σ_2 = apparent molar conversion cross section for the transition Pr → Pfr and of Pfr → Pr respectively and $\phi = \sigma_1/(\sigma_1 + \sigma_2)$ = the maximum Pfr/Ptot established by a given wavelength. The values of σ_1 and σ_2 for different wavelengths used, were derived from Butler's data by Bartley (1982) on the assumption that ϕ at equilibrium with red light (R) is 0.75 (see appendix I). Although recent publications suggest that ϕ for R may be somewhat higher (Vierstra and Quail 1982) it makes little quantitative differences to the calculated curves here since the population

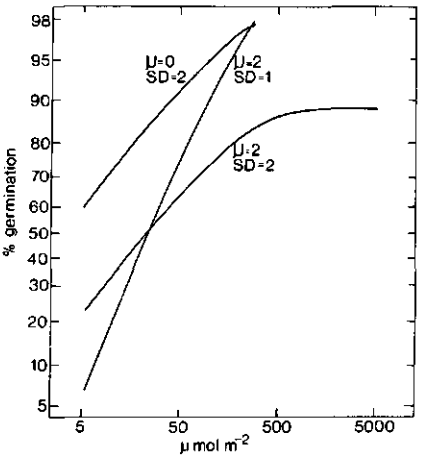

Fig. 3. Calculated fluence-response curves (660 nm) plotted on a probit scale for populations with different values for μ and SD, as indicated. In the calculations Ptot is 100.

is responding to ln Pfr. To express the equation in terms of percentage Pfr, Ptot can be substituted by 100%. Until now (Duke 1978) equation (3) has been used on the assumption that there is no pre-existing Pfr in the seeds, although experiments show that under the influence of environmental factors during ripening and drying of the seeds, the amount of Pfr can reach very significant levels (Kendrick 1976, Frankland 1976). Equation (3) can be modified to take account of pre-existing Pfr in the seeds, expressed as a fraction (ϕ_0) of Ptot.

$$Pfr = \left(1 - e^{-(\sigma_1 + \sigma_2) Nt \left(\frac{\phi - \phi_0}{\phi}\right)}\right) \phi \cdot Ptot \qquad (4)$$

This equation can be used not only for the appearance of Pfr, in the case of induction of germination but also for the disappearance of Pfr in the case of inhibition of germination. Figure 4 shows the relationship between ln fluence (660 nm) and Pfr/Ptot plotted on a logarithmic scale starting with different levels of pre-existing Pfr (ϕ_0). Since there is a linear relationship between the ln Pfr and the probit of germination (eq. 2), this reflects the relationship between the ln fluence and the probit of germination (Frankland 1976). The fluence-response curves for populations with different values of ϕ_0 are no longer parallel, the higher the pre-existing level of Pfr, the shallower

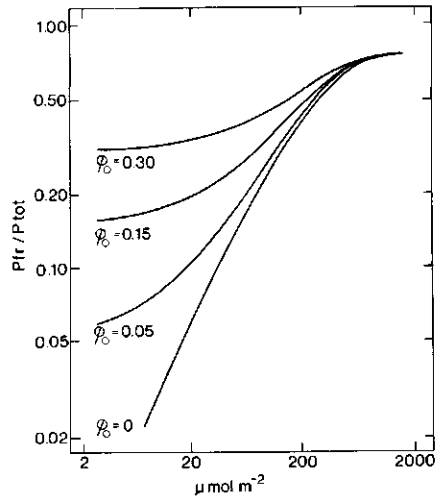

Fig. 4. Relationship between light fluence of 660 nm and the appearance of Pfr, plotted on a logarithmic scale with different amounts of pre-existing Pfr (ϕ_0), as indicated.

the slope of the curve. This relationship only approximates to a straight line when ϕ_0 is zero. Although Fig. 4 reflects the relationship between the ln fluence and the probit of germination, the actual shape of a fluence-response curve is also defined by μ and SD. A seed batch with a high sensitivity to Pfr (low μ) that has a certain level of pre-existing Pfr and a given SD will have a shallow fluence-response curve, because the pre-existing Pfr has a great influence on the slope of the fluence-response curve. Such a fluence-response curve is determined by the lower part of the ln fluence against ln Pfr/Ptot curves in Fig. 4, corresponding to $\mu \pm SD$ on the ordinate. A seed batch with a low sensitivity (high μ) with the same pre-existing Pfr and SD, will have a steeper fluence-response curve. In fig. 4 such a fluence- response curve is determined by the upper part of the ln fluence against ln Pfr/Ptot curves. It is therefore possible that extremely low levels of pre-existing Pfr can influence the shape of a fluence-response curve if the seeds are very sensitive to Pfr. This may be the reason why the fluence-response curves obtained with FR- and thermodormant lettuce seeds (Blaauw-Jansen and Blaauw 1975, Small et al. 1979)

have different slopes. Both prolonged FR and high temperature treatment reduce the pre-existing Pfr to a low level, while the high temperature treatment in addition makes the seeds extremely sensitive to Pfr. In the case of thermo-dormant lettuce seeds a level of 0.001% endogenous Pfr suffices to give a dramatic change in the slope of the fluence-response curves for promotion of seed germination. The fluence-response curve for promotion of FR-dormant lettuce seeds is steeper because these seeds are much less sensitive to Pfr. We feel this is a more plausible explanation than that put forward by Blaauw et al. (1976), who attempted to explain the difference in slope in terms of two processes having different numbers of quanta absorbed per active unit.

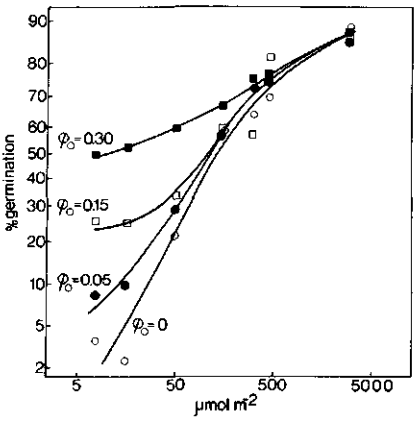

Fig. 5. Fluence-response curves for the induction of germination with red light (660 nm) of batch WT79 plotted on a probit scale, with different amounts of pre-existing Pfr (ϕ_0), as indicated. The pre-existing Pfr was established by 2.5 x 10^{-5} mol m^{-2} (ϕ_0 = 0.05), 7.1 x 10^{-5} mol m^{-2} (ϕ_0 = 0.15) and 1.7 x 10^{-4} mol m^{-2} (ϕ_0 = 0.30) red light (660 nm). The seeds were imbibed for 7 days at 7 °C, irradiated and incubated for 4 days at 20 °C in darkness.

Figure 5 shows the relationship between ln fluence (660 nm) and the probit of germination for seed batch WT79 in which different initial Pfr levels have been produced. This seed batch shows a low sensitivity to Pfr and has a low level of pre-existing Pfr. The initial levels of Pfr are established by pre-irradiation with calculated fluences of 660 nm using equation (3), assuming that there is no pre-existing Pfr in the seeds. These experimental results resemble the calculated curves for Pfr production (Fig. 4). To calculate fluence-response curves which coincide with the experimental curves, we have to utilize values for μ and SD in equation (2). The value of μ and SD can be calculated from the experimental fluence-response curve for no pre-existing Pfr in the seeds. When μ = 3.4 and SD = 0.9 the calculated curves (Fig. 6) coincide with the experimental curves of Fig. 5.

Pre-existing Pfr, also gives rise to fluence-response curves for different wavelengths that are not necessarily parallel (Fig. 7). The fluence-response curves for 660 and 690 nm are parallel when there is no pre-existing Pfr in the seeds. However when 8% Pfr is present, the curve for 660 nm is much steeper than that for 690 nm.

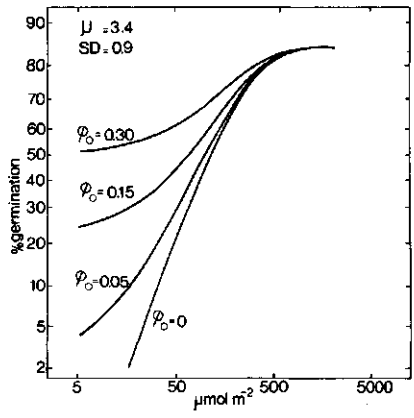

Fig. 6. Calculated fluence-response curves, plotted on a probit scale, for a population with μ = 3.4, SD = 0.9 and Ptot = 100 for different amounts of pre-existing Pfr (ϕ_0), as indicated.

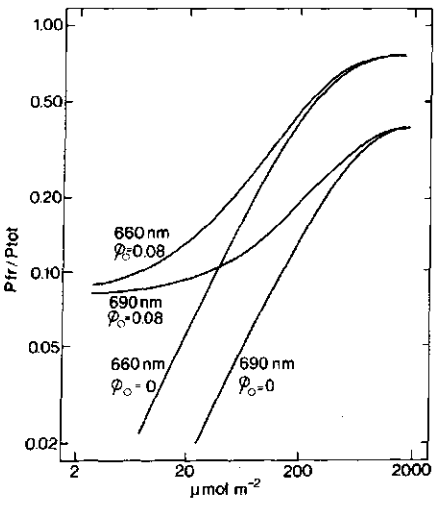

Fig. 7. Calculated relationship between light fluence of 660 nm and 690 nm and the appearance of Pfr plotted on a logarithmic scale, with different amounts of pre-existing Pfr (ϕ_0), as indicated. In the calculations Ptot is 100.

The level of dark germination can result from the Pfr requiring seeds being satisfied by their pre-existing Pfr or seeds can germinate as a result of some other factor, which is independent of the phytochrome system. Duke (1978) introduced the term 'overriding factor' for a non-phytochrome related process that influences germination. He only showed fluence-response curves for an overriding factor which influences the maximal percentage germination. In that case the factor prevents certain seeds from germinating and acts at random throughout the seed population irrespective of the seeds Pfr requirement for induction of germination. If a seed population has a normal distribution in ln Pfr requirement, it means that when a part of it germinates as a consequence of an overriding factor, the remaining part will still be respond normally with respect to ln Pfr requirement (Duke 1978). This means that although there is a high dark germination, the population will respond over the same ln Pfr range as without dark overriding factor germination. To calculate the influence of an overriding factor, the amount of Pfr, obtained by equation (3) or (4), must be expressed as percentage germination, using equation (2). The total percentage germination (y_z), caused by Pfr and the overriding factor

is:

$$y_z = y\ (1 - z) + (z \times 100) \qquad (5)$$

where z is the fraction of the population which germinates under the influence of the overriding factor. Calculated fluence-response curves for different percentages of the population germinating because of an overriding factor are shown in Fig. 8. For the calculations the values: μ = 2.0, SD = 2.0 and Ptot = 100 were used. The slopes of the fluence-response curves change under the influence of the overriding factor. If the maximum germination under the influence of Pfr is not 100%, the maximum germination in combination with an overriding factor will always be higher than without it, as can be seen in Fig. 8. A greater influence on the shape of the fluence-response curves will be obtained by a combination of an overriding factor and pre-existing Pfr. Duke (1978) calculated fluence-response curves for an overriding factor which reduced the maximal possible percentage germination. A combination of the two kinds of overriding factor, one promoting dark germination and the other reducing the light-induced germination, will be additive in reducing the slope of a fluence-response curve.

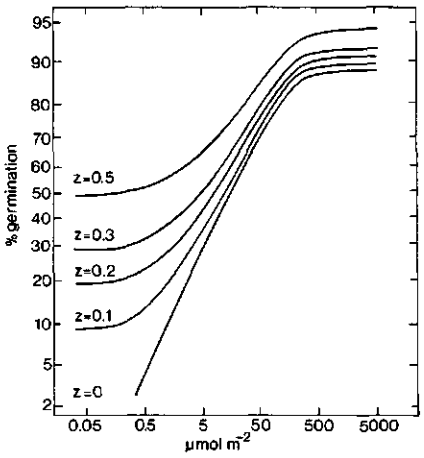

Fig. 8. Calculated fluence-response curves (660 nm) plotted on a probit scale, with different proportions of the population germinating in the dark because of an overriding factor (z), promoting germination by a non-phytochrome related process, as indicated. In the calculations μ = 2.0, SD = 2.0 and Ptot = 100.

Alteration in Ptot does not change the slope of a fluence-response curve (Duke 1978). However, this is only true when there is no pre-existing Pfr in the seeds and when there is no overriding factor active. Fluence-response curves were calculated with two different levels of Ptot combined with and without 20% dark germination because of an overriding factor (Fig. 9). While without the overriding factor (z = 0) the two curves are parallel, with the overriding factor the slopes of the fluence-response curves are not parallel. The same is true when dark germination occurs because the seeds contain pre-existing Pfr.

The so-called reaction partner of Pfr, X, can be considered to act as an overriding factor that prevents germination of a proportion of the light sensitive population. However, it is also possible that X acts as a limiting factor and effects the light sensitive population unequally. For example, a supply of X may only be found limiting in those seeds having the highest Pfr requirement (Frankland 1976, Duke 1978, Cone and Spruit 1983). In this case the fluence-response curves for induction of germination are predicted to be parallel and to level off at Pfr levels below that maximally possible depending upon the availability of X.

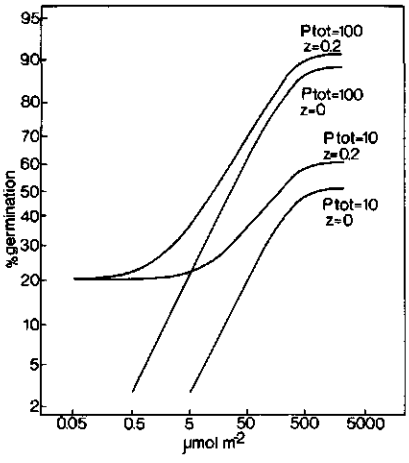

Fig. 9. Calculated fluence-response curves (660 nm), plotted on a probit scale, with different proportions of the population germinating in the dark because of an overriding factor (z) and different amounts of Ptot, as indicated. In the calculations μ = 2.0 and SD = 2.0.

Fluence-response curves were also determined for the inhibition of germination by FR (Fig. 10). The fluence-response curves for the two wildtype batches show a difference in sensitivity but are parallel. The fluence-response curves for the genotypes Hy-1 and Hy-2 are also parallel to each other but not with the wildtype curves. Before FR irradiation just enough R (660 nm) was given to induce maximal germination in each seed batch. Equation (4) can also be used to calculate fluence-response curves for the inhibition of germination induction. The value of ϕ_0 now is the Pfr level established by the pre-irradiation and ϕ is the final level of Pfr established by the FR irradiation. Figure 11 shows the relationship between the ln fluence of 730 nm and the logarithm of Pfr/Ptot for different initial amounts of Pfr/Ptot. The curves

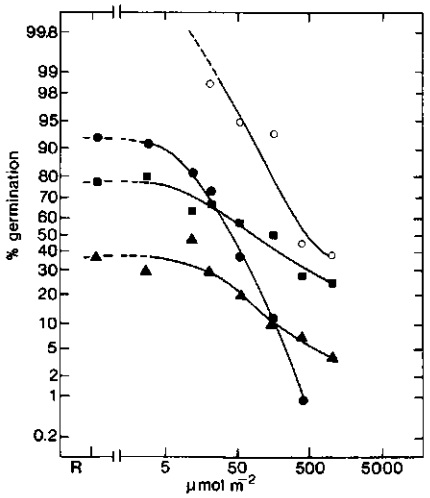

Fig. 10. Fluence-response curves, plotted on a probit scale, for the reversion of germination induction with far-red light (730 nm). Before the far-red the batches were pre-irradiated with 2.2 x 10^{-3} mol m^{-2} to WT79, 3.7 x 10^{-4} mol m^{-2} to WT81, 7.4 x 10^{-4} mol m^{-2} to Hy-1 and 1.5 x 10^{-3} mol m^{-2} to Hy-2 red light (660 nm). The batches WT79 (●) and WT81 (⊙) were imbibed for 7 days at 7 °C and Hy-1 (■) and Hy-2 (▲) 2 days at 7 °C, irradiated and incubated for 4 days at 20 °C in darkness.

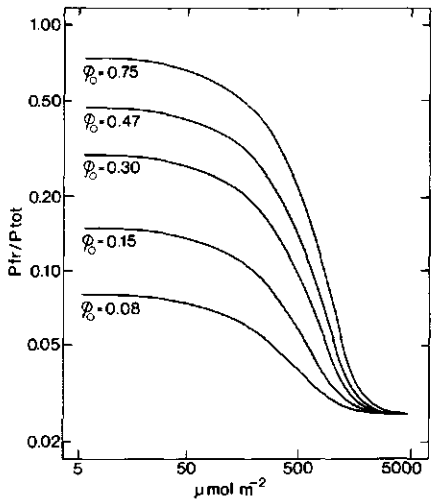

Fig. 11. Calculated relationship between light fluence of 730 nm and the disappearance of Pfr, plotted on on a logarithmic scale, with different amounts of initial Pfr (ϕ_0), as indicated.

obtained are not parallel. The curves with high initial Pfr/Ptot are steeper than those with low initial Pfr/Ptot. Here too the sensitivity and the range of Pfr requirement of a seed batch determines what part of these curves will be reflected in the fluence-response curves for inhibition of induction. Experimental fluence-response curves are presented in Fig. 12 for WT79, where germination induction is inhibited by 730 nm with different initial Pfr/Ptot, established by different fluences of R. The calculation of the initial Pfr level assumes that there is no significant pre-existing Pfr in the seeds. This batch only reaches maximal germination with the maximum possible Pfr. The fluence-response curves show minimal inhibition of germination at low fluences, because this seed batch is rather insensitive to Pfr.

Calculated fluence-response curves (Fig. 13) for the inhibition with the same values for μ, SD and Ptot as for the induction (see Fig. 6), do not coincide with the experimentally obtained curves of Fig. 12. Obviously the WT79 seeds need less FR for the inhibition than predicted. In the formula of Butler (1972) we are dealing with values of σ_1 and σ_2 which were determined in vitro. However in vivo P may have slightly different photochemical properties and also there is the possibility of other pigments which can differentially screen P. For instance the absorption of *Amaranthus retroflexus* seed coats changes dramatically between 600 and 800 nm (Taylorson and Hendricks 1971). R of

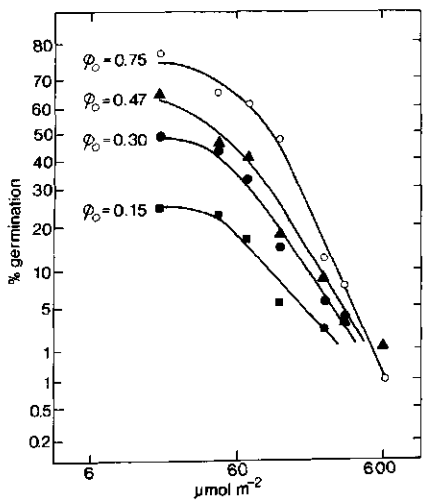

Fig. 12.
Fluence-response curves, plotted on a probit scale for the reversion with far-red light (730 nm) of batch WT79, with different amounts of initial Pfr (ϕ_0), as indicated. The initial Pfr level was established by 3.1×10^{-3} mol m^{-2} ($\phi_0 = 0.75$), 3.2×10^{-4} mol m^{-2} ($\phi_0 = 0.47$), 1.7×10^{-4} mol m^{-2} ($\phi_0 = 0.30$) and 7.1×10^{-5} mol m^{-2} ($\phi_0 = 0.15$) red light (660 nm)
The seeds were imbibed for 7 days at 7 °C, irradiated and incubated for 4 days at 20 °C in darkness.

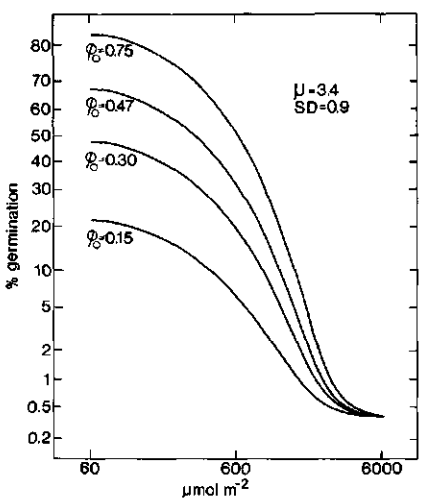

Fig. 13.
Calculated fluence-response curves, plotted on a probit scale, for the reversion of germination induction with far-red light of 730 nm of populations with different amounts of initial Pfr (ϕ_0), as indicated. In the calculations μ= 3.4, SD = 0.9 and Ptot = 100.

660 nm is absorbed c. 4 times more strongly than FR of 730 nm. Although we do not know the absolute differential attenuation of the light in the seeds for each wavelength, we can utilize a factor K, which is defined as the relative proportion of the quanta reaching P at a given wavelength with respect to 660 nm. We can introduce K into equation (4):

$$Pfr = \left(1 - e^{-(\sigma_1 + \sigma_2) Nt \,.\, K \left(\frac{\phi - \phi_0}{\phi} \right)} \right) \phi \,.\, Ptot \qquad (6)$$

When the exact absorption in the seeds is known, the factor K can be defined as the fraction of light transmitted to the site of P in the seed. Calculating fluence-response curves with a value for K of 5.6 for 730 nm produces curves (Fig. 14) which coincide with the experimental curves of Fig. 12.

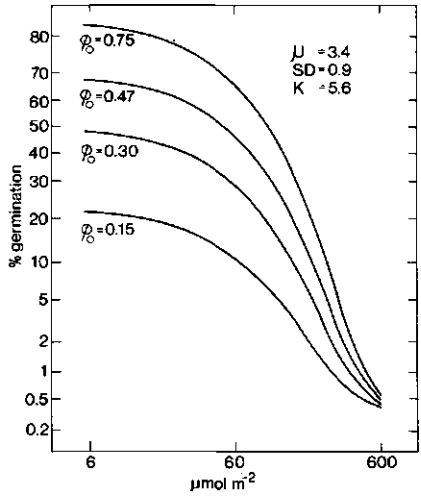

Fig. 14. Calculated fluence-response curves, plotted on a probit scale, for the reversion of germination-induction by far-red light (730 nm) of populations with different amounts of initial Pfr (ϕ_0), as indicated. K = relative differential attenuation of 730 nm to 660 nm light. In the calculations μ = 3.4, SD = 0.9, Ptot = 100 and K = 5.6.

Blaauw and Blaauw-Jansen (1975) showed that fluence-response curves for the inhibition of lettuce seed germination and promotion of mesocotyl growth after different fluences of R are not parallel. These workers attempted to explain their results by postulating different pools of Pfr, but clearly this is not necessary.

The fluence-response curves we obtained with different batches of *A. thaliana* seeds both for induction and inhibition of germination (Figs. 1 and 10) show differences in slope. The curves for Hy-1 and Hy-2 are shallow, both for the induction and for the inhibition of induction.

Since we pre-irradiated with FR, all the batches we used in induction experiments had the same low level of pre-existing Pfr. It is therefore unlikely that the pre-existing Pfr level can be responsible for the dramatic change in the slopes of the fluence-response curves of Hy-1 and Hy-2. Only in the case of wildtype batch WT81 it might have had an influence, because this batch is very sensitive to Pfr, and accordingly FR significantly promotes germination.

A more reasonable explanation of the shallow fluence-response curves of Hy-1 and Hy-2 is the fact that Ptot in these seeds is very low. The exact amount of P in these seeds is very difficult to determine, since it is near or below the detection limit of the spectrophotometer (Spruit 1970). The P content in Hy-1 and Hy-2 appears to be less than 20% of that of wildtype (Spruit et al. 1980). Certainly a combination of a low Ptot and an overriding factor which gives significant dark germination will result in a shallow fluence-response curve. This gives an explanation for the shallow curves for both induction and inhibition of germination. For the inhibition the seeds were pre-irradiated with just enough R to give maximal germination in each batch. It is unlikely that the different Pfr levels established by the different R fluences is responsible for the differences in the slopes. Even the lowest fluence of 3.7×10^{-4} mol m^{-2} for WT81 establishes 59% Pfr. All the other fluences utilized establish more than 70% Pfr. The fluence-response curves of Hy-1 and Hy-2 exhibit high dark germination as a result of an overriding factor. This combined with low maximal germination in the light results in a shallow fluence-response curve. There are two possible explanations of this low maximum germination. Firstly that the mutants are really depleted in Ptot as indicated by spectrophotometry or secondly that germination is inhibited by an overriding factor operating homogeneously throughout the light requiring population. Moreover, we do not know the value of μ and SD, which have a great influence

on the shape of the fluence-response curves. These parameters may be strongly dependent on the environmental conditions during seed maturation and harvest.

Fluence-response curves plotted as probit % germination against ln fluence are linear and parallel to each other at different wavelengths, only in special cases. When there is a certain level of pre-existing Pfr or an overriding factor, it means that a certain proportion of the seed population germinates in the dark and requires less light than predicted.

These experiments show that a seed population having a normal distribution for ln Pfr requirement for germination, does not necessarily have a normal distribution for ln light requirement.

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CHAPTER 4, ACTION SPECTROSCOPY

Introduction

Action spectroscopy is a non-destructive method for determining the absorption characteristics of the initial photoreceptor involved in mediating a photoresponse. This means that in a biological system a photoreceptorpigment can be visualized in vivo before it has been isolated. Action spectroscopy enabled the fine structure characteristics of the absorption spectra for the two forms of phytochrome, Pr and Pfr, to be predicted before the pigment was extracted and direct absorption measurements could be made. The methodology of making an action spectrum in principle is simple and needs no extensive instrumentation. The interpretation of action spectra however sometimes is more complex than hitherto anticipated.

How to make an action spectrum

In order to make an action spectrum one needs to obtain the values of the number of quanta (photonfluence) of different wavelengths required to produce a standard response. The best way to obtain these values is by determining fluence response curves for each wavelength (see chapter 3). The response is selected as some readily and precisely measurable variable, which in this study is seed germination after light exposure. The photonfluence is usually expressed in terms of numbers of incident quanta per unit area. The reciprocal of the photonfluence required to give a constant response for each wavelength examined is plotted against the wavelength, showing a peak for the most effective wavelength region. In order to make comparison of different action spectra easy, peak effectiveness is usually normalized to 100 percent (Shropshire 1972, Schäfer et al. 1983).

Factors influencing action spectra

In the experimental determination of a fluence-response curve and the construction of an action spectrum, there are a number of factors which must be taken into account.

Reciprocity (the Bunsen-Roscoe law), which means that for a given photonfluence (I x t) values of different irradiance (I) for different exposure times (t) result in a constant response (I x t = constant). However this may be difficult

to prove because fluence-response relationships often cover several orders of magnitude and one may be limited in finding a monochromatic source with sufficient output to produce a large response in a short time.

Errors can also be caused by screening. This can take the form of self-screening by the photoreceptor pigment or screening by another pigment. When a screening pigment is present, less of the applied quanta will reach the photoreceptor (be available for absorption), resulting in errors in calculating the relative effectiveness, the magnitude of which will depend on the absorption characteristics of the screening pigment. Self-screening results in an action spectrum that is broader than the actual absorption spectrum of the photoreceptor. A screen absorbing on one flank of the absorption spectrum of a photoreceptor will result in an action spectrum sharper than the absorption spectrum of the photoreceptor. Chlorophyll and seed coat pigments, that vary from one seed batch to another, dependent upon the conditions of seed maturation and dehydration, must be taken into account when considering action spectra for the photocontrol of seed germination. Seeds are optically very dense and little is known about the localization of phytochrome in relation to screening pigments and about the internal light distribution. The distribution of phytochrome molecules may give rise to self-screening, which may vary from one seed batch to another.

If the fluence rate of the actinic light is sufficiently high, the phytochrome phototransformations are much faster than the dark reactions (chapter 1). However, if the fluence rate of the actinic light is too low, the phototransformations become limiting and the dark reversion reactions cannot be neglected.

Very long irradiations should be avoided because of the possible interference of the high irradiance reaction (HIR). This is the case for irradiations of several hours or days. However, irradiations of a few minutes can also be influenced by the HIR. It is therefore advisable that irradiations for the low energy reaction (LER) are as short as possible and the irradiation time is kept constant.

Another large source of error in constructing fluence-response curves and

action spectra is the variability in the sensitivity of the responding system to light stimuli from day to day. These fluctuations are usually due to uncontrolled variables in the culture of the seeds and the seedlings. The variations can be reduced to a minimum by adequate control of nutrition, temperature, humidity and a uniform preparation of the material before irradiation. However, once these variations have been reduced to a minimum, changes in sensitivity may still occur. One way of minimizing errors is to measure with each set of experiments a standard dark control and the response to a standard fluence. Variations about the standard response and the dark control can be used to normalize values obtained at each wavelength. In the case of a seed batch where germination can change upon storage, it is necessary to keep these effects at a minimum and determine an action spectrum over the shortest time period possible.

Fluence-response curves

In the literature (e.g. Shropshire 1972) it is often argued that under ideal conditions, where there are no cooperative or interfering secondary effects, the fluence-response curves for each wavelength should have the same slope for all values between 20 and 80% of saturation. Therefore when the curves are parallel, the same action spectrum will be obtained for any size of response within this range and is independent of the sensitivity of the responding system. In chapter 3 it is shown for phytochrome responses that fluence-response curves under ideal conditions are not necessarily parallel. Deviations from parallel curves can result from differences in sensitivity and from screening pigments. This means that action spectra can be different depending upon the size of response selected. For comparison it is then important that the same response is always chosen for the construction of an action spectrum.

Theoretical calculations

In chapter 3 equations were given with which the amount of Pfr upon a subsaturating fluence can be calculated.

$$Pfr = (1 - e^{-(\sigma_1 + \sigma_2) Nt.K \left(\frac{\phi - \phi_0}{\phi}\right)}) \phi.Ptot \qquad (1)$$

where Nt = fluence in mol m^{-2}, σ_1 and σ_2 = apparent molar conversion cross section for the transitions Pr → Pfr and Pfr → Pr respectively, $\phi = \sigma_1/(\sigma_1+\sigma_2)$ = maximum Pfr/Ptot established by a given wavelength, Ptot = total amount of phytochrome and K = relative proportion of the quanta reaching phytochrome at a given wavelength with respect to 660 nm. If it is assumed that a seed population has a normal distribution in ln Pfr requirement for germination, the percentage of germination (y), as a consequence of a certain level of Pfr, can be plotted on a probit scale (see appendix II) with:

$$\text{probit } y = 5 + \frac{\ln Pfr - \mu}{SD} \qquad (2)$$

in which μ = ln Pfr required for 50% germination and SD = standard deviation of ln Pfr around μ. Corrections for an overriding factor (z) can be made with the equation:

$$y_z = y\,(1 - z) + (z \times 100) \qquad (3)$$

where z is the fraction of the population which germinates under the influence of the overriding factor and y_z is the total percentage germination.

Using equations 1 to 3, fluences (Nt) for each wavelength (λ) can be calculated which are required to give a certain germination response. Consequently an action spectrum representing the inverse of Nt as a function of λ can be calculated.

According to equation (1):

$$Nt = \frac{-\ln ((Pfr - Ptot.\ \phi) / Ptot\ (\phi_0 - \phi))}{(\sigma_1 + \sigma_2)\ .\ K} \qquad (4)$$

with: $Pfr = e^{(\text{probit } y - 5).SD + \mu}$ (5)

and

$$y = \frac{y_z - (z \times 100)}{1 - z} \qquad (6)$$

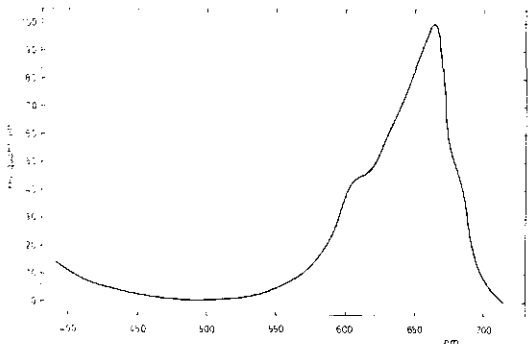

Fig. 1. Calculated action spectrum for the induction of 50% germination. In the calculations Ptot = 100, z = 0, μ = 2, SD = 2, ϕ_0 = 0 and K = 1. Rel. quant. eff. = relative quantum effectiveness.

To calculate an action spectrum some of the parameters must be given a value. The next example illustrates the simple situation at which pre-existing Pfr, screening pigment and overriding factor are absent (ϕ_o = 0, K = 1 and z = 0 respectively) and Ptot = 100, μ = 2 and SD = 2. The values of σ_1 and σ_2 for different wavelengths were derived from Butler's data (Butler 1972, Butler et al. 1964) by Bartley (1982) on the assumption that ϕ at equilibrium with R is 0.75 (see appendix I). For the induction of 50% germination (probit y = 5) the action spectrum of Fig. 1 is obtained with peak effectiveness at 665 nm and a shoulder at approx. 610 nm. Very little activity is seen between 400 and 550 nm and green light is particularly ineffective. Between 550 and 680 nm ϕ is constant. All the other parameters are wavelength independent (except K, see next section). This means that the fluence-response curves between 550 and 680 nm are parallel and that the same action spectrum in this range will always be obtained, irrespective of factors such as endogenous Pfr, overriding factor, sensitivity, range of Pfr requirement and Ptot (see chapter 3).

However beyond 680 nm fluence-response curves are no longer saturating at the same Pfr level (see chapter 3, Fig. 7), indicating that the size of response chosen influences the form of the action spectrum, as shown in Fig. 2. An action spectrum calculated for 85% germination (probit y = 6) is less effctive at long wavelengths than an action spectrum calculated for 15% germination (probit y = 4).

Beyond 680 nm, the shape of action spectra is also determined by the sensitivity (μ) of the seed batches (Fig. 3). A sensitive batch (low μ) shows more

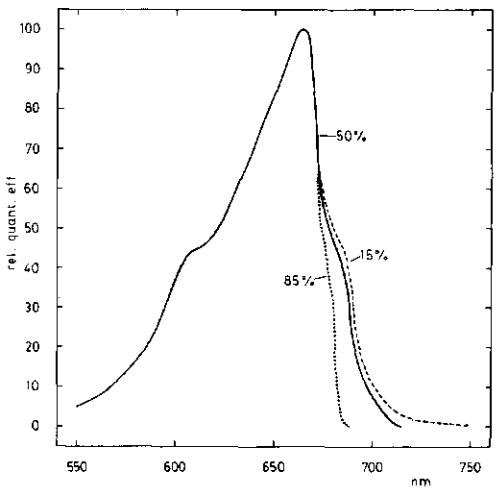

Fig. 2. Calculated action spectra for the induction of different percentages of germination. Rel. quant. eff. = relative quantum effectiveness. Further assumptions as in Fig. 1.

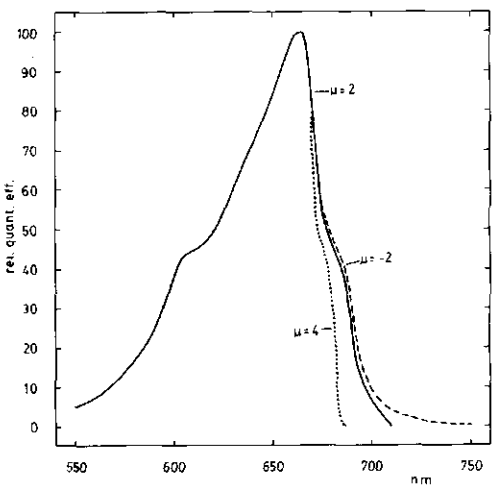

Fig. 3. Calculated action spectra for the induction of 50% germination for seed batches with different sensitivities to Pfr requirement (μ). Rel. quant. eff. = relative quantum effectiveness. Further assumptions as in Fig. 1.

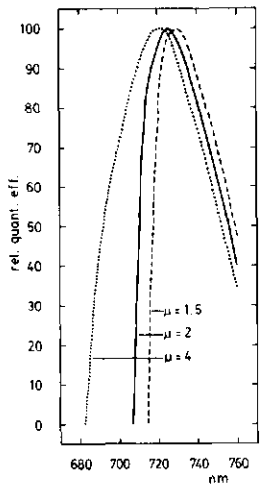

Fig. 4. Calculated action spectra for the reversion to 50% of germination induction for seed batches with different sensitivities to Pfr requirement (μ). Rel. quant. eff. = relative quantum effectiveness. ϕ_0 = 0.75. Further assumptions as in Fig. 1.

activity in the long wavelength region than an insensitive batch (high μ). Differences in the long wavelength tail of the action spectra also arise, due to differences in the endogenous Pfr, presence of an overriding factor promoting germination and Ptot. The range of Pfr requirement (SD) has no influence on the form of an action spectrum.

As a consequence of the before mentioned wavelength dependent factors, there is no standard spectrum for the induction of germination. Differences arise in the long wavelength tail of the spectra and are due to the properties of phytochrome and the seed batch characteristics.

Assuming a maximum Pfr level of 75% (ϕ_0 = 0.75), obtained after pre-irradiation with R, an action spectrum for the inhibition of germination induction can also be calculated. In Fig. 4 calculated action spectra for inhibition are shown with differences in sensitivity (μ). Spectra for batches with different sensitivities are not identical because ϕ is not constant beyond 680 nm. Even the peak position can shift. When μ = 4 (an insensitive batch), 720 nm is the most effective wavelength for inhibition of germination induction. However when μ = 1.5 (a sensitive batch), peak sensitivity is shifted to 730 nm. For inhibition of induction differences in the action spectra are also determined by differences in the endogenous Pfr, an overriding factor promoting germination and Ptot. This means that there is also no standard action spectrum for the inhibition of germination induction. The shape of the spectra is determined by the properties of phytochrome and the characteristics of the seed batch.

Screening by other pigments

To estimate the screening effect in *Arabidopsis thaliana* seeds, light transmission through the seeds was measured in an Aminco DW2a double wavelength spectrophotometer (American Instruments Company, Silver Spring, Maryland, USA). The spectrum was recorded in the double beam mode for a 2 mm layer of 16 h imbibed wildtype seeds against water as reference (Fig. 5). A very sharp rise in transmission from 600 to 740 nm is seen. Similar results were obtained by Taylorson and Hendricks (1971) who measured the transmission through individual seed coats of *Amaranthus retroflexus*. Besides the actual absorption by the seeds, there is also a scattering of the light. Short wavelength light scatters much more than long wavelength light. What phytochrome molecules perceive is the light transmitted through the seed coats. Figure 5 gives an impression of the relative amount of different wavelengths of light which reach phytochrome.

Influence of screening

Using the data of Fig. 5 the value of K for each wavelength can be estimated. With the equations (4), (5) and (6) an action spectrum can now be calculated in which K is taken into account. Figure 6 shows spectra for the induction of 50% germination with K = 1 (no differential screening) and with a wavelength dependent K, derived from Fig. 5. It appears that the spectrum in which K is taken into account is much narrower than the spectrum in which K = 1, and shifted to longer wavelengths. The peak position is shifted from 665 to 670 nm. Such a shift of an action spectrum has experimentally been observed by Blaauw-Jansen and Blaauw (1975) and Small et al. (1979), comparing action spectra for germination induction of very sensitive thermo-dormant lettuce seeds and insensitive FR-dormant seeds. The spectrum for the germination induction of thermo-dormant seeds was significantly shifted to longer wavelengths. They explained their results on the basis of a second R absorbing pigment (Blaauw-Jansen and Blaauw 1975) or by a second phytochrome process (Small et al. 1979). However it is also possible that the different pre-treatments result in changes of the concentration and characteristics of the screening pigments.

Action spectra for the inhibition of germination induction also show shifts to longer wavelength, when differential screening is taken into account (Fig. 7). Peak position is shifted from 725 to 735 nm. This effect can be amplified by the other factors, previously mentioned.

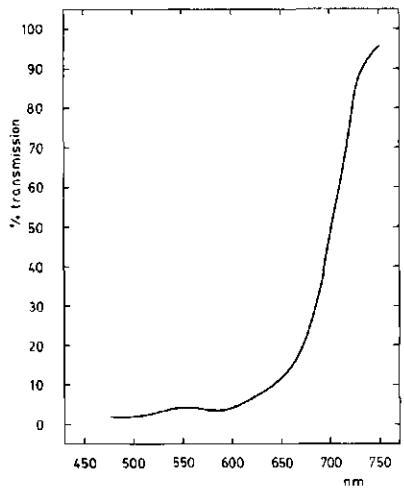

Fig. 5. Relative transmission through a 2 mm layer of A. *thaliana* seeds. The transmission at 800 nm was taken as 100.

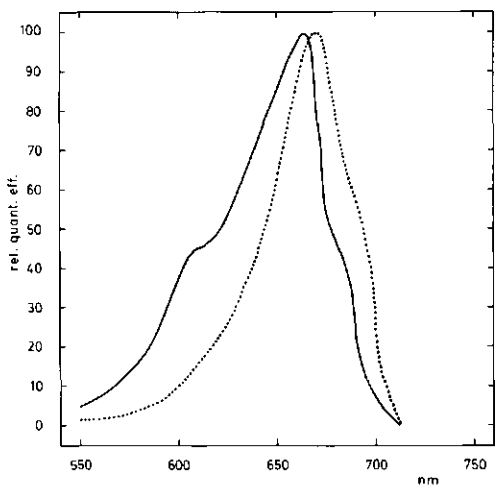

Fig. 6. Calculated action spectra for the induction of 50% germination with no influence of screening (solid line) and with the influence of wavelength dependent screening (dotted line) using values of K for each wavelength, derived from the data in Fig. 5. Rel. quant. eff. = relative quantum effectiveness.

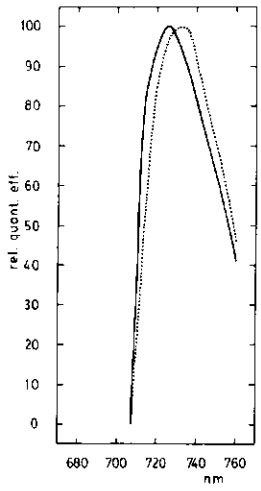

Fig. 7. Calculated action spectra for the inhibition to 50% of germination induction for a seed batch with no differential screening (solid line) and a seed batch with wavelength dependent screening (dotted line) using values of K for each wavelength, derived from the data in Fig. 5. Rel. quant. eff. = relative quantum effectiveness.

Conclusions

It is apparent that there is no standard action spectrum for a phytochrome response, both for the induction of seed germination as well as for the inhibition of the induction. The spectra are defined by the properties of phytochrome, but their shape is determined by the different properties and characteristics of the seed batch. One should therefore be careful to draw conclusions about the absorption characteristics of phytochrome on the basis of a small shift in the action spectrum. In particular the action spectrum for the inhibition of germination induction can be strongly modified. Moreovcer it is often very difficult to get a high degree of accuracy in experimentally determined action spectra. Normalizing action spectra for peak position is advisable for comparison of spectra. When the accuracy of the fluence-response curve at the peak position is low, it means that a small shift in this fluence-response curve has a large influence on the shape of the action spectrum.

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CHAPTER 5, IMBIBITION CONDITIONS AND SEED DORMANCY OF *Arabidopsis thaliana*.

Abstract

The optimal combinations of temperature in the range of 0 to 20 °C and duration (1 to 14 days) of imbibition for the induction of germination of *Arabidopsis thaliana* (L.) Heynh., ecotype 'Landsberg-erecta', by red light were investigated. At 2 °C, 10 days of imbibition are needed for loss of dormancy, whereas at higher temperatures, e.g. 15 °C, it is already lost after 1 or 2 days. It is proposed that the development of light-inducible germination is governed by two temperature dependent processes: the loss of primary or innate dormancy and the simultaneous induction of secondary dormancy. Data are discussed in terms of the availability of phytochrome, the availability of an unknown factor X and changes in sensitivity of the process of germination induction by the far-red absorbing form of phytochrome (Pfr).

Introduction

The self-fertile crucifer *Arabidopsis thaliana* (L.) Heynh. is widely used in plant research because of its small size, short life cycle and the availability of many mutants. Seed germination is a complicated process, influenced by many factors. Seed dormancy is highest in freshly harvested seeds and decreases during storage as after-ripening continues. During storage and imbibition, these processes are influenced by many factors such as temperature, humidity and light (Kugler 1951). Secondary dormancy is defined as the dormancy developing in seeds after harvest or dispersal (Duke et al. 1977, Karssen 1980/81). It develops in the absence of the factors necessary for germination such as light, oxygen, water, a suitable temperature or in the presence of an inhibitor. Seeds of several ecotypes of A. *thaliana* posses an innate dormancy which can be broken by cold imbibition. Routinely, seeds are imbibed for 4 to 7 days at a temperature of 2 to 5 °C (Redei 1970, Stokes 1965). Laibach (1956) observed that the germination percentage, 78 days after harvest of seeds of A. *thaliana*, was correlated with the length of the cold treatment at 5 °C. Comparable results were obtained by Rehwaldt (C.A. Rehwaldt, 1965. Thesis. Syracuse Univ., Syracuse, NY, USA) with seeds of the same species imbibed at 7 °C in the presence of 1 mM KNO_3.

A. thaliana seeds harvested from plants grown under long days are often heavily dormant, while those harvested from plants grown under short days are less dormant (Baskin and Baskin 1972). These authors also showed that daylength influences the rate of after-ripening of the seeds.

In the present experiments the optimum imbibition conditions to reach maximum germination were investigated for two batches of *A. thaliana* seeds, by varying the duration as well as the temperature of imbibition prior to the inductive irradiation with red light (R).

Materials and methods

See also chapter 2. *A. thaliana* seeds used in the present experiments were harvested from plants grown in late summer 1979 and winter 1981. R was obtained from a Leitz slide projector, using an interference filter of 667 nm. Three dishes were used for each treatment and the results are expressed as % germination $\pm$ SE. All treatments were repeated with qualitatively similar results.

Results

To determine the optimum conditions for germination, seeds of the 1979 harvest were imbibed for periods up to 14 days at temperatures in the range 0 to 20 ^{o}C. Imbibition for longer than 14 days led to fungal infection and drying out of the filter paper, even at the lower temperatures. Figure 1 shows the relationship between percentage germination and length of imbibition at various temperatures. In all cases the seeds received an inductive irradiation of 870 μmol m^{-2} R. No significant dark germination was observed. After one day of imbibition, high germination percentages are observed only when the seeds are imbibed at relatively high temperatures (15 to 20 ^{o}C). Imbibition for 1 or 2 days at 15 to 20 ^{o}C results in germination in excess of 50%.
It is therefore clear that these seeds, originally believed to be heavily dormant (Spruit et al. 1980), do not have an absolute cold requirement for loss of innate dormancy. On the other hand, seeds imbibed at lower temperatures fail to germinate after 1 or 2 days of imbibition and also after 3 days of imbibition at 20 ^{o}C. With an imbibition period of 3 days, the highest germination percentage is obtained when the seeds are imbibed at about 7 ^{o}C. Figure 1 shows that the longer the imbibition period, the lower the optimum temperature of imbibition

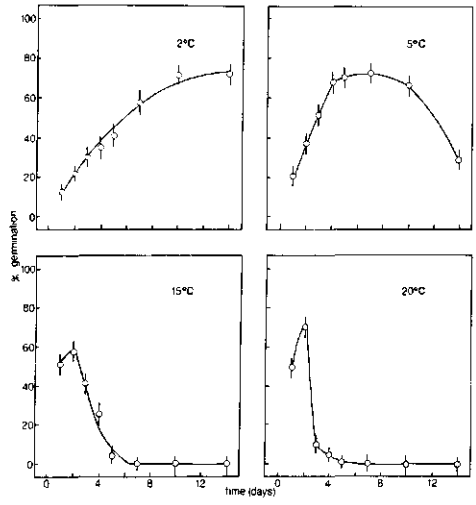


Fig. 1. Relationship between the time of imbibition and the percentage of germination at different imbibition temperatures for seeds of the 1979 batch. The seeds were imbibed in the dark and after irradiation with red light (870 μmol m^{-2}) incubated for 4 days in darkness at 20 °C. Dark germination is 0%.

needed for germination. At all imbibition temperatures above 0 °C, high germination percentages can be obtained, with each imbibition temperature having an optimum duration of imbibition. The higher the imbibition temperature, the shorter the period of imbibition needed. However, the optimum imbibition period is more critical at the higher temperatures.

In these experiments only one inductive fluence, found to be at or near saturation was used. More detailed fluence-response curves for seeds (summer 1979) imbibed at different periods at 2 °C are shown in Fig. 2, where the percentage germination is plotted on a probit scale. As found in other species, fluence-response curves plotted in this way are linear, indicating a normal distribution of ln Pfr requirement for induction of germination within the population (Frankland 1976, chapter 3). There is a great variation in the maximum inducible germination and the curves saturate between 150 and 1800 μmol m^{-2}. The longer the preceding imbibition at 2 °C, the higher the germination at higher fluences. In other words, upon prolonging the duration of imbibition, germination of an increasing fraction of the seeds can be induced by light.

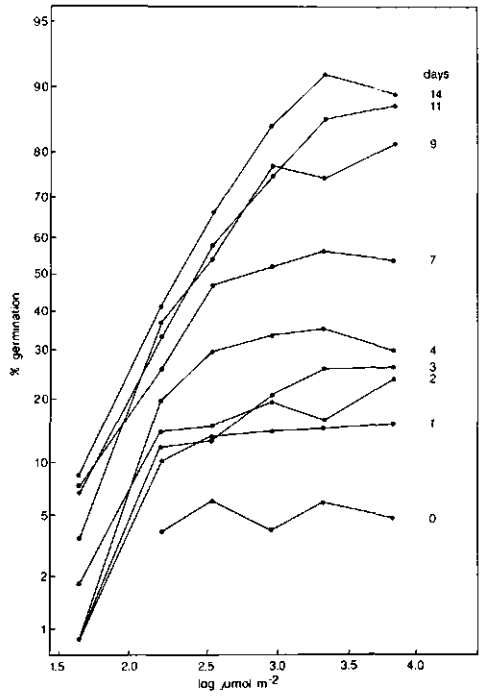


Fig. 2. Fluence-response curves for promotion of germination with red light. The seeds of the 1979 batch were imbibed at 2 °C for different numbers of days (0-14), as indicated on the right of each curve, irradiated and incubated for 4 days in darkness at 20 °C. The results are plotted as % of germination on a probit scale against the logarithm of the fluence. SE was less than ± 5%. Dark germination is 0%.

Whereas Fig. 2 shows fluence-response curves during the loss of primary dormancy upon prolonging imbibition at 2 °C, Fig. 3 shows fluence-response curves during the induction of secondary dormancy in the 1979 batch. The seeds were imbibed at 7 °C instead of 2 °C to induce the more rapid onset of secondary dormancy. In Fig. 3 the seeds all respond over the same fluence range, up to that establishing maximum Pfr (see chapter 3 and Fig. 2).

The response of seeds towards treatments intended to break dormancy also depends strongly upon the conditions during growth of the plants and ripening of the seeds. A batch of *A. thaliana* seeds harvested in the winter of 1981, proved very much more sensitive to light than those of plants grown during the summer of 1979. Figure 4 shows the response of 1981 seeds towards the duration of imbibition at two temperatures both for dark germination and for R-induced germination (standard fluence of 870 µmol m^{-2}). In contrast to the 1979 batch, not only is the germination response to light already close to 100% even after one day of imbibition, there is also considerable dark germination, especially at 2 °C.

The effect of alternating temperature cycles during imbibition was examined for the 1979 seed batch. A cycle, consisting of 8 h at 2 °C and 16 h at 20°C

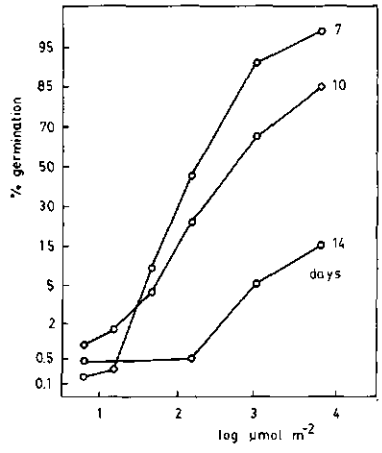


Fig. 3. Fluence-response curves for promotion of germination with red light. The seeds of the 1979 batch were imbibed at 7 °C for different numbers of days, as indicated on the right of each curve, irradiated and incubated for 4 days in darkness at 20 °C. The results are plotted as % of germination on a probit scale against the logarithm of the fluence. SE was less than ± 5%. Dark germination is 0%.

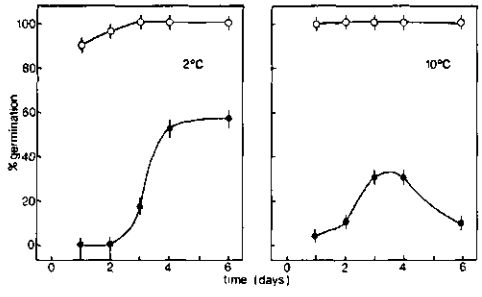


Fig. 4. Relationship between the duration of imbibition and the percentage of germination at imbibition temperatures of 2 and 10 °C of seeds of the 1981 batch. The seeds were imbibed in the dark and irradiated with 870 µmol m^{-2} red light (o) or kept in the dark (•). They were then incubated for 4 days in darkness at 20 °C.

proved more effective than one of 16 h at 2 °C and 8 h at 20 °C. However, neither of the two cycles gave rise to germination percentages as high as those obtained with seeds imbibed under constant temperatures (data not presented).

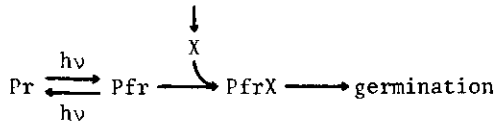
Discussion

The results in Fig. 1 can be understood by postulating two processes during the imbibition period. The first is a breaking of primary dormancy, which makes it possible for the seeds to germinate subsequently under favourable conditions. The second process, taking place simultaneously, is the induction of a secondary dormancy, which appears when germination conditions are not favourable, in this case the absence of light. Clearly both processes are temperature dependent. Other workers have shown a positive correlation between temperature and the induction of secondary dormancy in *Rumex sp.* (Le Deunff 1971, Totterdell and Roberts 1979), but failed to show temperature-dependent loss of primary dormancy. This may indicate different types of innate dormancy in seeds and the temperature-independent situation may be associated with seeds that are more heavily dormant and that require a period of stratification before germination can be induced under favourable conditions.

VanDerWoude and Toole (1980) studied the influence of pre-chilling on phytochrome-dependent seed germination in lettuce. They concluded that this treatment increases the sensitivity of the germination process to the pre-existing Pfr level. Our 1979 seed batch of *Arabidopsis* has an absolute requirement for light as shown by the absence of dark germination in all treatments. We may therefore conclude that these seeds do not contain sufficient Pfr to induce significant dark germination. Previous work has shown de novo phytochrome synthesis in seeds of *Amaranthus* to be absent at 0 ^{o}C (Kendrick et al. 1969) and we will assume that in all our seeds, the Ptot remains constant throughout imbibition at 2 ^{o}C. The response of seed batch 1981 (Fig. 4) differs from that of 1979 by a considerably lower Pfr requirement for germination, even at a very early stage of imbibition. We have not observed significant differences in the spectrophotometrically detectable phytochrome contents of seeds from different harvests of *Arabidopsis* when they are fully imbibed at 20 ^{o}C. Apparently, there is a certain level of pre-existing Pfr in the 1981 seeds, sufficient to induce considerable dark germination even after only 3 to 4 days imbibition at 2 ^{o}C. Also, the sensitivity to light is higher indicating that at a total phytochrome content comparable to that of the 1979 seeds, a much lower Pfr level suffices to saturate the germination process. Alternatively, the difference between the seed batches could be explained by the 1981 seeds having a higher pre-existing Pfr level than those of 1979. In addition, it is possible that the pre-treatment

of the 1981 seeds, makes a proportion of the seeds very sensitive to Pfr. This is further discussed in chapter 8.

The phytochrome control of germination has been considered by many workers in terms of Pfr interacting with a reaction partner X (Koller et al. 1964, Hartmann 1966, Karssen 1967, Frankland 1976, Duke 1978):



Clearly germination could then be influenced by the availability of (I) Pfr, (II) X and (III) the effectiveness of PfrX (e.g. the threshold of PfrX needed to promote germination of the average seed of the population). Theoretical analysis using this model (Frankland 1976, Duke 1978) enables certain predictions to be made about the relative importance of these parameters in the loss of primary dormancy and induction of secondary dormancy. Conditions affecting (I) and (II) will result in increased germination as the limiting factor becomes available during imbibition. Fluence-response curves are then predicted to coincide, attaining higher germination as an increased proportion of the population responds to the limiting factor. For all seeds in the population to germinate, the photostationary Pfr level established by R must be enough to satisfy germination. Although this holds for the seeds used here, it may not be so in heavily dormant populations. A parallel shift in fluence-response curves would support model (III), in that the threshold value of PfrX needed for promotion of the average seed in the population is shifted to a lower fluence as a result of prolonged dark imbibition. The data in Fig. 2 appear to show features of both situation (II) and (III). Unfortunately, the quantity of phytochrome detectable by spectrophotometry in fully hydrated seeds (Koornneef et al. 1980), proved too low to study in detail the development of photoreversibility during imbibition. However, even at 0 ^{o}C phytochrome apparently became fully photoreversible in a few hours. It would therefore appear that the kinetics of loss of primary dormancy, as derived from Fig. 2, are rather too slow to be explained by simple hydration of seed phytochrome. The observation that with increased imbibition, the fluence-response curves saturate at higher fluences suggest that germination is being limited by the availability of X, not by the Pr → Pfr phototransformations. The onset of secondary dormancy could result from the temperature-dependent loss of X or a decrease in effectiveness of PfrX (the requirement of a higher

PfrX threshold) which would both result in a progressive decline in final germination percentage, since saturation of the Pr → Pfr phototransformation would no longer satisfy the germination requirement of the whole population.

The characteristics of the fluence-response curves during the onset of secondary dormancy (Fig. 3) support the contention that it is the effectiveness of PfrX which becomes limiting, since the seeds all respond over the same fluence range, up to that establishing maximum Pfr, indicating that X is not the limiting factor. It is therefore possible on the basis of the fluence-response curves to conclude that the onset of secondary dormancy is not the opposite process to loss of primary dormancy.

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CHAPTER 6, THE TIME COURSE OF PHYTOCHROME ACTION

The promotive effect of red light (R) on seed germination can be nullified by subsequent far-red light (FR). However a certain time after R subsequent FR is no longer able to reverse its effect. Seeds have then escaped from FR photocontrol. The time needed for the seeds to escape from photocontrol is a measure of the time needed for action of the FR absorbing form of phytochrome (Pfr) to stimulate germination. This time course of Pfr action needed for germination is not constant. It is determined by the sensitivity of the seeds to light (Pfr) and by the initial amount of Pfr present. In this chapter it is shown that germination is not only a consequence of the initial amount of Pfr but also of the duration of its action. The average seed of a population with a short escape time germinates faster than the average seed of a population with a longer escape time.

Introduction

The far-red absorbing form of phytochrome (Pfr) stimulates germination. However, not only the initial amount of Pfr, but also the duration of its action determines the final germination percentage attained. The time needed to escape from photocontrol represents the time course of Pfr action. The escape time is the time after which the effect of R can no longer be reversed by FR. Experiments show that the escape time for 50% of the population for lettuce varies from 4 h (Gwynn and Scheibe 1972) to 9 h (Borthwick et al. 1954), for *Portulaca oleracea* L. from 1 to 3 h (Duke et al. 1977), for *Rumex crispus L.* from 8 to 35 h (Duke et al. 1977) and for *Chenopodium album* from 10 to 35 h (Karssen 1970). The escape from FR reversibility is defined by the pre-irradiation conditions that influence the level of dormancy. Duke et al. (1977) have taken the slope of the escape curves as a measure of the rate of Pfr activity. Bewley et al. (1968) showed that the duration of Pfr action, necessary for germination of lettuce seed, can be reduced to a few minutes by adding gibberellic acid at a concentration which itself had no influence on germination. After seeds have escaped from reversibility by short FR, germination can be inhibited by prolonged irradiation with light of a wide variety of spectral qualities, inclucing those, such as daylight, that promote germination when given as a short exposure (Bartley and Frankland 1982, Górsky and Górska 1979). While the promoting effect of light is mediated by Pfr, the mechanism of the inhibiting effect of prolonged

irradiation is less understood. Bartley and Frankland (1982) showed that the inhibiting reaction depends on the rate of phytochrome interconversion or 'cycling'. The inhibition of lettuce seed germination by prolonged irradiation after the seeds have escaped from short FR photocontrol show action maxima near 470 and 720 nm (Gwynn and Scheibe 1972).

In this chapter the escape time and the time course of germination was determined for different seed batches of *Arabidopsis thaliana*.

Materials and methods

General procedures are given in chapter 2. *Arabidopsis thaliana* seeds used in the present experiments were harvested from plants grown in late summer 1979 (WT79), winter 1981 (WT81), late summer 1981 (ttg) and spring 1983 (WT83). Saturating R (660 nm) was 8.4 mmol m^{-2}, given in 1 min. Saturating FR (730 nm) was 30 mmol m^{-2}, given in 2 min. Three dishes were used for each treatment and the results are expressed as % germination $\pm$ SE of the mean.

Results and discussion

The escape from FR reversibility after saturating R (escape time) of three different batches of *A. thaliana* was determined (Fig. 1). The seeds of batch WT81 escape rather rapidly from FR photocontrol. Fifty percent of the R responding seeds escaped within 1.5 h after R. The ttg seeds escaped in 2.5 h. Although the WT79 seeds can be induced to nearly 100% germination by a R pulse, their escape time is about 9 h.

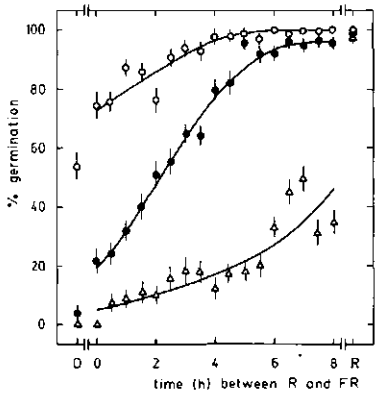

Fig. 1. The escape from far-red (FR) reversibility after saturating red light (R) of seed batches WT81 (o), ttg (●) and WT79 (△). D = dark germination. The seeds were imbibed for 7 days at 7 °C.

The dark germination in the batches WT79 and ttg is nearly zero and in WT81 50%. A saturating fluence of FR immediately after R stimulates the germination of WT81 to 70% and of ttg to 20%. This means that the batches WT81 and ttg are very sensitive to Pfr. The very low level of Pfr which is established by FR of 730 nm is apparently enough to stimulate germination of some of the seeds in the population (chapter 3). The batch WT79 is considerably less sensitive to Pfr.

The fast escape from reversibility by FR of WT81 seeds suggests that before irradiation with R, the low level of Pfr, already present in the seeds, has initiated significant phytochrome action. Consequently a very small fluence of R is sufficient to reach 100% germination. The slow escape of WT79 suggests that before irradiation little or no Pfr was present in the seeds and a greater fluence of R is required to reach 100% germination. Alternatively both could have the same Pfr level but one batch be more sensitive than the other to Pfr.

The escape time correlates well with the sensitivity of the seed batches to light, as shown in table 1. The less sensitive a seed batch is, which is indicated by the light fluence needed for 50% germination, the longer the escape time. Waddoups (1976) also reported that increased sensitivity to light correlated with increased escape from FR reversibility in *Synapsis arvensis* seeds.

seed batch	WT81	ttg	WT79
escape time (h)	1.5	2.5	9.0
fluence for 50% germination (μmol m^{-2})	4.4	18.7	193.0

Table 1. The time by which 50% of the seeds escaped from far-red reversibility and the fluence (660 nm) necessary for 50% germination.

The depth of dormancy (light sensitivity) is not only correlated with the escape time but also with the rate of germination (Fig. 2). The dormant batch WT79 needs 45 h for 50% of the population to germinate, while ttg seeds need 35 h and WT81 20 to 30 h. The biphasic character of the curve for WT81 can be explained by the fact that there is significant endogenous Pfr available to

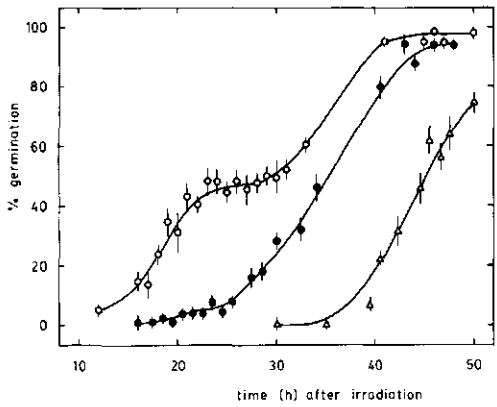

Fig. 2. The time course of germination after irradiation with saturating red light for seed batches WT81 (o), ttg (●) and WT79 (Δ). Dark germination is the same as shown in Fig. 1. The seeds were imbibed for 7 days at 7 °C.

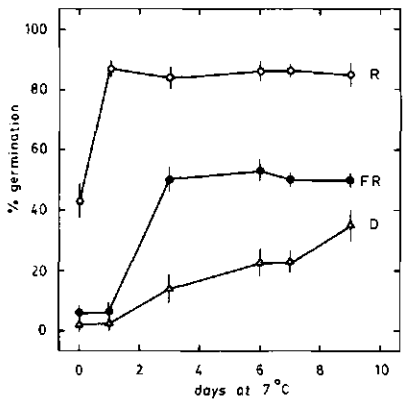

Fig. 3. Germination of seed batch WT83 in the dark (D), after saturating red (R) and far-red (FR) following different periods of imbibition at 7 °C.

promote germination of 40 to 50% of the population. Probably, during the imbibition period endogenous Pfr is active, satisfying germination of some seeds in the population. The upper part of the curve represents those seeds that germinate as a result of the R irradiation at time zero.

The dormancy of seed batch WT83 can be modified by changing the imbibition period (chapter 5). One day of imbibition at 7 ^{o}C results in 90% germination after saturating R (Fig. 3). Germination in the dark and after saturating FR is nearly zero. However, after 7 days, dark germination increases to 30% and germination after saturating FR to nearly 50%. The germination after R is not changed. The fact that the dark germination and FR germination increases after an increasing period at 7 ^{o}C shows that the sensitivity of the seeds to Pfr increases (the dormancy of the seeds decreases). Probably not only the sensitivity to Pfr increases but also the availability of the reaction partner of Pfr, X (Duke 1978, chapter 5).

In Fig. 4 the escape times of seeds of WT83 with different depths of dormancy are compared. Seeds pretreated with one day of imbibition at 7 ^{o}C are rather insensitive to Pfr, while seeds imbibed for 7 days at 7 ^{o}C are more sensitive. It is shown that the sensitive seeds escape very rapidly from FR reversibility, while the dormant seeds escape very slowly.

The rate of germination of WT83 seeds also depends on the depth of dormancy (Fig. 5), analogous to Fig. 2. The germination curve for the sensitive WT83 seeds is also biphasic. The initial part of the curve corresponds with that part of the population germinating in darkness (Fig. 4), endogenous Pfr being responsible for this germination. The dormant seeds need about 50 h, while the sensitive seeds need only 27 h for 50% germination. Probably the sensitivity of the seeds to Pfr depends on the availability of the reaction partner of Pfr, X, enabling Pfr to promote germination (chapter 5). The sensitive WT83 seeds, imbibed for 7 days at 7 ^{o}C might have more X available than the insensitive seeds imbibed for 1 day at 7 ^{o}C. The insensitive seeds therefore need a longer period of Pfr action to attain the same response as the insensitive seeds.

To show clearly that the rate of escape and the rate of germination is determined by the amount of Pfr present in the seeds, experiments could be carried out to determine the rate of escape and germination in one seed batch with different levels of Pfr established by subsaturating fluences of R. Unfortunately it proved impossible to find a batch that would germinate to 100% upon subsaturating levels of R and show no germination after FR. Although this experiment

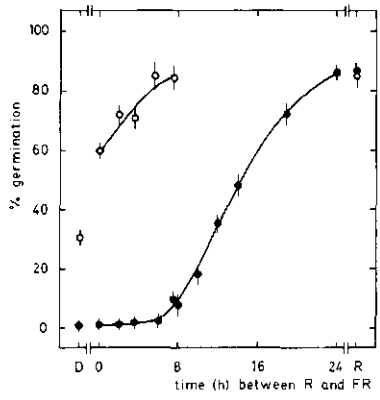

Fig. 4. The escape from far-red reversibility after saturating red light of seeds of batch WT83, pretreated with 1 (●) or 7 (o) days of imbibition at 7 °C. D = dark germination.

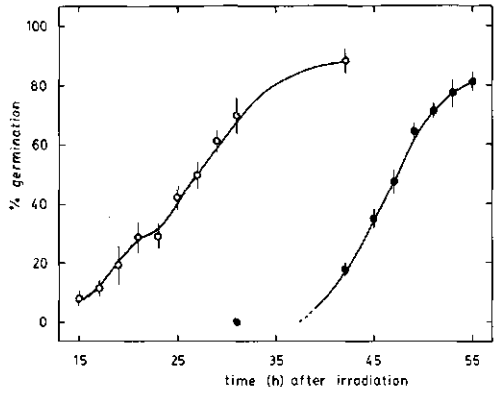

Fig. 5. The time course of germination after irradiation with saturating red light of seed batch WT83, pretreated with 1 (●) or 7 (o) days of imbibition at 7 °C. Dark germination is the same as shown in Fig. 4.

could not be done, the experiments described here show that not only the amount of Pfr determines the final germination level, but that also the duration of Pfr action is important. Germination is thus the result of the integral of Pfr action over time.

After seeds have escaped from FR reversibility, germination can still be inhibited by prolonged irradiation, by a cycling dependent phytochrome process (Bartley and Frankland 1982). Whereas short irradiation is not able to reverse germination initiation, prolonged irradiation is. Gwynn and Scheibe (1972) made an action spectrum for lettuce of the inhibition by prolonged irradiation after seeds escaped from short FR reversibility with maxima at 470 and 720 nm. To study the influence of prolonged irradiation on seed germination, the use of long-hypocotyl mutants of *Arabidopsis thaliana* (Koornneef et al. 1980) would at first sight appear to be useful to investigate the different pigments and processes responsible for the inhibition by prolonged irradiation. Unfortunately we were unable to get germination inhibition with prolonged irradiation after escape from short FR reversibility in *Arabidopsis thaliana* seeds.

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CHAPTER 7, ACTION SPECTRA FOR PROMOTION AND INHIBITION OF SEED GERMINATION IN WILDTYPE AND LONG-HYPOCOTYL MUTANTS OF *Arabidopsis thaliana*

Fluence-response curves were determined for the induction and inhibition of induction of germination by short exposure to light for wildtype and 4 mutants of *Arabidopsis thaliana*. The mutants show reduced photoinhibition of hypocotyl growth in white light compared to wildtype, suggesting they are either mutated with respect to phytochrome, the blue/UV absorbing photosystem or some other red absorbing photosystem. Action spectra constructed for germination induction and for the inhibition of induction for the different genotypes are qualitatively the same, having peaks of effectiveness at c. 660 nm and c. 730 nm respectively. In the blue region of the spectrum very little activity is seen in comparison with that of red light. Differences in bandwidth of effectiveness for induction of germination are attributed to different amounts and characteristics of screening pigments in the seed batches. The long-hypocotyl mutants therefore have a normal phytochrome system operative in the control of seed germination by short term irradiation and no other photosystem appears to be involved.

Introduction

Light can promote and/or inhibit seed germination of many plant species (Toole 1973, Frankland and Taylorson 1983). In most cases phytochrome has been shown to be the pigment involved in this response. A brief exposure to red light (R) transforms phytochrome to its far-red absorbing form (Pfr), promoting germination. Subsequent far-red light (FR) transforms Pfr back to the R absorbing form (Pr) and nullifies the effect of the R irradiation. The light responsivity of seeds varies greatly, depending on the conditions during seed ripening, seed storage and imbibition (Blaauw-Jansen and Blaauw 1975, Hand et al. 1982, Cone and Spruit 1983). Blue light (B) also has an influence upon seed germination, but this has not been extensively studied. In one report action spectra for the promotion of lettuce seed germination by short-term irradiation show interesting structure in the B spectral region (Small et al. 1979b). However these workers were not able to discriminate between phytochrome and a separate B light absorbing receptor pigment since phytochrome possesses a minor absorption

band in this spectral region. Prolonged B often inhibits germination (Kendrick et al. 1969, Gwynn and Scheibe 1972). It is thought that prolonged B is effective through the so called high irradiance reaction (HIR) of photomorphogenesis (Borthwick et al. 1969, Hartmann 1966, Mancinelli and Rabino 1978). None of the models explaining the HIR on the basis of phytochrome fully account for the high activity in the B region of the spectrum where phytochrome absorbs inefficiently. There are also some reports suggesting the existence of another R absorbing pigment, apart from phytochrome controlling photomorphogenesis (Jose and Vince-Prue 1977, Vanderhoef et al. 1979). Koornneef et al. (1980) implicated a number of different pigment systems in the prolonged irradiance inhibition of hypocotyl growth of *Arabidopsis thaliana* mutants. These mutants have long hypocotyls compared to wildtype when grown under white light. Mutants Hy-1 and Hy-2 have no spectrophotometrically detectable phytochrome in the seeds and the hypocotyls. Compared to wildtype these mutants have reduced sensitivity to continuous R and FR. Koornneef et al. (1980) conclude that the Hy-1 and Hy-2 genes regulate phytochrome synthesis. Mutant Hy-3 has a normal phytochrome content in the seedlings, but a reduced phytochrome content in the seeds. In hypocotyl growth, Hy-3 is sensitive to continuous FR, but compared to wildtype is insensitive to continuous R. Mutant Hy-4 has a normal phytochrome content, but reduced sensitivity in hypocotyl growth to continuous UV compared to wildtype. However it has a normal sensitivity to continuous R and FR, supporting the idea of a separate UV-B absorbing pigment involved in the photocontrol of hypocotyl elongation.

This chapter reports on the action spectra for the promotion and subsequent inhibition of seed germination by light of wildtype and these hypocotyl mutants of A. *thaliana*.

Materials and methods

Two batches of wildtype were used, one harvested in the summer of 1979 (WT79) and the other in the winter of 1981 (WT81). Dormancy is greatly influenced by the light conditions during seed maturation and WT79 therefore is more heavily dormant than WT81 (Baskin and Baskin 1972). The following long-hypocotyl mutants, harvested in the winter of 1981 were used: 21.84 (Hy-1), TO76 (Hy-2), Bo64 (Hy-3) and 2.23N (Hy-4). The wildtype batches were imbibed for 7 days at 7 ^{o}C (Cone and Spruit 1983, chapter 5), whereas the other batches were

imbibed for 2 days at 7 °C. As a precaution against any possible traces of long wavelength irradiation in the optical system, a 3 cm saturated $CuSO_4$-filter was placed between the light source and the seeds, when irradiated with wavelengths shorter than 550 nm. During the irradiation of the seeds, lids were removed from the petri-dishes. Irradiations were done in 1 min, except in the B part of the spectrum where the fluence rate proved to be too low, so irradiation times of up to 4 min were necessary. Reciprocity was shown to hold at all wavelengths for the longest irradiation time used. At least 3 dishes were used for each treatment and the response to a control fluence of 660 nm was determined with each experiment to test for any changes in sensitivity of the system. After irradiation the seeds were incubated in the dark for 4 days at 20 °C at the end of which the germination was monitored. For investigation of the photoinhibition, seeds were first pre-irradiated with saturing R (660 nm) followed by a 10 s dark interval before the inhibitory irradiation. Results are expressed as percentage germination on a probit scale against logarithm of fluence (see appendix II). SE's, always less than 5% of the mean, were omitted from the figures for clarity. The action spectra were constructed by plotting the reciprocal of the quantum requirement necessary for 50% germination for each wavelength. Quantum effectiveness is normalized to 100 for the maximum value (Shropshire 1972, Schäfer et al. 1983). Pfr levels, established by subsaturating fluences were calculated according to the modified version (Hartmann and Cohnen-Unser 1972) of Butler's (Butler et al. 1964) formula, using values of absorption cross sections of Pr and Pfr for different wavelengths derived from Butler's data by Bartley (1982) on the assumption that the maximum Pfr/Ptot is 0.75 (see chapter 3 and appendix I).

Results

Light stimulation of germination by light is maximal when 7 days of dark imbibition at 7 °C is given. The batches WT79 and WT81 have a high germination in the light and a low germination in the dark with this pre-treatment. The same imbibition period gave very high germination in the light for the long-hypocotyl mutants, but unfortunately this was accompanied by up to 80% dark germination in Hy-1. It was found that imbibing the seed batches of the long-hypocotyl mutants for 2 days at 7 °C reduced dark germination to an acceptable level while light still promoted the major part of the population. However,

imbibing seeds of WT79 for 2 days at 7 ^{o}C reduced the sensitivity to light resulting in only 30% germination for WT79 (chapter 5). In determining fluence-response curves for these seed batches it was impossible to use a standard imbibition period.

Figure 1 shows fluence-response curves for the induction of germination with R of 660 nm. The slopes of the fluence-response curves differ between the genotypes and fall into two groups. Hy-1. Hy-2 and Hy-3 have shallow slopes, while WT79, WT81 and Hy-4 have steep slopes. The fluence-response curves for the different wavelengths for a genotype are all parallel between 550 and 670 nm, as can be seen in Fig. 2 where fluence-response curves for the induction of germination of Hy-4 are shown. At shorter wavelengths than 550 and longer than 670 nm fluence-response curves are more shallow (see chapter 3). The different seed batches do not all have the same sensitivity towards light, as can be seen in Fig. 1. The sensitivity towards light depends greatly on the degree of dormancy and not on the kind of mutation, as is shown by the two wildtype batches. WT79 seeds need a much higher fluence than WT81 to get the same response.

The action spectra, calculated from the fluence-response curves are shown in Fig. 3 and 4. Figure 3 shows the spectra for the induction of germination for the spectral region 550 to 730 nm and Fig. 4 for the spectral region 390 to 550 nm. The spectra for WT79 and Hy-2 are not shown in Fig. 4 because these batches are so insensitive to B that 50% germination is not reached. The spectra calculated with and without correction of the fluence-response curves for the dark germination were found to be essentially identical. In Fig. 3 and 4 the reciprocal of the fluences which give 50% germination is taken for the standard response. The action spectra, both in the B and the R spectral regions are normalized to 100 for peak effectiveness at c. 660 nm. The spectra in the R spectral region show differences which result in differences in their half band width. These differences do not appear to be characteristic of the genotype since differences are also found between the two wildtype batches. Action spectra in the B spectral region show that there are no appreciable differences between the different genotypes. The activity of wavelengths shorter than 550 nm is in all the batches less than 2% of the activity of R.

That only the low energy reaction (LER) of phytochrome is involved is supported by the fact that the reciprocity law holds at all wavelengths throughout the spectrum and that induction of a large proportion of the population is inhibited by FR in all seed batches (data not shown).

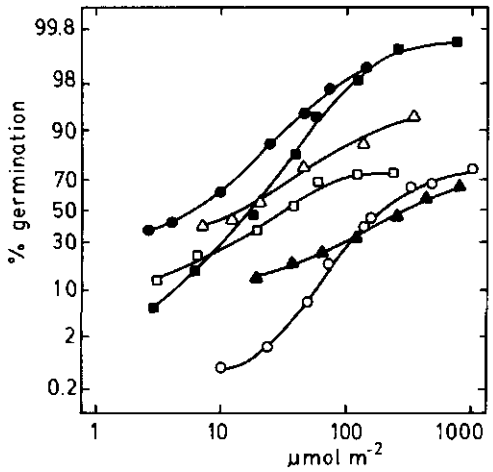

Fig. 1. Fluence-response curves for the induction of germination with red light (660 nm). The batches WT79 (o) and WT81 (●) were imbibed for 7 days at 7 °C and Hy-1 (Δ), Hy-2 (▲), Hy-3 (□) and Hy-4 (■) 2 days at 7 °C, irradiated and incubated for 4 days at 20 °C in darkness.

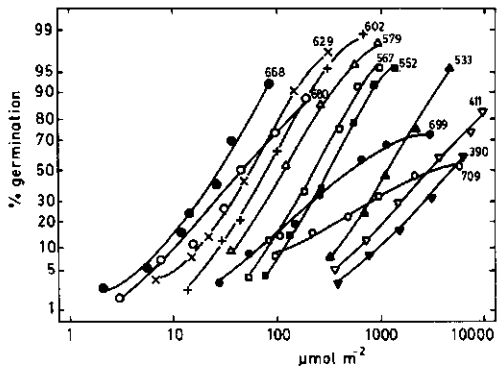

Fig. 2. Fluence-response curves for the induction of germination with different wavelengths for genotype Hy-4. The seeds were imbibed for 2 days at 7 °C, irradiated and incubated for 4 days at 20 °C in darkness.

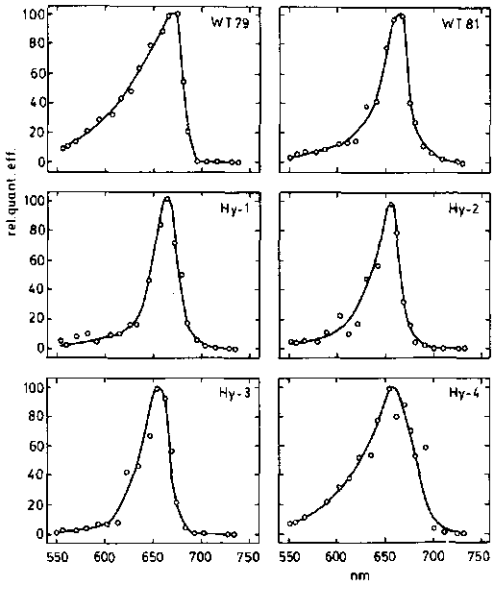

Fig. 3. Action spectra in the region 550 - 730 nm for the induction of germination for different genotypes of *Arabidopsis thaliana*, as indicated. Relative quantum effectiveness (rel. quant. eff.) for induction of 50% germination were determined. Peak effectiveness is normalized to 100%.

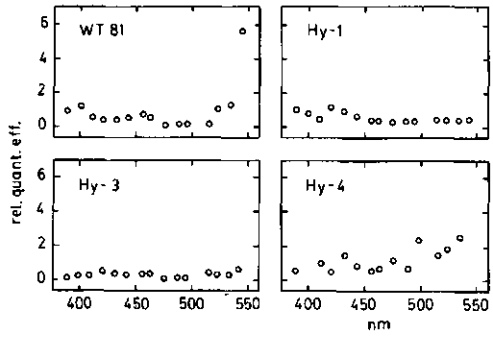

Fig. 4. Action spectra in the region 390 - 550 nm for the induction of germination for different genotypes of *Arabidopsis thaliana*, as indicated. Relative quantum effectiveness (rel. quant. eff.) for induction of 50% germination were determined. Peak effectiveness (c. 660 nm) is normalized to 100%.

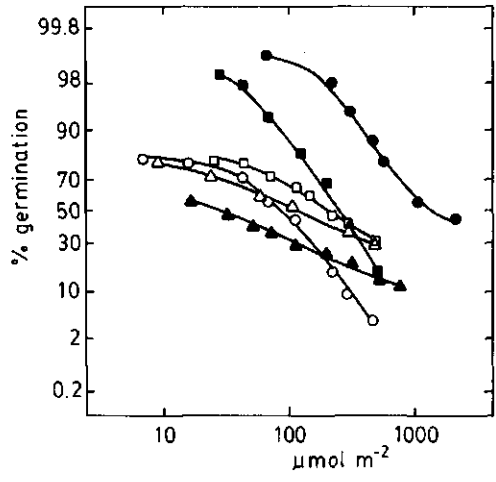

Fig. 5. Fluence-response curves for the inhibition of germination by far-red light (730 nm), pre-irradiated with a saturating fluence of red (660 nm). The batches WT79 (o) and WT81 (●) were imbibed for 7 days at 7 °C and Hy-1 (△), Hy-2 (▲), Hy-3 (□) and Hy-4 (■) 2 days at 7 °C, irradiated and incubated for 4 days at 20 °C in darkness.

Figure 5 shows fluence-response curves for the FR inhibition of germination after a saturating fluence with R (660 nm), for all the seed batches. Here again the fluence-response curves fall into two groups. Hy-1, Hy-2 and Hy-3 have shallow slopes and WT79, WT81 and Hy-4 have steep slopes. Two action spectra of the FR reversion, normalized to 100 for peak effectiveness, are shown in Fig. 6. These action spectra were constructed from fluence-response curves at different wavelengths using the fluence which inhibit germination to 50%. Although only the spectra for WT79 and Hy-2 are shown, all the other spectra have qualitatively the same shape with peak effectiveness at c. 730 nm.

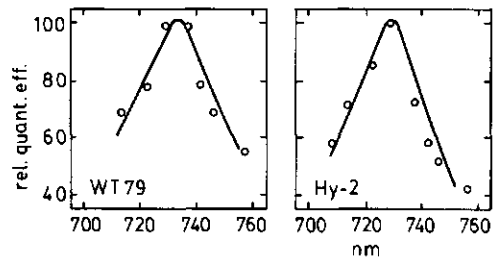

Fig. 6. Action spectra for the inhibition of the germination induced by red light for the genotypes WT79 and Hy-2 of *Arabidopsis thaliana*. Relative quantum effectiveness (rel. quant. eff.) for inhibition of 50% germination were determined. Peak effectiveness is normalized to 100%.

Discussion

The fluence-response curves both for the induction and inhibition of induction of Hy-1, Hy-2 and Hy-3 germination have shallower slopes than those of the wildtypes. In chapter 3 it was shown that the interaction of several factors influence the slope of a fluence-response curve, such as a pre-existing level of Pfr, an overriding factor, a change in the range of Pfr requirement of the seed population and differences in the total amount of phytochrome (Ptot). All the seed batches used had a low level of pre-existing Pfr, as indicated by the fact that FR (730 nm) was found to promote germination to some extent above the dark level. This also supports the view that they are relatively sensitive to Pfr. It is unlikely that the level of pre-existing Pfr alone can account for the very shallow slopes of the fluence-response curves for the induction of germination of Hy-1, Hy-2 and Hy-3. A small amount of pre-existing Pfr only influences the slope of the fluence-response curve when the seeds are very sensitive to light and consequently to small amounts of Pfr (see figs. 4 and 7, chapter 3). In batch WT81, the pre-existing Pfr may have had an effect, because this batch indeed showed a high sensitivity to light and a high level of dark germination. Ptot in the batches Hy-1. Hy-2 and Hy-3 is low and at or near the detection limit of the spectrophotometer (Spruit 1970). The amount of Ptot in Hy-1 and Hy-2 appears to be less that 20% compared to wildtype (Spruit et al. 1980). Hy-3 seems to have a little more phytochrome but always less than 40% of that of wildtype. A lower amount of Ptot alone does not result in a change of the slope of a fluence-response curve (see chapter 3, Fig. 9). This can only be expected when another factor, such as pre-existing Pfr or an overriding factor is involved. Since the level of pre-existing Pfr in our seeds is low, the influence of an overriding factor must be considered. The overriding factor is not expected to influence the slope of the fluence-response curves in WT79, WT81 and Hy-4 appreciably since these batches have a high Ptot level (see Fig. 9, chapter 3). The combination of an overriding factor and a low Ptot level could explain both the shallow curves for the induction and inhibition for Hy-1, Hy-2 and Hy-3. Another explanation could be a change in the range of Pfr requirement of the seed population (Duke 1978, chapter 3). A third possible explanation is that we are not dealing with normal phytochrome in the batches Hy-1, Hy-2 and Hy-3. If the mutation decreases the efficiency of phototransformations of Pr → Pfr and Pfr → Pr, the result will

be shallower fluence-response curves. However in this case the fluence-response curves will not level off at that fluence, which normally establishes maximum Pfr. With our seed batches this does not occur. We therefore do not think that the mutation in Hy-1, Hy-2 and Hy-3 has resulted in a modification of the phytochrome molecule itself.

Other investigators have shown a linear correlation between probit of germination and calculated logarithm of the Pfr produced by given fluences (Finney 1952, Duke 1978, Frankland 1976, chapter 3). We have calculated the % Pfr established by the different fluences of different wavelengths, using the modified formula of Butler et al. (1964) (see also chapter 3). Fig. 7 shows this relationship between probit of germination and calculated Pfr level for all the fluences used in the wavelength range 550 to 700 nm. The slopes of the curves of Hy-1, Hy-2 and Hy-3 appear shallower than of the other genotypes. In the case of Hy-3, the fact that the curve appears to levels off before the maximal possible Pfr is established suggests that a factor other than phytochrome is limiting, e.g. the reaction partner of Pfr, the factor X (Duke 1978). In batch

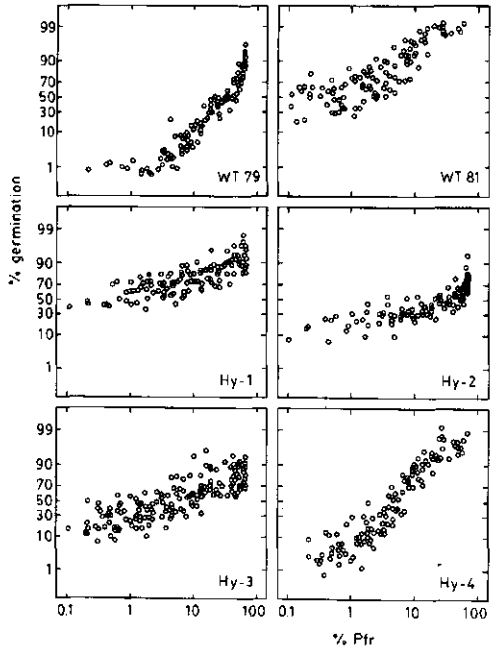

Fig. 7. Relationship between the logarithm of the Pfr as a percentage of total phytochrome and the probit of germination percentage of the different genotypes of *Arabidopsis thaliana*, as indicated. The data are derived from the individual fluence-response curves, made for determining the action spectra in the region 550 to 700 nm.

Hy-4 the seeds are sensitive to Pfr, resulting in nearly 100% germination at a Pfr level below the maximum possible. The few seeds which do not germinate may not be viable or the germination is blocked by an overriding factor (Duke 1978). The curve of Hy-2 suggests that there is a very high percentage Pfr needed for germination. This might be a consequence of the low level of Ptot or the insensitivity of the batch to Pfr. The fact that a relatively high percentage of the seeds germinate in some batches without light might be a consequence of the involvement of an overriding factor promoting germination (chapter 3).

Shropshire et al. (1961) determined the first action spectra for light induction and inhibition of germination of *A. thaliana* seeds. Although seed germination is a typical LER, they irradiated the seeds for 2.75 h for germination induction, while only a few seconds of high fluence rate light gave maximal germination with our seeds. Figures 3 and 4 show differences compared with the spectrum published by Shropshire et al. In particular we see less activity of wavelengths below 550 nm. In Fig. 4 the effectiveness of B is always less than 2% of the effectiveness of R. Fig. 4 provides no evidence for a B/UV absorbing pigment promoting germination, as well as no difference between the different genotypes. If a B/UV absorbing pigment additional to phytochrome was active in stimulating germination, the relative activity of B/UV light would be predicted to be higher in the phytochrome poor mutants Hy-1, Hy-2 and Hy-3 than in wildtype. Hy-4 also has a normal phytochrome action spectrum. If a separate B absorbing pigment is mutated in Hy-4, the relative activity of B would be reduced in this genotype with respect to wildtype. This does not appear to be the case. The fact that the seeds of the different batches germinate with short exposure to B is presumably due to direct absorption by phytochrome or indirect absorption by phytochrome through R fluorescence of the seeds and of the filter paper. Irradiations with wavelengths shorter than 550 nm showed fluorescence higher than 590 nm which was 0.3 to 0.45% of the total fluence. Small et al. (1979b) working with lettuce seeds were unable to discriminate between a separate B/UV absorbing pigment or energy transfer to phytochrome being involved.

The action spectra of Fig. 3 show differences in the region of 600 to 660 nm, which become apparent in bandwidth. These differences are not related to genotype since they are also seen in the two wildtype batches. Nevertheless the spectra are all qualitatively of the same shape and fit published spectra for *A. thaliana* (Shropshire et al. 1961) and lettuce (Blaauw-Jansen and Blaauw

1976, Small et al. 1979a). Using the formulae for calculation of Pfr levels established with subsaturating fluences (see chapter 3) and assuming a given normal distribution in ln Pfr requirement for germination throughout a seed population, fluences can be calculated required to give a certain germination response for each wavelength. This enables a theoretical action spectrum to be calculated, ignoring possible screening pigments. Between 550 and 660 nm the same theoretical action spectrum is always obtained (see chapter 4). The factors pre-existing Pfr, different levels of Ptot, sensitivity of the seeds to Pfr, the range of Pfr requirement and an overriding factor do not influence the shape of the action spectrum. Since ϕ (Pfr/Ptot) is essentially constant between 550 and 670 nm, ϕ will not influence the shape of the action spectra in this region. At wavelengths below 550 and above 670 nm ϕ does influence the shape of the spectra (Small et al. 1979a, chapter 4).

Taylorson and Hendricks (1971) showed that the light transmission of seed coats of *Amaranthus retroflexus* changes dramatically between 600 and 800 nm. This screening behaviour of the seed coat may be responsible for the differences in the action spectra of Fig. 3. Absorption by the seed coats and/or other screening pigments (e.g. chlorophyll) may vary from one seed batch to another. The fact that all the experimentally determined action spectra of Fig. 3 fall below the theoretical spectrum presented in Fig. 1 in chapter 5, suggests that screening pigments are present which absorb between 550 and 660 nm. This may also be the reason why in the literature most experimentally obtained action spectra are sharper than the absorption spectrum of phytochrome itself. This conclusion is supported by the data in Fig. 7 for calculated Pfr and germination response where a greater spread of the points is correlated with the sharp action peaks. Small et al (1979a) came to the conclusion that calculated action spectra do not differ between 550 and 660 nm. Beyond 660 nm the fall of the curve can be more or less sharp, depending on the sensitivity of the seeds to Pfr. An alternative possibility is that the extinction coefficients obtained for Pr and Pfr in vitro between 550 and 660 nm are in error. Only a minor adjustment suffices to yield calculated action spectra that conform more closely with those experimentally determined. However this does not explain the differences between the observed spectra of Fig. 3. Earlier Blaauw-Jansen and Blaauw (1976) attempted to explain the differences in the action spectra between 600 and 660 nm of FR-dormant and thermodormant lettuce seed on the basis that there were two different phytochrome species controlling germination.

The genotypes Hy-1 and Hy-2 have no spectrophotometrically detectable phytochrome (Koornneef et al. 1980), yet action spectra of Hy-1 and Hy-2 clearly indicate normal phytochrome action. The mutants are therefore not phytochrome-less but in addition to long hypocotyls in white light they have a reduced phytochrome content compared to wildtype in seeds and seedlings. Koornneef et al. (1980) concluded that the Hy-1 and Hy-2 genes regulate the synthesis of phytochrome, at least in the hypocotyl. In the case of Hy-2, our experiments suggest that it is possible that this gene could also regulate the synthesis of phytochrome that controls seed germination. The action spectra for the induction and inhibition of induction show that normal phytochrome is operative in all these mutants.

Koornneef et al. (1980) suggested that it might be possible that Hy-3 is mutated with respect to a R absorbing photosystem, other than phytochrome. Fig. 2 shows that the action spectrum of Hy-3 is the same as that of wildtype, and therefore it must be concluded that no pigment other than phytochrome is active in stimulating germination. Germination is a LER, while the inhibition of hypocotyl growth in white light also involves the HIR. It is possible that phytochrome action in the HIR alone is influenced in these mutants. This might also be true for a B/UV absorbing pigment, proposed on the basis of hypocotyl studies (Koornneef et al. 1980, Gaba and Black 1979, Meyer 1968). The genotype Hy-4 also shows a normal action spectrum compared to wildtype, supporting the conclusion that there is a single phytochrome reaction involved in the promotion of seed germination.

The action spectra of the inhibition by FR after an inductive R pulse for the different genotypes are qualitatively identical. All have a peak of effectiveness at c. 730 nm. We are therefore forced to conclude that the phytochrome system in these long-hypocotyl mutants is functionally normal in seed germination. While it is always possible that the mutants Hy-1, Hy-2 and Hy-3 have reduced synthesis of phytochrome that specifically controls hypocotyl elongation growth, it also remains possible that these mutants have a modification in a post photoreceptor point in the chain of reactions leading to inhibition of hypocotyl growth.

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CHAPTER 8, BIPHASIC FLUENCE-RESPONSE CURVES FOR LIGHT-INDUCED GERMINATION OF *Arabidopsis thaliana* SEEDS

With appropriate pretreatment of the seeds fluence-response curves for the induction of germination of *Arabidopsis thaliana* show two phases. A proportion of the population responds to very low fluence (VLFR), 10^{-4} - 10^{-2} µmol m^{-2}, establishing 10^{-4} to 10^{-2}% of the total phytochrome in the far-red absorbing form (Pfr) and a proportion of the population respond to low fluence (LFR), 1 - 1000 µmol m^{-2}, establishing 1 to 75% Pfr. The VLFR is not normally seen because the pre-existing Pfr level satisfies the Pfr requirement or use of green safelight establishes more Pfr than necessary to saturate the VLFR. Endogenous Pfr was depleted by a 24 h 35 °C treatment, presumably as a result of dark destruction and/or dark reversion to the red absorbing form (Pr), making it possible to visualize the VLFR. A short pulse of 35 °C treatment in combination with an appropriate temperature regime is also able to sensitize a proportion of the seed population. The proportion of the population showing the VLFR is determined by the duration of the cold imbibition pretreatment as well as the duration of the 35 °C treatment. Complex fluence-response curves were observed in which a proportion of the seeds being promoted in the VLFR range, were inhibited at higher fluences before being further promoted in the LFR range. This was particularly clear for seed batches being sensitized by a short 35 °C treatment. The VLFR may be of significance in the natural environment, enabling seeds buried in the upper layer of the soil to germinate, where the fluence rate falls off sharply and the LFR is not satisfied. A model is presented to explain the two phases in the fluence-response curves.

Introduction

Fluence-response curves for light-induced seed germination are normally sigmoid, when plotted as percentage germination against logarithm of fluence (chapter 3). However, fluence-response curves for light induced germination of lettuce seeds can be more complex and show biphasic response (Blaauw-Jansen and Blaauw 1975, 1976a, 1976b, Blaauw-Jansen 1981, Small et al. 1979a, 1979b, VanDerWoude 1983). Germination of sensitive seeds require c. 10^4 times less light than insensitive seeds. Sensitive seeds respond to very low fluences, 10^{-4} to 10^{-1} µmol m^{-2} 660 nm light, establishing 10^{-4} - 10^{-2}% Pfr, (VLFR), while

insenstive seeds respond to low fluences, 1 to 1000 μmol m^{-2} 660 nm light, establishing 1-75% Pfr (LFR). Blaauw-Jansen and Blaauw (1975), using seed batches of lettuce that germinate in darkness, induced dormancy and a subsequent light requirement either by prolonged exposure to far-red light (FR) during the imbibition period or by prolonged high temperature treatment. VLFR requires a very small amount of Pfr (10^{-4} to 10^{-2} % of total phytochrome as Pfr). Since seed batches usually contain low levels of endogenous Pfr, VLFR can only be visualized when this endogenous Pfr is removed. This can be done with different pretreatments, examples: (I) by supplying FR to transform Pfr to Pr followed by 24 h at 20 °C (VanDerWoude 1983) or (II) a high temperature treatment in order to stimulate Pfr destruction and/or dark reversion to Pr (Blaauw-Jansen and Blaauw 1975, Small et al. 1979a). FR-dormant seeds show normal fluence-response curves for light induced germination, while thermodormant seeds often show a biphasic fluence-response curve. The proportion of sensitive seeds in a population can be altered by dark storage, short high temperature treatment, cold imbibition and anesthetics (VanDerWoude 1983). Fluence-response curves for the very sensitive proportion of the population are more shallow than fluence-response curves for the less sensitive proportion of the population.

Until now, biphasic fluence-response curves for short term irradiation induced seed germination have only been reported for lettuce. In this chapter biphasic fluence-response curves are also demonstrated for promotion of *Arabidopsis thaliana* seed germination.

Materials and methods

The general germination procedure, described in chapter 2 was used. Prolonged high temperature treatment of the seeds was given by placing the seeds in darkness in an incubator at 35 °C. Short treatment at elevated temperature was given by floating the petri-dishes containing the seeds in darkness on the surface of a thermostatically controlled waterbath. Saturating fluences of red (R) and FR were obtained using interference filters with 660 and 730 nm transmission maximum and c. 10 nm bandwith at 50% of the transmission maximum (B40 type, Balzers, Liechtenstein). Saturating R was 6.2 mmol m^{-2} given in 1 min and FR was 22 mmol m^{-2} given in 2 min. The action spectra were constructed by plotting the reciprocal of the quantum requirement necessary

for 50% germination for each wavelength against the wavelength. Quantum effectiveness is expressed as percentage of the maximum value (Shropshire 1972, Schäfer et al. 1983, chapter 4).

Results

In the seed batches of A. *thaliana*, used in the present research, endogenous Pfr was removed by 35 °C treatment. Figure 1 shows the influence of time periods at 35 °C, following a cold imbibition period of 4 days at 7 °C, on the germination of WT81 seeds. The maximum germination after saturating R (1 min) is not significantly affected by a 35 °C treatment. However, dark germination decreases rapidly with an increasing period at 35 °C. This is interpreted as reflecting a fall in the endogenous Pfr in the seeds that is responsible for dark germination. After 16 h at 35 °C, the endogenous Pfr has fallen below

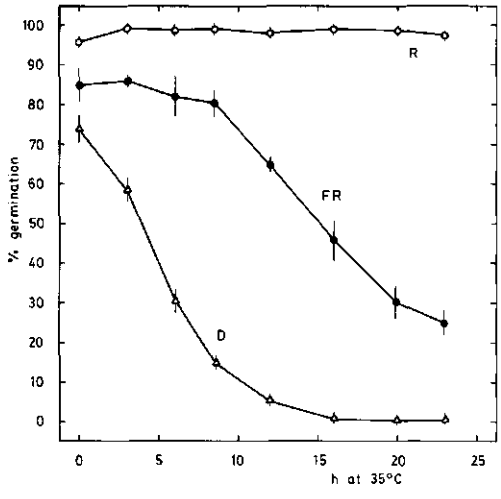

Fig. 1. The influence of duration (h) at 35 °C after 4 days at 7 °C, terminated by 1 min red (R) 2 min far-red (FR) or kept in darkness (D) on the germination of WT81 seeds. After irradiation, the seeds were incubated for 4 days at 20 °C in darkness.

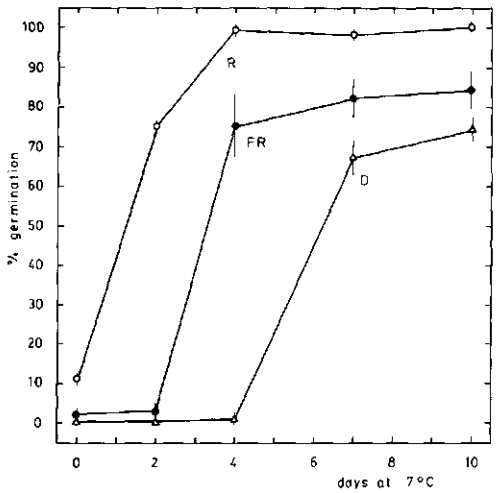

Fig. 2. The influence of duration (days) at 7 °C, followed by 16 h at 35 °C, terminated by 1 min red (R), 2 min far-red (FR) or kept in darkness (D) on the germination of WT79 seeds. After the irradiation the seeds were incubated for 4 days at 20 °C in darkness.

the threshold of the most sensitive seeds and dark germination is zero. From the germination induced by a saturating FR pulse (2 min), it is shown that the sensitivity of the seed batch to Pfr decreases simultaneously. Decrease in endogenous Pfr appears to start immediately after the beginning of the 35 °C treatment, whilst a decrease in sensitivity begins after 8 to 10 h. The saturating FR pulse of 730 nm establishes approximately 2.5% Pfr (see chapter 3). In lettuce seeds, this 2.5% Pfr establishes a percentage germination which lies between the VLFR and the LFR range (cf Small et al. 1979a). It is therefore expected that with A. *thaliana* seeds, that if a biphasic fluence-response exists, the germination after a saturating FR fluence represents the plateau between the VLFR and the LFR. The level of germination after FR in Fig. 1 is an indication of the proportion of the seed population responding to the VLFR. The germination after R immediately followed by FR was exactly the same as after FR alone (data not shown).

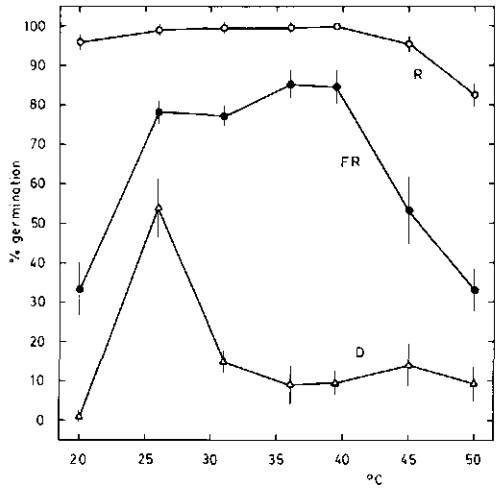

Fig. 3. The germination of Hy-5 seeds after 24 h at 20 °C, followed by 10 min at different temperatures and terminated with 1 min red (R), 2 min far-red (FR) or kept in darkness (D). After the irradiation the seeds were incubated for 4 days at 20 °C in darkness.

Differences in depth of dormancy cause a difference in sensitivity of the seeds to Pfr (chapter 5). In Fig. 2 differences in dormancy of WT79 seeds are obtained by different periods of imbibition at 7 °C, prior to the 35 °C treatment. It is shown that a prolonged imbibition period increases the sensitivity of the seeds to Pfr and/or the availability of the unknown reaction partner of Pfr (X), enabling Pfr to induce germination. After 4 days of imbibition dark germination also increases. It is possible that Pfr-action accumulates during the imbibition period at 7 °C, enabling the very low Pfr level remaining after the 35 °C treatment to satisfy germination (chapter 6) or alternatively that another factor than phytochrome becomes active, bypassing the Pfr requirement of seed germination (chapter 3). The germination after R, immediately followed by FR was the same as after FR alone (data not shown).

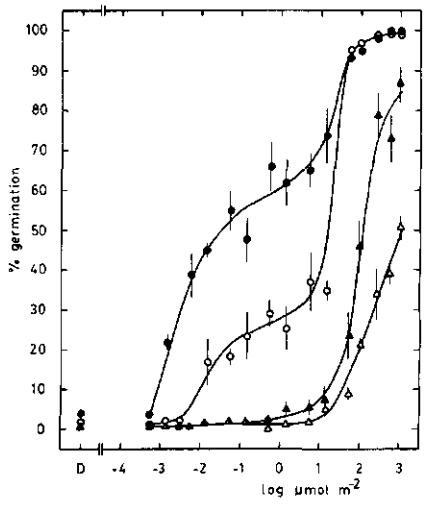

Fig. 4. Fluence-response curves for the induction of seed germination by 660 nm of WT81 seeds. Imbibition for 1 (△), 2 (▲), 4 (o) or 8 (●) days at 2 °C was followed by 24 h at 35 °C. After the irradiation, the seeds were incubated for 4 days at 20 °C in darkness.

Even a short pulse of 35 °C can sensitize a seed population without removing the endogenous Pfr completely (Takaki et al. 1985). When a seed batch contains very little endogenous Pfr, it is possible to visualize the VLFR with a short 35 °C pulse. In Fig. 3 the germination response of Hy-5 seeds is shown after a 10 min temperature pulse, following an imbibition period of 24 h at 20 °C. It is shown that the germination response after saturating R is scarcely affected by the temperature pulse and only at 45 °C is the maximum germination response reduced, presumably due to irreversible destruction of proteins and membranes. Dark germination shows that the seeds become sensitive even after a 10 min pulse of 26 °C, increasing the germination from 0 to 55%. Higher temperatures also sensitize the seed population, as shown by the FR response, but also result in a decrease of the endogenous Pfr as shown by decreased dark germination. The high level of germination after FR suggests that a great proportion of the population exhibits the VLFR. The response after R/FR was the same as after FR alone (data not shown).

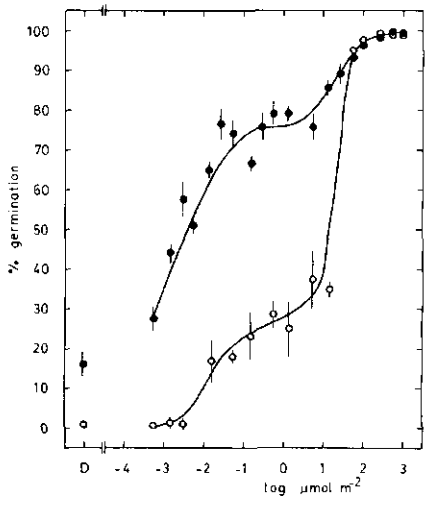

Fig. 5. Fluence-response curves for the induction of seed germination by 660 nm of WT81 seeds. Four days of imbibition at 2 °C was followed by 8 h (●) or 24 h (○) at 35 °C. After the irradiation, the seeds were incubated for 4 days at 20 °C in darkness.

Figure 4 shows fluence-response curves of WT81 seeds for the induction of seed germination by 660 nm light. Endogenous Pfr was removed by a 24 h period at 35 °C, resulting in a minimal dark germination (see Fig. 1). The level of dormancy of the seeds was varied by different imbibition periods at 2 °C (chapter 5). Biphasic fluence-response curves are obtained with a sufficiently long period of cold imbibition. After 1 or 2 days imbibition, the whole seed population exhibits the LFR. Obviously prolonged cold imbibition transfers an increasing proportion of the population to the VLFR range. The slight shift in the fluence-response curves is due to differences in sensitivity of the seeds to Pfr (chapter 3).

The proportion of the population exhibiting the VLFR is not only determined by the duration of the cold imbibition period but also by the duration of the 35 °C treatment. In Fig. 5 fluence-response curves for the induction of germination of WT81 seeds are shown with different periods of 35 °C treatment.

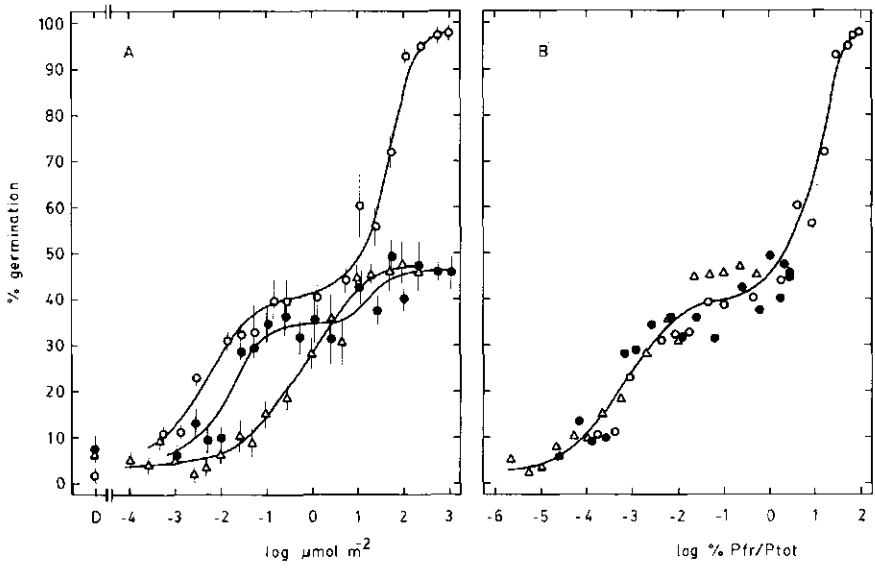

Fig. 6. (A) Fluence-response curves for the induction of seed germination by 660 nm (o), 730 nm (●) or 443 nm (Δ) of WT81 seeds. The seeds were imbibed for 8 days at 2 °C and followed by 24 h at 35 °C. After the irradiation, the seeds were incubated for 4 days at 20 °C. (B) Relationship between % Pfr and % germination calculated from the data of Fig. 6A (see also chapter 3). K, differential screening, for FR is 5.6 for R 1.0 and for B 0.2 (chapter 4).

Analogous to Fig. 1, prolonged 35 °C treatment decreases the amount of endogenous Pfr, resulting in a lower level of dark germination and a decreased proportion of the population responding in the VLFR range.

Small et al. (1979a, 1979b) and Blaauw-Jansen and Blaauw (1975) observed a minimum in some biphasic fluence-response curves for germination response that was between VLFR and LFR. Fluence-response curves for the induction of germination with FR light showed a remarkable optimum curve. VanDerWoude however, has also observed biphasic responses, but has not obtained a minimum in the fluence-response curves (personal communication). Figure 6 shows fluence-response curves for the induction of seed germination in WT81 seeds with R, FR and B light. The seeds were imbibed for 8 days at 2 °C, followed by a

24 h period of 35 °C. No decrease in response was observed upon increasing the fluence for the range of fluence rates and exposure times used and all the curves exhibit a plateau. This suggests that the fluence-response curves are only defined by the amount of Pfr established upon irradiation. Calculating the amount of Pfr, corresponding to the data in Fig. 6a, taking account of differential screening of the seed coat and tissues (chapter 3 and 4), give the same curve when germination is plotted as a function of % Pfr, produced by R, FR or B (Fig. 6b). This indicates that the germination response to these wavebands is only a consequence of the amount of Pfr they establish.

Figure 7 shows fluence-response curves for the induction of germination with 660 nm light of Hy-5 seeds which were pretreated with a 10 min 35 °C pulse following an imbibition period of 24 h at 20 °C. The seeds which are

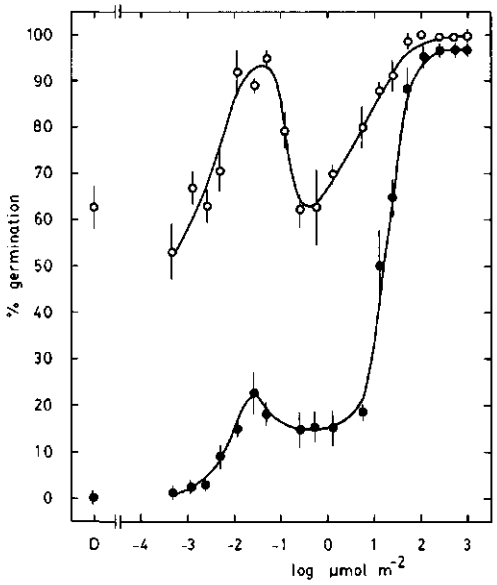

Fig. 7. Fluence-response curves for the induction of seed germination by 660 nm light of Hy-5 seeds. The seeds were imbibed for 24 h at 20 °C (•) or imbibed for 24 h at 20 °C, followed by 10 min at 35 °C (o). After the irradiation, the seeds were incubated for 4 days at 20 °C in darkness.

sensitized by 10 min 35 °C clearly show an optimum curve for the VLFR region of the fluence-response curve. Even seeds which are not 35 °C treated show a biphasic fluence-response curve and an indication of an optimum in the VLFR region of the curve. Obviously there are two processes working, one making the seeds extremely sensitive to Pfr, resulting in an increased dark germination and the other decreasing the sensitivity of the seeds to Pfr upon irradiation or upon the appearance of Pfr.

In order to show clearly that both the VLFR and the LFR are triggered by phytochrome, action spectra of both responses were calculated from fluence-response curves over the wavelength range 550 to 720 nm. It proved impossible to construct accurate action spectra of both the VLFR and the LFR of a seed batch with identical imbibition period and pretreatment. Therefore the action spectra of the LFR was made for Hy-5 seeds which were imbibed for 2 days at 7 °C and the action spectrum of the VLFR was made for Hy-5 seeds which were imbibed for 4 days at 7 °C and subsequently treated with 35 °C for 10 min. In Fig. 8 both action spectra show the characteristic action maximum of a phytochrome mediated response at 660 nm. Minor differences in the action spectra are probably not significant, due to errors in determining fluence-response curves in the VLFR range (see chapter 4). These results are consistent with those reported for lettuce by Blaauw-Jansen and Blaauw (1976a) and Small et al. (1979a).

VanDerWoude (1983) and Taylorson and Hendricks (1979) showed the promotive effect of anesthetics on the induction of germination of seeds of various species. VanDerWoude (1983) showed that ethanol was able to sensitize a proportion of a lettuce seed population depending on the period of ethanol treatment. These authors suggest that ethanol modifies membrane properties in the seeds, promoting germination. In *A. thaliana* seeds, ethanol was not able to promote germination (fig. 9). An increasing percentage ethanol in the growth medium inhibits the germination of WT81 seeds. Ethanol was given for 32 h at 20 °C before it was washed from the filter paper discs by suction. The seeds were subsequently treated for 16 h at 35 °C and irradiated with saturating R or FR. Not only ethanol proved to be poisonous but also other anesthetics such as glycerol, methanol, iso-propanol and acetaldehyde (data not shown). Inhibition of germination by ethanol is in agreement with data of Taylorson (1984) for

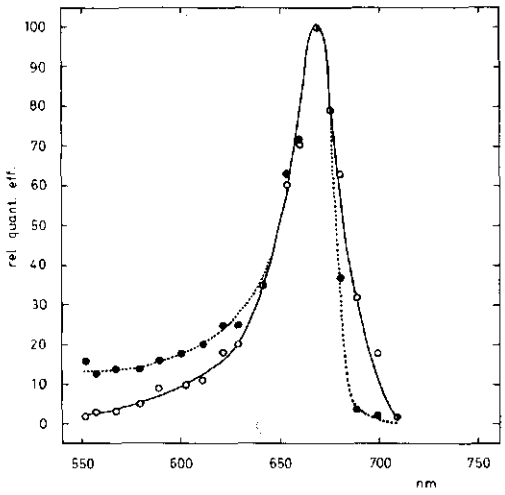

Fig. 8. Action spectra for the induction of seed germination of Hy-5 seeds. Seeds were imbibed for 2 days at 7 °C (o) or for 4 days at 7 °C, followed by 10 min at 35 °C (•). After the irradiation, the seeds were incubated for 4 days at 20 °C in darkness. Relative quantum effectiveness for the induction of 50% germination was determined. Peak effectiveness is normalized to 100.

Rumex crispus seeds. VanDerWoude (1983) and Taylorson (1984) report that ethanol might influence membrane properties, which affect the light sensitivity of germination. By comparing escape curves for FR light and ethanol Taylorson (1984) showed that for seeds in which ethanol inhibits, it might act at the same site as Pfr. Similar results have been obtained with A. *thaliana* Hy-5 seeds (Fig. 10). The escape time for 50% of the population from FR control is 5 h while the escape time from ethanol (2%) inhibition for 50% of the population is more than 20 h. Taylorson explains this difference in escape time by the fact that FR light reaches P immediately, while ethanol needs time to diffuse

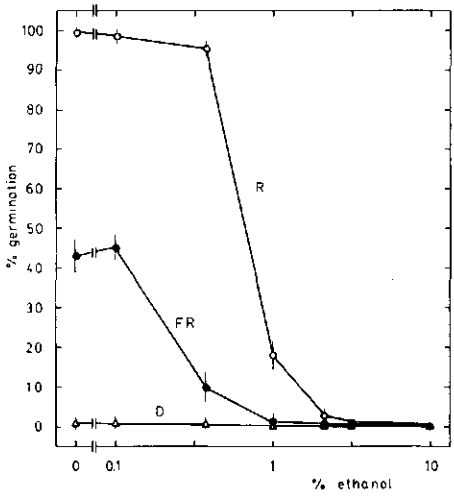

Fig. 9. The influence of different percentage ethanol in the growth medium on the germination response of WT81 seeds. The seeds were imbibed for 32 h at 20 °C, followed by 16 h at 35 °C and terminated by 1 min red (R), 2 min far-red (FR) or the seeds were kept in darkness. After the irradiation, the seeds were incubated for 4 days at 20 20 °C in darkness.

through the seed coat. However, if this is true, the escape from ethanol inhibition should occur at an earlier time than the escape from FR control. A prolonged escape in ethanol means that germination is inhibited at a point in the germination process which occurs much later than the germination inhibition by FR light. This does not exclude the possibility that in other or the same seeds, ethanol has a promotory effect on germination, at the site of Pfr action. One of the possibilities is that both ethanol and Pfr influence the membrane properties. The inhibition of germination by ethanol here is unlikely to be at the site of Pfr action.

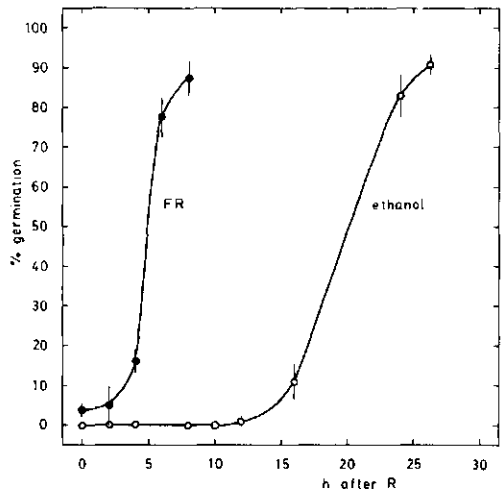

Fig. 10. The escape from far-red (FR) control (●) and from ethanol inhibition (○) after a saturating red fluence of Hy-5 seeds. Ethanol was given as a 2% solution. The seeds were imbibed for 2 days at 7 °C. After the irradiation, the seeds were incubated for 4 days at 20 °C in darkness.

Discussion

Biphasic fluence-response curves for the induction of seed germination have only been described previously for lettuce (Blaauw-Jansen and Blaauw 1975, Small et al. 1979a, VanDerWoude 1983). Here biphasic fluence-response curves are shown for the first time in another species, *Arabidopsis thaliana*. It appears that this type of response is not unusual, since it has also been recently demonstrated in *Rumex obtusifolius* (Kendrick and Cone, 1985). The fact that biphasic responses are rarely observed is probably due to endogenous Pfr stimulating

the germination of the VLFR seeds. Many workers also use green safelight, which establishes more than enough Pfr to saturate the VLFR. Biphasic fluence-response curves have not only been observed for the induction of seed germination but also for other phytochrome controlled processes, such as induction of rapid chlorophyll accumulation (Spruit et al. 1979), chloroplast movement in *Mougeotia* cells (Haupt and Seitz 1966) and elongation growth (Mandoli and Briggs, 1981).

Blaauw-Jansen and Blaauw (1975) showed that thermo-dormant lettuce seeds were able to respond in the VLFR range, while FR-dormant seeds were only able to respond in the LFR range. According to Blaauw-Jansen and Blaauw (1975), the observation of VLFR and LFR in one fluence-response curve suggests that there are two reactions controlling germination. The minimum in the fluence-response curves, they observed, were explained by assuming that there are two pigments interfering at the same reaction centre. The difference in slope of the fluence-response curves resembles the difference between one-hit and multi-hit Poisson probability curves (Blaauw et al. 1976). However with this model they were not able to explain the experimentally observed large increase of the threshold fluence between the VLFR and the LFR. In chapter 3 the difference in slope of the fluence-response curves has been interpreted in terms of the presence of a very small amount of endogenous Pfr.

In 1976 Blaauw-Jansen and Blaauw (1976a) published action spectra for the VLFR and the LFR. They suggest that two phytochrome species are operative in lettuce seeds. In a later paper Blaauw-Jansen and Blaauw (1976b) conclude that the inductive effect of FR is mediated by an abnormal form of Pfr, which is transformed into the normal form of Pfr by small FR fluences.

Small et al. (1979a) explain the difference between thermo-dormancy and FR-dormancy by assuming that the induction of germination in lettuce seeds is under the control of different processes. Under appropriate conditions two of these may be linked to phytochrome and act in series, the first being extremely sensitive to Pfr, the second much less. Induction of dormancy could consist of making one or the other decisive for germination. High temperature treatment is suggested to result in a bypass of the second less sensitive process, possibly by activating an essential metabolic process, otherwise under phytochrome control.

In 1983 Blaauw-Jansen put forward a hypothesis to explain the biphasic fluence-response curves by assuming the existence of a Pfr dependent Pfr-

destroying enzyme. Upon irradiation Pfr is formed, promoting germination. Above a certain critical threshold of Pfr, the Pfr-destroying enzyme becomes active, resulting in a decreased amount of Pfr, leading to a plateau or even a decrease in response. When irradiation increases, the Pfr-destroying enzyme is saturated and the Pfr level increases, promoting germination. This model could therefore explain the minimum seen in some fluence-response curves.

Recently VanDerWoude (1983) has presented a model in which membrane fluidity is altered by temperature treatments and anesthetics. He assumes that phytochrome exists as a dimer, which is only active when it is membrane bound. The activity of the heterologous dimer Pr:Pfr depends on membrane properties. While it is possible with this model to explain biphasic fluence-response curves, it is difficult to explain the minimum that is sometimes observed.

Before attempting to explain the existence of biphasic fluence-response, it is worthwhile to ask what is its possible advantage in the natural environment? It is shown that seeds buried near the soil surface are still able to germinate upon irradiation under favourable conditions (Frankland and Poo 1980, Frankland 1981). If seeds only responded to fluences in the LFR range, buried seeds would never be able to germinate if buried more than a few mm. Biphasic fluence-response curves enable a broader range of fluence to be used as an indication of the proximity of the soil surface. Experiments described here and by VanDer-Woude (1983) show that VLFR germination increases after an increasing period of cold imbibition. In a temperate climate, it is important for a seed to germinate in the spring after the cold of winter. Below deciduous plants, leaf canopy in the beginning of the spring is minimal and the R/FR ratio would play a minimal role. Viable seeds will germinate upon irradiation, indicating that the soil layer above the seeds is not too deep. The seeds which fall on the soil surface in the summer respond to the LFR and will only germinate when the R/FR ratio is sufficiently high, indicating they are not shaded by a leaf canopy.

In the explanation of Blaauw-Jansen (1983) it is possible that a seed under natural conditions is irradiated with a fluence, just enough to activate the Pfr-destroying enzyme but not enough to induce germination. This means that in such a seed there is a continuous synthesis of the enzyme and a continuous Pfr breakdown. However, it is shown (Karssen 1980/81, Wesson and Wareing 1969) that buried seeds can stay viable for several years. In the model of Blaauw-Jansen (1983) energy is needed for the continuous turnover of phytochrome

and therefore the situation is exessively wastefull for a seed where energy is at a premium. The other models of Blaauw-Jansen and Blaauw, proposed in an earlier stage of research also seem unlikely, since there is no good evidence for different forms of phytochrome or different pigment systems operating here. Figures 6 and 8 show clearly that both the VLFR and the LFR are triggered by Pr light absorption. Using long-hypocotyl mutants of A. *thaliana*, the influence of other pigments than phytochrome on seed germination could not be shown (chapter 7).

Findings of VanDerWoude (1983) and Taylorson (1984) indicate that membrane fluidity plays an important role in phytochrome mediated seed germination. In our experiments membrane properties may also alter upon cold treatment or short 35 ^{o}C treatment (Figs. 2, 3, 4 and 7). However we were not be able to show promotive effect of anesthetics in this system (Fig. 9). Figure 10 shows that in A. *thaliana* seed germination is inhibited by ethanol at a later time than that of Pfr action.

In chapter 3 a mathematical model is presented with which fluences needed for the induction and inhibition of seed germination can be calculated. Biphasic fluence-response curves imply that there are two ranges of Pfr sensitivity. The seed batch can be thought to consist of two different populations each with its own sensitivity range. However, this simple concept cannot explain the minimum observed in some curves (Fig. 7). Obviously sensitivity can change upon irradiation or upon the appearance of Pfr. The model in chapter 3 can be modified by assuming that for extreme low levels of Pfr, the sensitivity of the seed batch is Pfr dependent. We can think of a switch in the seeds that determines whether the seeds exhibit the VLFR or the LFR. An increasing amount of Pfr will switch seeds from the VLFR to the LFR range. Additionally chilling, short high temperature and anesthetics can switch the seeds from the LFR to the VLFR range. The extent of the VLFR can be the result of a balance between Pfr and factors influencing membrane properties. With this model it is also possible to explain the minimum in Fig. 7. If irradiation establishes a Pfr level enough to saturate the VLFR, an increasing amount of Pfr will switch the seeds from the VLFR to the LFR, possibly by changing membrane properties. When the sensitivity of the LFR is low, the Pfr level might not be enough to get the same response in the LFR mode as in the VLFR mode. In this model it is not necessary for phytochrome to be a dimer. Another explanation of the minimum in Fig. 7 is the involvement of a P cycling reaction that switches seeds from the VLFR to the LFR range. If a reaction dependent on

the amount of cycling played an important role in seed germination, the response after R/FR would be expected lower than after FR alone, but this was not observed.

Clearly the biphasic response seen in phytochrome responses is of great interest and provides an important indication of the underlying molecular processes involved in its action. Investigations of these responses at the molecular level in relation to membrane function are needed in order to elucidate fully the processes involved.

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Summary

This thesis reports research on the photocontrol of seed germination of wildtype and long-hypocotyl mutants of *Arabidopsis thaliana*. The mutants show reduced photoinhibition of hypocotyl growth in white light in comparison to that of wildtype. In monochromatic light some of the mutants also show no inhibition of hypocotyl growth by red and/or far-red light, while others show no inhibition in blue and UV light. It is proposed that these mutants might either be mutated with respect to red/far-red absorbing phytochrome, another red absorbing photoreceptor or a blue/UV absorbing receptor.

Preliminary studies on the breaking of dormancy and subsequent induction of germination by light are reported in chapter 5. It is shown that the imbibition treatment prior to irradiation has a great influence on the level of dormancy of the seed population. Prolonged imbibition results in secondary dormancy. By analysing fluence-response curves, it is shown that induction of secondary dormancy is not the opposite process as that of loss of primary dormancy. During loss of primary dormancy, the reaction partner of Pfr, X, appears to become less limiting, while during the onset of secondary dormancy Pfr appears to become limiting.

The seed germination behaviour of the long-hypocotyl mutants and wildtype was investigated. By determining action spectra of the induction and inhibition of induction of seed germination, information was obtained about the receptor pigments involved (chapter 7). It is demonstrated that phytochrome is the sole photoreceptor controlling seed germination. Although seeds can be induced to germinate by blue light, there appears to be no separate blue or UV receptor involved. The relative activity of blue light in the mutants Hy-1 and Hy-2, deficient in spectrophotometrically detectable phytochrome, is comparable to that of wildtype.

Although no significant differences in the action spectra of the mutants and wildtype were observed, the fluence-response curves both for the induction and inhibition of induction of germination show differences in form. The mutants Hy-1, Hy-2 and Hy-3 show a shallow fluence-response curve, while the mutants Hy-4 and Hy-5 and wildtype show steeper fluence-response curves.

To interpret the differences in the fluence-response curves of the different seed batches, a mathematical model was designed which allows theoretical fluence-

-response curves to be calculated (chapter 3). In this model it is assumed that there is a normal distribution for the logarithm of the Pfr requirement of individuals within a seed population. The validity of this assumption is supported by experiments in chapter 3, where theoretical and experimental fluence-response curves are shown to coincide. The known formula for the appearance of Pfr upon irradiation was modified to take account of pre-existing Pfr in the seeds. The model takes into account different levels of an overriding factor, affecting germination by a non-phytochrome related process, the total amount of phytochrome, the range of Pfr requirement in the population and differential screening. It is shown that these factors can have a great influence on the form and/or position of the fluence-response curves. Using this model it is suggested that the fluence-response curves of Hy-1, Hy-2 and Hy-3 are shallow because these seed batches have a low level of phytochrome and that the level of dark germination is the result of an overriding factor.

To show that the response to Pfr is not only a function of the amount of Pfr in the seeds, but also a function of the duration of Pfr action, the time course of phytochrome action was determined (chapter 6). It is shown that the escape from far-red photocontrol (time course of Pfr action) and the rate of germination is correlated with the sensitivity of the seeds to Pfr. Germination plots versus time can show two phases. The rapid phase of germination is due to those seeds having germination induction satisfied by their endogenous Pfr during imbibition.

The model in chapter 3 also enables theoretical action spectra to be calculated both for the induction and inhibition of induction of germination (chapter 4). It is shown that there is no standard action spectrum, the form and/or peak position of a spectrum being determined by the Pfr sensitivity of the seed population, pre-existing Pfr, overriding factor, total phytochrome and differential screening.

It is shown that seeds depleted of endogenous Pfr sometimes exhibit biphasic fluence-response (chapter 8). A part of the population is very sensitive to light, while the remaining part shows normal sensitivity. Imbibition conditions determine the proportion of the population responding to very low fluences. The model presented in chapter 3 was modified to fit the biphasic fluence-response curves by assuming that the sensitivity of the seeds to Pfr is determined by Pfr itself, at least at low levels of Pfr.

Samenvatting

Groene planten hebben licht nodig voor hun fotosynthese. Zonder licht zou er geen leven op aarde mogelijk zijn. Planten hebben licht niet alleen nodig om te groeien, maar ze hebben ook minimale hoeveelheden licht nodig voor de regulatie van allerlei groeiprocessen. Het reguleren van groeiprocessen door licht wordt fotomorfogenese genoemd. Een van de belangrijkste fotoregulerende pigmenten is fytochroom, dat onder invloed van licht van eigenschappen kan veranderen. Rood licht brengt fytochroom van de Pr in de Pfr vorm, dat fysiologisch actief is. Verrood licht daarentegen kan Pfr weer omzetten in Pr, de niet actieve vorm van fytochroom. De strategie van een plant is er ondermeer op gericht om met behulp van fytochroom de groei aan te passen aan de lichtomstandigheden. Zo zullen bijvoorbeeld veel zaden niet kiemen onder een bladerdak, omdat dan te weinig fotosynthetisch licht beschikbaar zal zijn voor een goede groei.

In dit proefschrift worden de resultaten beschreven van onderzoek naar de invloed van licht op de kieming van zaden van wildtype en lange-hypocotyl mutanten van de Zandraket (*Arabidopsis thaliana*). De remming van de hypocotylstrekking van de mutanten in wit licht is gereduceerd in vergelijking met dat van het wildtype. Sommige mutanten vertonen ook in monochromatisch rood en verrood licht geen remming van de hypocotylgroei, terwijl andere mutanten niet door blauw en UV licht geremd worden. Er wordt verondersteld dat de mutanten gemuteerd zijn in fytochroom, een ander rood absorberend pigment of een blauw/UV absorberend pigment.

Resultaten van onderzoek naar de breking van kiemrust en kieminductie door licht zijn beschreven in hoofdstuk 5. Imbibitie condities blijken een grote invloed te hebben op de gevoeligheid van de zaden voor licht. Door langere imbibitietijden wordt secondaire kiemrust geïnduceerd. Dosis-effect curven tonen aan dat de inductie van de secondaire kiemrust niet het tegenovergestelde proces is van dat van het verminderen van de primaire kiemrust. Tijdens de vermindering van de primaire kiemrust, is de reactiepartner van Pfr, X, limiterend, terwijl tijdens de inductie van de secondaire kiemrust Pfr limiterend is.

Het kiemingsgedrag van de lange-hypocotyl mutanten en wildtype is onderzocht. Door actiespectra van de kieminductie en -inhibitie te bepalen kan informatie verkregen worden omtrent de pigmenten die hierbij betrokken zijn (hoofdstuk 7). Het blijkt dat enkel fytochroom betrokken is bij kieming onder invloed van korte belichtingen. Hoewel de zaden ook kiemen in blauw licht, blijkt dat geen

apart blauw absorberend pigment actief is. De relatieve activiteit van blauw licht in de fytochroomarme mutanten Hy-1 en Hy-2 is vergelijkbaar met dat in wildtype.

Er werden geen significante verschillen tussen de actiespectra van de mutanten en wildtype gevonden. De dosis-effect curven vertoonden wel grote verschillen; de curven van de fytochroomarme mutanten Hy-1, Hy-2 en Hy-3 zijn minder stijl dan die van Hy-4, Hy-5 en wildtype.

Om de verschillen in de dosis-effect curven te kunnen verklaren is een mathematisch model opgesteld, waarmee theoretische curven berekend kunnen worden (hoofdstuk 3). In dit model wordt aangenomen dat binnen een zaadpopulatie een normaal verdeling bestaat voor de logarithme van de Pfr behoefte van individuele zaden. Dat deze aanname juist is blijkt uit proeven, beschreven in hoofdstuk 3, waar berekende dosis-effect curven overeen komen met experimentele curven. De reeds bekende formule voor de vorming van Pfr onder invloed van licht is zodanig aangepast dat ook rekening wordt gehouden met reeds aanwezig Pfr. Het model houdt ook rekening met de totale hoeveelheid fytochroom, de spreiding van de Pfr behoefte in de zaadpopulatie, afscherming door andere pigmenten en andere factoren dan fytochroom die een invloed hebben op de kieming. Het blijkt dat al deze factoren een grote invloed kunnen hebben op de vorm en/of positie van dosis-effect curven. Dit model suggereert dat de dosis-effect curven van Hy-1, Hy-2 en Hy-3 minder stijl zijn door de zeer geringe hoeveelheid fytochroom in de zaden en het feit dat enige kieming veroorzaakt wordt door een andere factor dan fytochroom.

Om aan te tonen dat kieming niet alleen een funktie van de hoeveelheid Pfr is, maar ook van de tijd dat Pfr aktief is, werd de tijdsduur van de Pfr werking bepaald (hoofdstuk 6). Het blijkt dat de tijd die nodig is om aan de remmende invloed van verrood licht te ontsnappen (d.w.z. de tijd dat Pfr actief is), gecorreleerd is met de gevoeligheid van de zaden voor Pfr. Dit geldt ook voor de snelheid van kieming. Wanneer de kieming tegen de tijd uitgezet wordt kunnen soms twee fasen onderscheiden worden. De snelle kieming wordt mogelijk veroorzaakt door endogeen Pfr dat reeds gedurende de imbibitie actief is.

Het model, zoals beschreven in hoofdstuk 3, maakt het mogelijk ook theoretische actiespectra te berekenen (hoofdstuk 4). De vorm en/of piekpositie van een actiespectrum wordt mede bepaald door de gevoeligheid van de zaden, endogeen Pfr, de totale hoeveelheid fytochroom, afscherming door andere pigmenten en andere factoren dan fytochroom die de kieming beïnvloeden.

In sommige gevallen kunnen zeer geringe hoeveelheden Pfr een aanzienlijke kieming veroorzaken (hoofdstuk 8). Het blijkt dat zaden, die geen endogeen Pfr bevatten, soms een dosis-effect curve vertonen met twee fasen. Een gedeelte van de zaden is extreem gevoelig voor licht. Het percentage zaden dat extreem gevoelig is voor licht wordt o.a. bepaald door de imbibitie condities. Het model, beschreven in hoofdstuk 3, kan aangepast worden om ook deze twee fasen te verklaren. Er wordt aangenomen dat, althans bij lage Pfr concentraties, de gevoeligheid van de zaden voor Pfr bepaald wordt door Pfr.

Appendix I

Tabel of the apparent molar conversion cross section for the transition Pr→Pfr (σ_1) and Pfr → Pr (σ_2) and the maximum Pfr/Ptot (ϕ) at different wavelengths (λ).

λ (nm)	σ_1	σ_2	ϕ	λ (nm)	σ_1	σ_2	ϕ
390	480	390	0.55	580	570	195	0.75
395	430	430	0.50	585	670	235	0.74
400	390	455	0.46	590	850	300	0.74
405	340	460	0.43	595	1115	365	0.75
410	300	450	0.40	600	1325	450	0.75
415	260	440	0.37	605	1530	520	0.75
420	230	420	0.35	610	1550	520	0.75
425	200	390	0.34	615	1570	525	0.75
430	170	345	0.33	620	1690	560	0.75
435	145	280	0.34	625	1865	610	0.75
440	120	240	0.33	630	2090	680	0.75
445	105	210	0.33	635	2270	740	0.75
450	95	190	0.33	640	2440	800	0.75
455	85	165	0.34	645	2660	900	0.75
460	75	145	0.34	650	3000	1015	0.75
465	65	125	0.34	655	3170	1040	0.75
470	50	100	0.33	660	3325	1025	0.76
475	40	65	0.38	665	3470	1155	0.75
480	30	45	0.40	670	2800	1050	0.73
485	25	30	0.45	675	1950	1025	0.66
490	25	25	0.50	680	1650	1155	0.59
495	25	20	0.56	685	1450	1235	0.54
500	28	20	0.58	690	850	1235	0.41
505	35	25	0.58	695	505	1285	0.28
510	40	25	0.62	700	325	1380	0.19
515	50	25	0.67	705	200	1475	0.12
520	60	30	0.67	710	125	1575	0.074
525	75	30	0.71	715	85	1670	0.048
530	90	35	0.72	720	70	1700	0.040
535	105	40	0.72	725	55	1680	0.032
540	125	40	0.76	730	40	1560	0.025
545	145	45	0.76	735	30	1430	0.021
550	175	55	0.76	740	20	1250	0.016
555	210	70	0.75	745	13	1090	0.012
560	250	80	0.76	750	5	915	0.005
565	315	105	0.75	755	2	740	0.003
570	400	130	0.75	760	0	570	0
575	490	160	0.75				

Appendix II

Probit transformation of % response

The relative frequency (f) for a normal distributed population is:

$$f = \frac{1}{SD\ \sqrt{2\pi}} \cdot e^{-(x-\mu)^2 / 2SD^2}$$

The percentage response (y) is:

$$y = \int_{-\infty}^{x} f(x)\,dx \cdot 100\ \%$$

Expressed in terms of probits, the response is:

$$\text{probit } y = 5 + \frac{x - \mu}{SD}$$

y	probit y	y	probit y	y	probit y	y	probit y
0	.....						
1	2.67	26	4.36	51	5.03	76	5.71
2	2.95	27	4.39	52	5.05	77	5.74
3	3.12	28	4.42	53	5.08	78	5.77
4	3.25	29	4.45	54	5.10	79	5.81
5	3.36	30	4.48	55	5.13	80	5.84
6	3.45	31	4.50	56	5.15	81	5.88
7	3.52	32	4.53	57	5.18	82	5.92
8	3.59	33	4.56	58	5.20	83	5.95
9	3.66	34	4.59	59	5.23	84	5.99
10	3.72	35	4.61	60	5.25	85	6.04
11	3.77	36	4.64	61	5.28	86	6.08
12	3.83	37	4.67	62	5.31	87	6.13
13	3.87	38	4.69	63	5.33	88	6.18
14	3.92	39	4.72	64	5.36	89	6.23
15	3.96	40	4.75	65	5.39	90	6.28
16	4.01	41	4.77	66	5.41	91	6.34
17	4.05	42	4.80	67	5.44	92	6.41
18	4.08	43	4.82	68	5.47	93	6.48
19	4.12	44	4.85	69	5.50	94	6.55
20	4.16	45	4.87	70	5.52	95	6.64
21	4.19	46	4.90	71	5.55	96	6.75
22	4.23	47	4.92	72	5.58	97	6.88
23	4.26	48	4.95	73	5.61	98	7.05
24	4.29	49	4.97	74	5.64	99	7.33
25	4.33	50	5.00	75	5.67	100	.....

Appendix II (continued)

y	probit y	y	probit y	y	probit y	y	probit y
0.1	1.91	2.6	3.06	95.1	6.65	97.6	6.98
0.2	2.12	2.7	3.07	95.2	6.66	97.7	7.00
0.3	2.25	2.8	3.09	95.3	6.67	97.8	7.01
0.4	2.35	2.9	3.10	95.4	6.68	97.9	7.03
0.5	2.42	3.0	3.12	95.5	6.70	98.0	7.05
0.6	2.49	3.1	3.13	95.6	6.71	98.1	7.07
0.7	2.54	3.2	3.15	95.7	6.72	98.2	7.10
0.8	2.59	3.3	3.16	95.8	6.73	98.3	7.12
0.9	2.63	3.4	3.18	95.9	6.74	98.4	7.14
1.0	2.67	3.5	3.19	96.0	6.75	98.5	7.17
1.1	2.71	3.6	3.20	96.1	6.76	98.6	7.20
1.2	2.74	3.7	3.21	96.2	6.77	98.7	7.23
1.3	2.77	3.8	3.23	96.3	6.79	98.8	7.26
1.4	2.80	3.9	3.24	96.4	6.80	98.9	7.29
1.5	2.83	4.0	3.25	96.5	6.81	99.0	7.33
1.6	2.86	4.1	3.26	96.6	6.83	99.1	7.37
1.7	2.88	4.2	3.27	96.7	6.84	99.2	7.41
1.8	2.90	4.3	3.28	96.8	6.85	99.3	7.46
1.9	2.93	4.4	3.29	96.9	6.87	99.4	7.51
2.0	2.95	4.5	3.30	97.0	6.88	99.5	7.58
2.1	2.97	4.6	3.32	97.1	6.90	99.6	7.65
2.2	2.99	4.7	3.33	97.2	6.91	99.7	7.75
2.3	3.00	4.8	3.34	97.3	6.93	99.8	7.88
2.4	3.02	4.9	3.35	97.4	6.94	99.9	8.09
2.5	3.04	5.0	3.36	97.5	6.96	100	.....

Curriculum vitae

John Cone werd geboren op 18 december 1955 te Ter Apelkanaal (Gr.). Na de lagere school werd de Rijksscholengemeenschap in Ter Apel bezocht, waar in 1974 het Atheneum-B diploma werd behaald. In datzelfde jaar werd begonnen met een studie biologie aan de Rijksuniversiteit van Groningen. In 1978 werd het kandidaatsexamen B4 behaald en in 1980 het doctoraalexamen met als hoofdvakken microbiologie en plantenfysiologie. In 1981 heb ik korte tijd gewerkt bij de milieu-inspectie van het Ministerie van Volksgezondheid en Milieuhygiëne te Groningen. Van juli 1981 tot juli 1984 was ik via een beurs van BION verbonden aan de vakgroep Plantenfysiologisch Onderzoek van de Landbouwhogeschool in Wageningen. Het onderzoek aan deze vakgroep, onder leiding van dr. C.J.P. Spruit (tot 1982), dr. R.E. Kendrick en prof. dr. W.J. Vredenberg, heeft geleid tot het schrijven van dit proefschrift. Vanaf februari 1985 ben ik verbonden aan de vakgroep Landbouwplantenteelt, waar in samenwerking met dr. B. Deinum en dr. F.M. Engels (PCM) onderzoek wordt gedaan naar de verteerbaarheid van maïs.