Genetic modification of shade-avoidance: overexpression of homologous phytochrome genes in tomato

Said Saleh Hindi Husaineid

Promotor:

Prof. Dr. L.H.W. van der Plas
Hoogleraar in de Plantenfysiologie, Wageningen Universiteit

Co-promotor:

Dr. A.R. van der Krol
Universitair hoofddocent bij de leerstoelgroep Plantenfysiologie, Wageningen Universiteit

Promotiecommissie:

Prof. Dr. Ir. M. Koornneef (Wageningen Universiteit/MPI, Keulen, Duitsland)
Prof. Dr. R.G.F. Visser (Wageningen Universiteit)
Dr. T. Hendriks (Université des Sciences et Technologies de Lille, Frankrijk)
Dr. Ir. E. Heuvelink (Wageningen Universiteit)

Dit onderzoek is uitgevoerd binnen de onderzoekschool voor Experimentele Plantenwetenschappen (EPW)

Said Saleh Hindi Husaineid

Genetic modification of shade-avoidance: overexpression of homologous phytochrome genes in tomato

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. Dr. M.J. Kropff
in het openbaar te verdedigen
op maandag 23 april 2007
des namiddags te vier uur in de Aula

Said S. H. Husaineid

Genetic modification of shade-avoidance: overexpression of homologous phytochrome genes in tomato (2007)

PhD thesis, Wageningen University, Wageningen, The Netherlands.
With references – with summaries in English, Dutch and Arabic

ISBN: 90 – 8504 – 512 - 6

Preface

I am deeply grateful to Great God "ALLAH" for the gain of power, knowledge and success. Also, this work would never have been possible without the contribution of many people around me.

In the Laboratory of Plant Physiology at Wageningen University I would like to thank first Prof. Dr. Linus H.W. van der Plas, my promotor. Dear Linus, at many stages in my work you turned an impossible situation to possible. The arrangement of the staying in Netherlands for my family financially and legally, the interest and support to my work and solving the problems related to the repetitive stress injury (RSI) and the valuable comments during the preparation of the thesis are all appreciated very much.

I feel honoured by having for my supervision in the first three years of the thesis work Dr. R. E. Kendrick. Dear Dick, I am pleased about your daily guidance and the discussion of the experimental setup and data analysis. In fact, most of my knowledge in photobiology is coming from you and the valuable books that you generously gave me, such as " *Pigment of imagination* " and "*Genetic and environmental manipulation of horticultural crops*" and many other valuable books. I am very grateful to Mrs. Chris Kendrick and you for your hospitality, concern about the schools of my children and your visit to my family in Wageningen.

I would like to thank my supervisor and co-promotor Dr. Sander van der Krol. Dear Sander, the ultimate step in this thesis was the writing phase during which the difficulties we faced required absolute concentration and understanding. I will be grateful to you forever for what you suffered during this phase. Besides you were interested in helping me to improve my Dutch language. You generously gave me the book " De dure auto van Said " and many other helpful materials. Thank you also for writing the Dutch summary.

Very special thanks to Mariëlle Schreuder for the great help with the light cabinets, sowing of seeds, measuring anthocyanin, western blotting, analyzing a great part of the data and the greenhouse work. Without your help this work would have been much more difficult.

I am grateful to Rosan Kok for her valuable help, discussion and sharing the results of the protein analysis.

Thanks to Bastiaan Brouwer for his help in the RT-PCR experiments and to the people at the greenhouse for the help with sowing and harvesting of seeds.

I am grateful to Dr. James Weller for the valuable information about the use of markers for the selection of homozygous lines.

Thanks to all the people of the Laboratory of Plant Physiology and the laboratory of Genetics at Wageningen University: Prof. Dr. Maarten Koornneef, Dr. Dick Vreugdenhil, Dr. Folkert Hoekstra, Dr. Henk Hilhorst, Wytske Nijenhuis, Trees van Nieuwamerongen, Jan van Kreel, Rob van der Vossen, Hans Dassen, Maurice Henquet, Corrie Hanhart, Diaan Jamar, Judith van de Mortel, Joost Keurentjes, John Verhees, Danielle Basten and Lidiya Sergeeva.

I would like to thank the management team of the project "Rassen onder glas met minder gas": Prof. Dr. Piet Stam, Dr. Theo Hendriks, Dr. Pim Lindhout, and Dr. Sjaak van Heusden for organizing the work discussions in the AIO group and giving valuable comments in relation to my work.

Life in Wageningen would not have been gone so smoothly for me, my wife and children without the help of many people in Wageningen. I would like to start with the Montessorischool were my children started to attend their classes from the first week of arrival in Wageningen. Thanks to Marijke and Jurre for the extra Dutch lessons they gave to my daughter and for their hospitality. Thanks also to Ruud and Ellen Meester for helping me and my family in the preparation for the Dutch Language examinations. Thanks to Louis Razoux Schultz who is helping me to better control the Dutch language and for the book "Wat weten we over de Islam ". Thanks to Jannie Biemond for her support and hospitality to me and my family. Thanks to our neighbour Maria Rijlaarsdam for her kindness and emotional support. Thanks to Kees Hopmans for his support to me and my family.

I also had the pleasure to share good moments with our friends in Wageningen; Hassan and Amal Sawalha and their daughters. Rafat and Rasha. Sami Mosad, Ahmed Arafat. Our meetings and celebration of the special days in the last two years were of great pleasure to me and my family.

In Palestine, I would like to thank our friends Issa Alfawaghra, Abdallah Barkat, Mahmoud Abo Ali, Mohamad Awad and their families. Our meetings, trips and work together were always special. Special thanks to Mahmoud Abo Ali for the valuable comments on the Arabic summary.

Most importantly my gratitude goes to my late father and my late brother; it is with much pain in my heart that I regret not to have this achievement witnessed by both of you who worked insistently to help me succeed. Now I can only say thank you.

Without my mother I would never have been where I am today. Her unconditional love made me work hard to try to live up to our dreams. You were the greatest mother one could have wished for.

I would like to express my deep heartily thanks to my brothers who came from Bethlehem/Palestine especially to attend my PhD graduation. Thank you,

Mahmoud, Ahmed and Mustafa for the determination to come to congratulate me in Holland and for your great support. Thanks to my brother Moneer and his family for their help and encouragement. Special thanks to my sisters Ezia, Seham and Intesar and their families for emotional support and kind wishes.

Special appreciation goes to my father in-law and to the spirit of my mother in-law. Thanks also to my brothers in-law Naeem and Noaman and their families and my sisters in-law Shalabia, Najeba, Afefa, Aida, Hajer, Iman and Huda. You were always close to me and my family.

In Holland: I not only worked for my PhD but I also worked for a great family. Without my family I cannot imagine my life and this PhD would not have been possible. Very deep and especial thanks to my lovely wife Adaweijeh for your continuous encouragement. At last I will thank my lovely daughter Amani and my lovely sons, Osameh, Mahmoud, Ahmed and Hamza. We worked together and our team work at home and in all life aspects resulted in this wonderful achievement.

Said Saleh Hindi Husaineid, April 2007

“Plant photomorphogenesis has clearer than many other fields of plant science demonstrated how complex, advanced and well adapted to their environment our green co-inhabitants of this planet are”

Kendrick, R.E. and Kronenberg, G.H.M. 1994. *Photomorphogenesis in Plants, 2nd Ed*. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Cover illustration: The cover illustrates the shade-avoidance response in high-density populations of wild type tomato plants (top) and transgenic tomato plants (bottom) after eight weeks in the greenhouse (see also Chapter 3, Figure 3.2, page 87).

In memory of my father

To my wife and children

Contents

Abbreviations

A	absorption
A_{535}	absorption at 535 nm
B	blue light
CAB	chlorophyll *a/b*-binding proteins
CaMV	cauliflower mosaic virus
cB	continuous blue light
cFR	continuous far-red light
cR	continuous red light
cry	cryptochrome mutant
cry	cryptochrome holoprotein
CRY	cryptochrome gene
CRY	cryptochrome apoprotein
D	darkness
DW	dry weight
EODFR	end-of-day far-red light
FR	far-red light
FR-HIR	far-red high irradiance response
FW	fresh weight
HIR	high irradiance response
hp	high pigment mutant of tomato
kDa	kilo Dalton, unit of atomic mass
LFR	low fluence response
LA	leaf area
LDW	leaf dry weight
LFW	leaf fresh weight
MAb	monoclonal antibodies
mRNA	messenger RNA
OE	overexpression
PAb	polyclonal antibodies
PAR	photosynthetically active radiation
phy	phytochrome mutant
phy	phytochrome holoprotein
PHY	phytochrome gene
PHY	phytochrome apoprotein
Pr	red light-absorbing phytochrome form
Pfr	far-red light-absorbing phytochrome form
Ptot	total spectrophotometrically detectable phytochrome
R	red light
R:FR	red light: far-red light ratio
RH	relative humidity
R-HIR	red light irradiance response
RT	reverse transcription
RT-PCR	reverse transcriptase-polymerase chain reaction
SAI	shade avoidance index

SAR	shade avoidance response
SDS-PAGE	sodium dodecylsulphate-polyacrylamide
SDW	stem dry weight
SFW	stem fresh weight
SE	standard error of the mean
UV-A	ultraviolet-A (320-400 nm)
UV-B	ultraviolet-B (280-320 nm)
VLFR	very low fluence response
v/v	volume/volume
w/v	weight/volume
WT	wild-type
WL	white light

Chapter 1

General introduction

1.1 Photomorphogenesis

Plants, being rooted in one place cannot escape from an environmental threat and therefore have developed mechanisms that allow them to sense environmental cues and modify their development where they are positioned. Light is important as source of energy for photosynthesis in green plants and plants have evolved with multiple photoreceptors to sense and respond to changes in their light environment. Photomorphogenesis is the way plants process the information about the light environment and modify their growth and development accordingly (Schäfer and Nagy, 2006). Plants develop differently in darkness (skotomorphogenesis) and in the light (photomorphogenesis). Characteristics of dark growth are elongated stems, undifferentiated chloroplasts, and unexpanded leaves (etiolated). Characteristics of light growth are the inhibition of stem elongation, the differentiation of chloroplasts and accumulation of chlorophyll, and the expansion of leaves (de-etiolated). Thus, the same stimulus causes opposite effects on cell elongation in leaves and stems. Photomorphogenesis can be induced by red light (R), far-red light (FR), and blue light (B). Figure 1.1 summarizes the strategies that plants follow throughout their life cycle and the role played by light during each of these processes. The large differences between etiolated and de-etiolated seedlings reflect how dramatic the effect of light can be on plant development. Light also plays a profound role at later stages of plant development, *e.g.* in shade-avoidance responses and in the control of the transition from vegetative to generative growth (flowering). Among other photomorphogenic responses two classes of responses are distinguished: (1) the phototropic responses, which involve the orientation of plant organs with respect to the direction of light; (2) the photoperiodic responses, which involve modification of various aspects of development to changes in the daily light/dark cycle, and involve a circadian timing mechanism (Baurle and Dean, 2006).

1.2 Plant photoreceptors

Plants possess four different types of photoreceptors dedicated to detect parameters of the light environment (Fig. 1.2): the R/FR absorbing phytochromes

(Neff *et al.*, 2000), the B/UV-A absorbing cryptochromes (Ahmad and Cashmore, 1993), phototropins (Briggs and Christie, 2002) and the UV-B absorbing photoreceptors (Beggs and Wellmann, 1994). All photoreceptors consist of a protein to which one or more molecules are coupled which enable light absorption (called chromophores; Davis *et al.*, 2001). A photoreceptor without its chromophore is referred to as the apoprotein and with the coupled chromophore as the holoprotein.

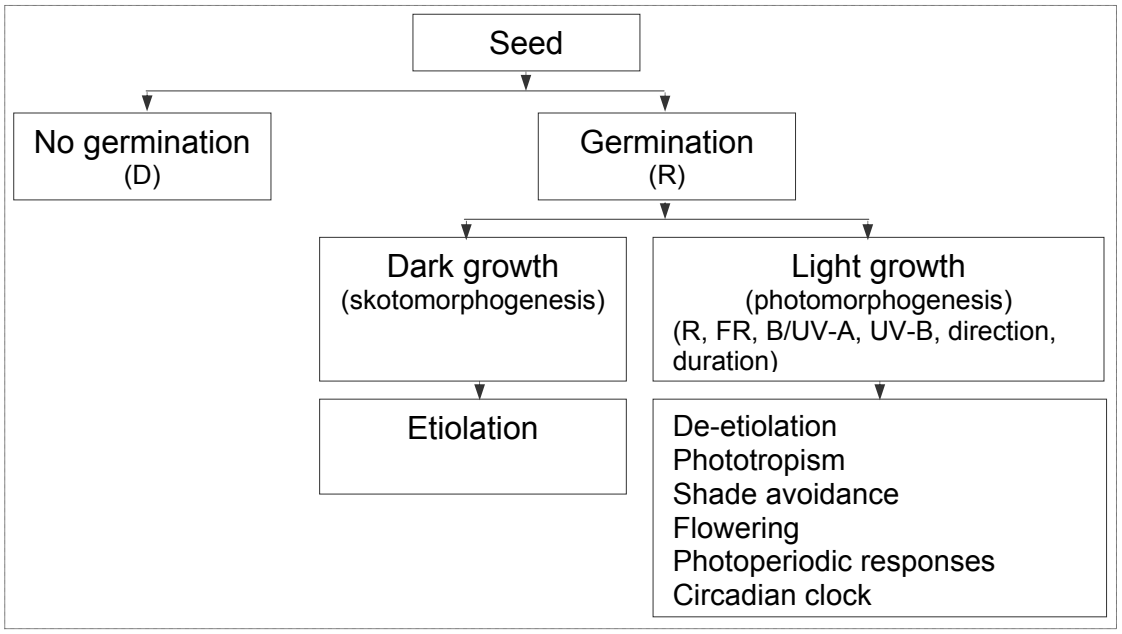


Figure 1.1. The role of light throughout the plant life cycle. D = dark, R = red light, FR = far-red light, B = blue light and UV-A/B = ultraviolet A/B light.

The molecular properties of these photoreceptors enable plants to perceive specific wavelengths of light and transduce this signal to downstream cellular components, which ultimately lead to responses (Quail, 2002a). Figure 1.2 gives a summary of some of the roles played by each type of photoreceptor in the different photomorphogenic responses. The scheme shows that there is substantial overlap in the targets of the signal transduction pathways, indicating that in many cases the action of a specific type of photoreceptor should be seen in the context of interaction and/or co-action with the other photoreceptors. This network of action and co-action is very important for plant survival, especially at the transition from dark to light after germination (de-etiolation) where the selection pressure is very strong.

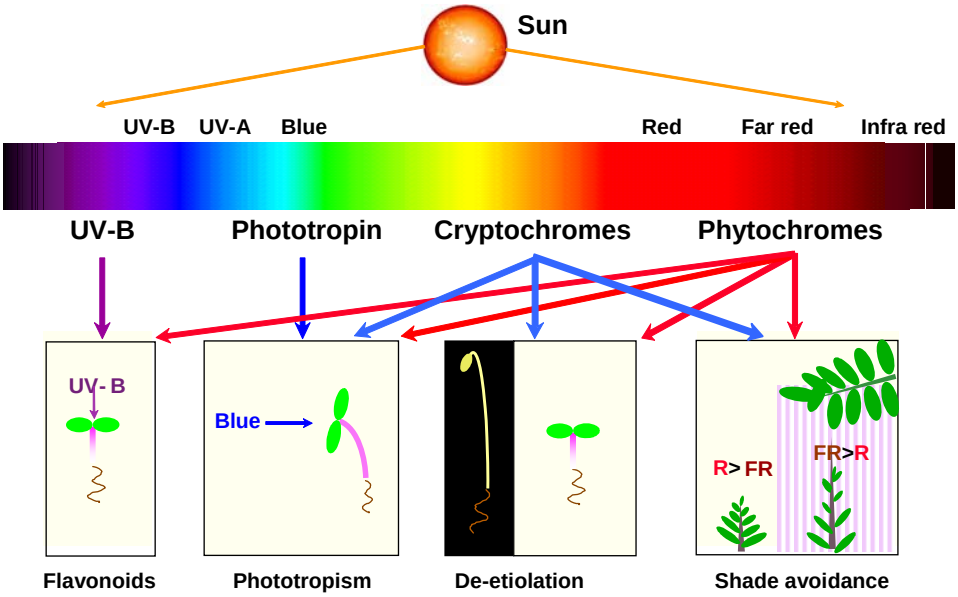


Figure 1.2. Summary of some of the photoreceptors and their co-action in specific responses at different stages of plant development. R = number of red light photons, FR = number of far-red light photons

1.2.1 Cryptochromes

Cryptochromes are B/UV-A absorbing photoreceptors found in both plants and animals. In *Arabidopsis*, three different cryptochrome (*CRY*) genes have been found: cryptochrome 1 (*CRY1*), cryptochrome 2 (*CRY2*; Ahmad and Cashmore, 1993; Lin and Shalitin, 2003) and cryptochrome 3 (*CRY3* or *CRY-DASH;* Kleine *et al*., 2003) which are structurally related to DNA photolyases, but do not possess DNA photolyase activity (Sancar, 2003). The cryptochrome apoproteins are around 75 kDa in molecular mass. Both cry1 and cry2 holoproteins are nuclear proteins, but cry1 may undergo light-dependent translocation to the cytosol. In the case of cry2 it is more or less constitutively localized in the nucleus (Ahmad, 1999; Kleiner *et al*., 1999; Lin and Shalitin, 2003). In tomato, four cryptochrome genes have been characterized: *CRY1a*, *CRY1b* and *CRY2* (Perrotta *et al*., 2001) and *CRY1-DASH* (Facella *et al*., 2006). The proteins encoded by the tomato *CRY1* and *CRY2* show greater similarity to their *Arabidopsis* counterparts than to each other, suggesting that duplication between *CRY1* and *CRY2* is an ancient event in the evolution of seed plants (Perrotta *et al*., 2000). In *Arabidopsis* cry1 is involved in the control of various aspects of plant development, most notably seedling de-etiolation and entrainment of the circadian clock (Lin and Shalitin, 2003), whereas cry2 is involved in the control of flowering time and hypocotyl elongation (El-Din El-Assal *et al*., 2003). In tomato, cry1 plays a role in seedling establishment, anthocyanin

accumulation and adult plant development (Weller *et al.*, 2001). A recent study of tomato lines overexpressing tomato *CRY2* shows that increased levels of cry2 result in a delay in flowering, overproduction of anthocyanin and chlorophyll in leaves and inhibition of hypocotyl and internode elongation (Giliberto *et al.*, 2005). There are numerous studies which indicate a genetic interaction between cryptochrome and phytochrome photoreceptor signalling (Ahmad and Cashmore, 1997; Ahmad *et al.*, 1998; Briggs and Huala, 1999; Cashmore *et al.*, 1999; Duek and Fankhauser, 2003; Lin and Shalitin, 2003; Ward *et al.*, 2005). For example, the interaction between the *Arabidopsis* photoreceptors phyB and cry2 controls flowering time, hypocotyl elongation (Hennig *et al.*, 1999a) and period of the circadian clock (Mockler *et al.*, 1999). This genetic interaction is based on an actual physical interaction between the cryptochrome and phytochrome photoreceptors that is of importance for their function (Ahmad *et al*., 1998; Más *et al*., 2000; Duek and Fankhauser, 2003).

1.2.2 Phototropins

Phototropins are B/UV-A absorbing photoreceptors which mediate phototropism, chloroplast movement and stomatal opening in higher plants (Briggs and Christie, 2002). The phototropins consist of a 120-kDa apoprotein to which a flavin mononucleotide (FMN) chromophore is coupled. Phototropins are characterized by having a serine/threonine (Ser/Thr) protein kinase domain in the C-terminal and two specialized domains designated light, oxygen and voltage (LOV) domains in the N-terminal end (Briggs and Christie, 2002). Two phototropin genes have been characterized in *Arabidopsis PHOT1* and *PHOT2* (Huala *et al*., 1997; Kagawa *et al*., 2001; Kinoshita *et al*., 2001; Sakai *et a*l., 2001). Little is known about the functional interaction between the phototropins and cryptochromes. However, a study of *phot1* and *phot2* mutants showed that cryptochromes may have a slight contribution to the phototropic response (Sakai *et al.*, 2001; Whippo and Hangarter, 2003). The molecular mechanism underlying the functional interaction of phototropins and phytochromes remains unknown. However, a recent study in tomato showed that tomato phyA is required for phototropic responses and this effect of phyA on phototropism is restricted to low fluence rates of B (Lariguet and Fankhauser, 2004; Srinivas *et al.*, 2004).

1.2.3 UV-B photoreceptors

A further group of photoreceptors are those absorbing UV-B radiation. Although the

non-specific effects of UV-B at high irradiance may be attributed to DNA damage, at low irradiances there are specific photomorphogenic responses observed in plants which suggest the presence of specific UV-B photoreceptor(s) (Kim *et al.*, 1998; Ulm *et al.*, 2004 and 2006; Brown *et al.*, 2005). For instance UV-B is an effective inducer of flavonoid or anthocyanin synthesis (Beggs and Wellmann, 1994). Several findings indicate that neither phytochrome nor cryptochrome function as UV-B photoreceptors (Kim *et al.*, 1998) However, the identity of UV-B photoreceptors is still unknown, as are the details of the downstream signal transduction components they activate.

1.2.4 Phytochromes

All higher plant phytochromes consist of an approximately 120-kDa polypeptide (apoprotein) to which a single chromophore is linked to the N-terminal domain, which conveys the R- and FR-absorbing properties of the photoreceptor. The chromophoric group is an open chain linear tetrapyrrole known as phytochromobilin (Quail, 1997). It is synthesised within the (pro)plastids and subsequently transported to the cytosol. The phytochrome apoproteins are synthesized within the cytosol and assemble autocatalytically with the plastid-derived chromophore. Figure 1.3 shows the various functional domains of the phytochrome photoreceptor. The 120-kDa polypeptide consists of several functional domains:

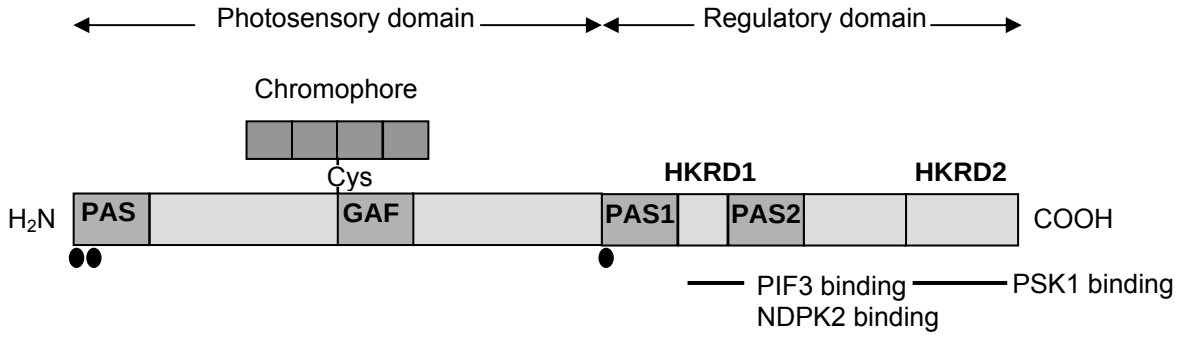


Figure 1.3. Domain structure and assembly of phytochrome. The N-terminal photosensory region contains the PAS domain and the chromophore-binding domain (GAF that harbours the conserved cysteine (Cys) residue) which confers the photosensory specificity to the molecule. The C-terminal domain contains two additional PAS (labelled PAS1 and PAS2) domains, regulatory domains and the histidine-kinase-related domains (HKRD1 and HKRD2). The C-terminal domain transmits signals to proteins that act downstream of the phytochrome. Closed circles are phosphorylation sites.

The N-terminal photosensory domain transmits a signal via nuclear translocation and interacting factors (Oka *et al.*, 2004) and contains a Per-Arnt-Sim (PAS; Kay *et al.*, 1997) domain and a cGMP phosphodiesterase/adenyl cyclase/FhlA (GAF) domain that harbours the conserved cysteine residue to which the chromophore is covalently attached (Sharrock and Mathews, 2006). Together with the GAF domain, other phytochrome motif domains are needed for the photochemical properties of the phytochrome photoreceptor (Fig. 1.3; Montgomery and Lagarias, 2002). The C-terminal domain of phytochrome contains a histidine kinase related domains (HKRD) and two motifs with homology to PAS signal-sensor domains (Sharrock and Mathews, 2006). Phytochrome molecules assemble as homodimers (Vierstra *et al.*, 1984), but recently evidence for the existence of heterodimeric phytochromes in *Arabidopsis* was found (Sharrock and Clack, 2004). A specific feature of the phytochrome photoreceptors is that they can act as photoreversible switches that are activated by R and inactivated by FR. See also Section 1.3.1.

1.3 Phytochrome stability, location and action

1.3.1 Phytochrome interconversion and photoequilibrium

Phytochromes are the most extensively studied and characterized photomorphogenic photoreceptors in higher plants. Early experiments with lettuce seeds showed that a pulse of R could activate germination and that this activation could be blocked by a subsequent pulse of FR. Moreover, several cycles of alternating R and FR result in multiple R/FR reversibility, germination depending on the last wavelength given (Borthwick *et al.*, 1952). The specific photoreceptor that was responsible for this photoreversible response was purified and was named phytochrome (Butler *et al.*, 1959). The first phytochrome cDNA was identified in 1984 by screening of an expression library of oat cDNA, using antibodies raised against purified phytochrome (Hershey *et al.*, 1984). Subsequently, phytochrome genes have been characterized from angiosperms, gymnosperms, ferns, mosses and algae (Schneider-Poetsch *et al.*, 1998). The phytochrome gene consists of four exons of the coding region interrupted by three introns (Lazarova *et al.*, 1998). When phytochromes assemble as holoproteins in the dark, they have an absorption spectrum with a maximum in the R region of the spectrum (called the Pr form). Upon absorption of R the conformation of phytochrome changes resulting in a shift in the absorption maximum to the FR (called the Pfr form). Upon absorption of FR the conformation of Pfr changes back to that of Pr (Quail, 1997). The R/FR reversible responses, which are mediated through phytochromes, are thus

explained by these two photoconvertible forms. The Pr form is thought to be biologically inactive and upon absorption of R is converted to the active Pfr form (Fig. 1.4).

Although the light-absorbing properties of Pr and Pfr are usually given as R and FR, respectively, actually both forms of phytochrome can absorb light of other wavelengths, albeit with lower efficiency. Since the absorption spectra of Pr and Pfr overlap, any wavelength of light establishes a photoequilibrium between Pr and Pfr. The dynamic photoequilibrium that is eventually reached between the Pr and Pfr forms at a given light wavelength is dependent on the difference in absorption efficiency of the two forms of phytochrome for that wavelength. Because of the very high efficiency of R and FR absorption by phytochromes, compared to that of other wavelengths, it is usually the R:FR of daylight (number of photons in the spectral band 655-665nm: number of photons in the spectral band 725-735 nm) that is most important in establishing the phytochrome photoequilibrium. For example, in the daylight the average R:FR is approximately 1.2, under which a photoequilibrium of about 60% Pfr/P_{tot} (P_{tot} = Pr + Pfr) is established (Kendrick and Kronenberg, 1994). Once this equilibrium is established the molecules continue to cycle between the two forms at a rate dependent upon the fluence rate of exposure. The Pfr form can also be reverted with low efficiency to Pr in the absence of light by a process called dark reversion (Butler and Lane, 1965; Fig. 1.4).

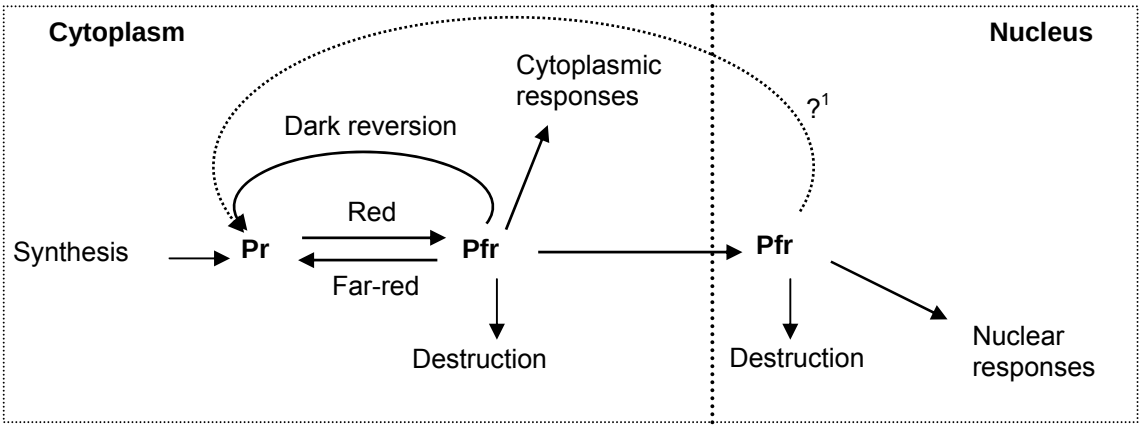


Figure 1.4. Simplified model of phytochrome photoconversion, destruction and localization.
[1]: it is not known whether a dark reversion of nuclear localized Pfr is associated with a translocation to cytosol.

1.3.2 The phytochrome gene family in *Arabidopsis* and tomato

Higher plants contain different phytochrome apoproteins encoded by a small gene

family. In *Arabidopsis* the family of phytochrome photoreceptors is encoded by five genes, designated *PHYA* to *PHYE* (Sharrock and Clack, 2002). Studies on the evolution of these *Arabidopsis PHY* sequences point to four major gene duplication events (Mathews and Sharrock, 1997). The earliest one generated the progenitors of the *PHYA/C* and *PHYB/D/E* types. Later, around the time of the origin of the flowering plants it appears that two more duplications occurred. The *PHYA* progenitor resulted in *PHYA* and *PHYC* types, and the *PHYB* progenitor resulted in *PHYB/D* and *PHYE*. The fourth and most recent duplication-event resulted in the *PHYB* and *PHYD* types. Tomato also contains five phytochrome genes, which have been designated as *PHYA*, *PHYB1*, *PHYB2*, *PHYE*, and *PHYF* (Hauser *et al.*, 1995). The amino acid sequence of the tomato *PHYA*, *PHYB1*, *PHYB2* and *PHYE* reveal an 88–89% identity with their *Arabidopsis* counterparts (Clack *et al.*, 1994). Although the tomato *PHYB1* and *PHYB2* show the highest homology with *Arabidopsis PHYB* and *PHYD*, respectively, they represent an independent duplication of *PHYB* within the Solanaceae (Pratt, 1995).

Phytochrome family members can be classified into two groups based on their stability in light: type I phytochrome is light labile (phyA), due to destruction after conversion to the Pfr form, and type II phytochromes are light stable (phyB-E). The *PHYA* gene, unlike the genes of the other phytochrome family members, has a high level of expression in darkness and the expression of *PHYA* is inhibited by light, resulting in a rapid decrease in *PHYA* mRNA, due to a continuous high degradation of *PHYA* mRNA both in the dark and the light (Hennig *et al.,* 2000). This downregulation of gene activity in the light is actually mediated through phyA and phyB signalling (Quail, 1994). In tomato it was shown that the *PHYB1*, *PHYB2*, *PHYE* and *PHYF* are subjected to relatively weak regulation by light (Hauser *et al.*, 1998). See Section 1.3.4.

1.3.3 Subcellular localization of phytochrome

Plant cell phytochromes can be found in both the cytoplasm and the nucleus. Studies on *Arabidopsis* have shown that in the dark Pr is present in the cytoplasm and upon conversion to Pfr undergoes a light-induced translocation to the nucleus (Nagy and Schäfer, 2002; Nagatani, 2004) where it interacts with signalling partners, inducing changes in the expression of target genes (see Section 1.3.5.3; Khanna *et al.,* 2004). There are also lines of evidence for cytosolic localization and action of Pfr: (1) specific targets of phytochrome-signalling are present in the cytosol, (*e.g.* the protein kinase substrates PKS1 and PKS2, (Lariguet *et al.,* 2003)),

(2) pharmacological studies have shown the involvement of cytosolic heterodimeric G-proteins in the control of phytochrome-dependent gene expression (Neuhaus *et al.,* 1993; Bowler *et al.,* 1994) and (3) some of the responses to light occur very rapidly and do not seem to involve changes in gene expression (*e.g.* changes in transmembrane potential and ion flux), suggesting that some light-signalling pathways may be located entirely in the cytoplasm.

Studies using phytochrome-green-fluorescent protein (GFP) fusions combined with immunochemical in situ localization techniques have shown that *Arabidopsis* phytochromes (Pfr form) have the potential to be imported into the nucleus in a light-quality and light-quantity dependent manner (Sakamoto and Nagatani *et al.,* 1996; Kircher *et al.,* 1999; Yamauchi *et al.,* 1999; Hisada *et al.,* 2000; Kim *et al.,* 2000; Kircher *et al.,* 2002; Huq *et al.,* 2003). Light-regulated phyB nuclear localization is a R/FR reversible response (Kircher *et al.*, 1999) and phyB accumulates in the nucleus as nuclear speckles (Más *et al.*, 2003a). Both the photoactivation and the nuclear import of *Arabidopsis* phyB are required for the biological activity of phyB (Huq *et al.*, 2003). The *Arabidopsis* phyA nuclear import was shown to be controlled by specific conditions of R, FR and B, while the *Arabidopsis* phyB, phyC, phyD, and phyE nuclear localization was regulated by R and WL (Kim *et al.*, 2000; Kircher *et al.*, 2002). Recently Mateos *et al*., (2006) showed that, as is the case for phyB, the N-terminal domain of phyA, when localized to the nucleus, triggered phyA responses. Also for phyC, phyD, and phyE the light-induced nuclear localization was correlated with their respective biological functions (Kircher *et al.*, 2002).

Besides being activated by light for their nuclear localization, another means of regulation seems to be the phosphorylation and dephosphorylation of phytochromes, which may be important as a means of fine tuning the light responsiveness mediated by phytochrome. In support of this idea, dephosphorylation of phyA, mediated by specific phosphatases such as PAPP5 (type 5 protein phosphatase) (Ryu *et al.*, 2005) and FyPP (protein phosphatase 2A) (Kim *et al.*, 2002), enhances plant photoresponsiveness. The molecular basis for these results was explained by an increase of both phyA affinity for its downstream molecular partners and phyA stability upon activation due to dephosphorylation, leading to enhanced photoresponses (Kim *et al.*, 2005; Rubio and Deng, 2005). Thus, the light dependent phosphorylation of one or more amino acid residues in the phytochrome could be a regulatory step for one or several aspects of phyA light

regulation. As for the phytochrome interacting partners, it was recently demonstrated that the induced intracellular phosphorylation of proteins such as PIF3 may represent the primary biochemical mechanism of phytochrome signaling (Al-Sady *et al.*, 2006). These studies demonstrate that this phosphorylation provide a molecular signal that flags the transcription factor for ubiquitination and degradation. Moreover these studies suggest that the phytochrome-induced phosphorylation of PIF3 may initiate rapid migration to nuclear speckles that may function as sites of ubiquitination and/or proteasomal degradation, providing the first direct evidence that phytochrome-mediated transphosphorylation does occur in the cell in a subcellular location which is necessary for phytochrome signaling (Al-Sady *et al.*, 2006).

1.3.4 Temporal and spatial regulation of phytochrome gene expression

Similarities and differences in spatial and temporal patterns of expression of individual members of the phytochrome gene family have been reported for *Arabidopsis* (Somers and Quail, 1995) and tomato (Hauser *et al.,* 1997; Hauser *et al.,* 1998). This could indicate that different phytochromes perform the same function at a different place or time, while redundancy of phytochrome function could occur at sites of overlapping expression. Alternatively, the differences in spatial and temporal expression could reflect differences in function between the members of the phytochrome gene family. Since one or more phytochrome genes are expressed in almost every plant tissue (Somers and Quail, 1995), almost every tissue has the potential for R/FR sensitive responses. The expression of most phytochrome genes is highest in vascular tissue, which may be related to a role for light signalling in phloem loading or unloading. The expression of the *Arabidopsis* phytochrome genes shows diurnal oscillations that persist under continuous light conditions, indicating that the expression of these genes is also under control of the circadian clock (Hall *et al.*, 2001; Toth *et al.*, 2001).

The tomato phytochrome genes are also expressed in almost every tissue, albeit at different levels (Hauser *et al.,* 1997). The *PHYB1* and *PHYB2* genes are expressed at a similar level in most tissues, but the expression of *PHYB2* is substantially elevated relative to *PHYB1* in fruits (Hauser *et al.*, 1997). A clear difference is also seen in their diurnal rhythms of expression in leaves, which differ in phase by about 10 h (Hauser *et al.*, 1998).

Despite a 10-fold down regulation of *PHYA* gene expression in the light, the

PHYA transcript levels in the light are still more abundant than the transcript levels of the other phytochrome genes (tomato: Hauser *et al.*, 1997; *Arabidopsis*: Clack *et al.*, 1994). Apparently, these high *PHYA* mRNA levels in the light are not sufficient for accumulation of high levels of active phyA. This is explained by the rapid destruction of the phyA in the Pfr form (Somers and Quail, 1995; Canton and Quail, 1999; Clough *et al.,* 1999).

1.3.5 Phytochrome signal transduction

Attempts to understand the transduction of light signals from photoreceptor to the different responses in plants has taken different approaches (see 1.3.5.1-1.3.5.5): (1) isolation and analysis of light-response mutants, yielding both photoreceptor-mutants and signal-transduction mutants (Møller *et al.*, 2002; Nagy and Schäfer, 2002; Quail, 2002a); (2) yeast two-hybrid screens to identify phytochrome-interacting proteins as potential primary signalling partners (Ni *et al.*, 1998; Fankhauser *et al.*, 1999; Choi *et al.*, 1999) and characterisation of phytochrome-binding proteins, using other phytochrome-protein interaction assays (Quail, 2002a; Quail, 2002b; Wang and Deng, 2004); (3) pharmacological complementation of phytochrome signalling (Bowler *et al.*, 1994; Okamoto *et al.*, 2001); (4) microarray-based expression profiling of transcriptional responses to R and FR signals (Ma *et al.*, 2001 and 2005; Salter *et al.*, 2003; Monte *et al.*, 2004; Tepperman *et al.*, 2004 and 2006; Jiao *et al.*, 2005; Mazzella *et al.*, 2005). All these studies have shown a complex network of actions and interactions that exist downstream of each phytochrome photoreceptor. Within the downstream network, there is evidence for redundancy, synergistic interaction and antagonism between the different phytochromes, as well as for phytochrome type-specific branches in the signal-transduction network, resulting in altered expression of hundreds of genes leading to specific responses.

1.3.5.1 Isolation and characterisation of light-response mutants

The distinct photomorphogenic responses have been used to screen mutant plant populations for specific impaired light responses. Such screens have yielded photoreceptor mutants, and mutants modified in genes that function in the signal-transduction pathway downstream of the photoreceptors. There are different types of photoreceptor mutants: mutants of the specific photoreceptor apoproteins and mutants which, are deficient in chromophore synthesis, the latter predicted to affect the whole class of photoreceptors. Because of the functional redundancy of some of

the phytochrome family members, not all type-specific phytochrome mutants display an obvious phenotype. There are several examples where only elimination of one or more photoreceptors reveals the role played by remaining members of the family. For example *Arabidopsis* plants deficient in phyD, only exhibit a pronounced phenotype in the phyB-null background (Aukerman *et al.*, 1997). Another example are *Arabidopsis* plants deficient in cry2, which only exhibit a pronounced effect on hypocotyl elongation in the cry1-null background (Aukerman *et al.,* 1997). Similarly, in tomato plants growing under white light (WL) the effect of phyB2 deficiency can only be seen in the phyB1-null background (Weller *et al.*, 2000).

Mutant screens in *Arabidopsis* have also led to the identification of a class of pleiotropic mutants which mimic many aspects of light-grown seedling development when grown in darkness (collectively referred to as *cop*/*det*/*fus* mutants). Like the light-grown seedling phenotype, the *cop*/*det*/*fus* mutants have a short hypocotyl, open cotyledons, and express light-inducible genes when grown in the dark. The *COP*/*DET*/*FUS* gene products thus act as active repressors of photomorphogenesis in darkness. Many of the *COP*/*DET*/*FUS* gene products encode proteins that are part of, or interact with, the SKP1, Cullin and F-box protein (SCF) complex, thus explaining the similar phenotype of these different mutations (Sullivan *et al.*, 2003). This SCF complex targets phytochrome signalling components such as long hypocotyl 5 (HY5; Cluis *et al.*, 2004) and phytochrome interacting factor 3 (PIF3; Bauer *et al.*, 2004; Park *et al.*, 2004; Monte *et al.*, 2004) for destruction by the 26S proteasome.

Tomato light-signalling mutants have been characterized, such as high pigment-1 (*hp1*) and high pigment-2 (*hp2*). The *hp1* and *hp2* mutants have been identified with exaggerated light responsiveness (Kendrick *et al.*, 1997). These mutations are responsible for an up to six-fold increase in R–induced anthocyanin biosynthesis in hypocotyls in *hp1* (Peters *et al.*, 1989) and *hp2* (Peters *et al.*, 1992). The *HP-2* gene was cloned and found to encode the tomato homolog of the *Arabidopsis* nuclear protein *DEETIOLATED1* (*DET1*) (Mustilli *et al.*, 1999). Recently, it was demonstrated that *hp1* is a mutation in a tomato *UV-DAMAGED DNA-BINDING PROTEIN 1* (*DDB1*) homolog, whose *Arabidopsis* counterpart interacts with *DET1* (Levin *et al.*, 2003; Lieberman *et al.*, 2004; Liu *et al.*, 2004). Interestingly, when these genes are mutated in tomato they do not cause a light phenotype in dark-grown seedlings. However, these mutant alleles are probably not null mutations for these loci. The difference between the phenotype(s) of ortholog genes in *Arabidopsis* and tomato indicates that, although plants use similar components for control of light responses, the ‘wiring’, and interactions of these

components may actually differ.

The role of the *Arabidopsis* constitutively nuclear localized transcription factor HY5 in promoting photomorphogenic development has been extensively characterized. HY5 is a basic leucine zipper (bZIP) which acts downstream of the photoreceptor network and activates transcription of light-induced genes, such as chalcone synthase or ribulose-1, 5-bisphosphate carboxylase / oxygenase (RBcS) by binding to the G-box motive in their promoter (Chattopadhyay *et al.*, 1998). Recently it was demonstrated that COP1 functions as an E3 ubiquitin ligase in the nucleus and is responsible for the ubiquitination and targeted degradation of a number of photomorphogenesis-promoting factors, including HY5, phyA, HER1 and SPA1 (Cluis *et al*., 2004) and cryptochromes (Wang *et al*., 2001). It is currently believed that blue light induces a conformational change in cryptochromes, leading to the deactivation of COP1 and thus to rapid accumulation of transcriptional factors such as HY5 (Yi and Deng, 2005). The phytochromes also interact with COP1 *in vitro*, which might inhibit COP1 activity and lead to the accumulation of transcription factors (Kim *et al.*, 2002; Yi and Deng, 2005). In transient assays in onion epidermal cells, it was reported that COP1 co-localized with phyA in the nucleus (Seo *et al.*, 2004). In addition the phyB, phyA and cry1 and cry2 photoreceptors were shown to inhibit the nuclear accumulation of COP1 resulting in increased accumulation of HY5. This was shown to be light-quality dependent: in R, phyB is primarily responsible for the accumulation of HY5, although phyA has a minor role; in FR, phyA and in B, cry1 and cry2 affect the abundance of HY5 (Osterlund *et al.*, 2000).

1.3.5.2 Different and overlapping roles for phytochrome family members in light responses

The roles played by different photoreceptors in response to natural light conditions are complex due to the multiple signals in natural light and interacting signal transduction pathways. The roles played by each phytochrome may be studied under R or FR. In *Arabidopsis,* mutants deficient in each of the five phytochromes have been studied alone, and in various combinations (Franklin *et al.*, 2003; Monte *et al.*, 2003). These studies provide insight into the phytochrome actions and interactions during the different types of phytochrome-mediated responses. For example, phyA plays no obvious role under continuous WL, but plays a dominant role in the germination response and mediates hypocotyl inhibition under FR (Nagatani *et al.*, 1993; Parks and Quail, 1993). Phytochrome A aids in B sensing (Neff and Chory, 1998), and acts in promotion of flowering (Johnson *et al.*, 1994). The phyB type of phytochrome functions in the inhibition of hypocotyl elongation

under R (Koornneef *et al.*, 1980; Reed *et al.*, 1994). Deficiency of phyB also results in pronounced petiole elongation, retarded leaf development, and early flowering phenotypes, which are all characteristic of the shade-avoidance responses (Smith and Whitelam, 1997). Recent microarray expression profiling has revealed that phyA, and not phyB, dominates in transducing continuous R (cR) signals to rapidly responding genes at the initiation of seedling de-etiolation (Monte *et al.*, 2004; Tepperman *et al.*, 2006). Studies on phytochrome-deficient mutants show that phyC is a weak R sensor, with a possible role in B sensing, while phyD functions, together with phyB and phyE in the shade-avoidance responses and light-regulated germination (Franklin *et al.*, 2003).

In tomato, mutants lacking specific phytochrome species such as the phyA-specific *fri* mutant (van Tuinen *et al.*, 1995b), phyB1-specific *tri* mutant (van Tuinen *et al.*, 1995a), and phyB2-specific mutants (Kerckhoffs *et al.*, 1999) have been isolated, characterized and studied alone, in double and in triple mutant combinations (Weller *et al.*, 2000). In these studies it was shown that the loss of phyB2 had a negligible effect on the development of WL-grown wild-type (WT) or phyA-deficient plants, but substantially enhanced the elongated pale phenotype of the *phyB1* mutant. Under continuous R, phyA action was largely independent of phyB1 and phyB2 in terms of the control of hypocotyl elongation, but antagonized the effects of phyB1 in the control of anthocyanin synthesis, indicating that photoreceptors may interact differently in the control of different traits. In the same study, results on the R fluence rate dependency of anthocyanin synthesis revealed that, this response consists of two components: the R-high irradiance response (HIR) component which is mediated by phyB1 and phyB2, and the phyA-mediated low fluence rate component, which is dependent on phyB2. Since specific mutants for any of the other tomato phytochromes have not been characterised, the function of these members of the phytochrome gene family in tomato can only be hinted at by indirect clues. For instance, the *phyA phyB1 phyB2* triple mutant retains residual responsiveness to supplementary daytime FR, indicating that at least one of the two remaining phytochromes plays a significant role in this response.

1.3.5.3 Characterisation of phytochrome-interacting proteins

Attempts to identify primary phytochrome signalling partners using yeast two-hybrid screens have revealed different putative phytochrome protein binding partners. These protein interaction assays have lead to the identification of a group of nuclear localized transcription factors having a basic helix-loop-helix (bHLH) structure: phytochrome-interacting factor 3 (PIF3: Ni *et al.,* 1998); PIF4 (Huq and Quail, 2002);

PIF1 (Huq *et al.*, 2004); PIF5 and PIF6 (Khanna *et al.*, 2004); PIF3-like 1 (PIL; Salter *et al.,* 2003); PIF3-like 5 (PIL5; Salter *et al.,* 2003 and Oh *et al.,* 2004); SPATULA (SPT; Penfield *et al.,* 2005) and long hypocotyl in FR (HER1; Fairchild *et al.,* 2000). Some of these binding partners seem to be shared between phyA and phyB (Ni *et al.*, 1998; Duek and Fankhauser, 2005). It has been shown that PIF1 and PIF3 proteins are stable in dark and are subjected to proteasome-mediated degradation which is dependent on the phytochromes (Bauer *et al.*, 2004; Huq *et al.*, 2004; Monte *et al.*, 2004; Shen *et al.*, 2005). The PIF3 protein can bind to both phyA and phyB. Genetic analysis of *pif3* mutants suggests that it acts as a negative regulator for both R and FR responses (Kim *et al.*, 2000). Overexpression studies with full-length PIF3 show that PIF3 negatively regulates phyB-mediated hypocotyl elongation and cotyledon opening, but promotes both phyA- and phyB-mediated chalcone synthase (CHS) induction (Kim *et al.*, 2003). Bauer *et al.*, (2004) suggested that light acts by enhancing nuclear import of phyA and phyB and promotes their interaction with transcription factors (e.g. PIF3) which triggers a signal transduction cascade that regulates the expression of about 2500 genes in *Arabidopsis*. Recently it was shown that phytochrome-induced phosphorylation of proteins such as PIF3 may initiate its degradation by tagging the transcription factor for degradation by the proteasome (Al-Sady *et al.*, 2006). The differences of affinity of bHLH proteins to phyA and phyB in *Arabidopsis* indicate that these photoreceptors in *Arabidopsis* have discrete and overlapping functions, *e.g.* PIF3 and PIF4 have a 6-fold higher affinity to phyB than to phyA (Huq *et al.*, 2004), whereas PIF1 has higher affinity to phyA than phyB (Martíacute-García *et al.*, 2000). The affinity of PIF1 for phyA is 10-fold higher than that of PIF3 and PIF4. The affinity of PIF1 and PIF3 for phyB is higher than that of PIF4 (Huq *et al.*, 2004). The differences in the interaction of phyA and phyB proteins and these PIF proteins in *Arabidopsis* indicate that, although both phyA and phyB use similar components for control of light responses, the level of the responses mediated by phyA and phyB can be very different.

Intense research efforts have identified many positive and negative components in the phyA and phyB signalling network in *Arabidopsis* (Chen *et al.*, 2004; Franklin and Whitelam, 2004; Wang and Deng, 2004). These intermediates were classified in three groups: (1) phyA signalling intermediates such as FHY1, FHY3 (Whitelam *et al.*, 1993; Hiltbrunner *et al.*, 2005), SPA1 (Hoecker *et al.*, 1998), FAR1 (Hudson *et al.*, 2003), FIN219 (Hsieh *et al.*, 2000), PAT1 (Bolle *et al.*, 2000), EID1 (Buche *et al.*, 2000), HFR1/RSF1/REP1 (Fairchild *et al.*, 2000; Fankhauser and Chory, 2000; Soh *et al.*, 2000), LAF1 (Ballesteros *et al.*, 2001) and LAF3 (Hare

et al., 2003); (2) phyB signalling intermediates which includes GI (Huq *et al.,* 2000), ELF3 (Liu *et al.,* 2001), ELF4 (Doyle *et al.,* 2002; Khanna *et al.,* 2003) and SRR1 (Staiger *et al.,* 2003); (3) both phyA and phyB signalling intermediates including PEF1 (Ahmad and Cashmore, 1996), PRR7 (Kaczorowski and Quail, 2003) and PFT1 (Cerdan and Chory, 2003).

Other phytochrome partners have been identified, such as PKS1 which is a cytoplasmic substrate for the phytochrome kinase activity of both the phyA and phyB protein (Fankhauser *et al.*, 1999) and a related protein PKS2 which has been shown to provide homeostasis to phyA signalling (Lariguet *et al.,* 2003). Plants overexpressing the *PKS1* gene showed reduced sensitivity to WL and R, suggesting that this gene functions as an inhibitor of phyB signal transduction (Fankhauser *et al.*, 1999). Besides these protein kinase substrates the enzyme nucleoside-diphosphate kinase 2 (NDPK2) was identified as a phytochrome-interacting protein using yeast two-hybrid screens. The enzyme NDPK2 was isolated as binding to the C-terminal domain of *Arabidopsis* phyA (Choi *et al.*, 1999; Im *et al.*, 2004). The NDPK2 is catalytically activated in the presence of the active Pfr phytochrome and appears to exert a positive effect on cotyledon unfolding and greening responses elicited by light and phytochromes (Choi *et al.*, 1999, Shen *et al.*, 2005). In their study Choi *et al*, 1999 show that the *ndpk2* mutant has shorter hypocotyl under continuous far-red light (cFR), consistent with the functional role in phyA signalling. The NDPK2 protein appears to localize to both nucleus and cytoplasm (Zimmerman *et al.*, 1999).

Studies of the involvement of phytochrome in B-signalling have shown considerable physiological and genetic evidence of interaction between the phytochrome and the cryptochrome signalling pathways in *Arabidopsis* (Más *et al.*, 2000; Neff *et al.*, 2000) and tomato (Weller *et al.*, 2001). In *Arabidopsis*, functional interaction between phyB, phyA and cry1 was demonstrated to control hypocotyl elongation and accumulation of anthocyanin and chlorophyll (Ahmad *et al.*, 1998; Neff and Chory, 1998). Evidence for phyB and cry2 interaction comes from co-immunoprecipitation experiments using plant extracts and from fluorescence resonance energy transfer (FRET) in plant cells (Más *et al.*, 2000). However, several groups have shown that the *phyAphyB* double mutant retains cry1-mediated responsiveness to B (Neff and Chory, 1998; Poppe *et al.*, 1998). Seedlings of the *cry1* mutant retain a normal anthocyanin accumulation response to low irradiance B. In an earlier section (1.3.3) we outlined the importance of the subcellular localization of phytochrome for its activity. It is thought that the direct physical interaction between phytochrome and cryptochrome is required for their activity. This

interaction, which could affect the activity of cryptochromes, would explain why the reported inhibition of hypocotyl elongation under B mediated by cryptochrome is dependent on the presence of phyA and phyB (Ahmad and Cashmore, 1997).

In tomato it was shown that both phyA and phyB1 contribute to B-induced anthocyanin accumulation (Kerckhoffs *et al.*, 1997) and inhibition of hypocotyl elongation (van Tuinen *et al.*, 1995a; van Tuinen *et al.*, 1995b). However, hypocotyl elongation and anthocyanin accumulation was retained in the *phyA phyB1* double mutant under B conditions (Kerckhoffs *et al.*, 1997), as a result of the co-action of other phytochromes and the cryptochromes.

1.3.5.4 Pharmacological complementation of phytochrome signalling

The tomato *aurea* mutant (Koornneef *et al.*, 1985) contains less than 5% of WT levels of phytochrome in etiolated seedlings due to a mutation in chromophore synthesis. This mutant has been used to study putative light signal-transduction intermediates, resulting in the identification of several components that are necessary for the light signal transduction (Neuhaus *et al.*, 1993). Micro-injection of oat phyA into hypocotyl cells of the *aurea* mutant rescued the mutant phenotype, resulting in a development of mature chloroplasts and accumulation of anthocyanin (Bowler *et al.*, 1994). In other complementation studies, cyclicGMP was found to mediate phyA-dependent anthocyanin biosynthesis and to participate, together with Ca^{2+} and Ca^{2+}/Calmodulin, in co-ordinating chloroplast development (Bowler *et al.*, 1994). Under similar conditions, micro-injection of oat phyB did result in development of mature chloroplasts, but did not induce anthocyanin accumulation (Kunkel *et al.*, 1996). Moreover, it was demonstrated that activators of heterotrimeric G (htG) proteins, and calcium (or activated Calmodulin) could trigger the same response, indicating that htG proteins and calcium are intermediates in the phytochrome-signalling pathway (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994; Okamoto *et al.*, 2001). A study by Jones *et al.*, (2003), using null mutants of *Gα* and *Gβ* subunits and a *Gα Gβ* double mutant, indicates that htG proteins play no direct role in the R and FR mediated inhibition of *Arabidopsis* hypocotyl growth. Moreover, in these experiments overexpression of the WT and constitutively active *Gα* subunit did not lead to altered light sensitivity (Jones *et al.*, 2003). It is known that the phytochrome signalling is a highly branched pathway; htG proteins apparently only affect a subset of the phytochrome responses.

1.3.5.5 Microarray-based analysis of transcriptional phytochrome responses

Besides direct cytoplasmic responses, many of the responses to phytochrome activation involve changes in gene expression (Tepperman *et al.*, 2001, 2004 and 2006; Casal and Yanovsky, 2005). Prominent examples of genes which have been used in many studies to follow phytochrome signalling, are the genes encoding light-harvesting components (*e.g.* chlorophyll *a/b*-binding protein: *CAB*), carbon fixation (*e.g.* small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase: *RBcS*) and anthocyanin biosynthesis (*e.g.* chalcone synthase: *CHS*). Some of the phytochrome responses also involve down regulation of gene activity, *e.g.* lipoxygenases, and nitrate reductase. In addition, expression of the *PHYA* gene itself is repressed through light signalling that involves both phyA and phyB (Kuno and Furuya, 2000).

With the development of microarray-based expression profiling, the response of the entire genome transcript level to phytochrome activation can now be followed. Results of such microarray experiments show that hundreds of genes change expression in response to R or FR treatment to dark-grown seedlings (Fankhauser and Staiger, 2002; Quail, 2007). By performing this transcription profiling of R or FR responses in the different phytochrome mutants, the overlap and differences in the specific phytochrome-signalling pathways can be identified. Microarray experiments of transcriptional responses to treatments that mimic the shade-avoidance response (EODFR treatment) have shown that PIL1, HFR1 and TOC1 proteins are all involved in the accelerated growth associated with the shade-avoidance response (Devlin *et al.*, 2003; Salter *et al.*, 2003; Sessa *et al.*, 2005). The PIL1 transcription factor shows an over 50-fold increase in expression within minutes in response to an end-of-day FR (EODFR) treatment, and seems to be a key regulator of the shade-avoidance response (Salter *et al.*, 2003). Whether phytochromes also directly bind to PIL1 is not known. However, PIF1, PIF3 and PIF4, a group of BHLH proteins to which PIL1 belongs, were reported to bind to phyA and phyB in *Arabidopsis* with different affinity (Huq *et al.,* 2004); see Section 1.3.5.3).

1.4 Ectopic expression of phytochrome genes in plants

The cloning of phytochrome genes from various plant species has allowed the creation of transgenic plants with ectopic expression of homologous or heterologous phytochrome genes. Such plants provide novel ways to study phytochrome action such as specific aspects of dimerization, *in vivo* light absorption properties and

phytochrome stability. Several factors influence the phenotypic effect that may result from the overexpression of a phytochrome gene: (1) the strength and cell specificity of the promoter that drives phytochrome transgene expression. In most studies the constitutive *35S*-promoter from the cauliflower mosaic virus (CaMV) is used, which may not only result in higher than normal expression level of the phytochrome, but also in expression in tissues where this phytochrome is normally absent. (2) The availability of chromophore for the assembly of phytochrome holoprotein. For instance, ectopic expression of a phytochrome gene in cells in which no or only very low levels of chromophore are available will have no or only limited effects. (3) The specific combination of ectopic expressed phytochrome gene and host plant. When the ectopic expression of a phytochrome gene is in the homologous host (*e.g.* tomato phytochrome genes in tomato) one can assume that the extra phytochrome is recognized by all endogenous phytochrome-interacting proteins, and thus potentially all branches of the phytochrome-signalling pathway can be affected. Moreover, it is assumed that also the endogenous degradation machinery efficiently recognizes a homologous phytochrome. In contrast, overexpression of a heterologous phytochrome may show limited or even different interactions with the set of phytochrome interacting proteins in the new host. Differences in the affinity for the phytochrome- binding partners in the host may result in differential effects on branches of the phytochrome-signalling network. For instance, this may lead to higher stability of the heterologous phytochrome in its new host, due to reduced recognition by components of the phytochrome-degradation machinery. (4) The dominant negative effect of the introduced phytochrome, either by silencing of the endogenous WT gene or by inactivation of the WT phytochrome due to a 'non-productive interaction' of the accumulated phytochrome with cellular reaction partners.

1.4.1 Ectopic expression of *PHYA*

There are numerous publications on the effect of ectopic expression of *PHYA*, both in dicot and monocot plant species, in order to test the phyA function in more detail. In these studies the *PHYA* was studied in heterologous and homologous systems. The heterologous oat *PHYA* has been expressed in tobacco (Keller *et al.,* 1989; McCormac *et al.,* 1992; Ballaré *et al.,* 1994; Casal and Sánchez, 1994; Casal *et al.,* 1995; Robson *et al.,* 1996; Halliday *et al.,* 1997; Mazzella *et al.,* 1997), in *Arabidopsis* (Boylan and Quail, 1991; Whitelam *et al.,* 1992; Boylan *et al.,* 1994), in tomato (Boylan and Quail, 1989; McCormac *et al.,* 1992; Casal *et al.,* 1995) and in

potato (Boylan and Quail, 1989; McCormac *et al.,* 1992; Casal *et al.,* 1995); compare Table 5.1. Also the heterologous oat *PHYA* has been expressed in the monocots rice (Clough *et al.,* 1995; Casal *et al.,* 1996) and in wheat (Shlumukov *et al.,* 2001). In addition the heterologous *Arabidopsis PHYA* has been expressed in rice (Clough *et al.,* 1995; Casal *et al.,* 1996), the heterologous rice *PHYA* has been expressed in tobacco (Clough *et al.,* 1995; Casal *et al.,* 1996) and the heterologous rice *PHYA* has been expressed in *Arabidopsis* (Halliday *et al*., 1999). On the other hand the homologous tobacco *PHYA* has been expressed in tobacco (Sharkey *et al*., 1991), the homologous potato *PHYA* has been expressed in potato (Heyer *et al*., 1995; Yanovsky *et al*., 1998 and 2000) and the homologous *Arabidopsis PHYA* has been expressed in *Arabidopsis* (Casal *et al*., 1996). In some cases overexpression of *PHYA* results in a strong suppression of hypocotyl elongation under cFR (McCormac *et al*., 1992; McCormac *et al*., 1993). However, in contrast to the role of endogenous phyA under cR or WL, overexpression of *PHYA* results in short hypocotyl phenotype and extreme dwarfism in adult plants under cR or WL (Boylan and Quail, 1989; Boylan and Quail, 1991; Boylan *et al*., 1994; Robson *et al*., 1996). This was explained as an effect of persistence of high levels of the heterologous phyA protein in the green transgenic plants as a result of the ectopic expression of the foreign gene driven by the *35S* promoter and reduced recognition by the degradation machinery.

1.4.2 Ectopic expression of *PHYB*

The B-type phytochrome genes from several plant species have been used for overexpression in both homologous or heterologous host plants (Wagner *et al*., 1991 and 1996; McCormac *et al*., 1993; Jackson *et al*., 1996; Halliday *et al*., 1997; Jordan *et al*., 1997; Clough *et al*., 1999; Hennig *et al*., 1999b; Thiele *et al*., 1999; Casal *et al*., 2000b; Zheng *et al*., 2001; Wallerstein *et al*., 2002; Boccalandro *et al*., 2003; Sharrock *et al*., 2003; Fernandez *et al*., 2005; Zheng *et al*., 2006); compare Table 5.1. In most instances the phenotypes conferred by overexpression are consistent with exaggeration of the normal activities of these photoreceptors, indicating that phyB signalling levels are normally limiting for most responses (Robson and Smith, 1997; Boccalandro *et al*., 2003; Sharrock *et al*., 2003). Overexpression of *Arabidopsis PHYB* in potatoes resulted in a modified plant architecture, increased photosynthetic performance and increased tuber yield (Boccalandro *et al*., 2003; Schittenhelm *et al*., 2004). Overexpression of tobacco *PHYB1* in chrysanthemum results in shorter plants with greener leaves compared to

WT (Zheng *et al.*, 2001). Overexpression of tomato phytochrome genes (*PHYB1* or *PHYB2*) in *Arabidopsis* plants with phyB null background rescued the *phyB* phenotype indicating that both tomato genes are functionally related to *Arabidopsis PHYB*. Ectopic expression of these tomato *PHYB1 and PHYB2* genes resulted in an exaggerated response to R in *Arabidopsis* WT plants (R. Kok, pers. comm.).

1.4.3 Ectopic expression of other *PHY*-genes

Arabidopsis PHYC was overexpressed in *Arabidopsis* (Qin *et al.*, 1997) and resulted in plants with larger primary leaves. Similarly, overexpression of the *Arabidopsis PHYC* gene in tobacco also affected leaf development and resulted in plants with significantly larger mature leaves (Halliday *et al.*, 1997). No clear phenotype was reported when *Arabidopsis PHYD* was overexpressed in *Arabidopsis* (Sharrock *et al.*, 2003). Ectopic expression of *PHYD* in an *Arabidopsis phyB* mutant background failed to reduce any aspect of the mutant *phyB* related phenotype. In contrast, ectopic expression of *Arabidopsis PHYE* resulted in enhanced hypocotyl elongation responses, both in WT and in an *Arabidopsis phyB*-mutant background (Sharrock *et al.*, 2001; Sharrock *et al.*, 2003). These results indicate that phyE, but not phyD, can substitute for phyB in *Arabidopsis*.

1.5 Phytochromes and time sensing

In order to give an appropriate response to the onset of the daily photoperiod and overall length, plants not only need photoreceptors to detect the light, but also some kind of time-measuring mechanism, to measure the daily duration of illumination and/or darkness. Time is measured in plants by an endogenous circadian clock and many processes are regulated by this mechanism (hypocotyl growth, cotyledon movement, leaf conductivity, gene expression). Clock regulation of processes results in anticipation of changes in the light environment and optimises growth and development in the 24-h day/night cycle (Dodd *et al.*, 2005). Effects of the circadian clock on gene expression have been monitored in planta using the promoter of a chlorophyll *a/b*-binding protein fused to the luciferase reporter gene (*CAB::LUC*) (Millar *et al*., 1995; Devlin and Kay, 2000; Hall *et al*., 2001; Toth *et al*., 2001; Hall *et al*., 2002; Más *et al*., 2003a). Using this reporter it has been shown that the periods of the circadian clock are affected by light intensity or phytochrome-signalling. Reduced light perception and reduced illumination have similar effects on the period length of the circadian clock: under normal light and phytochrome conditions the

period of the clock is around 24 h, whereas, under reduced light or with impaired light perception by phytochromes the period length increases and approaches that of full darkness (or absence of light perception by photoreceptors), a period length of over 30 h (Devlin and Kay, 2000; Toth *et al*., 2001; Hall *et al.*, 2002; Más *et al.*, 2003b; Salome and Mcclung, 2005). Several molecular components for the plant circadian clock have been identified (reviewed in Salome and Mcclung, 2005). The clock consists of a set of positive and negative feedback regulation networks of specific transcription factors. The expression of these factors is influenced by phytochrome signalling, resulting in a daily phytochrome-mediated entrainment of the clock (Somers *et al.*, 1998; Salome and Mcclung, 2005; Baurle and Dean, 2006). In turn, the circadian clock contributes to a cyclic expression of phytochrome genes and cyclic light responses, resulting in a phenomenon named “gating of the light response” (Salter *et al.*, 2003). This gating is, for instance, important for the shade-avoidance response (see Section 1.8).

1.6 Phytochrome-mediated photomorphogenic responses to light

Phytochrome-mediated responses to light can be grouped in different ways: by light induction, by photoperiod length, or by light quality (R:FR). Light-induced responses are divided into (1) very low fluence responses (VLFR) (2) low fluence responses (LFR) and (3) high irradiance responses (HIR). The VLFR responses are mediated by phyA and are not R/FR reversible, since even FR can elicit enough Pfr to induce the response. The VLFR are established in situations in which all phytochrome is present in Pr form (e.g in a dark-grown seedling) and the role of phytochrome in these cases apparently is to sense the quantity of light rather than its quality. The LFR, which can be induced by a short pulse of low irradiance R, shows the classical R/FR reversibility. The LFR are mediated by phytochromes other than phyA. The relationship between LFR and Pfr/Ptotal is logarithmic, and saturation of the response often occurs at low levels of Pfr/Ptotal (Smith, 1982). The HIRs require continuous irradiation of R (R-HIR) or FR (FR-HIR) and therefore they are not R/FR reversible (Casal *et al.*, 2003). The fluence-rate dependency of HIRs is usually explained by the fluence rate dependent phytochrome cycling between Pr and Pfr and it seems that under these conditions the phytochrome acts as a photon counter.

The photoperiod responses are usually divided into short-day and long-day responses. A typical response to photoperiod treatment of plants is flowering; some plant species only flower when short days (long nights) are given (*e.g.*

chrysanthemum), other species show the opposite response, and flower only under long days (short nights) (*e.g.* lettuce). Some plants form flowers regardless of daylength (day neutral plants, *e.g.* tomato). Because the length of the circadian period in darkness is measured by an endogenous circadian clock, which in turn is entrained by phytochrome signals, phytochromes also influence the photoperiodic responses. Finally, responses that are related to changes in R:FR are the EODFR (Smith, 2000) and the shade-avoidance response (see Section 1.8).

1.7 Phytochrome signalling and plant hormones

Alterations in hormone levels and hormone sensitivity have been implicated in some of the light-growth responses. However, little is known about the mechanisms through which the phytochromes control changes in elongation growth. It is possible that such a mechanism involves changes in the local levels of one or more plant hormones at some point. Gibberellins (GAs) are known to regulate phytochrome-mediated shoot elongation (Weller *et al*., 1994; López-Juez *et al*., 1995). In their study, López-Juez *et al*. (1995) showed that the growth of seedlings whose endogenous GA biosynthesis is inhibited by uniconazole and the differential response of those seedlings to the application of exogenous GAs in the presence or absence of active phytochrome, are best described as an altered GA responsiveness controlled by phytochrome. This means that GAs must be present to drive cell elongation and that the extent of such GA-driven growth is modulated by the current phytochrome status in which GAs act as a driving force for elongation, and Pfr acts as a brake. GAs also play a role in the repression of photomorphogenesis when plants are grown in dark (Alabadi *et al.*, 2004). GA inhibitors and *gal-3* mutant seedlings grown in the dark have light grown seedling phenotypes such as shorter hypocotyls and expanded cotyledons (Alabadi *et al.*, 2004). Recently it was demonstrated that phytochromes regulate GA metabolism as well as abscisic acid metabolism during seed germination in *Arabidopsis* (Seo *et al.*, 2006, Oh *et al.*, 2006). In addition it was shown that phyA and/or cry1 act redundantly in pea to induce the rapid down regulation of GA levels after transfer to B (Foo *et al.*, 2006b). The study of the involvement of auxin in the EODFR response in the stem of pea and *Rumex palustris* plants suggests that auxin also might be involved in some photomorphogenic responses (Behringer *et al.*, 1992; Cox *et al.*, 2004). Exogenous application of auxin can mimic light-related phenotypes, such as an increase in cell elongation in the hypocotyl and a reduction in cotyledon size

(Saibo *et al.*, 2003). In conjunction with auxin, it appears that the hormone ethylene is also involved in adult plant responses such as the shade-avoidance response. Ethylene is rapidly produced by *Arabidopsis* rosette leaves in response to decreased light intensities (Vandenbussche *et al.*, 2003). It was also shown that ethylene-insensitive plants have reduced leaf angle responses to neighbouring vegetation (Pierik *et al.*, 2004). Another piece of evidence shows that transgenic ethylene-insensitive tobacco (*Nicotiana tabacum*) plants have reduced shade-avoidance stem-elongation in response to neighbours (Pierik *et al.*, 2003). Both experiments indicate that ethylene affects growth responses, while it was also shown that low R:FR enhances ethylene production in tobacco (Pierik *et al.*, 2004). Recently Foo *et al*., (2006a) investigated the role of ethylene in pea (*Pisum sativum*) mutant plants. This study demonstrates that phyA appears to play a role in the regulation of ethylene production, as phyA mutant plants exhibit a phenotype consistent with elevated ethylene production.

1.8 Near-neighbour, shade-avoidance and end-of-day responses

The success of competitive interactions between plants determines the chance of survival of individuals and eventually of whole plant species. Shade-tolerant plants have adapted their photosynthesis to function optimally under low-light conditions (Franklin and Whitelam 2006). These plants are therefore capable of long-term survival under the shade cast by other plants. In contrast, shade avoiders adapt their growth to catch maximum sunlight and therefore rapidly dominate gaps in a canopy. This latter adaptation is achieved by a set of responses including enhanced internode and petiole extension growth, increased apical dominance, retarded leaf development, and an acceleration of flowering. These physiological adaptations are accompanied by, or result in changes in the distribution of assimilates between leaves, stems, and roots (Halliday *et al.*, 1997; Smith and Whitelam, 1997). In order for the plants to precisely measure changes in the light environment they should have photoreceptors which have the capacity to measure these changes (Vandenbussche *et al.*, 2005). Daylight contains roughly equal proportions of R and FR (R:FR = ~1.2), but within vegetation that ratio is lowered as a result of the R absorption by photosynthetic pigments. In an open environment, the R:FR of light reflected from neighbours is an accurate signal of neighbour proximity (Ballaré *et al*., 1987; Ballaré *et al*., 1990; Gilbert *et al*., 2001; Vandenbussche *et al.*, 2005). Plants have sophisticated sensing mechanisms operating through the

phytochromes that perceive the R:FR as an indicator of neighbour proximity. The roles played by the phytochromes in shade-avoidance responses have been investigated in *Arabidopsis* and tomato. In *Arabidopsis,* multiple mutant analyses have revealed that the perception of low R:FR in *Arabidopsis* is mediated solely by phyB, D and E, acting in a functionally redundant manner (Devlin *et al.*, 1998; Devlin *et al.*, 1999; Franklin *et al.*, 2003).

In tomato, WL-grown WT plants respond to supplementary FR both by increased stem elongation and by reduction of anthocyanin accumulation (Fig. 1.5). The physiological roles of phyA and phyB have been characterised by transgenic and mutant analysis (Kendrick *et al.*, 1997). In tomato, phyB1 contributes strongly to de-etiolation under continuous R and thus has a similar role as *Arabidopsis* phyB (van Tuinen *et al.*, 1995a). However, *phyB1* mutants still respond strongly to EODFR and supplementary daytime FR (van Tuinen *et al.*, 1995b). Tomato phyB2 acts redundantly with phyB1 to control development (Weller *et al.*, 2000). Weller *et al.* (2000) showed that tomato triple mutant plants lacking phyA, phyB1, and phyB2 still retained a strong residual response to supplementary daytime FR, indicating that at least one of the two remaining phytochromes plays a significant role in tomato photomorphogenesis.

WL WL+ FR

Figure 1.5. Shade-avoidance response of wild-type tomato seedlings simulated by addition of supplementary far-red light (FR) to the white light (WL) source, lowering the photon ratio of red to far-red from 6.3 to 0.1 (Weller *et al.*, 2000).

Downstream of the phytochromes, information about the components involved in the SAR control is limited. In section 1.3.5.5 we discussed the use of microarray technology in characterizing transcription factors such as *PIL1* and *HFR1*, which are quickly and reversibly regulated by simulated shade (Devlin *et al.*,

2003; Salter *et al.*, 2003; Sessa *et al.*, 2005). It is possible that the PIL1 and HFR1 are candidate factors potentially representing the entry point for the phytochrome signalling into the shade-modulated transcriptional cascade. Oligo-array analysis, aimed to investigate the transcriptional response in *Arabidopsis* to EODFR treatment showed that the circadian clock gates the rapid shade-avoidance response to this treatment (Salter *et al.*, 2003).

Phenotypically similar growth responses to those described for SAR can be observed when plants are submerged in water (Pierik *et al.*, 2005). The resemblance in the morphological and biochemical response to low light and submergence might be explained by separate signal-transduction cascades that both affect a common downstream component (Voesenek *et al.*, 2006). However, the study of Mommer *et al*., (2005) demonstrates the possibility of additive effects when both low light and submergence signals are present. Submerged plants growing in water experience both low light intensities and reduced gas exchange (Voesenek *et al.*, 2006). In order to restore contact with the atmosphere, plants growing under water try to bring the leaf tips closer to the water surface. This avoidance response is similar to the situation in which plants try to bring leaves closer to the light which enhances plant performance and fitness (Schmitt *et al.*, 2003). It was also shown that ethylene-insensitive plants have reduced leaf angle responses to neighbouring vegetation (Pierik *et al.*, 2004). Recently Foo *et al*., (2006a) investigated the role of ethylene in pea (*Pisum sativum*) mutant plants. This study demonstrates that phyA appears to play a role in the regulation of ethylene production, as phyA mutant plants exhibit a phenotype consistent with elevated ethylene production. The study of Mommer *et al*., (2005) demonstrates that for the photosynthetic acclimation the responses of SAR and submergence are not the same. Moreover, the R:FR increases in submerged environment because water specifically absorbs the longer wavelengths suggesting that the submergence-induced response takes place under conditions under which SAR can not be induced.

1.8.1 Manipulation of the shade-avoidance response

Genetic manipulation of many different target genes have been aimed at bringing about marked and sustainable increases in crop yield and quality (Boccalandro *et al*., 2003; Davuluri *et al*., 2004; Liu *et al*., 2004; Giliberto *et al*., 2005). Experiments aimed at testing the hypothesis that the shade-avoidance response is an adaptive phenotypic response, using plants with phenotypes manipulated by altering R:FR

(Dudley and Schmitt, 1996) or using phytochrome mutants and phytochrome overexpressing plants (Schmitt *et al.*, 1995), demonstrated that elongated plants were more fit at high density and suppressed plants were more fit at low density (Schmitt *et al*., 1995; Dudley and Schmitt, 1996; Schmitt *et al*., 2003). In other experiments, which aimed at improving tomato yield under field conditions using plastic mulches of different colours, which selectively reflect different wavelengths of light into the plant canopy (changing R:FR), it was demonstrated that this method can reduce the negative effects of low R:FR and results in better plant production in the field at little extra cost to the grower (Kasperbauer, 1992; Kasperbauer and Hunt, 1992 and 1998; Kasperbauer and Loughrin, 2004). In their study Robson *et al.* (1996) show that the transgenic overexpression of oat *PHYA* in tobacco suppresses the shade-avoidance response. This causes proximity-induced dwarfing (conditional dwarfing). When the transgenic plants are grown at high densities in the field this enhances allocation of assimilates to leaves resulting in an increase in the leaf harvest index. It demonstrates that transgenic suppression of shade avoidance provides the potential to modify crop plant architecture conditionally upon density caltivation. In potato plants the overexpression of *Arabidopsis PHYB* increased branching and tuber number independent of plant density (Boccalandro *et al.*, 2003) and reduced plant height. In rice plants the overexpression of *Arabidopsis PHYA* under the rice *rbcS* promoter resulted in shorter plants and increases grain yield (Garg *et al.*, 2006). These examples demonstrate that manipulation of the shade-avoidance response is an attractive approach to manipulate horticultural production and provides a reliable means to reduce crop height.

Since shade avoidance results in allocation of resources to parts of the plant both harvestable and non-harvestable, any benefit for growers from manipulation of the shade-avoidance response in a crop plant will depend on the plant concerned and the part of plant to be harvested. Significant improvement in crop yield might be achieved by a better understanding of the roles phytochromes play in the regulation of responses to the light environment.

1.9 Greenhouse tomato production

Tomato is a member of the family Solanaceae that includes several additional economically important crops such as potato, pepper and eggplant and as such represents the most valuable plant family in terms of vegetable crops with important contributions to human health and nutrition. In terms of human health, tomato fruit is

a major component of daily meals and constitute an important source of minerals, vitamins and antioxidant compounds. Tomato is one of the most important greenhouse vegetable crops grown in the Netherlands. To meet the future challenge for a sustainable glasshouse tomato production, a steady annual increase of greenhouse tomato yield is a real challenge for physiological research. However the greenhouse tomato production in the Netherlands is one of the main greenhouse heat energy sinks and therefore of environmental concern. More efficient energy use in greenhouse production can be achieved through increase of plant production using the same energy input or/and decrease in energy input with the same production. Besides growing tomatoes for commercial purposes in the greenhouse in such a way that, the grower gets the largest return compared to the total annual cost is very important. The largest return to the grower can be achieved by: (1) Improvement of the fruit quality in order to meet the increasing demand of consumers in a highly competitive fresh market and (2) Enhancement of the efficiency of a crop in a greenhouse in order to increase tomato yield. Enhancement of the energy efficiency in production of greenhouse tomato plants may be achieved by manipulation of the shade-avoidance response, leading to a larger investment of the plant in leaves or fruits and enabling more plants to be grown per unit area.

1.10 Outline of the thesis

This thesis reports on the manipulation of phytochrome levels in tomato to investigate the effect on growth and development, with special attention to the shade-avoidance responses. Suppressing or changing the shade-avoidance response in tomato may enable denser cultivation in greenhouses and thus have a favourable effect on total energy cost when calculated as crop-yield per unit area. Tomato has become a well- established model species complementary to the *Arabidopsis* model system. The experiments presented in this thesis with tomato also enhance our understanding of phytochrome signalling in tomato and can reveal which effects are similar to those observed in the *Arabidopsis* model and which effects of phytochrome signalling are species specific. Moreover, because of the importance of tomato as a crop plant, the insights may be directly applicable in tomato breeding programs.

Chapter 2 presents a first molecular and physiological characterisation of tomato lines overexpressing *PHYA*, *PHYB1*, and *PHYB2* of tomato. In Chapter 3 the shade-avoidance response and resource allocation in selected tomato lines

overexpressing *PHYA*, *PHYB1*, and *PHYB2* are presented. Chapter 4 describes the analysis of plants with combinations of overexpression of different phytochrome genes or combinations of *PHY* and *CRY* overexpression. In Chapter 5 the results of the previous chapters are discussed in relation to each other and the approach to develop a tomato high-density cultivation system by manipulation of the shade avoidance response through phytochrome overexpression is evaluated.

Chapter 2

Characterization of tomato lines with ectopic expression of the tomato *PHYA*, *PHYB1,* and *PHYB2* genes *

Husaineid, S.H., Kok, R.A., Schreuder, M.E.L., van der Plas, L.H.W. and van der Krol, A.R.

Abstract

Transgenic tomato (*Lycopersicon esculentum* Mill.) lines, overexpressing the tomato phytochrome genes *PHYA*, *PHYB1* and *PHYB2*, under the control of the double *CaMV-35S* promoter have been generated to test the level of saturation in individual phytochrome-signalling pathways in tomato. For the detection of PHYA, PHYB1 and PHYB2, specific antibodies were produced and characterised. Analysis of RNA using RT-PCR and analysis of protein using Western blots confirmed the presence of elevated phytochrome mRNA and phytochrome protein levels in dark-grown seedlings of the respective *PHY* overexpressing (*PHY*OE) lines. Exposure to 4 h of red light (R) resulted in a decrease in PHYA in the *PHYA*OE lines, indicating that the excess of PHYA is also targeted for regulated destruction. This indicates that the extra phytochromes are assembled into holoproteins and that chromophore availability is not limiting. The elongation and anthocyanin accumulation responses under white light, R and far-red light (FR) were used for characterization of selected *PHY*OE lines. In addition, the anthocyanin biosynthesis during a 24-h period of monochromatic R and FR at different fluence rates in 4-day-old dark-grown seedlings has been studied. The elevated levels of phyA in the *PHYA*OE lines (PHYA increased up to 32-fold) had little effect on seedling and adult plant phenotype. However *PHYA*OE in *phyA* mutant background rescued the mutant phenotype, proving that expression of the transgene results in biologically active phyA. Similarly, *PHYB2*OE was able to rescue the *phyB1phyB2* double mutant phenotype. The *PHYB1*OE lines (PHYB1 levels increased up to 32-fold) showed only a mild effect on inhibition of stem elongation and anthocyanin accumulation, and showed little or no effect on the R high irradiance response (R-HIR). In contrast, in the *PHYB2*OE lines (PHYB2 level increased up to 200-fold), *PHYB2*OE resulted in a strong inhibition of elongation, enhancement of anthocyanin accumulation and a strong amplification of the R-HIR.

*This chapter has been published, together with some of the results of chapter 3 in *Journal of Experimental Botany* (2007), **58**: 615-626.

2.1 Introduction

Phytochromes are the most extensively characterized photomorphogenic photoreceptors in higher plants. The molecular properties of these photoreceptors enable them to perceive and transduce red light (R) and far-red light (FR) signals to downstream cellular components, ultimately leading to modulation of the expression of genes responsible for photomorphogenesis (Quail, 2002a). Tomato contains five phytochrome genes, designated *PHYA*, *PHYB1*, *PHYB2*, *PHYE*, and *PHYF* (Hauser *et al.*, 1995). The phytochrome apoproteins (PHYA, PHYB1, PHYB2, PHYE and PHYF) are synthesized within the cytosol and assemble autocatalytically with the plastid-derived chromophore to form the phytochrome holoproteins (phyA, phyB1, phyB2, phyB2, phyE and phyF). Two types of phytochrome exist: type I phytochrome, which is readily degraded in the light and is abundant in dark (D)-grown tissue (phyA) and type II phytochromes, which are relatively stable in the light (all other phytochromes). The phyA pool is type I and the other phytochromes are type II. The phytochrome holoprotein has two different light-absorbing conformations (Quail, 1997). The R-absorbing form (Pr) is thought to be inactive, and upon absorption of R is converted to the activated FR-absorbing form (Pfr). General classes of phytochrome responses have been defined, of which the FR high irradiance response (FR-HIR) and R very low fluence response (VLFR) were attributed to phyA. The R low fluence response (LFR), which is R/FR reversible, R-HIR and the shade-avoidance response are attributed to other phytochromes (Mancinelli, 1994).

Mutants impaired in specific phytochrome genes have been used to study the roles of individual phytochromes during tomato development (Kendrick and Kronenberg, 1994). From these studies it has been concluded that phyB1 is mainly responsible for mediating the de-etiolation response of seedlings to R (inhibition of hypocotyl elongation, enhancement of anthocyanin accumulation, unfolding of the hypocotyl hook, cotyledon expansion [Kerckhoffs *et al.*, 1997]). The contribution of phyB2 to seedling de-etiolation was only seen in the absence of phyB1, suggesting that in a *phyB2* mutant the action of other stable phytochromes compensate for the loss in phyB2 signalling (Weller *et al.*, 2000). Discrete functions of different members of the tomato phytochrome gene family for anthocyanin biosynthesis have been reported (Kerckhoffs *et al.*, 1997). The anthocyanin response during de-etiolation shows a strong tissue and cell specificity and normally is mainly restricted to the lower epidermis of leaf and a single sub-epidermal cell layer of the hypocotyl. However, injection of oat phyA in hypocotyl epidermal cells showed that potentially

phytochrome signalling can also induce anthocyanin biosynthesis in these cells (Neuhaus *et al.*, 1993).

Anthocyanin biosynthesis in seedlings under cR of different fluence rates is regulated by phyA, phyB1 and phyB2. This response was shown to include two components: a low fluence rate component and the R-HIR. The phyA mediates the low fluence rate component and was shown to be dependent on phyB2, while both phyB1 and phyB2 mediate the R-HIR (Weller *et al.*, 2000). The FR-HIR was shown to be mediated only by phyA (Kerckhoffs *et al.*, 1997). In de-etiolation under cR it was demonstrated that there is a strong negative effect of phyA on phyB2-mediated anthocyanin accumulation. However, a positive interaction of phyA with phyB1 is seen in the enhancement of anthocyanin synthesis by pre-treatment with FR (Weller *et al.*, 2000).

Plants overexpressing particular phytochrome genes may display different phenotypes that will provide new ways to study phytochrome action, such as enhanced *in vivo* light absorption properties, phytochrome dimerization and target protein interaction and cell specificity. The introduction of a *PHY*OE transgene may result in elevated levels of a particular phytochrome, leading to an enhanced phenotype. Alternatively, the gene product could dimerize with other phytochrome family members, resulting in novel interactions which may interfere with the function of endogenous phytochromes (*i.e*. cause a dominant negative effect). Ectopic expression of a phytochrome transgene may result in phytochrome accumulation in cells, which in wild type (WT) do not express phytochrome. The question is whether the phytochrome apoprotein can assemble into a biologically active holoprotein in such cells and thus whether the effect of elevated levels of phytochrome are limited to those cells in which endogenous *PHY* genes are already active. If overexpression of phytochrome genes does not lead to an enhanced phenotype it is important to distinguish between non-functional elevated levels of phytochrome (*e.g*. lack of assembly into phytochrome holoprotein) or a lack of action of additional biologically active phytochrome, (*e.g.* due to saturation of the signalling pathway). Assembly of biologically active phytochrome, encoded by the introduced transgene can be most precisely assessed in a mutant plant, which is deficient in the phytochrome gene family member concerned.

In this chapter the activities of elevated levels of phyA, phyB1, and phyB2 in the control of tomato plant growth and development have been compared, using a transgenic assay, based on overexpression of the tomato *PHY* genes under control of the constitutive *CaMV-35S* promoter. The effect of *PHYA*OE, *PHYB1*OE and *PHYB2*OE in WT and mutant backgrounds is presented and discussed in relation to

the different potential actions and interactions of phyA, phyB1 and phyB2 in tomato photomorphogenesis.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

Tomato *phy*-mutants and *PHY*OE lines studied were all in the tomato (*Lycopersicon esculentum* Mill.) WT cv MoneyMaker (MM) background. The mutant lines used here have been described previously: the *phyA*-null mutant (*phyA-1* [*fri*^{1}]; van Tuinen *et al*., 1995a), the *phyB1*-null mutant (*phyB-1* [*tri*^{1}]; van Tuinen *et al*., 1995b), the *phyB2*-null mutant (*phyB2-1* [70F]; Weller *et al*., 2000) and *phyB1phyB2* double mutant (Weller *et al.*, 2000). Seedlings were grown in a peat-based compost in trays placed in growth cabinets at constant temperature (25°C), a relative humidity (RH) of 70% and light conditions as indicated. Plants were watered once a day. The dark control plants were watered under dim green safelight.

2.2.2 Cloning, plant transformation and line selection

Full-length cDNA clones of the tomato *PHYA* (Lazarova *et al.*, 1998a), *PHYB1* (Lazarova *et al.*, 1998b), and *PHYB2* (Kerckhoffs *et al.*, 1999) were fused to the *CaMV-2x35S* promoter and cloned into the binary vector pKYLX 7 (Schardl *et al.*, 1987) using standard molecular techniques.

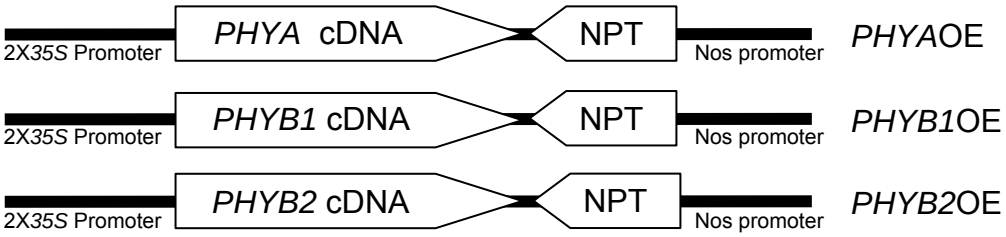


Figure 2.1. Chimeric *PHY*OE gene constructs used for transformation of tomato. NPT: Neomycin phosphotransferase gene, Nos: Nopaline synthase, *2x35S*: *CaMV-35S* promoter with double enhancer region.

The binary vectors with *PHY*-constructs (Fig. 2.1) were introduced into *Agrobacterium tumefaciens*, which was subsequently used for transformation of tomato (McCormick, 1991). Primary transformed tomato T_0 shoots were selected on medium containing kanamycin (50 mg/l). Tetraploid regenerated transformants were

removed. Single insert *PHY*OE lines were identified by segregation analysis of T_1 seedlings after germination on medium containing kanamycin. From the kanamycin resistant single-insert seedlings the shorter seedlings were selected for growth to maturity and collection of T_2 seeds (to increase the possibility of selecting homozygous plants). Homozygous *PHY*OE lines were identified by segregation analysis of T_2 seed germination on medium containing kanamycin. The homozygous *PHY*OE lines were crossed with *phyA* or *phyB1phyB2* double mutant plants. The subsequent F_2 progeny was grown on kanamycin medium under monochromatic FR or R to select plants for further analysis. Plants homozygous for the *phyA* or *phyB1phyB2* double mutant background were identified by phenotype and their identity was confirmed by PCR analysis on genomic DNA. Screening using specific PCR primer sets designed for the identification of WT and mutant phytochrome alleles was carried out as described by Weller *et al*., (2000). Segregation analysis of kanamycin resistance in F_3 plants identified those lines homozygous for the *PHY*OE transgene.

2.2.3 Analysis of gene expression by real time quantification RT-PCR

Total RNA was isolated using the TRIZOL kit (Invitrogen Co., Carlsbad, CA, USA) from D-grown seedlings (4 days) and D-grown seedlings subsequently treated with 4 h R. All RNA samples were treated with DNAase (Invitrogen) to remove potential genomic DNA contamination, after which RNA was converted into cDNA using M-MLV reverse transcriptase (Invitrogen), all according to instructions of the supplier. Specific primer pairs for the cDNA sequences of *PHYA*, *PHYB1*, and *PHYB2* were designed using the program "Taqman® Primer Design" (Applied Biosystems, Foster City, CA, USA) (Table 2.1). Primer concentrations giving the lowest threshold cycle (C_t) value were selected for further analysis. Detection of real-time RT-PCR products was done in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) by the incorporation of the fluorescent DNA dye SYBR green (Applied Biosystems) following the manufacturer's recommendations, using 16-fold dilution in sterile water of first-strand cDNA reaction mixes. All reactions were heated to 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The expression of the constitutively expressed tomato cyclophilin gene was used as a reference between samples. All calculations were performed as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems). The mean and standard error (SE) of results from two RT-PCR experiments based on two independent RNA extractions of the same plant material,

was calculated.

Table 2.1. The PCR primers used in the RT- PCR reactions, DNA sequences are listed 5' to 3'.

Gene	Forward	Reverse
PHYA	AGGCAGGCTAACAAAAGATCGTATA	TGCATCGGCTTCACTACCAA
PHYB1	GGTGGGTAGAGATCCAACTTCG	TCGCCAGGGCATATAATCCT
PHYB2	GGTTTTCGCTGATTTCTTACAGATTA	ACCAGGGCAAACAATCCTGA
Cyclophilin	GAGTGGCTCAACGGAAAGCA	CCAACAGCCTCTGCCTTCTTA

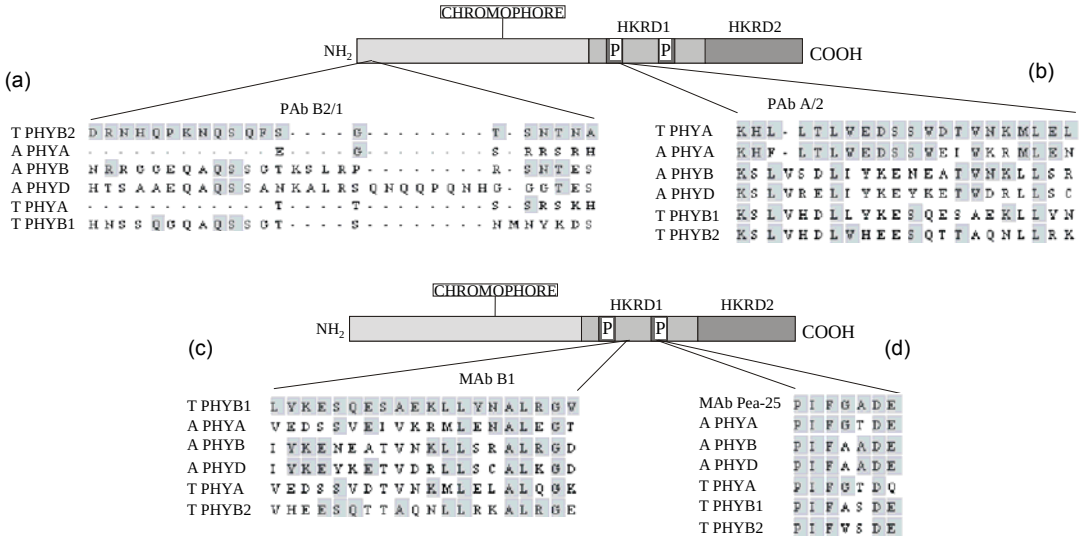


Figure 2.2. Epitope regions used for anti-phytochrome antibody production. Indicated are the selected amino acid (AA)-sequences within the phytochrome protein, which were used for production of synthetic peptides that were used for immunisation. (a) The selected AA-sequences from the tomato (T) PHYB2 are compared to related AA-sequences from T PHYA and T PHYB1 and the *Arabidopsis* (A) PHYA, A PHYB and A PHYD. (b) The selected AA-sequences from the T PHYA are compared to related AA-sequences from T PHYB1 and T PHYB2 and to A PHYA, A PHYB and A PHYD. (c) The selected AA-sequences from the T PHYB1 are compared to related AA-sequences from T PHYA and T PHYB2 and to A PHYA, A PHYB and A PHYD. (d) AA-sequence for the epitope that is recognized by the MAbPea-25 antibody, which falls within the conserved PAS domain (P). The AA-sequence is compared to related AA-sequences from T PHYA, T PHYB1and T PHYB2 and to A PHYA, A PHYB and A PHYD. HKRD1 and HKRD2: Histidine Kinase related domains.

2.2.4 Generation of new tomato PHYA, PHYB1 and PHYB2 specific antibodies

Synthetic peptides (20-mers) based on selected amino acid (AA)-sequences of tomato PHYA, PHYB1 and PHYB2 (Fig. 2.2a–d) were used to raise monoclonal antibodies (MAb) in mice and polyclonal antibodies (PAb) in rabbits. This yielded a polyclonal antibody against PHYA (PAb A/2), a monoclonal antibody against PHYB1 (MAb B1) and a polyclonal antibody against PHYB2 (PAb B2/1). For production of phytochrome apoprotein, the full-length cDNA of tomato *PHYA*, *PHYB1* or *PHYB2* was cloned into the pET28b (+) expression vector (Novagen, Madison, Wisconsin, USA) using standard molecular techniques. The *PHYA*, *PHYB1* or *PHYB2* expression constructs were introduced in *E. coli* strain BL21-DE3 and expression was induced according to supplier. Crude *E. coli* extracts were used for characterization of the antibodies. The extract of induced *E. coli* with an empty expression vector was used as a negative control.

The MAbPea-25 (Cordonnier-Pratt *et al.*, 1986a; 1986b) recognizes a very highly conserved PAS domain in phytochrome (Fig. 2.2d) and therefore this antibody can be used for detection of the total phytochrome pool in different species.

2.2.5 Protein extraction and immunoblot analysis

Proteins were extracted from whole tomato seedlings (hypocotyl plus cotyledons; D-grown seedling (4 days) and D-grown seedlings subsequently treated with 4 h R or 8 days WL(16 h WL/8 h D) grown seedlings) or tomato leaves (2-5 cm in length). Plant material was immediately frozen in liquid nitrogen and proteins extracted using the EZ extraction protocol as described (Martínez-García *et al.*, 1999). In addition, a protease inhibitor cocktail (Sigma, St. Louis, Missouri, USA) (10 μl/ml EZ buffer) was used to prevent protein degradation during the extraction. Protein concentration was determined using the DC protein assay kit (BIO-RAD, Hercules, CA, USA). Proteins (30-300 μg) were size-separated in 7.5% (w/v) SDS-polyacrylamide gels and subsequently electro-blotted onto an Immun-Blot PVDF® membrane (BIO-RAD) in 25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol, pH 8.3. The membranes were blocked in blocking solution containing 1% (w/v) skim milk powder in 50 mM Tris, 150 mM NaCl, and 0.05% (v/v) Tween solution (TBST). For the detection of phytochrome proteins, PAbA/2, MAbB/1, PAbB2/1 and MAbPea-25 were used. The MAbPea-25 detects the total phytochrome pool and was used in cases where PAbB2/1 failed to give a signal. Western blots were

incubated with primary antibody in blocking solution for 65-105 min. After washing 4 times in TBST, membranes were incubated with the secondary antibody (anti-mouse immunoglobulins for MAbs and anti-rabbit immunoglobulins for PAbs) IgG-POD, Lumi-light^{PLUS} Western Blotting Kit (Roche Molecular Biochemicals, Mannheim, Germany) in 1% blocking solution for 30-75 min, and then rinsed 4 times in TBST. The membrane was then incubated in the Lumi-light^{PLUS} substrate (Lumi-light^{PLUS} Western Blotting Kit (Mouse/Rabbit); Roche). An intensified CCD camera (C2400-77, Hamamatsu Photonics, Hamamatsu, Japan) was used to image the light emission. Intensity of the protein bands on the blot was quantified using the Argus 50 software (Hamamatsu Photonics).

2.2.6 Anthocyanin assay

For determination of anthocyanin accumulation, samples (whole seedling, hypocotyl or young leaves 1.0-1.5 cm in length) were extracted with 1.2 ml acidified (1% HCl, v/v) methanol for 24 h in D. Subsequently, a partitioning was performed by the addition of 0.9 ml H_2O and 2.4 ml chloroform to each extract. The samples were centrifuged for 30 min at 3600 rpm. The absorption of the aqueous (top) phase was determined spectrophotometrically (DU-64; Beckman Instruments, Fullerton, CA, USA) at 535nm (A_{535}). The relative values of the anthocyanin content were either expressed as A_{535} per 10 seedlings, as A_{535} per 5 hypocotyls or as A_{535} per g fresh weight (FW).

2.2.7 Broad-band red and far-red experiments

The continuous red light (cR; 600-700 nm) and continuous far-red light (cFR; 700-800 nm) experiments were performed as described by Kerckhoffs *et al.* (1997). Forty seeds per tray were pre-incubated in D at 4°C for 48 h to synchronize germination. Subsequently, seeds were placed in D at 25°C for 72 h. The irradiation with cR or cFR (both at 3 μmol m^{-2} s^{-1}) started just before the seedlings emerged through the soil surface. The length of 30-40 hypocotyls was measured after 14 days from sowing. The mean hypocotyl length of 30-40 seedlings grown in D was also measured. In addition to hypocotyl length, the anthocyanin level was determined in samples of 5 hypocotyls. The mean values and SE of 4 groups were calculated.

2.2.8 White-light experiments

Seeds were sown in trays and transferred to a phytotron with a 16 h white light (WL) / 8 h D diurnal treatment (WL; 150 μmol m^{-2} s^{-1} photosynthetically active radiation) at 25°C. After 11 days the hypocotyl length of the seedlings was measured. The seedlings were transferred to individual rock wool blocks and moved to the greenhouse and grown for 4 weeks after which the plant height and anthocyanin content from young developed leaves (1.0-1.5 cm in length) were measured.

2.2.9 Narrow-band fluence rate experiments

The R and FR fluence rate experiments were performed in a threshold box set-up as previously described by Kerckhoffs *et al.*, (1997). Briefly, seeds were sown on filter paper moistened in germination buffer (10 mM phosphate buffer pH 7.5 containing 1 mM KNO_3) in plastic boxes with transparent lids. In each box four filter papers on which 30 seeds of WT and three other transgenic lines were sown separately. In the experiment, seeds from each genotype were incubated in D for 90 h (WT seeds) or 114 h (transgenic seeds) at 25°C in order to get an equal seedling development at the start of the experiment. Subsequently, seedlings were exposed to 24 h continuous monochromatic R (using interference filter with peak transmission at 680 nm; Baird-Atomic, Bedford, USA,16.6 nm band width at 50% of the maximum transmission) or FR (using interference filter with peak transmission at 730 nm; Baird-Atomic, Bedford, USA,10.9 nm band width at 50% of the maximum transmission) of different fluence rates (Kerckhoffs *et al.*, 1997). The level of anthocyanin was determined at the end of 24 h light treatment in sets of 10 whole seedlings. The mean values and SE of four independent experiments were calculated.

2.3 Results

2.3.1 Generation of tomato *PHYA*OE, *PHYB1*OE, and *PHYB2*OE lines

For construction of the tomato *PHYA*OE, *PHYB1*OE, and *PHYB2*OE lines, the tomato *PHYA*, *PHYB1* and *PHYB2* cDNAs were cloned behind the double *CaMV 35S*-promoter in a binary vector and the chimeric genes were introduced into tomato by *Agrobacterium tumefaciens* mediated transformation (Fig. 2.1). Transformed tomato shoots (T_0) were selected on kanamycin, resulting in 15

*PHYA*OE, 12 *PHYB1*OE, and 33 *PHYB2*OE independent primary transformants. From each T_0 plant, seeds (T_1) were harvested and segregation analysis of T_1 seedlings indicated 10 *PHYA*OE, 5 *PHYB1*OE, and 13 *PHYB2*OE lines with a single locus insertion. Heterozygous and homozygous progeny plants were distinguished from each other by size difference of T_1 plants and through segregation analysis of T_2 seedlings on medium containing kanamycin. For each transgene representative homozygous lines were selected for collection of T_3 bulk seed batches which were used for the analysis of plant phenotype under different physiological conditions (A/1, A/3 for *PHYA*OE; B1/2, B1/4 for *PHYB1*OE and B2/4, B2/8, B2/9 for *PHYB2*OE). In addition, the A/3 and B2/9 lines were crossed with the *phyA* mutant and *phyB1phyB2* double mutant, respectively. The F_2 Seeds were harvested from the F_1 progeny plants and kanamycin resistant seedlings were analysed for presence of the WT and the mutant *phy*-locus/loci through PCR analysis of genomic DNA, using specific primers. These analyses identified individual seedlings homozygous for the *phyA* or *phyB1phyB2* loci. The selected plants were grown and seeds harvested. Segregation analysis of seedlings from these seed batches germinated on kanamycin revealed the plants homozygous for both the transgene and the phytochrome mutant locus/loci (A/3*phyA* and B2/9*phyB1phyB2*).

2.3.2 Phytochrome mRNA levels in WT and *PHY*OE lines

Quantitative RT-PCR was used to determine the level of overexpression in the *PHYA*OE, *PHYB1*OE, and *PHYB2*OE lines. The mRNA level was related to that of cyclophilin mRNA in each sample and values were normalized to those in D-grown WT. The results in Figure 2.3 show the mean ± SE of two independent RNA extractions of the same plant material. Figure 2.3a shows that in D-grown seedlings the *PHYA* mRNA levels in A/1 and A/3 are not significantly increased relative to WT. It has been demonstrated for oat *PHYA* that transcription of the endogenous *PHYA* gene, which is very high in D-grown seedlings, is suppressed in the light, resulting in a rapid decline of *PHYA* mRNA level due to the intrinsic high turnover of this messenger (Seeley *et al.*, 1982; Lissemore *et al.*, 1987). Similarly, a 6-fold reduction in the *PHYA* mRNA level in response to a light pulse of D-grown seedlings was observed in tomato, with an apparent half-life of 3.5 h of the *PHYA* mRNA (Hauser *et al.*, 1998). Therefore the *PHY* mRNA levels in D-grown seedlings treated with 4 h R were also analysed. Figure 2.3a shows an indication of a decrease in mRNA levels after 4 h R treatment in both WT and in the *PHYA*OE lines, but after 4 h R *PHYA* mRNA levels in A/3 are higher than in WT.

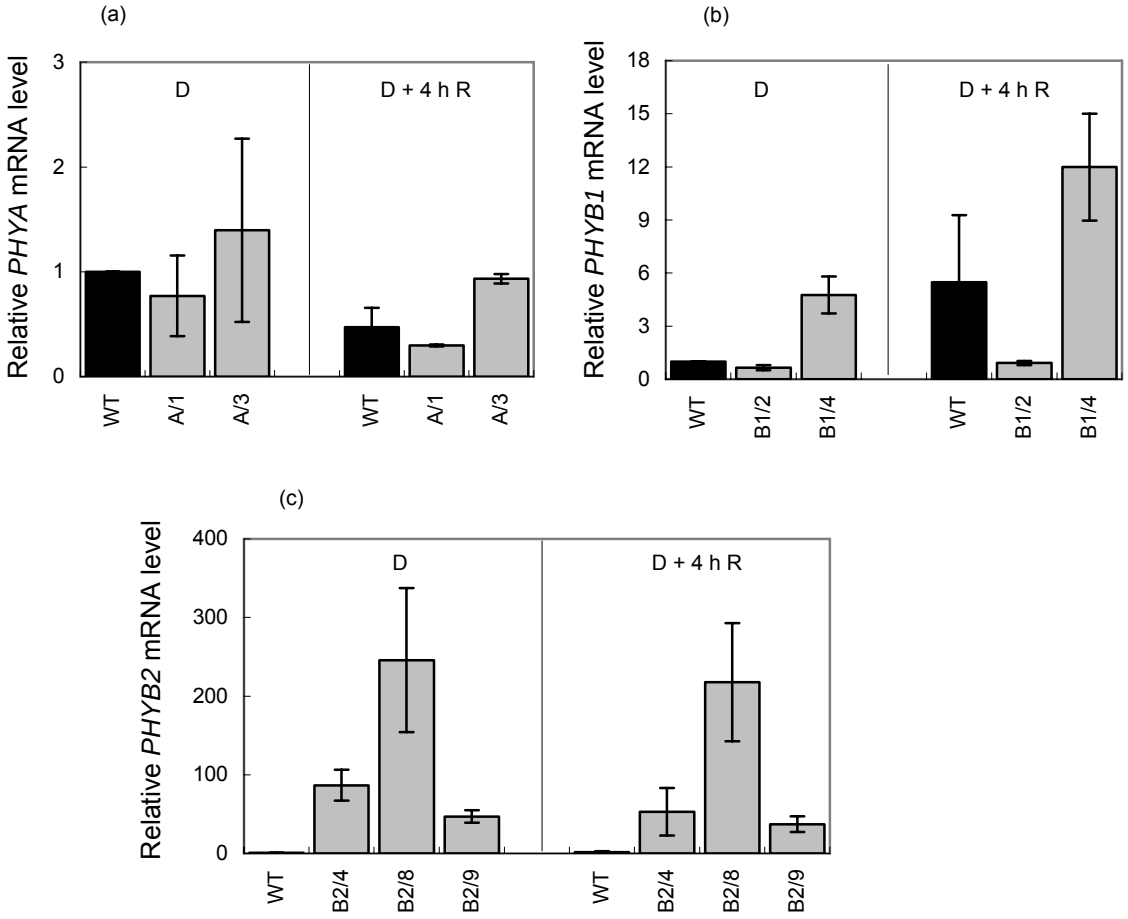


Figure 2.3. Relative *PHY* mRNA level in *PHY*OE lines normalized to that in D-grown WT. The expression of the phytochrome genes was related to the level of expression in each sample of the constitutively expressed tomato cyclophilin gene. The results are the mean ± SE of two independent RNA extractions. (a) Relative *PHYA* mRNA level in A/1 and A/3 lines. (b) Relative *PHYB1* mRNA level in B1/2 and B1/4 lines. (c) Relative *PHYB2* mRNA level in B2/4, B2/8 and B2/9 lines. D: 4-day-old dark-grown seedlings, D + 4 h R: 4-day-old dark-grown seedlings subsequently treated with 4 h red light.

The results of the quantification of the *PHYB1* mRNA level in D-grown seedlings show that in the B1/2 line there is no significant increase in *PHYB1* mRNA level compared to WT, while in the B1/4 line a 4-fold increase (Fig. 2.3b) is observed. This figure shows a 2-fold increase in mRNA level in both WT and the B1/4 line after 4 h R treatment. Figure 2.3c shows the quantification of *PHYB2* mRNA. The results show that in WT the signal for *PHYB2* mRNA was very low and that *PHYB2* mRNA in D-grown seedlings is increased about 90-fold in B2/4, about 250-fold in B2/8 and about 50-fold in B2/9 compared to that in WT. No significant change in the *PHYB2* mRNA was seen after 4 h of R treatment (Fig. 2.3c).

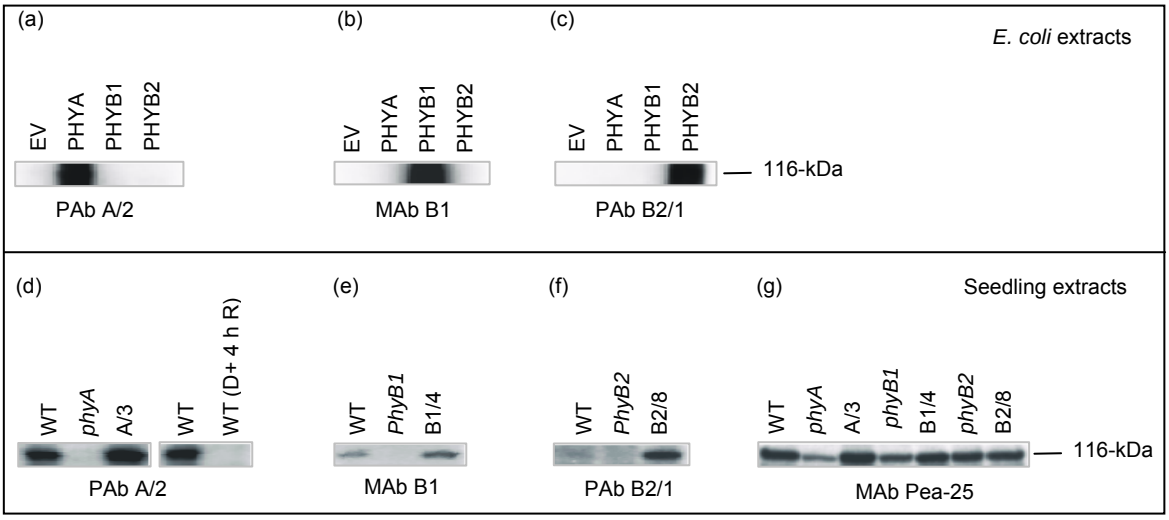


Figure 2.4. Characterization of antibodies specific for tomato phytochromes.(a-c) Western blots with protein extracts of induced *E. coli* cultures (10 µg) with empty vector (EV) and tomato *PHYA*-expression vector (PHYA), tomato *PHYB1*-expression vector (PHYB1) or tomato *PHYB2*-expression vector (PHYB2), (d-g) Western blots with protein extracts (300 µg protein) of tomato seedlings, probed with different tomato anti-PHY antibodies: (a and d) the anti-PHYA antibody PAb A/2, (b and e) the anti-PHYB1antibody MAb B1, (c and f) the anti-PHYB2 antibodies PAb B2/1 and (g) Characterization of anti-PHY antibody MAbPea-25. All protein extracts are from dark (D)-grown seedlings or from D-grown seedling plus 4 h of red light (D + 4 h R). WT: wild type; *phyA*: mutant *phyA*; A/3: WT with *CaMV 2x35S-PHYA*. *phyB1*: mutant *phyB1*; B1/4: WT with *CaMV 2x35S-PHYB1*; *phyB2*: mutant *phyB2*; B2/8: WT with *CaMV 2x35S-PHYB2*.

2.3.3 Characterization of antibodies specific for tomato phytochrome proteins.

For the characterization of phytochrome protein levels in plants new specific antibodies directed against PHYA, PHYB1 and PHYB2 were generated. Figure 2.2 shows the selected AA-sequences and the comparison to the related AA-sequences of tomato and *Arabidopsis* phytochromes that were used as a template for the production of specific synthetic peptides. The synthetic peptides were used to immunise mice or rabbits to raise a polyclonal antibody (PAb) against PHYA (PAbA/2), a monoclonal antibody (MAb) against PHYB1 (MAbB1) and a polyclonal antibody against PHYB2 (PAbB2/1). The specificity of each antibody was determined by probing Western blots with the tomato PHYA, PHYB1 and PHYB2 apoproteins produced in *E. coli* (Fig. 2.4a-c). The figure shows that each of these antibodies specifically recognizes the corresponding PHY. The antibodies were subsequently tested in tomato seedling extracts (Fig. 2.4d-g). Western blots with protein extracts of 4-days-old D-grown WT tomato seedlings, phytochrome mutants (*phyA*, *phyB1* or *phyB2*) and transgenic lines with overexpression of *PHYA*, *PHYB1*

or *PHYB2* (lines A/3, B1/4 and B2/8, respectively) were probed with the different tomato anti-PHY antibodies. The PAb A/2 antibody detected a protein product of expected size (116-kDa) in WT and A/3 line. The band was not detected in the *phyA* mutant, while in A/3 the 116-kDa band was more intense compared to WT (Fig. 2.4d). When D-grown seedlings are given 4 h of R the intensity of band is much reduced compared to D, indicating that the detected protein is labile in the light (Fig. 2.4d). Combined these results confirm that the 116-kDa band which is detected on Western blots of plant extracts is indeed PHYA. The tomato anti-PHYB1 antibody MAb B1 detects a 116-kDa band in extracts from WT, but not in extracts from the *phyB1* mutant. Moreover, the intensity of the 116-kDa band was higher in the *PHYB1*OE line B1/4, confirming the specificity for PHYB1 of this antibody in plant extracts (Fig. 2.4e).

Although the tomato anti-PHYB2 antibody PAb B2/1 was highly specific for detection of PHYB2 produced in *E. coli* (Fig. 2.4c), it failed to detect PHYB2 with high reproducibility in WT tomato seedling extracts. However, with this antibody a 116-kDa band was detected in the *PHYB2*OE line B2/8, indicating that with this antibody PHYB2 may be detected in seedling extracts with elevated levels of PHYB2 (Fig. 2.4f).

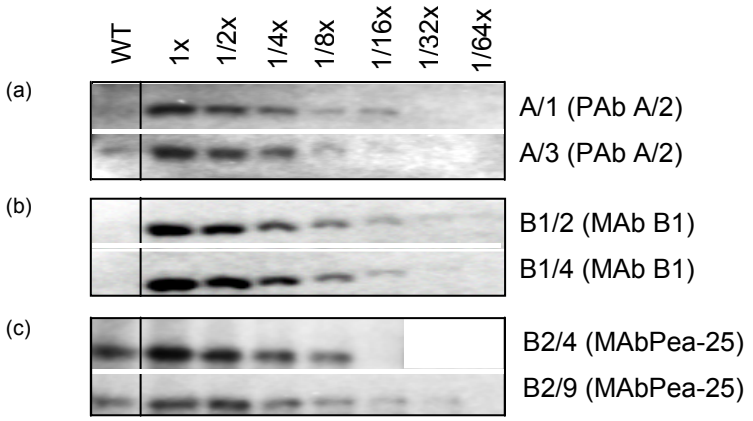


Figure 2.5. Quantification of PHYA, PHYB1 and PHYB2 levels in the *PHY*OE lines. Detection of PHYA, PHYB1 or PHY levels in dilution series of extracts from 4-day-old dark (D)-grown seedlings of: (a) the *PHY*OE lines A/1and A/3 using anti-PHYA PAb A/2. (b) the *PHYB1*OE lines B1/2 and B1/4 using anti-PHYB1 MAb B1. (c) the *PHYB2*OE lines B2/4 and B2/9 using anti-phytochrome MAbPea-25. WT: 300 μg of D-grown seedling extract; each *PHY*OE dilution series starts with 300 μg of D-grown seedling extract (1x) which was subsequently diluted 1/2, 1/4, 1/6, 1/8, 1/16, 1/32 or 1/ 64 times.

2.3.4 Quantification of phytochrome protein level in the *PHY*OE lines

Using the new PHY antibodies the relative PHYA and PHYB1 level in the *PHYA*OE and the *PHYB1*OE lines could be determined reproducibly. For the quantification of PHYA and PHYB1 levels a dilution series of protein extracts from *PHYA*OE and *PHYB1*OE plants was made for comparison to the signal in WT (Fig. 2.5a and b). The results show that extracts from D-grown seedlings from A/1 and A/3 lines have to be diluted approximately 8- to16-fold, to obtain a signal similar to that in WT D-grown seedlings, while the extracts from D-grown B1/2 and B1/4 seedlings have to be diluted at least 32-fold before the signal was similar to that in WT. The PHYB2 levels in the *PHYB2*OE lines had to be quantified indirectly, using the anti-phytochrome antibody MabPea-25. Figure 2.4g shows that MAbPea-25 recognizes a 116-kDa band in all extracts from D-grown seedlings. The intensity of this band is only 10 % of that in WT in the *phyA* mutant, indicating that approximately 90% of the Ptot pool in WT D-grown seedlings is from PHYA and 10% is from the other phytochromes. From the dilution series, the signal of Ptot in B2/4 was estimated to be at least 4-fold, and in B2/9 to be at least 8-fold higher than in WT (Fig. 2.5c). There is no quantitative information of the total phytochrome protein content or PHYB2 protein content in tomato seedlings. However, the *PHYB2* transcript levels in D-grown seedlings was shown to be 4.4% of the total *PHY* transcript level in D-grown seedlings (Hauser *et al*.,1998). For our calculations, we therefore assumed the PHYB2 to be approximately 4% of the Ptot pool in D-grown seedlings. Assuming that all the extra protein recognized by the MAbPea-25 is from *PHYB2*OE, this implies that PHYB2 levels in the B2/4 and in the B2/9 are at least 100 to 200 times higher than the PHYB2 level in WT. In Figure 2.6a-c the quantifications of the relative PHY level in extracts of D-grown seedlings and extracts from D-grown seedlings that were subsequently treated with 4 h R are shown. Since no phytochrome could be detected in WT in this series of experiments the results of the *PHY*OE lines are expressed in relative units. The results show that when D-grown seedlings are exposed to 4 h R, the PHYA levels are reduced to approximately 25% of the signal from D-grown seedlings (Fig. 2.6a). This figure shows that the same reduction of PHYA level was observed when A/3 is overexpressed in WT or in *phyA* mutant background. Figure 2.6b shows that PHYB1 levels do not change significantly when D-grown seedlings are exposed to 4 h R. In Figure 2.6c the results of direct quantification of PHYB2 levels, using the anti-PHYB2 antibody are shown. This figure shows that after 4 h R treatment the PHYB2 levels do not change significantly. No difference is observed when the B2/9

transgene was overexpressed in WT or in the *phyB1phyB2* double mutant background (Fig. 2.6c).

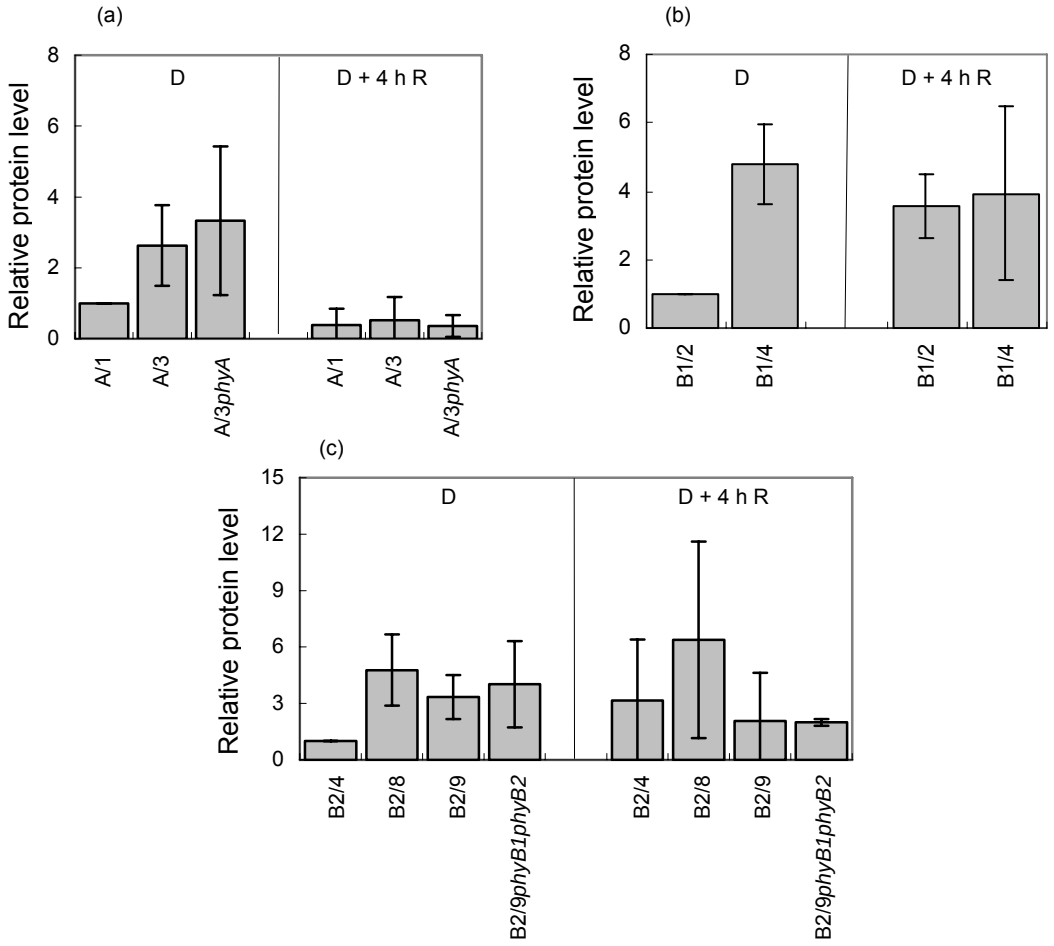


Figure 2.6. Relative PHYA, PHYB1 and PHYB2 protein levels. Extracts are from 4-day old D-grown seedlings and 4-day-old D-grown seedlings, subsequently treated with 4 h red light (R). PHYA was measured using anti-PHYA PAb A/2. PHYB1 was measured using anti-PHYB1 MAB B1. PHYB2 was measured using anti-PHYB2 PAb B2/1 (a) The PHYA protein levels normalized for those in A/1 (mean ± SE, n = 5, loaded protein 30 μg in two blots and 300 μg in three blots). (b) The PHYB1protein levels normalized for those in B1/2 (mean ± SE, n = 4, loaded protein 30 μg in two blots and 300 μg in two blots) and (c) The PHYB2 protein levels normalized for those in B2/4 (mean ± SE, n = 2, loaded protein = 30 μg). The results are based on analysis of 2-5 Western blots which for phyA and phyB1 also include biological repeats. In the blots where 30 μg protein was loaded to avoid saturation of the phytochrome signal in the *PHY*OE lines the WT levels were below detection limit.

2.3.5 Characterization of plants grown under white light

The phenotypes of WT and the different *PHY*OE lines grown under WL conditions were compared (16 h WL/8 h D; Figure 2.7a-c). The results show that there is only a small effect of *PHYA*OE and *PHYB1*OE on seedling development compared to the effect of *PHYB2*OE (Fig. 2.7a). The *PHYB2*OE lines are shorter and have

smaller primary leaves compared to WT. In addition, the figure shows that the phenotype of the *phyB1phyB2* double mutant (long stem, light green leaves) was rescued in the B2/9*phyB1phyB2* line. Figure 2.7b shows a cross section of the hypocotyl of a WT, a *phyB1phyB2* double mutant and a B2/4 seedling grown for 11 days under WL and indicates that anthocyanin accumulation is restricted to the sub-epidermal cell layer both in WT and B2/4, but is enhanced in B2/4 compared to WT. In the *phyB1phyB2* double mutant only very limited anthocyanin accumulation is seen in the same cell layer. The adult phenotype of WT, B2/4 and B2/8 plants, grown for 8 weeks in the greenhouse, is shown in Figure 2.7c. This figure shows that also at this stage of development the B2/4 and B2/8 plants are shorter than WT. In addition, the leaves of the transgenic plants appeared greener than those of WT plants.

The hypocotyl length of 11-day-old plants and plant height and anthocyanin levels in 8-week-old plants grown under WL were quantified. Figure 2.8a shows that the hypocotyl length of A/1, B1/4 and all *PHYB2*OE lines are significantly reduced compared to WT. However, this reduction in length was only very small in the A/1 line, but more pronounced in B1/4 and the *PHYB2*OE lines. When the plants are 8 weeks old, the *PHYA*OE in the A/3 line and *PHYB1*OE in the B1/4 line resulted in a significant reduction of plant height. In the 8-week-old plants the effect of *PHYB2*OE was even more pronounced than at the seedling stage, resulting in up to 50% suppression of stem elongation compared to WT (Fig. 2.8b). The results in Figure 2.8c show that *PHYA*OE and *PHYB1*OE have little or no effect on anthocyanin accumulation compared to WT. In contrast, a strong enhancement of leaf anthocyanin accumulation was observed in the *PHYB2*OE lines. In the B2/8 line the enhancement of anthocyanin accumulation was about 12-fold and in the B2/9 and B2/9*phyB1phyB2* lines this was about 10-fold compared to WT (Fig. 2.8c). The results in Figure 2.8a-c also show that the *phyB1phyB2* double mutant has a clear phenotype for hypocotyl length, plant height and anthocyanin accumulation compared to WT. All these aspects of the mutant phenotypes were rescued by B2/9 overexpression in the *phyB1phyB2* double mutant background, confirming that biologically active phyB2 is produced as a result of expression of the B2/9 transgene.

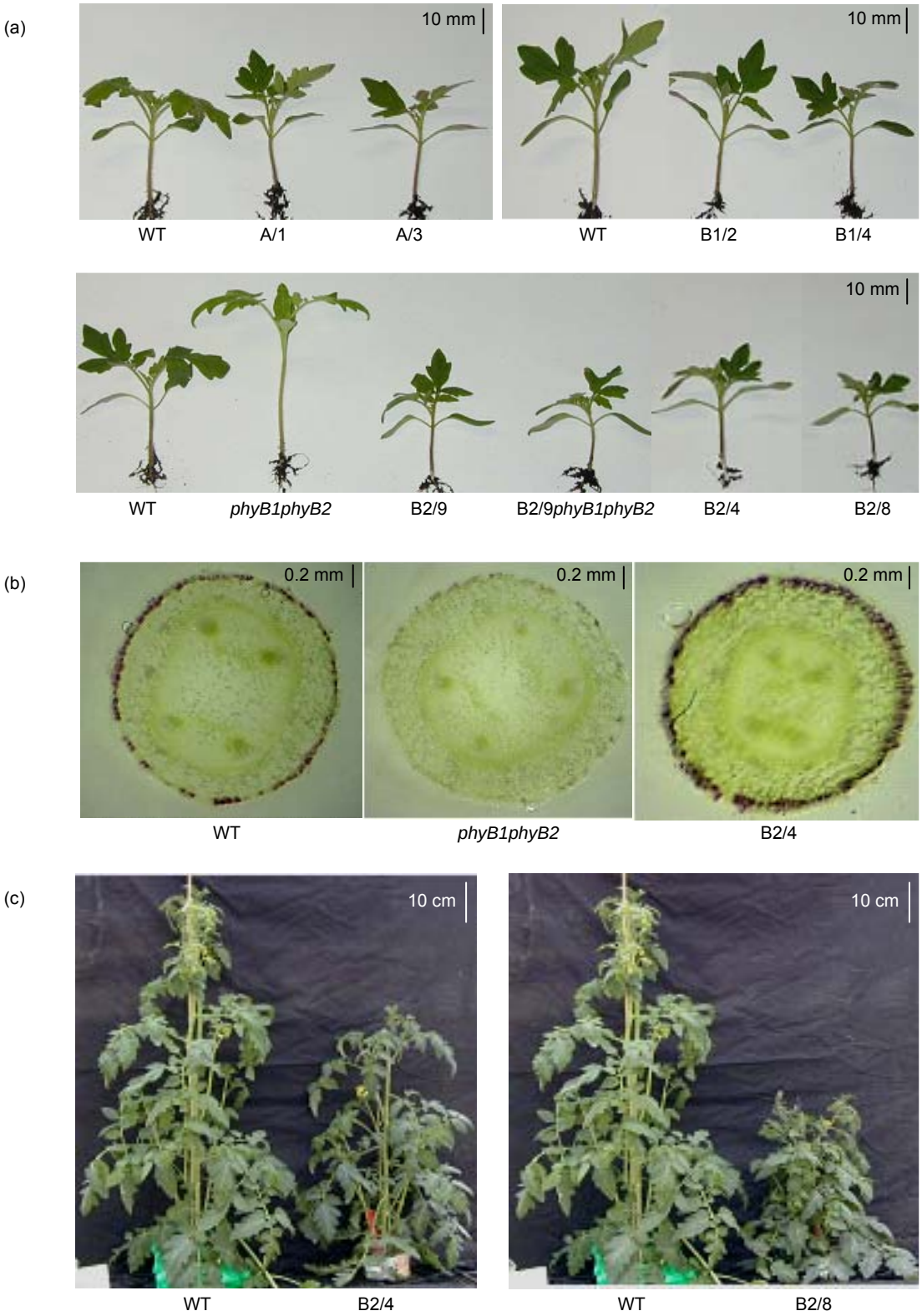


Figure 2.7. Phenotype of wild type (WT) and transgenic tomato lines. (a) Seedling phenotypes after 11 days growth in phytotron under diurnal white light (WL) (16 h WL/8 h D). (b) Anthocyanin localization in hypocotyl cross section of WT, *phyB1phyB2* double mutant and transgenic line B2/4. (c) Comparison of the plant phenotype of the B2/4 and B2/8 lines after 8 weeks growth in the greenhouse with the WT control demonstrates the influence of *PHYB2*OE on adult plant height.

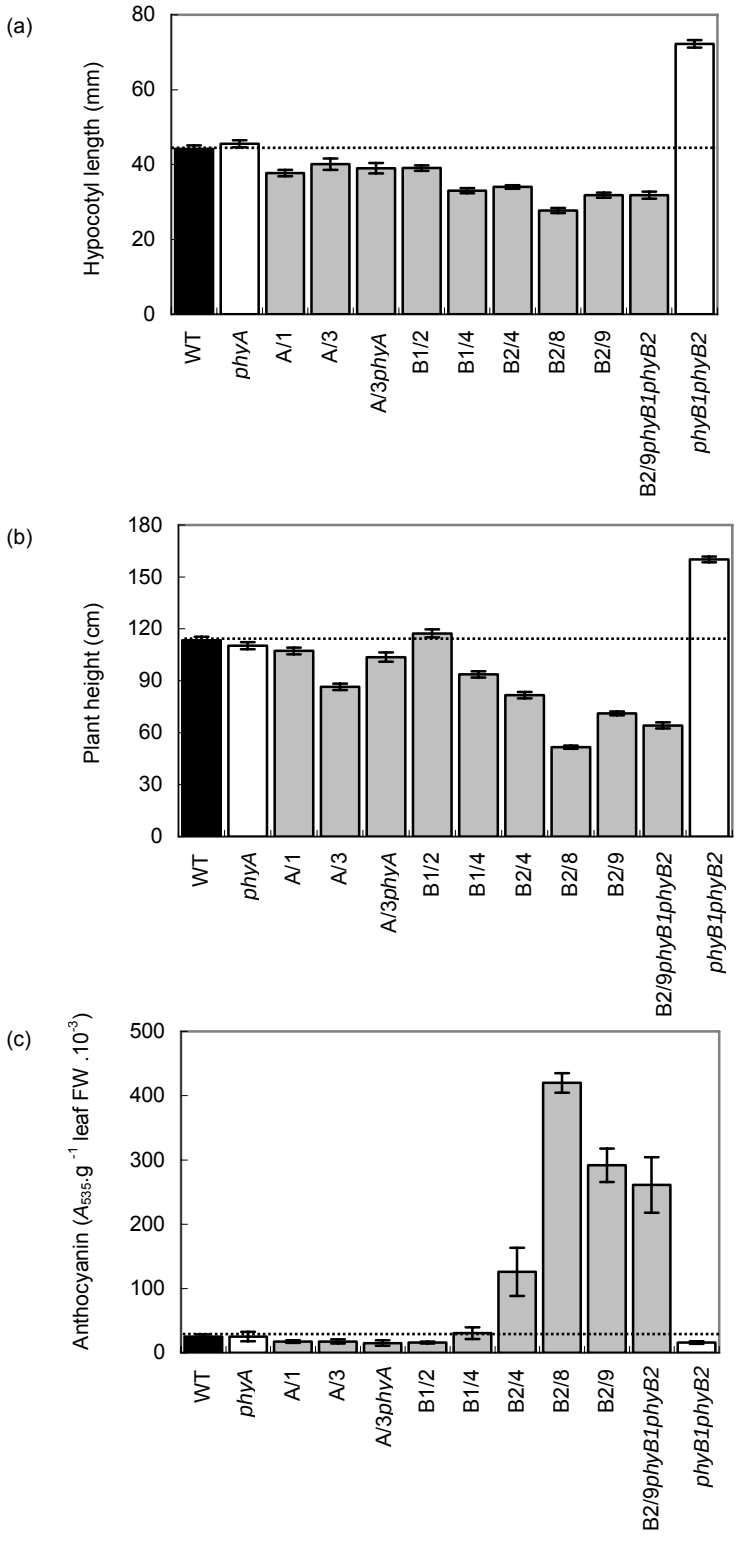


Figure 2.8. Quantification of phenotypes of white light (WL) (16 h WL/8 h D)-grown tomato plants. (a) Hypocotyl length of plants grown in the phytotron, 11 days from sowing, means ± SE (n = 40). (b) Plant height of plants grown in the greenhouse, 8 weeks from sowing, means ± SE (n = 15). **(**c) Anthocyanin content of young leaves of plants grown in the greenhouse, 8 weeks from sowing. The experiment was repeated with qualitatively similar results.

2.3.6 Broad-band red and far-red light experiments

The effect of increased phytochrome levels in the overexpressing transgenic lines may be more pronounced under light conditions where specific photoreceptors are activated (*e.g.* under R or FR where phytochromes are activated independent of B-absorbing photoreceptors). In order to specifically quantify the contribution of phytochrome to growth, seedlings from WT, *PHY*OE lines and from two *phy*-mutant lines (*phyA* and *phyB1phyB2*), were grown under broad-band cR (600-700 nm) and cFR (700-800 nm). The inhibition of hypocotyl elongation after 14 days cR and cFR of these lines was compared to that in WT (Fig. 2.9a-c). The results show the mean ± SE of measurements of 30-40 seedlings and are expressed as percentage inhibition of hypocotyl elongation, relative to D-grown control seedlings. Figure 2.9d shows that the hypocotyl length of the *PHY*OE seedlings is similar to the length of WT seedlings when seedlings are grown for 14 days in darkness.

When grown under cR the transgenic *PHYA*OE lines displayed no significant increase in inhibition of hypocotyl elongation, relative to WT plants (Fig. 2.9a). Surprisingly, when *PHYA*OE lines are grown under cFR, where phyA plays a dominant role, we also detected no significant change in the inhibition of hypocotyl elongation compared to WT plants (Fig. 2.9a). As predicted the *phyA* mutant is essentially blind to FR, resulting in inhibition of hypocotyl elongation and this phenotype is fully rescued when combined with *PHYA*OE (A/3*phyA*; Fig. 2.9a). The anthocyanin content in the seedlings grown under cR and cFR is given in Figure 2.10a-c. This figure shows that under cFR there is a pronounced reduction in anthocyanin accumulation in A/1 and A/3 lines compared to WT (Fig. 2.10a). This figure also shows that in the *phyA* mutant anthocyanin accumulation is enhanced under cR and strongly inhibited under cFR. Both these aspects of the mutant phenotype were rescued by *PHYA*OE in the *phyA* mutant background (A/3*phyA*).

Both under cR and cFR the *PHYB1*OE had no effect on hypocotyl elongation compared to WT plants (Fig. 2.9b). However, in the *PHYB2*OE lines there was a strong effect on inhibition of hypocotyl elongation under cR compared to WT (80% and 60% inhibition, respectively), while no phenotype for hypocotyl elongation was observed in the *PHYB2*OE lines under cFR (Fig. 2.9c). Under cR the anthocyanin accumulation in the *PHYB1*OE line B1/2 was similar to WT, but in the B1/4 line the anthocyanin level under cR was lower than WT (Fig. 2.10b). Similarly, under cFR, the anthocyanin accumulation in the B1/2 line was not different from WT, while in the B1/4 line anthocyanin accumulation was reduced by 40% compared to WT (Fig. 2.10b). In the *PHYB2*OE lines the anthocyanin level was strongly affected under cR

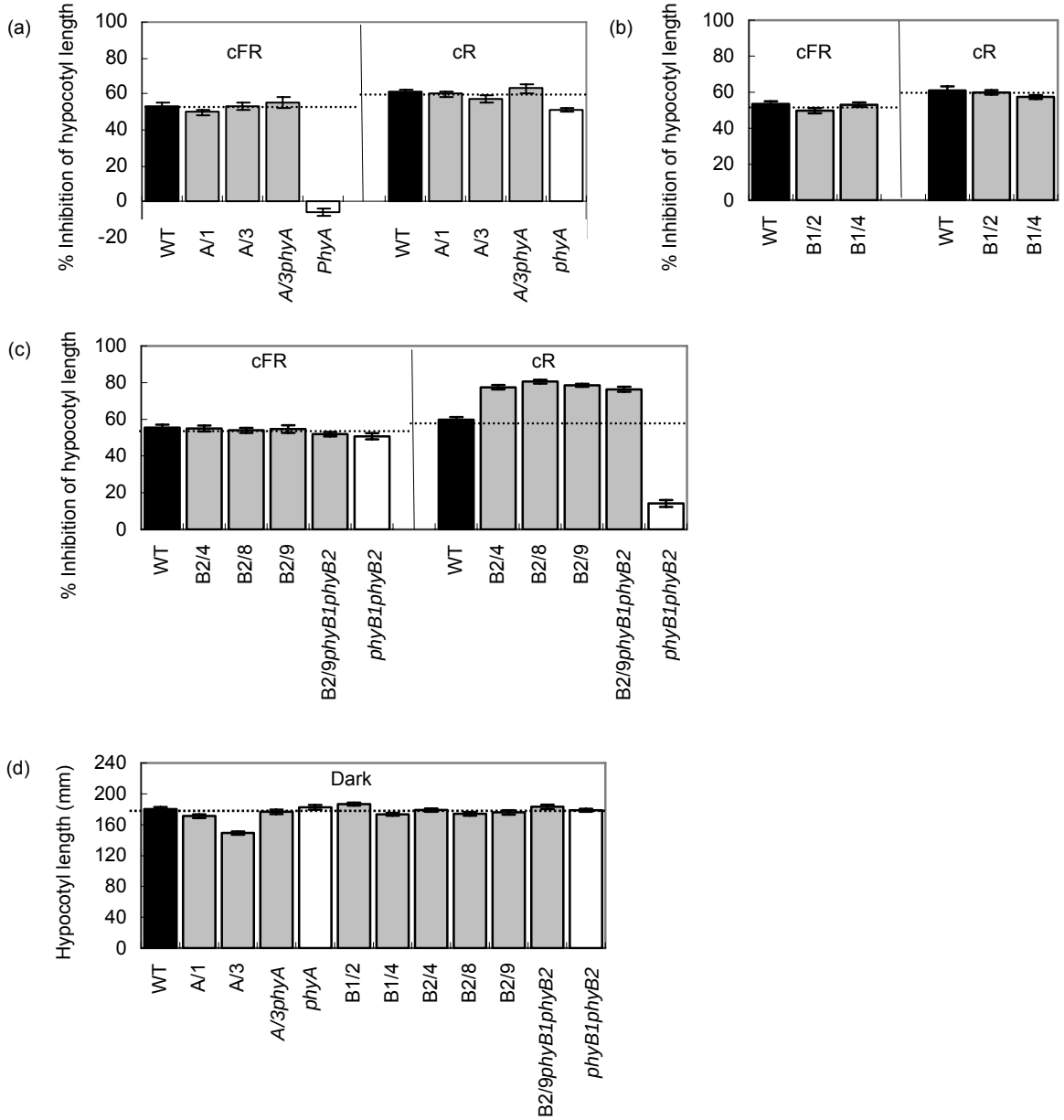


Figure 2.9. Inhibition of hypocotyl elongation under cR and cFR: (a) *PHYA*OE lines, (b) *PHYB1*OE lines and (c) *PHYB2*OE lines. (d) Hypocotyl length (mm) of D-grown seedlings. The mean values of the length of 30-40 hypocotyls under broad band cR and cFR (3 μmol m^{-2} s^{-1}) were used to calculate the inhibition of hypocotyl elongation relative to the dark control (d). All measurements were taken 14 days after sowing. Values are means ± SE (n = 30-40). The experiments were repeated with qualitatively similar results.

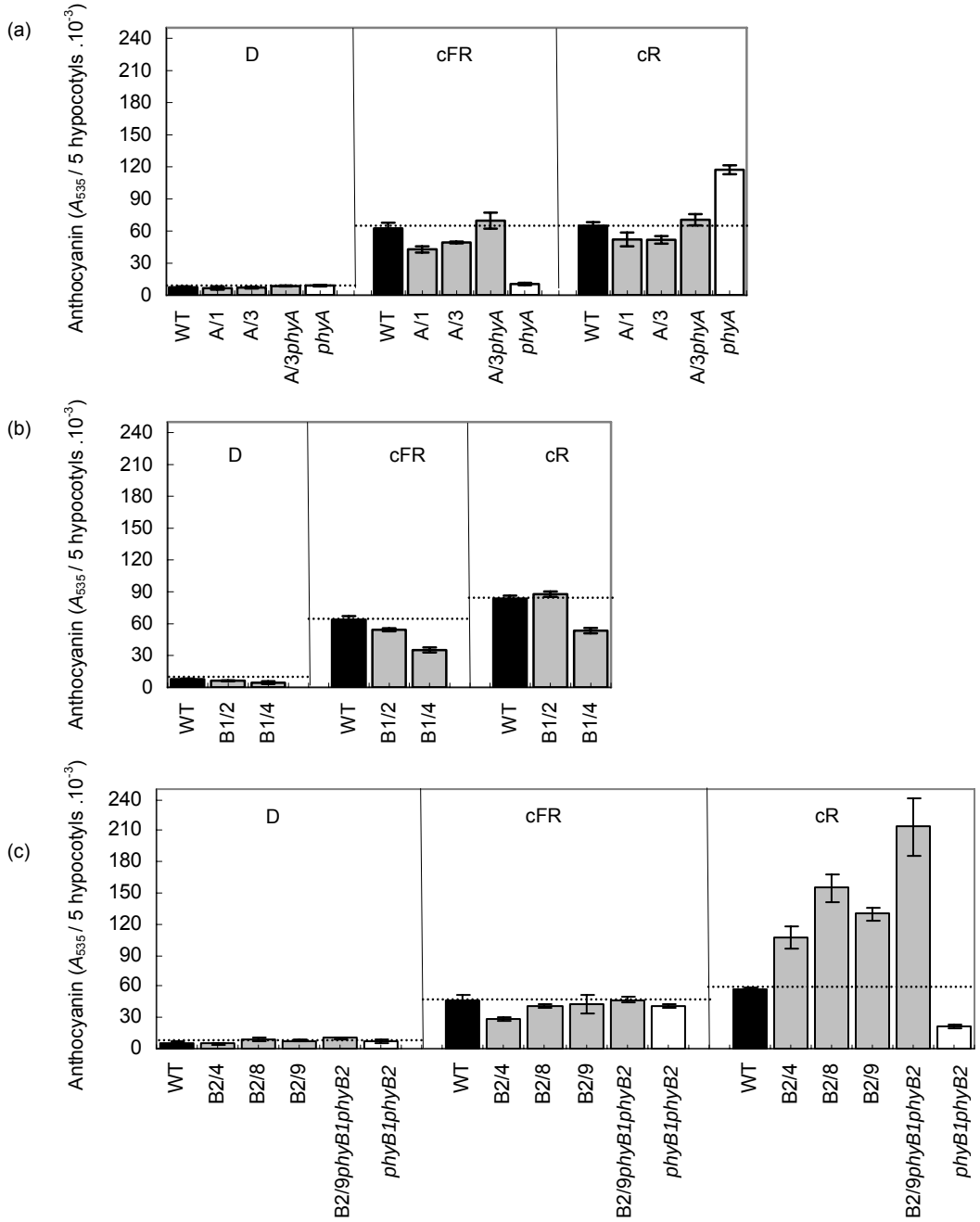


Figure 2.10. Anthocyanin accumulation under cR, cFR and in D: (a) *PHYA*OE lines. (b) *PHYB1*OE lines. (c) *PHYB2*OE lines. All measurements were taken 14 days after sowing and the anthocyanin levels in 5 hypocotyls were measured. Values are means ± SE (n = 4). The experiments were repeated with qualitatively similar results.

and up to 3-fold higher compared to that in WT (Fig. 2.10c). Under cFR there was no effect on anthocyanin accumulation in the B2/8 and B2/9 lines, but in the B2/4 line the accumulation of anthocyanin was lower than in WT (Fig. 2.10c).

The results in Figures 2.9c and 2.10c show that for both hypocotyl and anthocyanin the phenotype of the *phyB1phyB2* double mutant is rescued by the overexpression of *PHYB2* in the B2/9*phyB1phyB2* line.

2.3.7 Narrow band R and FR fluence rate responses

The fluence rate response relationships for anthocyanin synthesis in the *PHY*OE lines during 24-h continuous R (680 nm) or FR (730 nm) treatment to 4-day-old D-grown seedlings were determined. The results for R show that overexpression of A/3 in the *phyA* mutant background (A/3*phyA* line) could rescue the *phyA* mutant phenotype by restoring the low fluence rate component to a level similar to that in WT (Fig. 2.11a). This suggests that the effective phyA level, resulting from overexpression in the A/3 line, is at least similar to the phyA level in WT. However, the same expression of A/3 in WT background resulted in a slight decrease rather than an increase of the response to low fluence rate compared to WT (Fig. 2.11a). The same *PHYA*OE lines were tested under cFR. The results in Figure 2.12 show that, as expected, after 24 h of cFR the *phyA* mutant shows no response, while in the A/3*phyA* line the response was restored to a level above that in WT. The response of A/3 in the WT background under cFR was lower than in WT at the higher fluence rates, indicating that a specific optimum in phyA level is required for maximal response to FR.

Characterization of the response in the *PHYB1*OE lines shows that the R-HIR was little affected compared to WT (Fig. 2.11b). Overexpression of *PHYB1* also had no effect on the low fluence rate component in the B1/2 line, but resulted in a small reduction of the low fluence rate component in line B1/4 (Fig. 2.11b). Figure 2.11c shows that both the low fluence rate and R-HIR components are absent in the *phyB1phyB2* double mutant. When the B2/9 transgene is expressed in the *phyB1phyB2* double mutant background (B2/9*phyB1phyB2*) the results show a R-HIR (Fig. 2.11c) that is even higher than the WT at low fluence rates. This indicates that the expression of *PHYB2* in the B2/9 line rescues the lack of both a functional phyB1 and phyB2 in the *phyB1phyB2* double mutant. A similar amplification of the R-HIR was observed when the B2/9 transgene is expressed in WT (Fig. 2.11c). A strong amplification of the R-HIR was observed in the two other *PHYB2*OE lines B2/4 and B2/8 (Fig. 2.11d). The results in Figure 2.11c and d show a shift of the WT R-HIR response to lower fluence rates in the *PHYB2*OE lines.

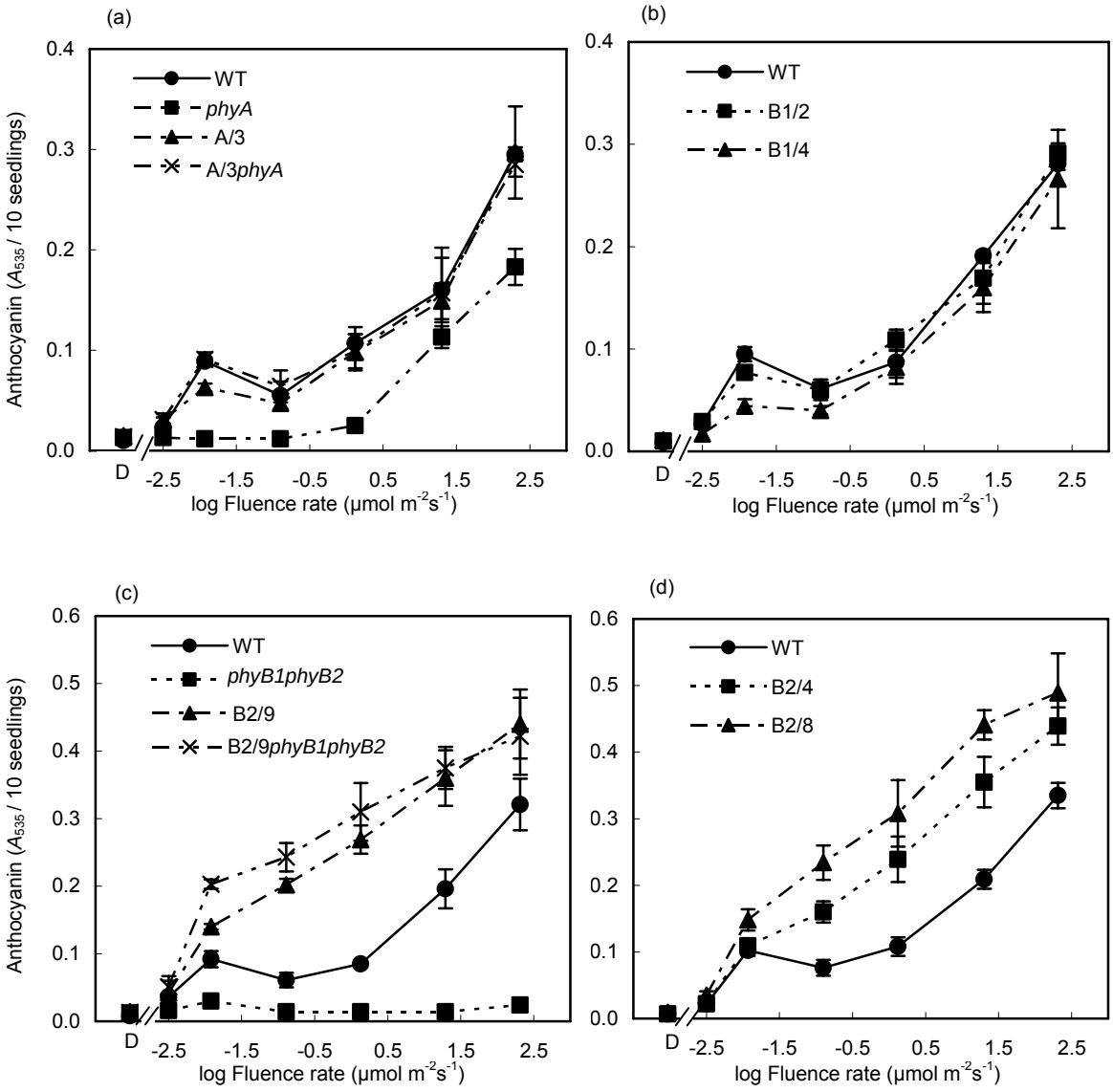


Figure 2.11. Anthocyanin accumulation (A_{535}/10 seedlings ± SE, n = 4) in WT and (a) *PHYA*OE lines and *phyA* mutant, (b) *PHYB1*OE lines and (c and d) *PHYB2*OE lines and *phyB1phyB2* double mutant. Dark-grown 4-day-old seedlings were given a 24-h irradiation with 680 nm of different fluence rates. D = dark control.

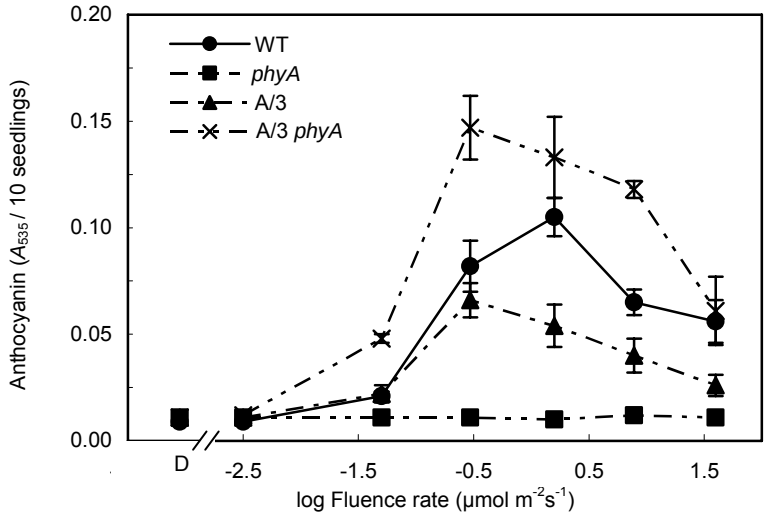


Figure 2.12. Anthocyanin accumulation (A_{535}/10 seedlings ± SE, n=4) in WT, *phyA* mutant and *PHYA*OE seedlings in response to different fluence rates of FR. Dark-grown 4-day-old seedlings were given a 24-h irradiation with 730 nm of different fluence rates. D = dark control.

2.4 Discussion

2.4.1 Elevated levels of biologically active phytochrome in tomato seedlings

The phytochrome protein level in tomato plants with ectopic expression of the homologous *PHYA*, *PHYB1*, and *PHYB2* genes was characterized (Fig. 2.1), using antibodies specific for PHYA, PHYB1 and PHYB2 that were generated for this purpose. Despite the high specificity of these antibodies, they proved of limited use in quantifying phytochrome protein levels in extracts of WT plants. This either indicates that: (1) these antibodies have a low titre; (2) they only bind to the phytochrome proteins with low affinity or (3) the phytochromes are present at extremely low levels in the protein extracts from WT plants. However, these antibodies did prove to be useful for the analysis of phytochrome protein levels in the *PHY*OE lines.

The effect of *PHYA*OE on *PHYA* mRNA levels was small compared to the *PHYA* mRNA levels in D-grown WT seedlings (Fig. 2.3a). Despite this, the protein analysis suggests that the PHYA level in A/1 and A/3 lines is elevated 8- and 16-fold, respectively, compared to that in WT. The PHYA level in the A/3*phyA* line was similar to that in A/3 (Fig. 2.5a). With the detection of phytochrome proteins on Western blots we cannot distinguish between phytochrome apo-protein or biologically active holo-protein. However, the ~15-fold increased levels of PHYA in A/3*phyA* rescued the *phyA* mutant phenotype, indicating that at least part of the

detected PHYA is biologically active and assembles into holoprotein (Fig. 2.9a, Fig. 2.10a, Fig. 2.11a, and Fig. 2.12). Moreover, the fact that the bulk of excess of PHYA in D-grown seedlings is light labile, indicates that the bulk of excess PHYA is assembled into phyA (Fig.2.4d and Fig.2.6a).

No significantly elevated levels of *PHYB1* mRNA were detected in the *PHYB1*OE lines. However, a minimum 32-fold increase in PHYB1 level compared to WT, both in B1/2 and B1/4 was detected on Western blots. These *PHYB1*OE lines show a small, but distinct enhanced inhibition of hypocotyl elongation and a reduction in anthocyanin accumulation (Fig. 2.8a, 2.10b, and 2.11b), indicating that at least part of the excess of PHYB1 is biologically active and is assembled into phyB1. However, elevated levels of phyB1 have opposite effects on the elongation and the anthocyanin accumulation response.

The B2/9 transgene rescues the *phyB1phyB2* double mutant phenotype (B2/9*phyB1phyB2*, Fig. 2.7a, Fig. 2.8, Fig. 2.9c, Fig. 2.10c and Fig. 2.11c), indicating that PHYB2 is assembled into biologically active phyB2. The level of phyB2 in the *PHYB2*OE lines relative to endogenous level is up to 200-fold (see section 2.3.4). Presumably this is because the expression level of the endogenous *PHYB2* promoter is extremely low compared to the strength of the 2x*35S* promoter of the transgene. If the effectiveness of endogenous levels of phyB1 and phyB2 in suppression of plant elongation are similar, the phenotype of the *PHYB2*OE lines is expected to be stronger than that of *PHYB1*OE lines (up to 200-fold increase in *PHYB2*OE versus only up to 32-fold in PHYB1, respectively), which is indeed the case (Fig. 2.7a, Fig. 2.8a-c, Fig. 2.9b and c, Fig. 2.10b and c and Fig. 2.11b-d). However, this is not consistent with the fact that loss of phyB2 function has little effect on plant elongation. It could be that phyB2 is inherently more efficient than phyB1 in signalling, but that in the WT phyB2 level is low compared to that of phyB1.

2.4.2 The *PHYA*OE has little effect on plant growth and development.

The *PHYA*OE lines used here show little phenotype under all the experimental conditions, despite the fact that PHYA was up to 8 to 16-fold elevated in these lines (Fig. 2.5a and 2.6a). The overexpression of the A/3 transgene in the *phyA* mutant background rescued aspects of the mutant phenotype (see A/3*phyA*, Fig. 2.10a). However, despite a ~15-fold increase of PHYA in A/3*phyA* lines, only a WT phenotype was seen in this line. These results suggest that in WT phyA levels are already saturated for phyA mediated responses, even under FR. Overexpression of

the same A/3 gene in WT resulted in a slight decrease of the low fluence rate component suggesting that extreme high phyA levels lead to a dominant negative effect (Fig 2.11a). A similar negative effect was seen in the *PHYA*OE lines in the FR fluence rate response (Fig. 2.12) and in the cFR experiment (Fig. 2.10a). In both these experiments accumulation of anthocyanin was reduced in the *PHYA*OE lines A/1 and A/3. This dominant negative effect could be due to titration of an essential factor by the excess PHYA (*e.g.* inactivation of phytochrome function by formation of mixed dimers with and without a chromophore).

In contrast to the results of the homologous *PHYA*OE in tomato presented here, the overexpression of heterologous oat *PHYA* in tobacco and tomato results in extreme dwarfism plants (Boylan and Quail, 1989; Robson *et al.*, 1996). An explanation for the differences in effect of homologous and heterologous *PHYA*OE could be that in the case of the homologous system phyA is more efficiently recognized by the endogenous degradation machinery. Indeed, in contrast to extracts from D-grown *PHYA*OE seedlings (Fig. 2.6a), no phytochrome protein was detected with MAb A2 in extracts of *PHYA*OE lines grown in the light (data not shown), suggesting a rapid turnover of the constitutively expressed PHYA under these conditions. In contrast, in systems with heterologous *PHYA*OE, PHYA was detected in extracts from light-grown plants (Boylan and Quail, 1989; Robson *et al.*, 1996).

2.4.3 *PHYB2*OE results in the most severe effect on plant elongation and pigmentation

The elevated levels of phyB1 and phyB2 in the *PHYB1*OE and *PHYB2*OE lines resulted in shorter stem and higher anthocyanin levels compared to WT, both in seedlings and adult plants, when grown under WL (Fig. 2.8). However, the elevated levels of phyB2 in the *PHYB2*OE lines gave the most pronounced suppression of hypocotyl and stem elongation and enhancement of anthocyanin accumulation. The phenotype of the B2/9*phyB1phyB2* line resembles that of the B2/9 line, suggesting that at higher expression levels the B2/9 action is saturated.

The results of *PHYB1*OE and *PHYB2*OE in tomato are similar to overexpression of *Arabidopsis PHYB* under the *CaMV-35S* promoter in *Arabidopsis*, which also correlates with short hypocotyls under WL and R (Wagner *et al.*, 1991). This indicates that the *Arabidopsis* phyB and both tomato phyB1 and phyB2 target similar responses under the same light conditions. Indeed, overexpression of either the tomato phytochrome *PHYB1* or *PHYB2* in the *Arabidopsis* phyB mutant, can

rescue the *phyB* phenotype (Kok, R., pers. comm.).

2.4.4 *PHYB1*OE and *PHYB2*OE and the red high irradiance response.

It has been shown that the fluence-rate response for anthocyanin synthesis during 24-h continuous R treatment is composed of two components in WT plants: a low fluence rate component and a HIR component. The low fluence rate component has been shown to be phyA-mediated and co-dependent on phyB2, while the R-HIR component has been shown to be both phyB1 and phyB2 dependent (Kerckhoffs *et al.*, 1997; Weller *et al.*, 2000). Here we demonstrate that the extra phyB1 in the *PHYB1*OE lines leads to little change in the R-HIR compared to WT (Fig. 2.11b). In contrast, the higher elevated levels of phyB2 in B2/4, B2/8 and B2/9 and B2/9*phyB1phyB2* lines result in a strong amplification of the R-HIR (Fig. 2.11c and d). These results again indicate a possible difference in the efficiency of phyB1 and phyB2.

The R-HIR depends on the cycling of the phytochrome pool, which presumably is directly related to the fluence rate. In this concept the cycling between Pfr and Pr operates as a 'photon counter' (Kendrick and Kronenberg, 1994). The effective output of cycling phytochrome may thus be enhanced in two ways: through an increase of the total stable phytochrome pool, or through an increase in fluence rate, which results in a higher cycling rate (Kendrick and Kronenberg, 1994). Although in our experiments the levels of phyB1 or phyB2 have been increased, only the very high levels of phyB2 result in an amplified R-HIR. The shift towards lower fluence rates can be used to estimate the effective level of stable phytochrome pool size (Ptot) that is reached in the *PHYB2*OE lines. In the B2/4 line there is a shift to approximately ten-fold lower fluence rates and in B2/9 there is a shift to approximately 100-fold lower fluence rates (Fig. 2.11c and d). This corresponds to the lower *PHYB2* expression level in B2/4 compared to B2/9. In the *PHYB2*OE lines the shift of the R-HIR to lower fluence rates causes an overlap of the R-HIR component with the phyA-dependent R low fluence rate component. When this is taken into account, the phyA-dependent low fluence rate component appears to be little affected in these lines. In the *PHYB1*OE lines the apparent decrease in the R low fluence rate component would cause the R-HIR component to start at higher fluence rates. However, apparently this is compensated by a shift of the R-HIR to lower fluence rates by the amplification of the R-HIR mediated by *PHYB1*OE.

2.4.5 Cell specific effects by constitutive overexpression of *PHYB2*

Analysis of hypocotyl cross sections show that, although a constitutive promoter was used for overexpression of *PHYB2*, the enhancement of anthocyanin accumulation in response to light is only seen in the same sub-epidermal cell layer which is pigmented in WT seedlings (Fig. 2.7b). The increased level in phyB2 signalling towards anthocyanin biosynthesis therefore only seems to be effective in this particular sub-epidermal cell layer and might be limited in other cell types by downstream components of the light signalling pathway that targets anthocyanin biosynthesis. It could be that also the effect of *PHY*OE on elongation is only from enhanced phytochrome signalling in the sub-epidermal cell-layer: the suppressed elongation of the sub-epidermal cells could prevent elongation of other cells within the tissue in which *PHY*OE is less efficient.

2.4.6 *phy*-mutants vs. *PHY*OE: complementary approaches reveal roles of phytochromes in tomato photomorphogenesis.

Mutant studies in tomato show that phyB1 contributes more strongly to de-etiolation than phyB2 under continuous R (van Tuinen *et al.*, 1995a). However the even stronger phenotype of the *phyB1phyB2* double mutant, indicates that phyB2 acts redundantly with phyB1 to control development (Weller *et al.*, 2000). The results presented here show that when the PHYB1 level is increased up to 32-fold, the plant phenotype was little affected. This might indicate that the endogenous phyB1 levels are saturated and that other factors, which are specifically downstream of phyB1 signalling become limiting in *PHYB1*OE plants. When the PHYB2 level is increased by 100-fold the phenotype was strongly affected under all the conditions used, indicating that at least downstream factors for phyB2 signalling are not limiting.

Although mutant analysis indicated that the phyA-dependent low fluence rate component is co-dependent of phyB2 and independent of phyB1 (Weller *et al.*, 2000), an increase of phyB1 above the WT level reduces the response under low fluence rates (Fig. 2.11b). This indicates that phyB1 not only is required for the low fluence rate response, but that at elevated levels an antagonistic role can be assigned to phyB1. High levels of phyB1 (Fig. 2.10b) also appear to inhibit responses under FR, which normally are ascribed to phyA. Combined these results suggest a negative interaction between phyA and phyB1 at elevated levels of phyB1. This reduced low fluence rate response in the *PHYB1*OE plants could affect

the anthocyanin accumulation response over the whole range of fluence rates that was tested: the small reduction in anthocyanin accumulation due to lower response at low fluence could mask the small increase in anthocyanin accumulation due to a shift in the R-HIR towards lower fluence rates in B1/4.

In conclusion, the results presented here demonstrate the success in overexpressing phytochrome genes in tomato. The analysis of the effect of *PHY*OE complements previous studies using type specific phytochrome mutants. For inductive responses in WT, overexpression sometimes results in dominant negative effects. In the case of the HIR reaction for anthocyanin accumulation, overexpression results confirm the prediction that the fluence rate response relationship for such a response would be shifted to lower fluence-rates. This enabled an estimate to be made of the increase in the number of phytochrome molecules in relevant overexpression lines compared to WT. In the next chapter analysis of the shade-avoidance responses under different physiological conditions of these *PHY*OE lines will be presented

Chapter 3

Shade-avoidance responses in tomato lines ectopically expressing tomato *PHYA*, *PHYB1* and *PHYB2* *

Husaineid, S.H., Schreuder, M.E.L., van der Krol, A.R. and van der Plas, L.H.W.

Abstract

To study the effect of enhanced phytochrome levels on the shade-avoidance response (SAR) in tomato, plants were produced with constitutive overexpression (OE) of the tomato *PHYA*, *PHYB1*, and *PHYB2* genes. Effects of the *PHY*OE on the SAR were tested in a proximity assay and an end-of-day-FR (EODFR) experiment. In the proximity assay, wild type (WT) and transgenic *PHYA*OE, *PHYB1*OE, and *PHYB2*OE lines were grown in high-density stands in the greenhouse and the differential effect on growth of inner and outer positioned plants was quantified. In the EODFR experiment, WT and *PHYA*OE, *PHYB1*OE and *PHYB2*OE lines were grown in growth cabinets, with and without a 20 min EODFR treatment. Growth and anthocyanin levels were quantified in treated (+EODFR) and non treated (–EODFR) plants. In the *PHYA*OE lines, little effect on the SAR and + and –EODFR responses was observed, but the shade-avoidance index (SAI) was reduced in the A/1 line compared to WT. The *PHYB1*OE and *PHYB2*OE resulted in a suppression of the SAR, leading to dwarf plants. However, these plants still respond to shade conditions, resulting in a similar or even higher SAI than in WT. The suppression of elongation and the enhancement of anthocyanin accumulation are largely eliminated by 20 minute EODFR treatment both in *PHYB1*OE and *PHYB2*OE lines as well as in WT plants. Compared to WT, *PHYB2*OE reduced biomass in shoot, stem and root of 8-week old plants, while in one *PHYB1*OE line allocation of biomass towards the roots was preferred over allocation towards stem. The effects of *PHY*OE are discussed in relation to theoretical predictions on the effect of increased phytochrome levels on the SAR and SAI.

* Part of the results in this chapter is published together with results of Chapter 2 in the *Journal of Experimental Botany* (2007), **58**: 615-626. The other part of the results is in preparation and to be submitted for publication.

3.1 Introduction

Plants have evolved different strategies in order to enhance their survival prospects under conditions of competition for light. One of these strategies, shade tolerance, allows plants living in deep shaded environment to capture and utilize light very efficiently. Another strategy is shade avoidance, which allows plants to position the leaves out of the shade in order to maximize the capture of light. The shade avoidance response (SAR) involves the perception of specific light signals of canopy shade or of light reflected from neighbouring plants and is manifested by a rapid and dramatic increase in the extension growth of stem and petioles at the expense of leaf growth and storage organ production. Responses to shade may also involve effects on leaf angle, anthocyanin accumulation and flowering time. Collectively these responses are called the SAR or shade avoidance syndrome and perception of the red light: far-red light ratio (R:FR) by phytochrome plays an important role in this SAR. In direct daylight the R:FR is approximately 1.2. Within plant communities, however, this ratio may be reduced up to 0.01 depending on plant density, due to absorption of R by chlorophyll. The responses of plants to changes in R:FR are mediated by the phytochrome family of photoreceptors (Smith, 2000). Phytochromes can occur in an inactive (Pr) and active form (Pfr). Since absorption of FR by Pfr results in a conversion into Pr and the absorption of R by Pr results in a conversion into Pfr, the level of active phytochrome(s) is set by the dynamic equilibrium between Pr and Pfr, which in turn is determined by the R:FR in the ambient light perceived by the plant. Sunlight filtered through a plant canopy and/or light reflected from neighbouring plants, has a lower R:FR than direct sunlight and consequently results in a shift of the Pr $\rightleftharpoons$ Pfr equilibrium towards Pr. Since Pfr suppresses plant extension growth (Smith, 1982), a reduction in Pfr levels will result in an enhanced plant elongation.

In higher plants phytochromes are encoded by a small gene family and tomato has one light labile (phyA) and four light-stable phytochromes (phyB1, phyB2, phyE and phyF) (Hauser *et al.*, 1995). The role of phytochromes levels and type in SAR can be evaluated by growing plants with different levels of individual phytochromes at high density (proximity assay). However, in dense populations differences between shaded and non-shaded plants may also derive from limitations of photosynthetic light or other non-phytochrome related factors that differ between shaded and non-shaded plants (*e.g*. changes in ethylene concentration or blue light fluence). These factors can be avoided experimentally by comparing plants with or without supplementary FR during the day (Smith, 1982) or

by comparing plants with and without FR at the end-of-the-day (EODFR treatment, Kendrick and Kronenberg, 1994).

In tomato, it has been shown that phyB2 acts redundantly with phyB1 in SAR (Weller *et al.*, 2000). However, a tomato triple mutant lacking phyA, phyB1, and phyB2 still showed residual responsiveness to supplementary daytime FR. This indicates that at least one of the two remaining phytochromes in tomato plays a significant role in tomato SAR (Weller *et al.*, 2000). Previously, mutant studies have shown that tomato phyA does not contribute to the EODFR response (van Tuinen *et al.*, 1995a), while phyB1 has a strong contribution to the response to EODFR (van Tuinen *et al.*, 1995b)

The SAR results in re-allocation of resources of the plant towards the stem (Libenson *et al.*, 2002) and therefore may affect leaf, fruit or seed yield. For some crop plants suppression of the SAR in plants grown at high densities could result in a higher proportion of biomass allocated to the harvestable organs (Smith and Whitelam, 1997). The response to R:FR was reduced by overexpression of oat *PHYA* in tobacco, resulting in a reduced stem: leaf fresh weight ratio for plants grown at high density (Robson *et al.*, 1996). Potato with ectopic expression of the *Arabidopsis PHYB* showed increased tuber yield in isolated pots and especially when grown at high density (Boccalandro *et al*., 2003; Thiele *et al*., 1999). Overexpression of oat *PHYA* results in dwarf plants in tomato (Boylan and Quail, 1989) and it is not established whether this will result in a reallocation of resources towards fruits in these plants.

Another approach towards suppression of SAR is overexpression of tomato phytochromes in tomato. In Chapter 2 it was shown that *PHYB1*OE lines resulted in a mild suppression of stem elongation, while *PHYB2*OE lines have a strong suppression of plant height, for plants grown under white light (WL). This could be interpreted as a mild and strong effect of phyB1 and phyB2, respectively, on suppression of SAR. In this chapter the effect of overexpression of tomato *PHYA*, *PHYB1* and *PHYB2* on SAR and shade-avoidance index (SAI) (see Section 3.2.2) of tomato plants in a close-proximity assay was studied. In these experiments lines expressing a *CaMV 2x35S-PHYA* (A/1 and A/3), a *CaMV 2x35S-PHYB1* (B1/2 and B1/4), or a *CaMV 2x35S-PHYB2* (B2/4, B2/9, B2/9*phyB1phyB2* and B2/8) transgene were used. From these plants the SAR, SAI, and EODFR response was quantified, and the effect of *PHY*OE on biomass allocation to the different plant organs was determined.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

The different tomato genotypes that were used in the experiments were all in the genetic background of *Lycopersicon esculentum* Mill. cultivar MoneyMaker (MM), which served as wild type (WT). Construction and initial characterisation of transgenic tomato lines with *PHYA*OE, *PHYB1*OE and *PHYB2*OE have been described in Chapter 2. The homozygous line B2/9*phyB1phyB2* was obtained by screening the T_2 progeny of a cross of the *PHYB2*OE line B2/9 (Chapter 2) with the *phyB1phyB2* double mutant (Weller *et al*., 2000).

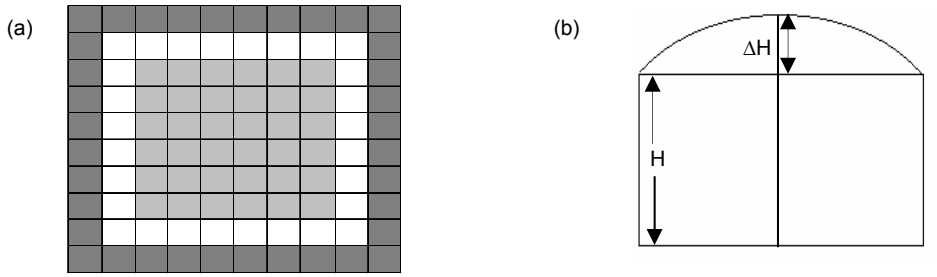


Figure 3.1. (a) Plain view of the block of 10 x 10 plants in the proximity assay experiment. The 36 plants-out (dark grey) and 36 plants-in (light grey) were used to study shade avoidance responses and shade avoidance index. (b) Schematic drawing of cross section through the plant population. h = height of plants-out. ΔH = [height of plants-in] – [height of plants-out]. The shade-avoidance index (SAI) = ΔH/H.

3.2.2 Proximity experiment

The experiments were designed to study the SAR of plants grown at a density of 100 plants m^{-2}. One hundred plants of WT, A/1, A/3, B1/2, B1/4, B2/4, B2/8 and B2/9 were grown in 10X10 Rock wool blocks in the period of June and July 2002 and June and July 2003, in a greenhouse under natural light conditions. The blocks of 10x10 plants were surrounded by a wall of black plastic foil 40 cm high at a distance of 40 cm from the plants to prevent interference from surrounding plant populations. The plants were grown for 6 weeks, after which all plants were harvested and analysed for plant height, leaf area (LA; all leaves longer than 0.5 cm), leaf fresh weight (LFW; all leaves longer than 0.5 cm) and leaf dry weight (LDW; all leaves longer than 0.5 cm), number of leaves, stem fresh weight (SFW) and stem dry weight (SDW), and stem diameter. From each measurement the

mean values and SE are calculated of 36 plants grown at the centre of the population (plants-in) and 36 plants grown at the outer edge of the population (plants-out) (Fig. 3.1a). From the mean height values the SAI was calculated using the formula: ([height plant-in] minus [height plant-out]) / (height plant-out): $\Delta H/H$ (Fig. 3.1b). Experiments were carried out in sets of three (WT plus two *PHY*OE lines) and each *PHY*OE line was tested twice over the period 2002 to 2003. In two out of the eight experiments the response of the WT control plants deviated from the norm (SAI < 0.02) and consequently the results from these two replicate experiments were omitted from the analysis.

3.2.3 Root biomass allocation experiment

Tomato seeds were germinated in trays of peat-based compost until the emergence of primary leaves, at which time the seedlings were transferred to small plastic pots filled with 4.8 mm clay beads (Substraat Korrels; JongKind Grond Aalsmeer-Holland, the Netherlands). The pots were half covered with circulating nutrient solution on a greenhouse bench, under ~25°C day/18°C night. After 28 days the roots were carefully removed from the clay beads, and root fresh and dry weight was measured. In addition the plant height, SFW and SDW and LFW and LDW were measured.

3.2.4 End-of-day far-red light experiment.

Seeds were sown directly in trays of peat-based compost (40 seeds per tray) and grown for 10 days in a phytotron in a 16 h WL (160 µmol m^{-2} s^{-1}) / 8 h D cycle at 25˚C, at a relative humidity of 65-70 %. On day 10, the plants were transferred to growth cabinets and allowed to adjust to the lower level of WL (120 µmol m^{-2} s^{-1}) for one day. The subsequent 18 days the plants received 20 min FR (3 µmol m^{-2} s^{-1}) irradiation, directly following the daily 16 h WL period (+EODFR), while controls received 16 h WL / 8 h D and no FR irradiation (–EODFR). Plant height, first internode length, hypocotyl length, and anthocyanin content of young leaves (1.0-1.5 cm) were measured at the end of the treatment. From these measurements the mean values ± standard error (SE) of 35 +EODFR plants and 35 –EODFR were calculated. The EODFR index was calculated using the formula: ([height +EODFR plants] minus [height of –EODFR plants], divided by [height of –EODFR plants]).

3.2.5 Anthocyanin assay

Samples of young leaves (1.0 -1.5 cm in length) were harvested, weighed and subsequently anthocyanin was extracted as described in 2.2.6. The relative anthocyanin level was determined by measuring the absorption of the extract at 535 nm (A_{535}). The anthocyanin content was expressed as (A_{535} per g FW).

3.2.6 Estimation of the stable phytochrome pool size.

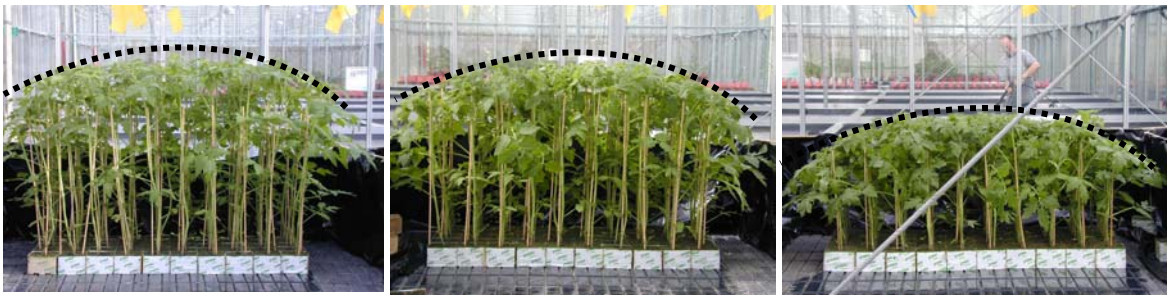
The total phytochrome Ptot (Ptot = Pr + Pfr) levels in WT and transgenic 4-day-old dark (D) grown seedlings were determined using MAb Pea-25. Using this antibody it was demonstrated that the Ptot pool sizes in the B1/2 and B1/4 lines were approximately 8-fold higher than that in WT. Similarly, the Ptot pool size in the line B2/4 was estimated to be at least 4-fold, while both in B2/8 and B2/9 the Ptot pool size was estimated to be 8-fold higher than in WT (see Chapter 2). Because the part of the Ptot signal in WT which is from type II phytochromes is less than 10% of the Ptot signal from dark-grown seedlings extracts (Hauser *et al.,* 1997), we estimate the stable Ptot pool size in the B1/2, B1/4, B2/8 and B2/9, to be about 80-fold higher than in WT, and the stable Ptot pool size in the B2/4 was estimated to be at least 40-fold higher than WT.

3.3 Results

3.3.1 Effects of *PHY*OE on shade-avoidance responses in tomato

In Chapter 2 it was shown that the overexpression of *PHYA* and *PHYB1* has only limited effects on plant height in tomato, while overexpression of *PHYB2* resulted in dwarf plants. This could be interpreted as a suppression of the elongation response of SAR due to phyB2 accumulation in the *PHYB2*OE lines. To analyse the effect of *PHY*OE on SAR in more detail, the *PHYA*OE lines A/1 and A/3, *PHYB1*OE lines B1/4 and B1/2, and *PHYB2*OE lines B2/4, B2/8 and B2/9 were selected for characterisation of SAR and SAI in a proximity-assay. Plant populations for each genotype were grown at a density of 100 plants m^{-2} and plant growth parameters were determined for plants inside and plants at the outer edge of each population. Experiments were carried out in 2002 and repeated in 2003. Figure 3.2 shows the phenotype of WT and the B1/4 and B2/9 plants in cross section through the centre of the plant population. Plants growing at the outer edge of the population (which

receive less canopy shade and reflected light from neighbours than plants within the population) are shorter than plants on the inside, in WT as well as in B1/4 and B2/9 lines. Figure 3.3 shows the plant height, LA, LDW and SDW of plants grown in each population (results from experiments carried out in 2002). Comparison of height of plants-in and plants-out in each population shows that for all genotypes the plants on the outside are shorter than plants on the inside. (Fig. 3.3a), indicating that in all *PHY*OE lines there is still a measurable response to shade.



WT B1/4 B2/9

Figure 3.2. Shade-avoidance responses in high-density populations. The phenotype of a cross section at the middle of the population of 8-week-old WT, B1/4, and B2/9 tomato plants, which were grown at high density in the greenhouse in Rock wool blocks, is shown. The dotted curve above the plant population illustrates the differential growth of the plants at the outside and inside of the population.

In WT as well as in all transgenic lines, there was no significant difference in LA of plants grown on the inside and the outside, although there was a general tendency for leaves to be larger on plants growing at the outside (Fig. 3.3b). Compared to WT the LA of plants-in and plants-out of the *PHYA*OE and *PHYB1*OE plants was not affected in a consistent way. The LA of both plants-in and plants-out of the B2/4 and B2/8 transgenic lines was reduced compared to WT, both for experiments carried out in 2002 and in 2003 (Fig. 3.3b and results not shown). The reduction in LA in these transgenic lines is not only from reduced cell elongation, but also from reduced biomatter accumulation in leaves, because also LDW was reduced in these lines compared to WT (Fig. 3.3c and results not shown). The results in Figure 3.3c show that in general the allocation of biomatter towards leaves is lower for plants on the inside compared to plants growing on the outside of the population. Compared to WT, the overall allocation of biomatter to stems is reduced in the *PHYB2*OE lines. The reduced SDW in these populations correlated well with the reduced height of plants in each population, both for the experiments carried out in 2002 and 2003 (compare Fig. 3.3a and Fig. 3.3d and results not shown).

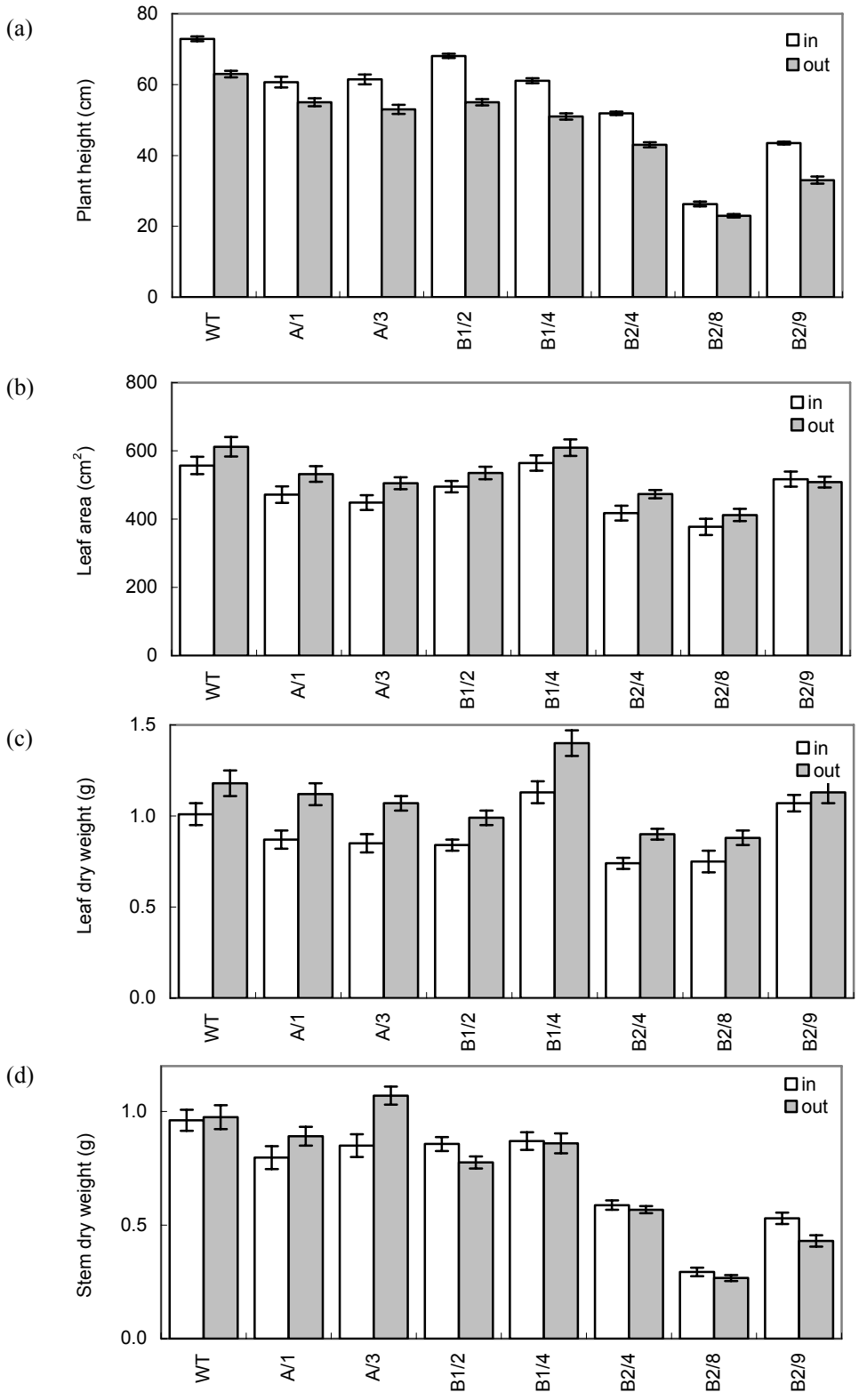


Figure 3.3. Plant growth parameters for plants-in (in) and plants-out (out) in the high density population experiment. The plant height (a), leaf area (b), leaf dry weight (c) and stem dry weight (d) were determined for plants-out (out) and plants-in (in). Results are the mean ± SE (n = 36).

3.3.2 Responses of wild type and *PHY*OE lines to end-of-day FR

In the previous section the SAR was studied for plants grown in close proximity in the greenhouse. Under those conditions, it is possible that factors other than phytochrome contribute to the differences between plants grown in the inside or outside of each population. In order to nullify the effects of these factors, the same lines were tested in an EODFR experiment. In addition we studied the B2/9*phyB1phyB2* line to see if it could complement the *phyB1phyB2* double mutant phenotype. Figure 3.4 shows the phenotype of the WT plants and the B1/4 and B2/9 lines after 18 days of + or –EODFR treatment. The EODFR treatment results in a strong increase in plant height compared to the controls, both in WT and the *PHY*OE lines. The total plant height, first internode length and hypocotyl length of plants after 18 days treatment with and without EODFR were quantified (Fig. 3.5a-c). The results in Figure 3.5a,b show that for the A/1 and A/3 lines there were no large differences in the plant height and the length of the first internode compared to WT, but the A/1 line clearly had a significantly taller hypocotyl than WT (Fig. 3.5c). The absolute difference in total plant height, first internode length and hypocotyl length between EODFR treated and non-treated plants is significantly increased in the *PHYB1*OE and in the *PHYB2*OE lines except for the B2/8 line and is smaller in the *phyB1phyB2* double mutant compared to WT (Fig. 3.5a-c). The phenotype of the *phyB1phyB2* double mutant was complemented by the overexpression of *PHYB2*, resulting in similar hypocotyl, internode and plant length as in B2/9 (Fig. 3.5a-c).

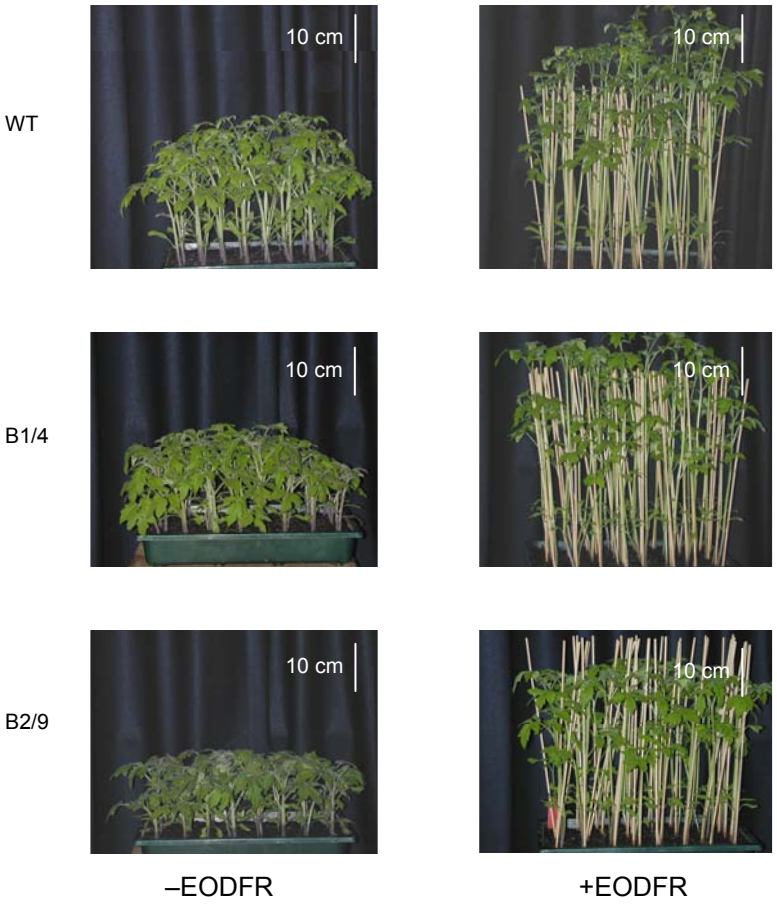


Figure 3.4. Phenotypes of WT, B1/4, and B2/9 tomato plants after 18 days of + and – end-of-day-far-red light (EODFR) treatment. For +EODFR treatment the plants received 20 min FR (3 µmol m^{-2} s^{-1}) irradiation after the daily white light period, before the dark period. The –EODFR were grown under similar conditions but received no FR.

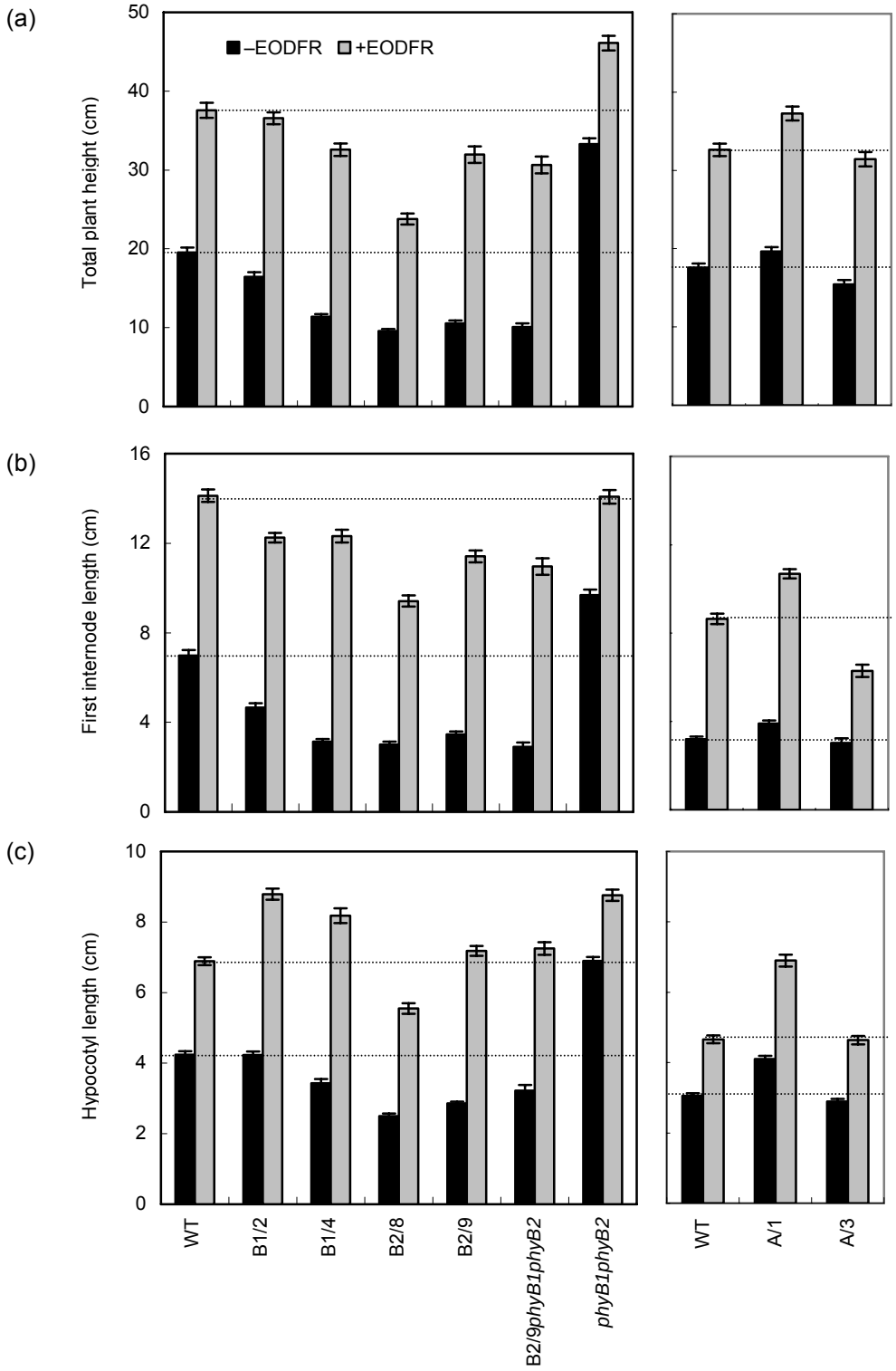


Figure 3.5. Plant elongation response after 18 days of + and – end-of-day-far-red light (EODFR) treatment. (a) Plant height, (b) First internode length and (c) hypocotyl length of WT and transgenic lines + and –EODFR treatment. Results are the mean values ± SE (n = 35). The results in the right panels are from a separate experiment performed at a different time of the year, which resulted in a slower growth rate (dotted lines show the control levels in wild type (WT) in each set of experiments).

The anthocyanin levels in immature leaves (1.0-1.5 cm in length) were also quantified in plants after 18 days + or –EODFR treatment (Fig. 3.6). The results show that in response to the EODFR treatment the anthocyanin levels are strongly reduced in all genotypes. Figure 3.6 shows that the residual anthocyanin levels after a +EODFR treatment is higher in the *PHYB2*OE lines compared to WT and lower in the *PHYA*OE lines compared to WT. The absolute differences in anthocyanin accumulation in + and –EODFR treated plants are larger for B1/4 and all *PHYB2*OE lines. Although the level of anthocyanin in the *phyB1phyB2* double mutant is much reduced compared to WT, a response to the EODFR treatment could still be observed. This effect of the absence of functional phyB1 and phyB2 in the *phyB1phyB2* double mutant was rescued by the overexpression of *PHYB2* alone (see B2/9*phyB1phyB2*; Fig. 3.6) and results in a phenotype similar to B2/9 in WT.

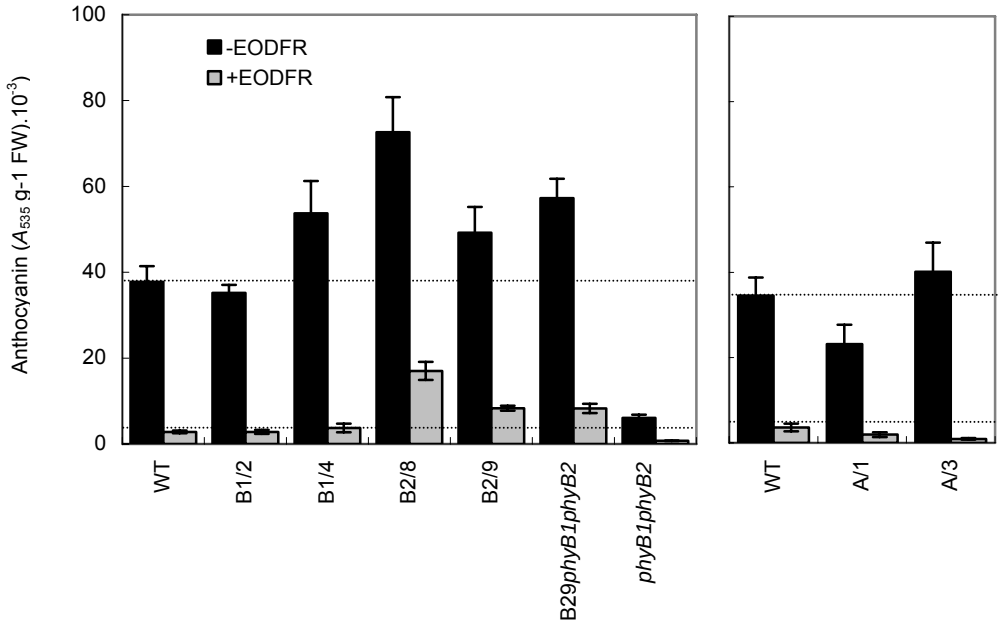


Figure 3.6. Anthocyanin accumulation responses to + and – end-of-day-far-red light (EODFR) treatment. Samples were harvested and extracted after 18 days treatment + or –EODFR. Results are mean ± SE (n = 4). The results in the right panels are from a separate experiment performed at a different time of the year (dotted lines show the control levels anthocyanin in wild type (WT) in each set of experiments).

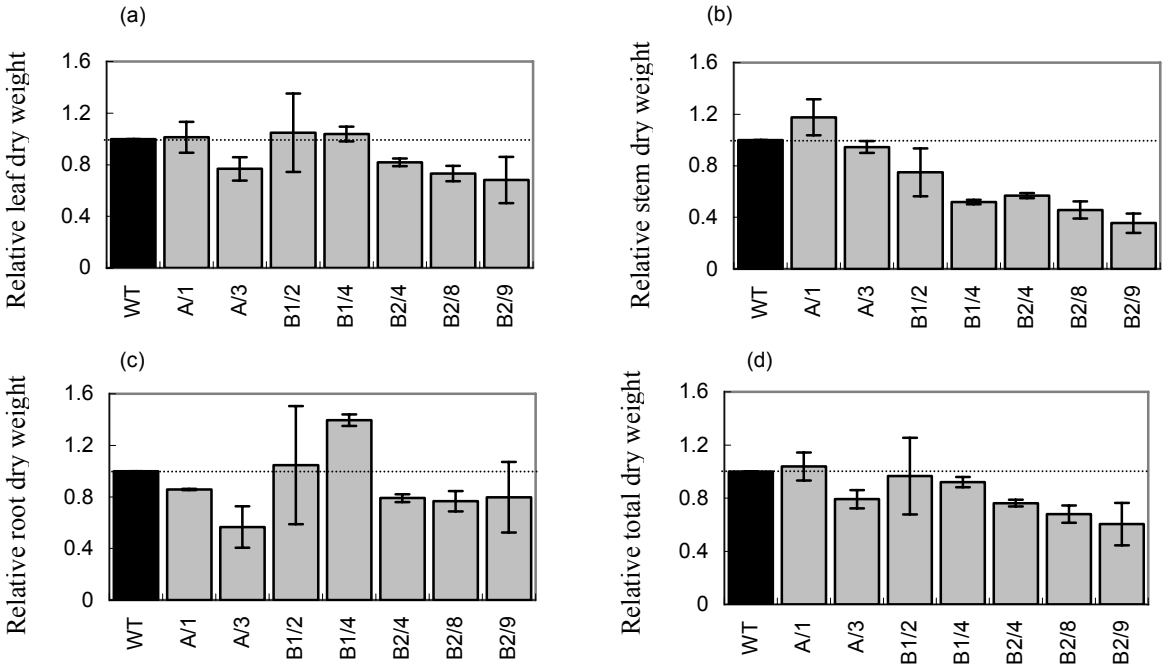


Figure 3.7. Biomass allocation in wild type (WT), *PHYA*OE, *PHYB1*OE and *PHYB2*OE lines. The dry weight of leaf, stem, root and total dry weight were determined for WT, *PHYA*OE, *PHYB1*OE and *PHYB2*OE lines. Values were normalized to those of WT (mean WT values: LDW = 3.4 g ± 0.3; SDW = 1.4 g ± 0.1; RDW = 0.6 g ± 0.04, TDW = 5.5 g ± 0.4). The average of two independent sets of measurements (each based on 5 plants) is shown. Results are mean ± SE (n = 2). (a) Relative leaf dry weight (LDW); (b) Relative root dry weight (RDW); (c) Relative stem dry weight (SDW); (d) Relative total dry weight (TDW).

3.3.3 Effects of *PHY*OE on biomass allocation

In the proximity assay experiment (Section 3.3.1) it was demonstrated that the dry matter accumulation in leaf and stem is reduced in some of the *PHY*OE lines. This indicates that allocation to different plant organs may be affected by *PHY*OE. Therefore the effect of *PHY*OE on biomass allocation to leaf, stem and roots was studied in separate experiments. Plants in the greenhouse of WT, *PHYA*OE, *PHYB1*OE and *PHYB2*OE lines were grown in hydroponics culture and dry weight of leaf, stem and root and total dry weight were determined after 4 weeks. The values of the plant growth parameters were normalized to those of the WT in each independent set of experiments (Fig. 3.7a-d). In the *PHYA*OE lines A/1 showed no significant difference with WT for LDW and SDW, but a small decrease in RDW compared to WT. The A/3 line showed a significant reduction in both LDW and RDW compared to WT (Fig. 3.7a-c). The results in Figure 3.7 show that in the *PHYB1*OE lines the B1/2 line has similar LDW, SDW, RDW and TDW compared to the WT whereas in the B1/4 line there was less biomass allocation to stem, but an

increase in biomass of roots (Fig. 3.7b-c). For the *PHYB2*OE lines the dry matter accumulation in all plant organs decreased, but relatively more in stem compared to leaves and roots (Fig. 3.7a-c). Figure 3.7d shows that the sum of these effects is a reduced total dry weight for the *PHYA*OE line A/3 and all *PHYB2*OE lines, while overall biomass accumulation in A/1 and the *PHYB1*OE lines B1/2 and B1/4 was not significantly affected.

3.4 Discussion

3.4.1 Phytochrome levels and shade-avoidance index.

It has previously been shown that there is a negative linear relationship between the extension growth rate of plants and the proportion of Ptot which is maintained as Pfr (Pfr/Ptot; Smith and Holmes, 1977). Because overexpression of *PHY* in several plant species resulted in an increased suppression of plant elongation (Boylan and Quail, 1989; Casal and Sánchez, 1994; Robson *et al*., 1996), plant height is most likely affected by the absolute amount of Pfr and not the ratio of Pfr: Pr. In Figure 3.8 a linear relationship between Pfr and plant height is plotted, with the slope determined by the experimental values for WT tomato plants in shade and non-shade as presented in Figure 3.3a. We calculated the amount of Pfr in shade and non-shade for different Ptot pool sizes, assuming a R:FR of 0.6 for shaded plants and R:FR of 1.0 for non shaded plants (Table 3.1). Given the hypothetical relationship between R:FR and Pfr/Ptot (Smith and Holmes 1977), these R:FR conditions result in 40% of the Ptot in the Pfr form for shaded and 50% of the Ptot in the Pfr form non-shaded plants, respectively. Each Pfr value gave a predicted plant height from Figure 3.8. From these values we calculated the SAI for different Ptot pool sizes (Table 3.1 and Fig. 3.9). The graph shows that the shade avoidance index can increase up to 10-fold when the Ptot pool size is doubled. Above a doubling of the WT Ptot pool size (for instance by *PHY*OE) the SAI will actually decrease to zero, while this decrease in SAI is also expected at very low Pfr levels (for instance in a *phy* mutant which mimics shade avoidance).

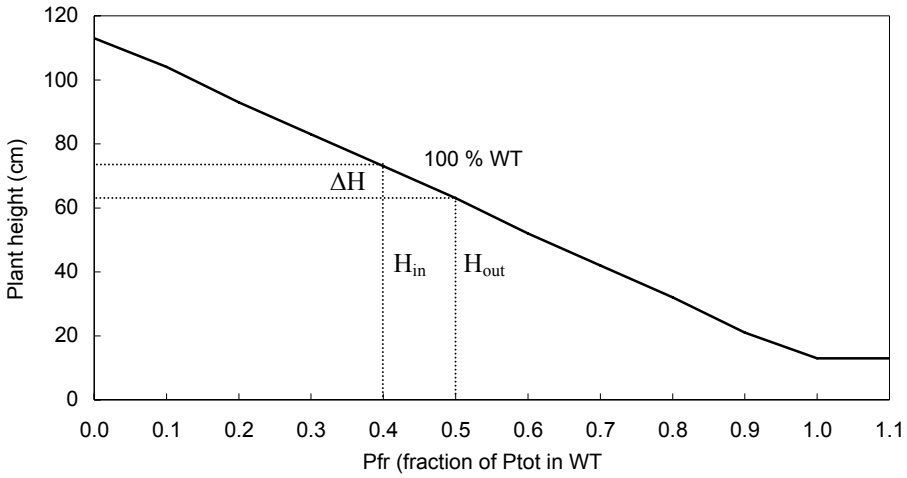


Figure 3.8. Hypothetical relationship between plant height and amount of stable Pfr for tomato plants. The WT plant height values of shaded and non-shaded plants in Figure 3.3a were used to set the slope. The Pfr concentration is given as fraction of Ptot in WT. The minimum plant height is 13 cm because this was the plant height at the start of the experiment.

3.4.2 Variable effects of *PHYA*OE on the shade-avoidance response.

It was noted that the effect of *PHYA*OE in A/1 on plant height varied between experiments: small reduction in plant height in two proximity assays (Fig 3.3a); small increase in plant height in two EODFR experiments (Fig 3.5a); small decrease in plant height in the experiments described in Chapter 2. The elongation response to proximity in the *PHY*OE lines was used to calculate the SAI index relative to that of WT, for each set of experiments (Figure 3.10). In 2002 the SAI in A/1 was lower than in WT and the SAI of A/3 was similar to WT. However, in the replicate experiment of 2003 with A/1, A/3 and B1/4, the response of the WT control plants deviated from the norm (SAI < 0.02), so that no firm conclusions can be drawn for the effect of homologous *PHYA*OE on SAI. Although phyA is degraded in the light, the constitutive expression of the *35S*::*PHYA* transgene could still result in a low steady state level of Pfr^{phyA} in the light, adding to the suppression of plant elongation. The response to proximity for A/1 and A/3, given in Figure 3.3a was used to calculate the SAI index (Fig. 3.10). The calculation shows that in the A/3 line the SAI is not significantly affected, but in the A/1 line a reduction of the SAI was seen in two independent experiments (Fig. 3.10). In the *PHYA*OE lines the constitutive overexpression of homologous *PHYA* apparently can still contribute to the Ptot pool size in the light, causing a reduction in the elongation in line A/1 compared to WT (Fig. 3.3a). Because phyA is relatively more stable under low R:FR, the contribution of *PHYA*OE to the total Pfr pool under shade conditions

could be relatively larger than under non-shade conditions, resulting in reduction in SAI compared to the SAI of WT. The R:FR-dependent effect on phyA stability is even stronger in the case of heterologous *PHYA*OE, resulting in a negative SAI (conditional dwarfing, Robson *et al*., 1996).

Table 3.1. Calculation of the Shade Avoidance Index for different Ptot pool sizes.
The values are based on a Pfr fraction of 0.4 in shade (R:FR=0.6) and a Pfr fraction of 0.5 in non-shade (R:FR=1.0) (Smith and Holmes, 1977). The Pfr concentration is given as fraction of the stable phytochrome pool (Ptot) in WT. Values of plant height are from figure 3.8. The minimum plant height is 13 cm because this was the plant height at the start of the experiment. SAI = $\Delta H / H_{out}$

% Ptot in WT	Pfr	Pr	H_{in}	H_{out}	$(\Delta H) / H_{out}$
0.2	0.08	0.1	106	104	0.02
0.4	0.16	0.2	98	93	0.05
0.6	0.24	0.3	89	83	0.07
0.8	0.32	0.4	81	73	0.11
1	**0.4**	**0.5**	**73**	**63**	**0.16**
1.2	0.48	0.6	64	53	0.23
1.4	0.56	0.7	56	42	0.33
1.6	0.64	0.8	48	32	0.50
1.8	0.72	0.9	40	21	0.90
2.0	0.8	1.0	32	13	1.46
2.2	0.88	1.1	23	13	0.77
2.4	0.96	1.2	13	13	0

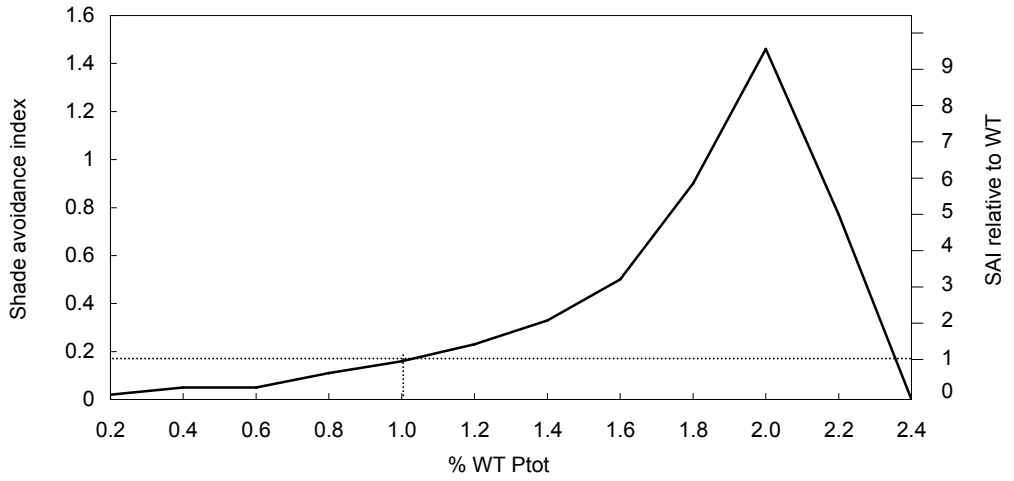


Figure 3.9. The SAI (left Y-axis) or relative SAI (right Y-axis) for different stable Ptot pool sizes (for calculations see table 3.1).

The effect of *PHYA*OE on plant length was largely eliminated by an EODFR treatment. The response to EODFR for A/1 and A/3, given in Figure 3.6, were used to calculate the EODFR index for elongation (Fig. 3.11). The EODFR index was not affected in A/1 and only slightly increased in A/3. Combined, the results indicate that homologous *PHYA*OE has no consistent or very little effect on the response of plants to shade conditions.

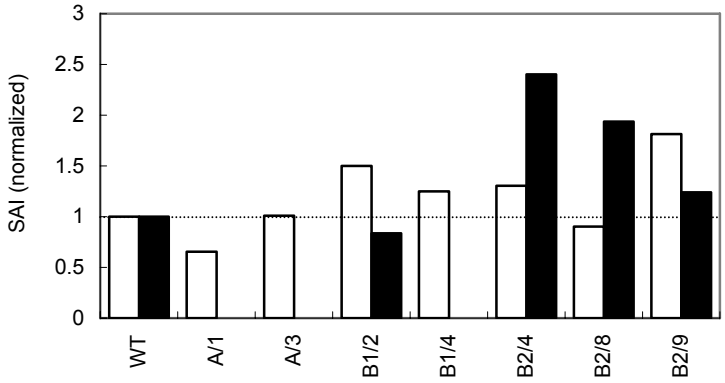


Figure 3.10. The shade-avoidance index for plant height, normalized to that of WT. SAI = ∆H/H; Fig. 3.1). White bars: SAI calculated from results in 2002 (Figure 3.3a). Black bars: SAI calculated from results in 2003 (not shown). Replicates of A/1, A/3 and B1/4 from 2003 were omitted due to irregularities in the WT control.

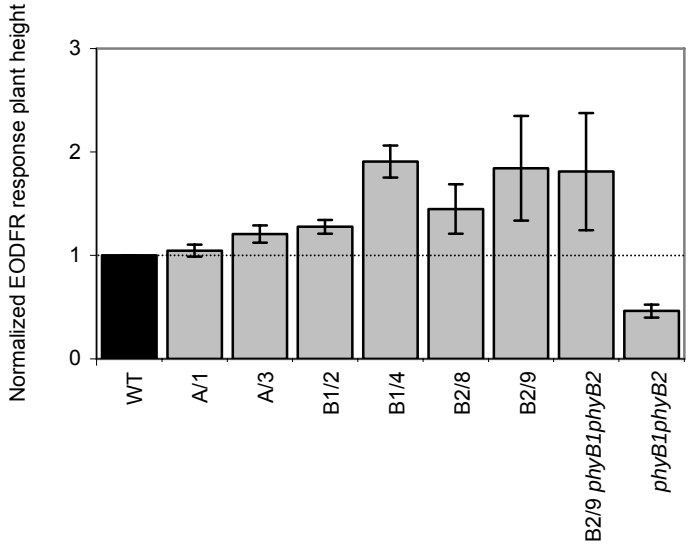


Figure 3.11. The EODFR index of Plant height. All values are normalised for the EODFR index in WT. The results are the means ± SE (n = 2). The EODFR index was calculated using the formula: [height ''+EODFR'' plants] minus [height of ''–EODFR ''plants], divided by [height of –EODFR plants], normalized for WT.

3.4.3 *PHYB1*OE and *PHYB2*OE mostly increase the shade-avoidance index

The excess of phyB1 and phyB2 in tomato plants results in overall shorter plants and an increase in the absolute differences in length (Fig. 3.3a and 3.5) and difference in anthocyanin accumulation in plants with and without EODFR treatment (Fig. 3.6). From the values in Figure 3.3 and 3.5 the SAI and EODFR index of the *PHYB1*OE and *PHYB2*OE lines were calculated (Fig. 3.10 and 3.11). Both *PHYB1*OE and *PHYB2*OE lead to suppression of elongation and in most cases in an increased SAI (Fig. 3.9). However, in 2002 the SAI in B2/8 was decreased, while in 2003 it was increased compared to WT (Fig. 3.10). Figure 3.9 shows that these results are consistent with the prediction that the SAI first increases upon *PHY*OE, but then decreases above a certain phytochrome level. The calculations show that for Ptot pool size larger than 2xWT the SAI is sensitive to small changes in Ptot, which may explain the variation in SAI calculated from experiments in 2002 and 2003 (Fig. 3.10). The increase in the EODFR index in the *PHYB1*OE and *PHYB2*OE lines (Fig. 3.11) confirms that the effect of *PHYB1*OE and *PHYB2*OE on plant height is mediated by an altered response to a given R:FR.

The SAI results of 2002 can be used to estimate the increase in Ptot pool size. Each SAI corresponds to two different Ptot pool sizes in the graph of Figure 3.9. The plant length (H_{out}) was used to determine which of these two Ptot pool sizes was most likely the correct one. For B1/2 and B1/4 the SAI (relative to WT) was 1.5 and 1.2, while H_{out} was still quite similar to WT (55 cm and 51 cm, respectively). This indicates that the effective increase in Ptot in these *PHYB1*OE plants is between 1.1x and 1.2x WT levels. For the lines B2/4 the SAI is ~1.3, while H_{out} is 42 cm, suggesting a ~1.2-fold increase in Ptot pool size compared to WT. Similarly, the SAI of 1.8 (Fig. 3.9) and H_{out} of 33 cm (Fig. 3.3) of B2/9 suggest a 1.3-fold increase in the Ptot levels in B2/9 compared to WT. The SAI in B2/8, which was shown to have the highest level of *PHYB2*OE (chapter 2), is 0.9x WT. This suggests that Ptot in B2/8 is ~2.3x Ptot in WT and that in B2/8 plants elongation has been fully suppressed. However, the plants of B2/8 are 20 cm long, so still have increased in size compared to the start of the experiment (Fig. 3.3), perhaps as a consequence of developmental processes leading to elongation that were already initiated before the transfer of the plants to the high-density situation. Analysis of total phytochrome levels in *PHYB2*OE lines using Western blots, indicated a ~8-10 fold increase in Ptot in B2/9 and B2/8 (Chapter 2 and Section 3.2.6). However, these estimated Ptot levels were measured in D-grown seedlings, while the estimation of the Ptot pool size from the change in length and change in SAI is from

light grown plants. Phytochrome was not detected in WL-grown plants, and also not in plants with the constitutive overexpression of the type II phytochrome genes (Chapter 2 and unpublished data). This indicates that there is a high turnover of PHY in WL-grown plants, but that the overall contribution of the constitutive *PHY*OE results in an approximately two-fold effective increase in Ptot pool size.

3.4.4 Effect of *PHY*OE on biomass allocation

The overexpression of *PHYA* had relatively little effect on biomass allocation to leaf and stem (Fig. 3.3c and d, Fig. 3.7a and b). Both in line A/1 and A/3 the dry weight of the roots was lower compared to WT, suggesting that the *PHYA* expression may interfere with the root sink function (Fig. 3.7c). In contrast, lowering the R:FR resulted in re-allocation of resources to stems in sunflower (Libenson *et al.*, 2002), while overexpression of oat *PHYA* in tobacco resulted in an increased leaf index (LDW:TDW) (Robson *et al*., 1996). The difference in effect of homologous and heterologous *PHYA*OE may be due to differences in stability of the phyA protein in a homologous or heterologous host plant (Robson *et al*., 1996).

The TDW was most affected in the *PHYB2*OE lines (reduced to 60-80% of WT TDW, Fig. 3.7d). This could be a combined effect of reduced root size and/or reduced leaf size in *PHYB2*OE plants and an effect of altered responses to R:FR by *PHYB2*OE on the optimized position of leaves for photosynthesis. It has been demonstrated that leaf orientation and other architectural parameters may affect the ability of the plant to maintain photosynthetic tissue in crowded populations (Ballaré *et al.*, 1997).

The effect of *PHYB1*OE and *PHYB2*OE in tomato on biomass accumulation differs from the effect of *Arabidopsis PHYBOE* in potato (Thiele *et al*., 1999; Boccalandro *et al*., 2003; Schittenhelm *et al.*, 2004). In potato the *PHYBOE* resulted in an increased leaf, root and tuber yield. In rice plants the overexpression of *Arabidopsis PHYA* under the rice *rbcS* promoter resulted in shorter plants and increases grain yield (Garg *et al.*, 2006). In subsequent experiments the lines B2/9, A/3 and B1/4 were selected for the investigation in the greenhouse of fruit biomass accumulation and the results showed, that for all these lines fruit biomass was about 50% lower than WT (A. van der Ploeg, unpublished data).

3.4.5 Concluding remarks

In this study it was demonstrated that *PHYB1*OE and *PHYB2*OE affect SAR in tomato. The overexpression of the *PHYB1* and *PHYB2* genes decreased plant elongation, but mostly increased the SAI, depending on the expression level that was reached. Overall, the results were in agreement with the presented hypothetical relationship between Pfr pool size and plant height (Fig. 3.8) and between Ptot pool size and SAI (Fig. 3.9). The results from the homologous *PHYA*OE and *PHYB*OE in heterologous host plants suggest that effects are not only obtained by expression level but also by phytochrome stability.

Chapter 4

Interaction between ectopic overexpression of tomato phytochrome and cryptochrome genes in tomato

Husaineid, S.H., Schreuder, M.E.L., van der Krol, A.R. and van der Plas, L.H.W.

Abstract

Transgenic tomato (*Lycopersicon esculentum* Mill.) lines, with ectopic overexpression of the homologous *PHYA*, *PHYB1*, *PHYB2*, *CRYI* and *CRY2* genes have been produced. Here we studied the level of saturation in phytochrome and cryptochrome signalling by comparing hemizygous with homozygous transgenic plants. Also we produced different combinations of *PHY*OE or combination of *PHY*OE with *CRY*OE, to study the interaction between *PHY's* and *PHY* and *CRY* at elevated levels of expression. The growth characteristics of seedlings under red, far-red, and blue light were determined, as well as those of greenhouse grown adult plants. Of the plants with different combinations of *PHY*OE, only the combination of *PHYB1*OE/*PHYB2*OE resulted in a small additional suppression of stem elongation, but no significant increase in anthocyanin accumulation was observed in these plants. Combining *CRY1*OE or *CRY2*OE with *PHY*OE in most instances had little additional effect. However, the combination of *PHYB2*OE/*CRY2*OE resulted in a substantial shorter stem and increased anthocyanin level in leaves, when plants were grown in WL. The results show that the effects of *PHYA*OE, *PHYB1*OE and *CRY1*OE on plant growth are not limited by the expression level of the other endogenous photoreceptors, while both for *CRY2*OE as well as *PHYB2*OE the effects are limited by the WT expression level of *PHYB2* and *CRY2*, respectively.

4.1 Introduction

Phytochrome and cryptochrome photoreceptors in tomato

Plants have developed different types of photoreceptors to adapt to light conditions that may occur during growth and development. In tomato this has lead to a family of five phytochromes, designated phyA, phyB1, phyB2, phyE and phyF (Hauser *et al.*, 1995; see also chapter 1), for the perception of red light (R) and far-red light (FR). For the perception of blue light (B), tomato contains four cryptochrome photolyase-related flavoprotein photoreceptors (cry1, cry1a, cry2 and CRY-DASH; (Ninu *et al*., 1999; Perrotta *et al*., 2000; Perrotta *et al*., 2001; Giliberto *et al*., 2005; Facella *et al*., 2006). In addition, B can be perceived by phototropin, a photoreceptor with a specific role in the perception of unilateral light (Briggs and Huala, 1999). Phytochromes also absorb B, albeit with low efficiency. Indeed it was shown that both phyA and cry1 are the major photoreceptors that mediate de-etiolation in tomato seedlings under low and high B irradiance (Weller *et al*., 2001).

Mutants impaired in specific phytochrome genes (Kerckhoffs *et al*., 1999; van Tuinen *et al*., 1995a and b) have been used to identify the roles of individual phytochromes in tomato photomorphogenesis. In these studies, specific light responses have been classified, of which the FR high irradiance response (FR-HIR), and very low fluence response (VLFR) are attributed to phyA, while the R/FR reversible low fluence response (LFR), the R-HIR and the shade-avoidance response (SAR) were mainly attributed to the other phytochromes (Mancinelli, 1994). The phyA accumulates to very high levels in dark (D) grown seedlings, making it the most prominent phytochrome involved in the initial response to light. After prolonged exposure to light the reduced expression of *PHYA* and the specific degradation of phyA results in a rapid decline in phyA levels and the role of other phytochromes become more dominant. This was demonstrated recently using microarray expression profiling, which revealed that phyA has a dominant role in transducing continuous R (cR) signals to rapidly responding genes, at the initiation of seedling de-etiolation, and that phyB has a large role after prolonged exposure to cR and after reduction of the phyA levels (Tepperman *et al.*, 2006). In tomato, phyB1 was identified as the main phytochrome responsible for mediating the de-etiolation response of seedlings to continuous cR, which is marked by inhibition of hypocotyl elongation, enhancement of anthocyanin accumulation, unfolding of the hypocotyl hook and cotyledon expansion (Kerckhoffs *et al*., 1997). Studies using different combinations of phytochrome mutations have shown that phyB2 acts redundantly with phyB1 in the specific response to shade conditions (SAR):

However, a tomato triple mutant lacking phyA, phyB1 and phyB2 still showed some residual responsiveness to supplementary daytime FR indicating that at least one of the two remaining phytochromes in tomato may also contribute to SAR (Weller *et al.*, 2000).

Mutants of the *CRY1* gene in tomato display only a small increase in hypocotyl length and a decrease in anthocyanin accumulation, for seedlings grown under B and a reduced sensitivity for B at higher irradiances (Weller *et al*., 2001). No *cry2* mutants of tomato have been described, but partial suppression of *CRY2* expression has been obtained through virus induced gene silencing (Giliberto *et al*., 2005). The partial silencing of *CRY2* expression results in an increased internode elongation and a reduction in flowering time (Giliberto *et al*., 2005).

Interaction between photoreceptors

Genetic analysis of double and triple photoreceptor mutants in *Arabidopsis* and tomato has revealed different types of interaction between the photoreceptors. Each photoreceptor tends to modify the response mediated by other photoreceptors either directly or indirectly, and depending on the species, developmental stage and specific process. For instance, a contribution of phyB2 to seedling de-etiolation was only seen in the absence of phyB1, suggesting that in a *phyB2* mutant the action of other phytochromes may compensate for the loss in phyB2 signalling (Weller *et al*., 2000). Anthocyanin biosynthesis in seedlings during a 24-h period of cR at different fluence rates was shown to include two components: a low fluence rate component and the R-HIR component. The low fluence rate component is phyA-mediated, but was shown to be co-dependent on either phyB1 or phyB2 (Weller *et al*., 2000). During de-etiolation under cR there is a strong negative effect of phyA on phyB2-mediated anthocyanin accumulation. However, a positive interaction of phyA with phyB1 is seen in the enhancement of anthocyanin synthesis by pre-treatment with FR (Weller *et al*., 2000). It was shown in *Arabidopsis* that the interactions which have been observed between type II phytochromes may in part be based on the potential heterodimer formation (Sharrock and Clack, 2004). It is unclear whether such heterodimer formation also plays a role in the interaction of phyB1 and phyB2 signalling in tomato.

In addition to interaction between different phytochromes, interaction also occurs between the different phytochromes and the cryptochromes, both for the elongation responses (Casal and Mazzella, 1998; Neff and Chory, 1998; Hennig *et al.*, 1999a; Casal, 2000a; Más *et al.*, 2000; Folta and Spalding 2001; Jarillo *et al*., 2001; Mazzella and Casal, 2001; Weller *et al*., 2001) as well as the anthocyanin

responses (Mancinelli, 1990; Mancinelli *et al*., 1991; Wade *et al*., 2001; Weller *et al*., 2001). The analysis of multiple photoreceptor mutants has revealed the significance of the combined action of different photoreceptors in *Arabidopsis* and tomato plants. For example organogenesis is very slow in the *phyAphyBcry1cry2* mutant in *Arabidopsis* (Mazzella and Casal, 2001). The case in tomato was even more impressive, since the *phyAphyB1phyB2cry1* mutant is effectively lethal (Weller *et al*., 2001). In tomato, studies on the interaction of phytochromes and cry1 identified phyA as the major photoreceptor for low irradiance B and cry1 as the major photoreceptor for high irradiance B mediated responses, but phyB1 and phyB2 also contribute to B sensing, depending on the light conditions or the process under study (Weller *et al*., 2000; Weller *et al*., 2001). The effect of phytochromes on cryptochrome signalling under B may be based on co-activation of the cryptochrome and phytochrome signal transduction pathways (Weller *et al*., 2001). In addition, the interaction between phytochrome and cryptochrome signalling may be based on the formation of functional phytochrome-cryptochrome protein complexes. In *Arabidopsis* it was shown that the physical interaction between phyB and cry2 is required for import into the nucleus (Más *et al.*, 2000). In the nucleus phyB interacts with the transcription factor PIF3 (Ni *et al*., 1998). The direct interaction between phyB and cry2 may thus serve to modulate light regulated transcription related to the control of flowering time, hypocotyl elongation and SAR (Casal, 2000a) and the entrainment of the circadian clock (Casal and Mazzella, 1998; Neff and Chory, 1998; Somers *et al*., 1998; Mockler *et al*., 1999; Más *et al*., 2000).

Exploring the potential of photoreceptor signalling in tomato

We previously characterized tomato lines overexpressing *PHYA*, *PHYB1* or *PHYB2* (chapter 2 this thesis). Results showed that *PHYB2*OE results in the most severe effect on plant elongation and anthocyanin accumulation, both at the seedling and adult stage of plant development. Here we studied the phenotype of homozygous and hemizygous plants of selected *PHY*OE lines to get insight into the level of saturation in phytochrome signalling reached in these plants. Phytochrome signalling in lines with *PHY*OE may be limited when it depends on a functional interaction with other phytochromes or cryptochromes, which are expressed only at WT levels. Therefore we also made plants with different combinations of overexpression of *PHYA*, *PHYB1* and *PHYB2* and different combinations of *PHY*OE with *CRY1* or *CRY2* (Giliberto *et al.*, 2005) to explore the potential of photoreceptor

signalling at elevated photoreceptor levels. The plants with multiple *PHY*OE or combinations of *PHY*OE with *CRY*OE were characterized under WL, R, FR, and B.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

Tomato *phy*-mutant and *PHYOE* plants were isolated in tomato (*Lycopersicon esculentum* Mill.) wild type (WT) cv MoneyMaker (MM). The mutant lines used here have been described previously: *phyA* (*fri*[1]: [van Tuinen *et al*., 1995a-b]), *phyB1* (*tri*[1]*:* [van Tuinen *et al*., 1995b]), *phyB2* (70F: [Weller *et al*., 2000]), *phyB1phyB2* double mutant (Weller *et al.*, 2000). The *PHYOE* tomato lines has been described in chapter 2 (this thesis) and *CRY2*OE has been described previously (Giliberto *et al.*, 2005). The *CRY1*OE line C1 was donated by Giovanni Giuliani (ENEA, Trisaia Research Canter, Rotondella, Italy) and has not previously been described. Seedlings were grown in peat-based compost in trays placed in growth cabinets at constant temperature (25°C), a relative humidity (RH) of 70% and light conditions as indicated. Plants were watered once a day. The dark control plants were watered under dim green safelight.

Different combination of crosses between selected homozygous lines of *PHYA*OE (A/1 and A/3), *PHYB1*OE (B1/2 and B1/4) and *PHYB2*OE (B2/4, B2/8 and B2/9) were carried out in the greenhouse in 2002. For the *PHY*OE-combination crosses the plant with the strongest phenotype was used as a mother plant (phenotype: WT<A/1<A/3; WT<B1/2<B1/4; WT<B2/4<B2/9<B2/8). Crosses for the production of hemizygous *PHY*OE/WT plants were made on the transgenic plants, using WT pollen as donor. In most cases the phenotype of the progeny from the crosses with WT was less severe than in the homozygous line, in agreement with a hemizygous state of the progeny plant genotype (table 4.1).

In 2004 selected homozygous lines of *PHY*OE were crossed with selected homozygous *CRY*OE lines: for *PHYA*OE the line A/1, for *PHYB1*OE the line B1/4, for *PHYB2*OE the line B2/9, for *CRY1*OE the line C1 and for *CRY2*OE the line C2 was used. For the *PHY*OE/*CRY*OE-combination crosses the C1 and C2 plants were used as a mother plants. The double hemizygous F1 lines A/1C1, A/1C2, B1/4C1, B1/4C2, B2/9C1 and B2/9C2, which were generated from these crosses, were used for further investigation (table 4.1). The parent lines and the hemizygous (table 4.1) overexpression lines (A/1WT, B1/4WT, B2/9WT, C1WT and C2WT) were used as controls.

4.2.2 Anthocyanin assay

Seedling or leaf material was harvested and subsequently anthocyanin was extracted as described in 2.2.6. The anthocyanin level was determined by measuring the absorption of the extract at 535 nm (A_{535}) and the anthocyanin content was expressed as A_{535} per 5 seedlings or as A_{535} per g leaf FW.

4.2.3 Broad band red, far-red and blue light experiments

The irradiation with cR and continuous FR (cFR) (3 μmole m^{-2} s^{-1}) was as described in section 2.2.7. In addition seeds and seedlings were treated with 24h of continuous B (cB) (3μmole m^{-2} s^{-1}) under similar conditions as described in 2.2.7.

4.2.4 White light treatment

Seeds were sown in trays and transferred to a phytotron with a 16h white light (WL) / 8h D diurnal treatment (WL, 150 μmol m^{-2} s^{-1} photosynthetically active radiation [PAR]) at 25°C. After 11 days the hypocotyl length of the seedlings was measured. The seedlings were transferred to individual Rock wool blocks and moved to the greenhouse and grown for 4 weeks after which the plant height and anthocyanin content from young developed leaves (1.0-1.5 cm in length) were measured.

4.3 Results

4.3.1 Characterization of *PHY*OE gene dosage effect

To determine the level of saturation in specific phytochrome signalling in selected *PHYA*OE, *PHYB1*OE and *PHYB2*OE lines, the elongation and anthocyanin accumulation responses of homozygous and hemizygous *PHY*OE lines grown under 16h WL and 8h D were compared (Fig. 4.1a-c). Figure 4.1a shows that hypocotyl length of hemizygous A/3WT and the homozygous A/3 is not or only slightly reduced compared to WT. However, in 8-week old plants the effect of both hemizygous A/3WT and the homozygous A/3 on the reduction of stem length compared to WT has significantly increased (Fig. 4.1b). The results show that the hypocotyl length in the hemizygous B1/2WT and B1/4WT does not significantly differ from WT, but in the homozygous B1/2 and B1/4 lines the hypocotyl is shorter than in WT (Fig. 4.1a). This indicates that the additional response to WL may be

threshold dependent. Figure 4.1b shows that in 8-week old plants an effect of *PHYB1*OE was no longer visible in homozygous or hemizygous B1/2, but stem length in homozygous and hemizygous B1/4 was significantly reduced compared to WT (Fig. 4.1b). The hypocotyl length of the hemizygous B2/4WT and B2/9WT was shorter than WT and did not differ from homozygous B2/4 and B2/9. This indicates that the single copy of B2/4 and B2/9 is already sufficient to saturate this response to WL in seedlings (Fig. 4.1a). However, at adult stage of development the reduction in stem length was stronger in homozygous B2/4 and B2/9 than in hemizygous B2/4WT and B2/9WT respectively (Fig. 4.1b), indicating that over developmental time there still is a dosage effect of *PHYB2*OE.

A second response by which phytochrome signalling can be quantified is the accumulation of anthocyanin in young leaves of mature plants. Figure 4.1c shows that *PHYA*OE and *PHYB1*OE had little or no additional effect on anthocyanin accumulation compared to WT, both in hemizygous and homozygous plants. In contrast, a strong enhancement of anthocyanin accumulation was observed in the *PHYB2OE* lines. The enhancement of anthocyanin accumulation in homozygous B2/4 and B2/9 compared to WT was about 5-fold and 12-fold respectively (Fig. 4.1c). However, the enhancement of anthocyanin accumulation in the hemizygous B2/4WT and B2/9WT plants was approximately half of that in the homozygous plant, 2.5-fold and 7.5-fold respectively (Fig. 4.1c). The results show that in WL the stem elongation response is closer to saturation both in seedlings and mature *PHYB2OE* plants, while the anthocyanin accumulation response remains sensitive to *PHYB2* gene expression level and/or gene dosage. However, here should be noted that this dosage effect is less clear or absent, when light conditions cause intrinsic higher anthocyanin levels in these *PHYB2OE* plants (see e.g. Fig. 4.4b)

The increased light signalling potential in the *PHY*OE lines was subsequently characterised under R (600-700 nm) or FR (700–800 nm) light conditions. Seedlings from homozygous and hemizygous *PHY*OE lines were grown for 14 days under cR or cFR, after which the hypocotyl length was determined and compared to WT (Fig. 4.2 and Fig. 4.3).

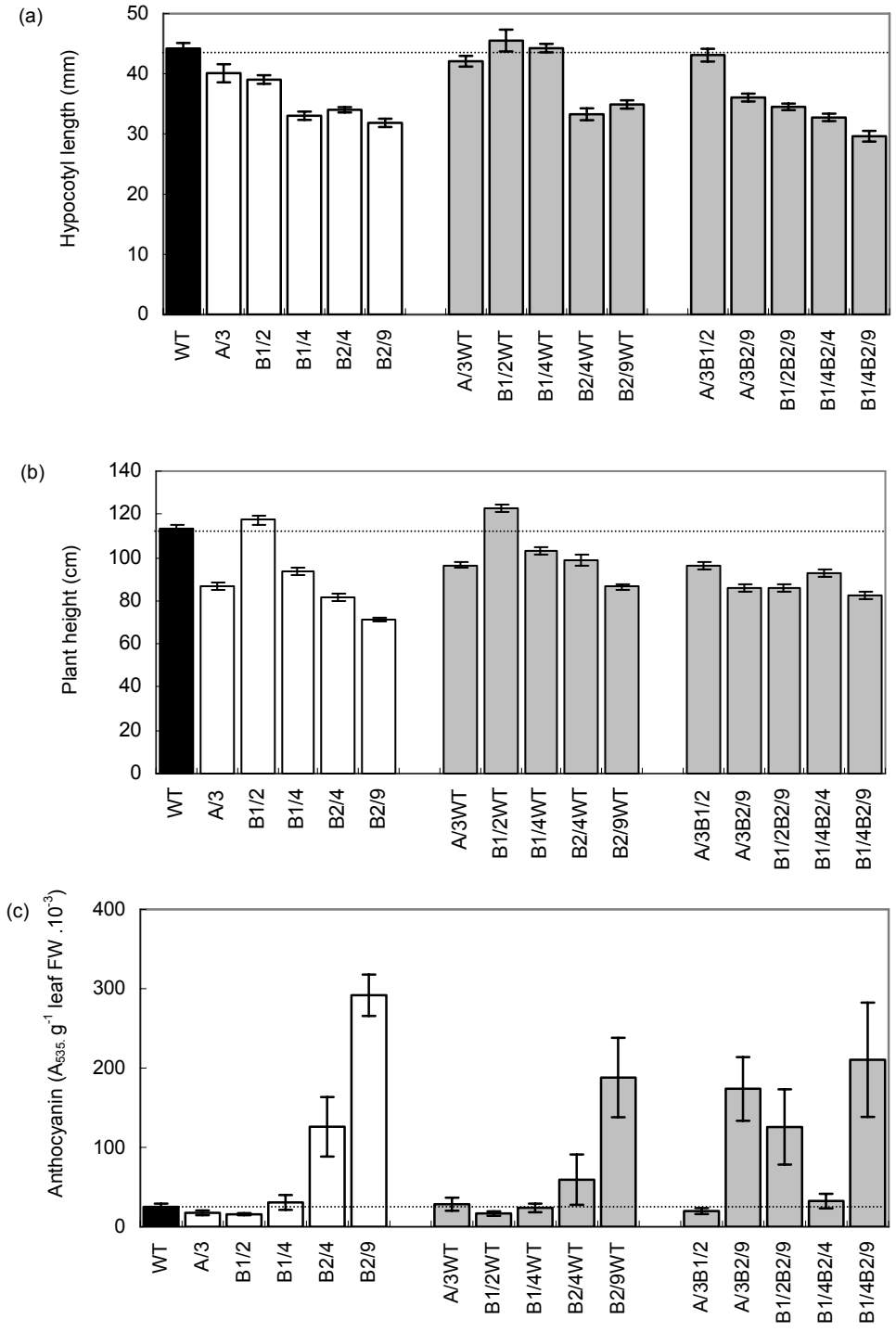


Figure 4.1 Phenotypes of WL-grown tomato plants homozygous or hemizygous for *PHY*OE. (a) Hypocotyl length (mm) of seedlings grown for 11 days under continuous WL. Results are the mean of 35 seedlings ± SE. (b) Plant height (cm) of plants grown in the greenhouse, 8 weeks from sowing, results are mean of 15 plants ± SE (c) Anthocyanin content of young leaves of plants grown in the greenhouse, 8 weeks from sowing, results are mean of 4 leaves ± SE.

Under cR the hypocotyl length of homozygous A/3 seedlings, but not of hemizygous A/3WT, was reduced compared to WT (Fig. 4.2a), indicating a dosage dependent enhanced suppression of elongation under this condition. Similarly, only homozygous A/3 had an effect on anthocyanin accumulation under cR (Fig. 4.2b). Unexpectedly anthocyanin accumulation was lower than WT, indicating a dosage dependent reduced phytochrome signalling. A gene dosage dependent response for elongation and anthocyanin accumulation was also observed for seedlings grown under cFR (Fig. 4.3).

Under cR there was no clear effect on hypocotyl length of *PHYB1*OE in the hemizygous lines, but in the homozygous B1/4 a significant decrease in hypocotyl length for seedlings was observed (Fig. 4.2a). Under cFR the hypocotyl length of B1/2WT and B1/4/WT was slightly longer than WT, while the homozygous lines showed either no effect on hypocotyl length (B1/2), or a reduced hypocotyl length (B1/4) (Fig, 4.3a).

In the *PHYB2OE* lines, similar effects in homozygous (B2/4 and B2/9) and hemizygous (B2/4WT and B2/9WT) lines on hypocotyl length under cR were observed (both have 60% of WT hypocotyl length; Fig. 4.2a). This indicates that, as during growth under WL, under cR the phyB2 signalling is saturated in the hemizygous plants. No clear effect was observed on hypocotyl length when plants were grown under cFR, in both homozygous and hemizygous plants with *PHYB2*OE (Fig. 4.3a). The anthocyanin contents in the seedlings grown under cR and cFR are given in Figure 4.2b and 4.3b. Under cR and cFR the anthocyanin accumulation in the homozygous B1/2 plants was similar to that in the hemizygous B1/2WT plants, indicating saturation in signalling towards anthocyanin accumulation. Under cR and cFR, the homozygous B1/4 showed lower anthocyanin levels compared to the hemizygous B1/4WT line (Fig. 4.2b and Fig.4.3b).

The anthocyanin accumulation was equally affected by cR in the homozygous and hemizygous *PHYB2*OE lines, resulting in up to 2-fold higher level than in WT (Fig. 4.2b). Under cFR anthocyanin accumulation was similarly reduced in homozygous and hemizygous B2/4, while levels in B2/9 plants were not significantly different from that in WT (Fig. 4.3b).

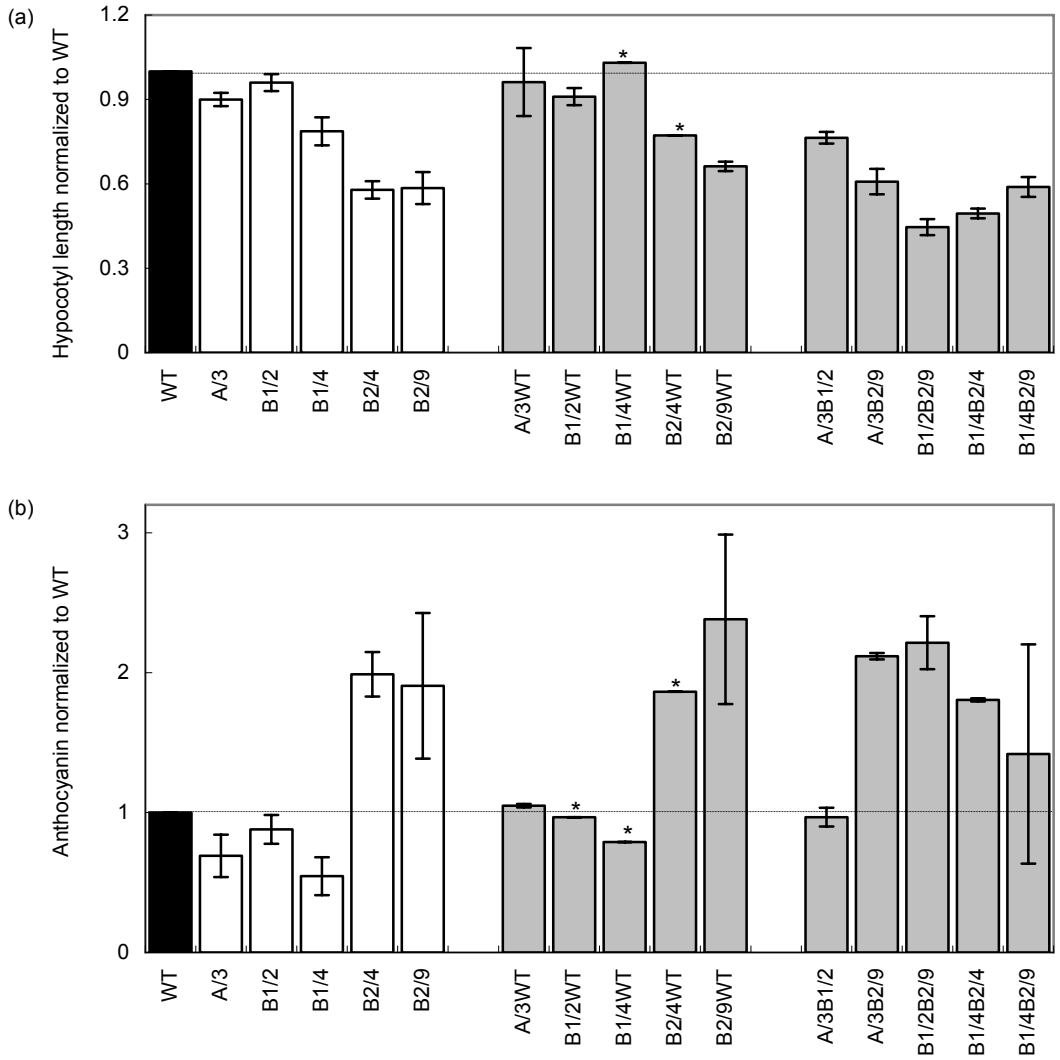


Figure 4.2 Quantification of phenotype of continuous R (cR) grown tomato seedlings. (a) Hypocotyl length of seedlings grown for 14 days from sowing. Values are means of two independent experiments (except for lines with *) ± SE and normalized to the mean length of 35 WT seedlings. In each experiment for each genotype the hypocotyl length of 35 seedlings was determined. (b) Anthocyanin accumulation. Values are means of two independent experiments (except for lines with *) ± SE and normalized to the mean anthocyanin accumulation in 4x5 WT seedlings. In each experiment for each genotype the anthocyanin accumulation in 4x5 seedlings was determined.

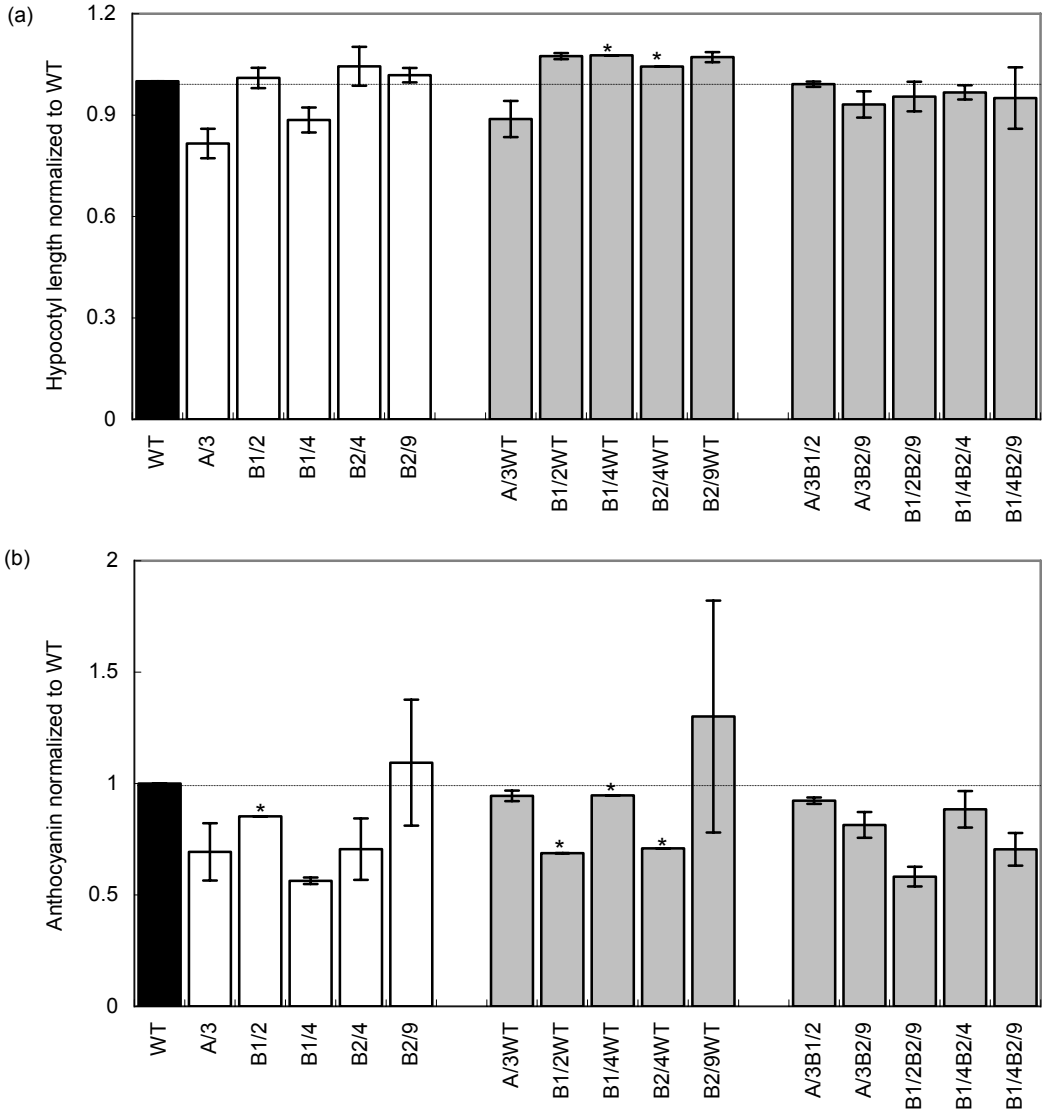


Figure 4.3 Quantification of phenotype of continuous FR (cFR) grown tomato seedlings. (a) Hypocotyl length of seedlings grown for 14 days from sowing. Values are means of two independent experiments (except for lines with *) ± SE and normalized to the mean length of 35 WT seedlings. In each experiment for each genotype the hypocotyl length of 35 seedlings was determined. (b) Anthocyanin accumulation. Values are means of two independent experiments (except for lines with *) ± SE and normalized to the mean anthocyanin accumulation in 4x5 WT seedlings. In each experiment for each genotype the anthocyanin accumulation in 4x5 seedlings was determined.

4.3.2 Interactions between *PHYA*OE, *PHYB1*OE and *PHYB2*OE

We tested whether addition of a second *PHY*OE gene can further enhance the phenotype of a single *PHY*OE gene. Selected homozygous transgenic lines with *PHY*OE were crossed to WT and to one another. The phenotypes of the plants hemizygous for a single *PHY*OE gene (A/3WT, B1/2WT, B1/4WT, B2/4WT and B2/9WT) were compared to those of plants hemizygous for two different *PHY*OE genes (A/3B1/2, A/3B2/9, B1/2B2/9, B1/4B2/4 and B1/4B2/9). For analysis of the effects on plant elongation and anthocyanin accumulation the plants were grown under WL (Fig. 4.1), cR (Fig. 4.2) and cFR (Fig.4.3). The results indicate that there is no additional effect from the addition of *PHYA*OE to either *PHYB1*OE or *PHYB2*OE, for seedlings grown under WL (Fig. 4.1a).

For seedlings grown under cR the hemizygous *PHYA*OE or the hemizygous *PHYB1*OE had no significant effect on hypocotyl length (Fig. 4.2). However, in the double hemizygous plant the hypocotyl length was significantly shorter than either *PHYA*OE or the hemizygous *PHYB1*OE (A/3B1/2; Fig. 4.2a). In contrast, the anthocyanin accumulation response in A/3B1/2 did not differ from that in A/3WT and B1/2WT (Fig. 4.2b). When seedlings were grown under cFR the hypocotyl length of double hemizygous A/3B1/2 seedlings was as WT, suggesting that the small decrease in hypocotyl length observed in hemizygous *PHYA*OE was compensated by the small increase in hypocotyl length observed in hemizygous *PHYB1*OE (Fig. 4.3).

Because of the redundancy in phyB1 and phyB2 signalling that was deduced from mutant studies (Weller *et al*., 2000), and the potential of type II phytochromes to form heterodimers (Sharrock and Clack, 2004) , it was of special interest to see the effect of combining *PHYB1*OE with *PHYB2*OE. Three different combinations were tested (B1/2B2/9, B1/4B2/4 and B1/4B2/9); however, the effect of phyB2 on plants grown under WL could not be increased by the addition of *PHYB1*OE (Fig. 4.1). Only when plants were grown under cR an additional enhancement of the suppression of hypocotyl elongation was noted compared to a single *PHYB2*OE or *PHYB1*OE gene (Fig. 4.2a). However, this additional effect of *PHYB1*OE on *PHYB2*OE on hypocotyl elongation under cR was not seen for the anthocyanin accumulation response (Fig. 4.2b).

4.3.3 Interactions between *PHY*OE and *CRY*OE in tomato.

It was subsequently tested whether the effect of individual *PHY*OE was limited by the endogenous *CRY1* or *CRY2* expression level. For this purpose, selected

homozygous *PHY*OE lines were crossed with homozygous *CRY*OE plants to create the double hemizygous plants A/1C1, B1/4C1, B2/9C1 and B2/9C2. The *CRY*OE plants contain either the tomato *CRY1* or *CRY2* gene under control of the double-35S *CaMV* promoter. The homozygous *CRY2*OE plants (C2) have been described before (Giliberto *et al*., 2005). For characterisation of the phenotype, plants were grown under WL and under cB, as both conditions may activate phytochromes as well as cryptochromes. The results show a similar suppression of hypocotyl elongation under WL in C2 and C2WT (Fig. 4.4a), suggesting that for the C2 plants a single *CRY2*OE gene is already close to saturation of this response. In contrast, the results in Figure 4.4b show that the hemizygous *CRY2*OE (C2WT) has no effect on anthocyanin accumulation, whereas the anthocyanin accumulation in the homozygous *CRY2*OE (C2) plants increased compared to WT, suggesting a cry2 threshold-dependency for this response.

The *CRY1*OE line C1 has not previously been described. Our results here show that C1 has an increased stem elongation compared to WT, for plants grown under WL (Fig. 4.4a). However, this effect is gene dosage dependent, since the hemizygous C1WT has no significant effect on hypocotyl length compared to WT (Fig. 4.4a).

The double hemizygous A/1C1 has no additional effect on hypocotyl length and anthocyanin accumulation for plants grown under WL (Fig. 4.4). Similarly the hypocotyl length of the double B1/4C1 was similar to that of C1/WT and B1/4WT (Fig. 4.4). In contrast an additive or synergistic effect of cry1 and cry2 on phyB2 was observed in the double hemizygous B2/9C1 and B2/9C2 compared to any of the single hemizygous plants in both elongation response and anthocyanin response (Fig. 4.4a).

Analysis of the hypocotyl elongation and anthocyanin accumulation under cB are given in Figure 4.5. The results show that homozygous *CRY2*OE (C2) has a stronger suppression of hypocotyl elongation than the hemizygous C2WT under cB (Fig. 4.5a), suggesting a dependency for this response on cry2 dosage. However, enhancement of the anthocyanin accumulation response under cB was similar in the homozygous plants and in the hemizygous plants (Fig. 4.5.b). This indicates that the single copy of C2 is already sufficient to saturate this response to cB. In contrast to cry2, both homozygous *CRY1*OE (C1) and hemizygous *CRY1*OE (C1WT) has only a limited effect on hypocotyl elongation and anthocyanin accumulation under cB (Fig. 4.5). These results indicate that at low intensity of cB (3 μmole m^{-2} s^{-1}) the *CRY2*OE has a bigger effect on seedling growth and pigmentation than *CRY1*OE.

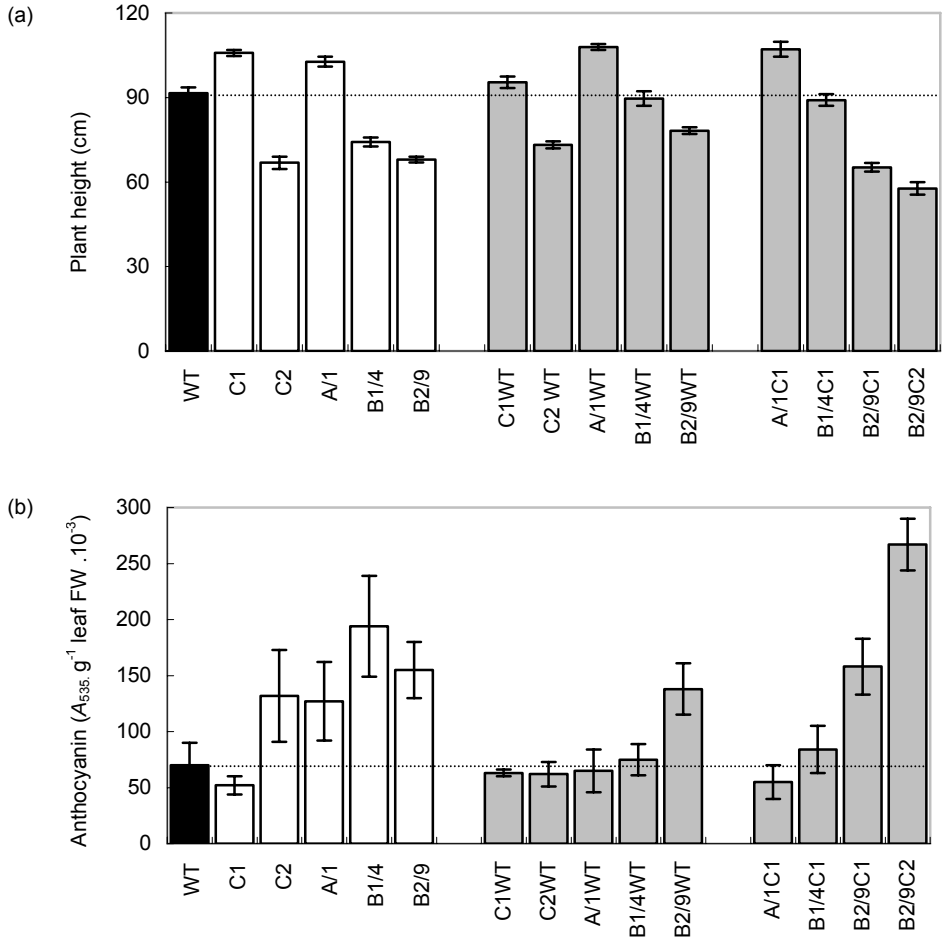


Figure 4.4 Phenotype of tomato with *PHY*OE, *CRY*OE or a combination of *PHY*OE/*CRY*OE under WL (a) Plant height of plants grown in the greenhouse. Measurements were taken after 8 weeks from sowing. Values are means of 15 plants ± SE (b) Anthocyanin responsiveness. Anthocyanin levels in young leaf. Values are means of 4 leaf samples ± SE. The experiments were repeated with qualitatively similar results.

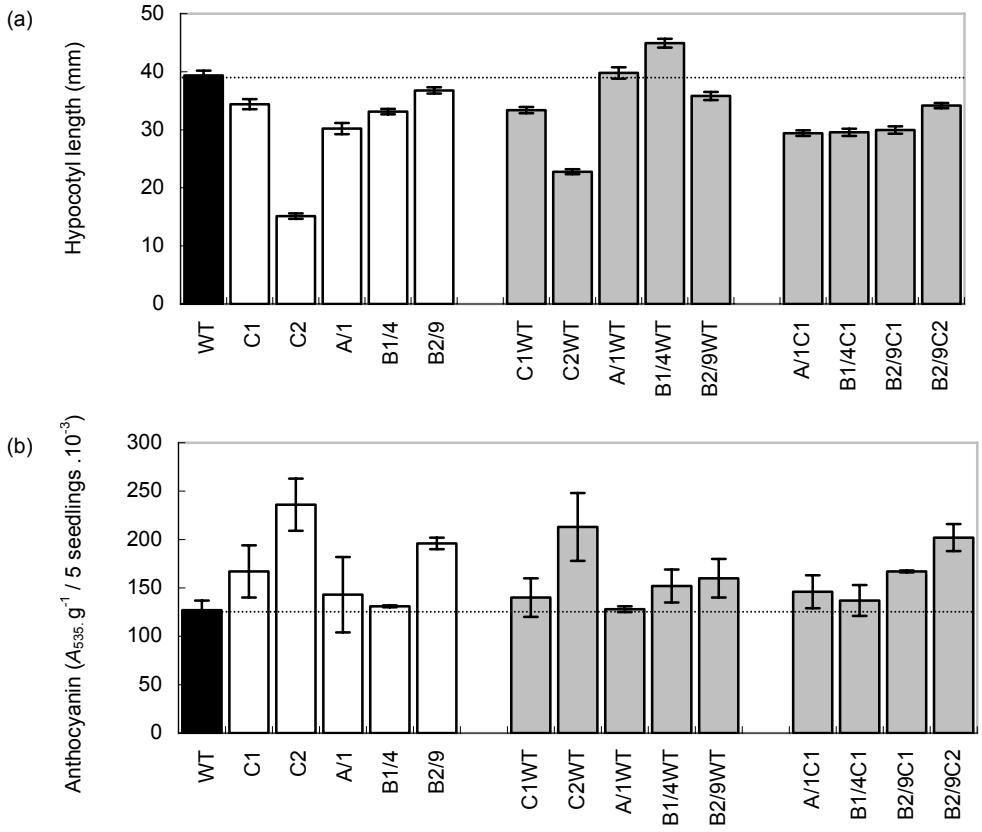


Figure 4.5 Blue light responsiveness of phytochrome and cryptochrome overexpressing lines (a) Hypocotyl length of seedlings grown under continuous Blue light (cB). Measurements were taken after 14 days from sowing. Values are means ± SE n = 35 (b) Anthocyanin responsiveness under continuous blue light (cB) (3 μmol m-2 s-1). Measurements were taken 14 days after sowing and the anthocyanin levels in 5 hypocotyls were measured. Values are means ± SE n = 4. The experiments were repeated with qualitatively similar results.

A small synergistic interaction between *PHYA*OE and *CRY1*OE was observed for suppression of hypocotyl elongation when seedlings were grown under cB (compare C1WT and A1WT with A/1C1; Fig. 4.5a). Similarly, a synergistic interaction was observed between *PHYB1*OE and *CRY1*OE (Fig. 4.5a). Combining *PHYA*OE or *PHYB1*OE with *CRY1*OE had little effect on anthocyanin accumulation under cB conditions (Fig. 4.5b).

The effect of elevated levels of *CRY2*OE on hypocotyl elongation was actually antagonised by the effect of *PHYB2*OE (Fig. 4.5a). In contrast to the positive interaction between *PHYB2*OE and *CRY2*OE that was observed for anthocyanin accumulation in seedlings grown under WL (Fig. 4.4b), no interaction was observed between *PHYB2*OE and *CRY2*OE when plants were grown under cB (Fig. 4.5b).

4.4 Discussion

4.4.1 Gene dosage effect in *PHY*OE lines

In Chapter 2 homozygous lines for *PHY*OE were used to study the effect of *PHYA*OE, *PHYB1*OE and *PHYB1*OE on plant elongation and anthocyanin accumulation at both seedling and adult stage of plant development. Here we analysed whether phytochrome-signalling in these lines is saturated, by comparing homozygous and hemizygous tomato plants with *PHY*OE. The results show that at the seedling stage the suppression of hypocotyl elongation in plants with *PHYA*OE or *PHYB1*OE is threshold dependent so that effects are only significant in plants which are homozygous for *PHYA*OE or *PHYB1*OE and when phytochrome is maximally activated (e.g. under cR) (Fig. 4.1a and Fig. 4.2a). In contrast, in the *PHYB2*OE lines, the signalling is already near saturation for suppression of hypocotyl elongation, as no large differences between homozygous and hemizygous *PHYB2*OE plants were observed under the different light conditions tested (Fig. 4.1, Fig. 4.2 and Fig. 4.3). Under WL only *PHYB2*OE plants show a strong enhancement of anthocyanin accumulation, in one case with gene dosage effect (Fig. 4.1c), and in one case without gene-dosage effect (Fig. 4.4b). Under cR only *PHYA*OE plants (A/3; Fig. 4.2b) show a gene dosage dependency for suppression of anthocyanin accumulation, whereas under cFR this is only shown by one of the two *PHYB1*OE lines (B1/4; Fig. 4.3b).

The anthocyanin accumulation response shows higher variation than the elongation response (see for instance the anthocyanin levels under WL in B1/4 in Figure 4.1 and 4.4) and therefore seems to be more susceptible for changes in environmental conditions. This may explain the variable gene dosage effects on anthocyanin accumulation in *PHYB1*OE plants (Fig. 4.1c and Fig. 4.4b). The light and average temperatures during the eight weeks of the experiment 1 (shown in Fig. 4.1) were higher than during the eight weeks of experiment 2 (shown in Fig. 4.4). More important for anthocyanin accumulation may be the average photoperiod during the experiment, this was longer during experiment 1 than during experiment 2.

Mutant studies have identified phyB1 as the major phytochrome involved in the induction of anthocyanin accumulation (Weller *et al*., 2000). For instance, when phyB1 is present the loss of phyB2 had no effect on anthocyanin accumulation. In contrast to these findings from mutant studies, our *PHY*OE results show that *PHYB1*OE has little effect, while *PHYB2*OE has a major effect on anthocyanin accumulation (Fig. 4.1b and 4.2b). Signalling of phyB1 towards anthocyanin

accumulation may therefore be fully saturated at WT phyB1 levels. Although *PHYA*OE (homozygous) results in reduction in anthocyanin accumulation under cR (Fig. 4.2b), this effect is dosage dependent and does not occur in the hemizygous plant A/3WT (Fig. 4.2b). The suppression of anthocyanin accumulation in *PHYA*OE plants may therefore be different from the mechanism responsible for suppression of anthocyanin accumulation at WT phyA levels.

4.4.2 Interactions between *PHYA*, *PHYB1* and *PHYB2*.

4.4.2.1 Little interaction between PHYAOE and PHYBs

The interaction between different phytochrome signalling pathways at elevated levels of expression was studied by combining the *PHYA*OE with *PHYB1*OE or *PHYB2*OE and by combining *PHYB1*OE with *PHYB2*OE. The combined elevated levels of *PHYA* and *PHYB2* had no big additional effect on hypocotyl elongation or anthocyanin accumulation for seedlings grown under WL, R or FR (Fig 4.1, 4.2 and 4.3), indicating that *PHYA* can not further enhance the signalling from *PHYB2*OE. On the other hand, in A/3B1/2 seedlings grown under R, the hypocotyls were significantly shorter compared to those of A/3WT and B1/2WT (Fig. 4.2a and b), indicating an additional effect of *PHYA*OE on phyB1 signalling. When seedlings were grown under FR, the small effect of *PHYB1*OE was compensated by the small opposite effect of *PHYA*OE on hypocotyl elongation (Fig. 4.3a).

4.4.2.2 Effects of PHYB2OE on elongation are limited by WT phyB1 levels

Mutant studies have shown that in tomato phyB1 and phyB2 function redundantly in controlling adult plant response to changes in R: FR in the environment. In contrast to these mutant studies, which show that phyB1 has relatively more effect on elongation and anthocyanin accumulation responses than phyB2 (Weller *et al*., 2000), the *PHY*OE studies show that *PHYB2*OE results in more severe effects on plant growth and pigmentation than *PHYB1*OE (Chapter 2). The results in Figure 4.1a show that under WL conditions, overexpression of the *PHYB1* gene in combination with *PHYB2*OE further enhances the *PHYB2*OE effect on plant elongation responses, especially in B1/4B2/9. This indicates that endogenous phyB1 levels are limiting for the action of *PHYB2*OE in elongation responses under WL and R.

When *PHYB1*OE was combined with *PHYB2*OE the anthocyanin accumulation was not further enhanced under R or under WL (Fig. 4.1c and Fig. 4.2b). The different effects of the combination of *PHYB1*OE with *PHYB2*OE on

elongation and anthocyanin accumulation indicate different levels of saturation of these two different responses.

4.4.3 Interactions between *PHY*OE and *CRY*OE.

*4.4.3.1 Only small interaction effect of PHYA*OE *and CRY1*OE *under B*

There are several lines of evidence that phytochromes and cryptochromes interact, not only at a genetic level, but also by direct physical interaction. For instance, it has previously been shown that in *Arabidopsis* phyA can bind to and phosphorylate cry1 and cry2 (Ahmad *et al*., 1997 and 1998). It was also demonstrated that the interaction between phyA and cry1 depends on both the light quality and irradiance, both for the elongation responses (Casal and Mazzella, 1998; Poppe *et al*., 1998; Casal, 2000a; Folta and Spalding 2001; Mazzella and Casal, 2001), as well as the anthocyanin accumulation responses (Mancinelli, 1990; Mancinelli *et al*., 1991; Wade *et al*., 2001). Moreover, in *Arabidopsis* both phyA and cry1 act redundantly in control of the period length of the biological clock under low cB, but it is not known how this affects the elongation response (Somers *et al*., 1998; Poppe *et al*., 1998; Casal, 2000a; Folta and Spalding 2001; Mazzella and Casal, 2001)

Assuming that such interactions are also important for the phyA/cry1 and phyA/cry2 function in tomato, the effect of *PHYA*OE may be limited by the expression level of either endogenous cry1 or cry2. For the elongation response, there was only a small synergistic interaction between elevated levels of phyA and cry1 when plants were grown under cB, indicating that only under this condition WT phyA levels limit the suppression of elongation by *CRY1*OE.

Mutant studies in tomato demonstrated that phyA and cry1 are the main photoreceptors mediating anthocyanin accumulation under B at irradiances above 3 μmole $m^{-2}s^{-1}$, while at irradiances below 3 μmole $m^{-2}s^{-1}$ phyA is the main mediator of the anthocyanin accumulation response (Weller *et al*., 2001). The experiments presented in this chapter, which were carried out under greenhouse conditions and at 3 μmole $m^{-2}s^{-1}$ of B, show that *CRY1*OE does not contribute to the effect of *PHYA*OE on anthocyanin accumulation under WL or cB (Fig. 4.4b and Fig. 4.5b). This indicates that for these light conditions and for this response WT phyA and cry1 levels may already be saturating.

4.4.3.2 Interactions of PHYBOE and CRY2OE

The results presented in this Chapter show that *PHYB1*OE may have an additional effect on anthocyanin accumulation when plants are grown under WL, but only in homozygous plants (B1/4 and B1/4WT in Fig. 4.1c and Fig. 4.4c). No additional effect was observed in B1/4C1 under WL (Fig. 4.4b) or under cB (Fig. 4.5b). This suggests that the contribution of cry1 towards the anthocyanin accumulation response may already be saturating in WT, while the contribution of phyB1 towards anthocyanin accumulation is similar over a range phyB1 levels (WT to 16-fold WT phyB1 levels), but an enhanced response may occur near 32-fold WT phyB1 levels.

Hemizygous *PHYB1*OE or hemizygous *CRY1*OE had no effect on the elongation response for plants grown under WL (Fig. 4.4a). However, for plants grown under cB, *PHYB1*OE in hemizygous plants resulted in increased elongation, which is usually associated with reduced photoreceptor signalling, while hemizygous *CRY1*OE suppressed elongation more than WT (Fig. 4.5a). The elongation was stronger suppressed in the double hemizygous plant B1/4C1 than in the single hemizygous C1WT plant. As B presumably results in a less efficient activation of phyB1 than WL, the effects of interaction between phyB1 and cry1 thus seem to be Pr^{phyB1}-dosage dependent.

Mutant studies on tomato seedlings have shown that both phyB1 and cry1 contribute to the inhibition of hypocotyl elongation and accumulation of anthocyanin under cB (Weller *et al*., 2001). In these studies Weller *et al* (2001) demonstrated that when the four photoreceptors phyA, phyB1, phyB2 and cry1 are mutated, no anthocyanin accumulates in response to cB. However, anthocyanin accumulation is increased in homozygous *CRY2*OE plants, indicating that also cry2 may be of importance for anthocyanin accumulation (Fig. 4.4b). Combined these results indicate that the action of cry2 towards the anthocyanin accumulation response may depend on the co-action of other photoreceptors. The effect of *PHYB2*OE on hypocotyl elongation and anthocyanin accumulation was significantly enhanced by *CRY2*OE under WL (Fig. 4.4). In contrast, for plants grown under B, the effect of B2/9C2 on hypocotyl elongation was less pronounced than in the C2WT, indicating that under this light condition *PHYB2*OE and *CRY2*OE act antagonistically (Fig. 4.5a).

Table 4.1. Effects of homozygous and hemizygous *PHY*OE and *CRY*OE on plant elongation and anthocyanin accumulation. Tomato seedlings grown under different light conditions as indicated. For production of hemizygous plants, WT was used as pollen donor. 0 = WT response. + = response stronger than WT, – = response weaker than WT and N = not measured.

	Plant elongation				Anthocyanin			
	WL	**R**	**FR**	**B**	**WL**	**R**	**FR**	**B**
WT	0	0	0	0	0	0	0	0
A	+	+/0	++	++	0	–	–	0
A/WT	+	0	+	0	0	0	0	0
B1	++	+	+	++	0	–	0/–	0
B1/WT	+/0	0	0	0	0	0/–	0/–	0
B2	+++	+++	0	+	+++	+++	0	++
B2/WT	+++	+++	0	+	++	+++	0	+
C2	+++	N	N	+++	++	N	N	++
C2/WT	+++	N	N	++	0	N	N	++
C1	–	N	N	+	–	N	N	+
C1/WT	0	N	N	+	0	N	N	0
A/C1	–	N	N	+	–	N	N	0
B1/C1	0	N	N	+	0	N	N	+
B2/C1	+++	N	N	+	++	N	N	+
B2/C2	++++	N	N	+	+++	N	N	++

4.4.4 Concluding remarks

The effects of *PHY*OE and *CRY*OE under different light conditions are summarised in table 4.1. The table shows that under WL, the largest effects on suppression of hypocotyl elongation and on enhancement of anthocyanin accumulation are from *PHYB2*OE and *CRY2*OE. For the other *PHY*OE and *CRY*OE lines the effects can be positive or negative, depending on the light conditions used. The results show that conclusions drawn from mutant studies are not easily transferable to situations with elevated levels of photoreceptors (Guo *et al.*, 1998).

The interactions between the different photoreceptor signalling pathways may be from direct physical interactions between photoreceptors (Más *et al.*, 2000), but may also be based on interactions between light signalling and the endogenous biological clock. For instance, it has been noted that in *Arabidopsis* both *PHY* and *CRY* genes are under control of the endogenous circadian clock (Toth *et al*., 2001),

while at the same time, both type of photoreceptors are also involved in entrainment of this circadian clock (Millar, 2003). The overexpression of a single phytochrome-gene may therefore affect the phase or amplitude of expression of endogenous *PHY* and/or *CRY* genes directly, or through modulations in endogenous clock signalling. Only limited information is available on circadian clock signalling in tomato. For instance, it has been shown that the endogenous tomato phytochrome-genes and CRY-DASH are under control of the circadian clock machinery in tomato (Hauser *et al*., 1997 and 1998, Facella *et al*., 2006). In future studies the transgenic tomato plants, which have been characterised here will be used to analyse the effects of *PHY*OE on clock regulated gene expression in tomato.

Acknowledgements

The authors gratefully acknowledge Prof. Giovanni Giuliani (ENEA, Trisaia Research Canter, Rotondella, Italy) for providing the seeds of the tomato cryptochrome overexpression line (*CRY1*OE; C1 line).

Chapter 5

General discussion

Abstract

This thesis reports on the manipulation of phytochrome levels in tomato to investigate the effect on growth and development, with special emphasis on the shade-avoidance responses. This thesis project was part of a larger program ('gewassen onder glas met minder gas') aimed at reducing heating cost and related CO2 emissions associated with the Dutch tomato cultivation system. The project started out with several attempts to manipulate shade avoidance responses in tomato by introducing different types of transgenes into tomato, varying from photoreceptor genes, a mutant ethylene receptor gene (*ETR1-1*) and genes affecting plant hormone balance (*ROLC*). The overall aim was to enable higher density cultivation of tomato in greenhouses and thus reduce heating costs for a given production of tomatoes. From the different transgenic plants that were produced, the lines with overexpression of homologous phytochrome genes were selected for further analysis, to see which of the *PHY*OE lines results in control of density dependent dwarfing and would thus enable growing more tomato plants per m^{-2}. In addition, the differential effects of individual *PHY*OE in tomato has added to the insight in the individual functions of the tomato *PHYA*, *PHYB1* and *PHYB2* genes. In this chapter, the generation and characterization of tomato lines overexpressing tomato phytochrome genes *PHYA*, *PHYB1*and *PHYB2* and the experiments carried out in the preceding chapters are discussed in relation to other published work on photomorphogenesis. The advantages and limitations of manipulation of the phytochrome genes in tomato are addressed, as well as some of the practical consequences of the results obtained for phytochrome overexpression in tomato.

5.1 Photomorphogenesis and phytochromes

Higher plants perceive environmental light signals with a quality, intensity, duration, direction and periodicity which provides plants with information not only on the ambient light conditions but also on other elements such as neighbouring plants and seasonal changes (Casal *et al.*, 2003). Plants develop differently in darkness (skotomorphogenesis) and in the light (photomorphogenesis). Characteristics of dark growth are elongated stems, undifferentiated chloroplasts, and unexpanded (etiolated) leaves. Characteristics of light growth are the inhibition of stem elongation, the differentiation of chloroplasts and accumulation of chlorophyll, and the expansion of leaves (de-etiolation) (Schäfer and Nagy, 2006). Light also plays a profound role at later stages of plant development, *e.g.* in shade-avoidance responses and in the control of the transition from vegetative to generative growth (reviewed in Smith, 2000). In higher plants light signals are perceived and transduced by a network of phytochromes, cryptochromes, phototropins and their downstream signalling elements (Chapter 1; reviewed in Sharrock and Mathews, 2006). Higher plants contain a small collection of structurally similar phytochromes (Wagner *et al*., 2005) and tomato contains five phytochrome genes, which have been designated *PHYA*, *PHYB1*, *PHYB2*, *PHYE*, and *PHYF* (Hauser *et al.*, 1995). The amino acid sequence of the tomato PHYA, PHYB1, PHYB2 and PHYE reveals an 88–89% identity with their *Arabidopsis* counterparts (Clack *et al.*, 1994). Within the set of different photoreceptors the phytochromes are unique in being reversibly photochromic photoreceptors.

5.2 Phytochrome structure / function relationship

Phytochromes are homodimeric chromoproteins, with each subunit consisting of a linear tetrapyrrole chromophore covalently attached to each polypeptide monomer. Once bound to the apoprotein, the chromophore enables detection of red light (R) and far-red light (FR) by photointerconversion between two conformations, a R-absorbing Pr form and a FR-absorbing biologically active Pfr form (Sharrock and Mathews, 2006). The phytochromes contain PAS and GAF domains (see Fig.1.3, Chapter 1). The interaction between the PAS and GAF domains causes an efficient photoconversion between Pr and Pfr by R and FR, respectively. Recent studies provide evidence that light quality and quantity-dependent translocation of phytochrome to the nucleus represents a major regulatory step in light dependent signalling (Matsushita *et al.*, 2003; Mateos *et al*., 2006). Chen *et al*., (2005)

proposed a molecular mechanism for phytochrome nuclear localization in which a nuclear-localization signal within the C-terminal PAS domain is masked by the chromophore containing N-terminal domain and is unmasked by the light-dependent conformational changes. One of the two PAS domains is important for phytochrome homodimer formation, while type II phytochromes may also form heterodimers (Sharrock and Clack, 2004). The other PAS domain is important for interaction with downstream targets of phytochromes, e.g. the interaction with phytochrome-interacting factor (PIF3; Ni *et al.*, 1998). The interaction with PIF3 is dependent on the photoconversion of phytochrome from Pr to Pfr and leads to a phosphorylation and subsequent degradation of this transcriptional repressor and thus, indirectly, to induction of light-regulated gene expression. Within the nucleus both phytochromes and PIF3 localise to nuclear speckles, presumably the site where PIF3 degradation takes place (Nagatani, 2004).

5.3 Ectopic phytochrome levels and photomorphogenesis effects

Studies in *Arabidopsis* and tomato showed that phytochromes are expressed in every plant tissue and within one tissue multiple members of the phytochrome gene family may be expressed (*Arabidopsis*: Pratt, 1995; Sharrock and Clack, 2002 and tomato: Hauser *et al*., 1997 and 1998). Photoreceptors work in concert to achieve a homeostasis or balance in plant growth and development (Casal, 2006). For instance, the effects of extreme variation in the natural light environment on plant growth and development are significantly less with the full complement of photoreceptors than after mutation of one or more photoreceptor. As a consequence, manipulation of the concentration of individual phytochromes through genetic engineering may alter this photoperception equilibrium. This may lead to severe effects on phenotype, some of which, however, may be beneficial for agronomically important traits. Experiments in which the levels of a phytochrome have been changed in different plants, using either homologous or heterologous phytochrome species, are listed in table 5.1. In this thesis we investigated the effects of the elevated levels of tomato phyA, phyB1 and phyB2 on tomato growth and development with particular interest in potential beneficial effects on tomato greenhouse cultivation.

Expression of the different *PHY*OE genes was confirmed by protein analysis and indicated an up to 16-fold increase in PHYA, 32-fold increase in PHYB1 and 200-fold increase in PHYB2 levels in transgenic *PHYA*OE, *PHYB1*OE and

*PHYB2*OE tomato seedlings, respectively (Chapter 2). Phytochrome levels are from extracts from D-grown seedlings, as both endogenous and ectopically expressed phytochrome could not be detected with our antibodies in extracts from light-grown plants. Especially *PHYB2*OE resulted in a large significant shift in the R-HIR response to lower fluence rates compared to that in WT (Chapter 2). This shift to approximately 100-fold lower light intensities was in agreement with the 200-fold increase in PHYB2 as estimated from western blots, and indicates that phyB2 is the major cR-sensing phytochrome for growth and pigmentation responses in tomato. Although phyA was previously shown to be the dominantly active phytochrome for cFR responses (Kendrick *et al.*, 1997), the ~16-fold, increase in PHYA levels in transgenic tomato plants resulted in only a small inhibition of hypocotyl elongation and enhancement of anthocyanin accumulation in tomato seedlings grown under cFR. When plants were grown in close proximity, the ~16-fold increase in PHYA has some effect on stem length but very little effect on shade avoidance responses (SAR; Chapter 3). From this we concluded that, even though the expression directed by the 35S promoter is independent of the feedback regulation that down-regulates endogenous *PHYA* promoter activity (Kay *et al*., 1987), the constitutively produced phyA is rapidly degraded (Clough *et al*., 1999) and limits accumulation of ectopically produced homologous phyA in light.

The elevated levels of PHYB1 and PHYB2 in the *PHYB1*OE and *PHYB2*OE lines resulted in shorter stems and higher anthocyanin levels compared to WT, both in seedlings and adult plants (chapter 2). The *PHYB2*OE resulted in the most pronounced effects on growth, also when plants are grown in the greenhouse in the proximity assay (Chapter 3). This effect of phyB1 and phyB2 on growth and pigmentation was demonstrated to be largely photoreversible by an EODFR treatment and thus proven to be mediated by ectopically produced biologically active phytochromes (Chapter 3). *PHYB2*OE was also very effective in complementing the *phyB1phyB2* double mutant, indicating that phyB2 molecules are able to complement the loss of phyB1 molecules in tomato plants. Because loss of phyB2 function has little effect on elongation or anthocyanin accumulation, the effectiveness of phyB2 on plant growth and pigmentation was underestimated from the mutant studies (Kerckhoffs *et al.*, 1997; Weller *et al.*, 2000). This could indicate that phyB2 is inherently more efficient than phyB1 in signalling towards the elongation and anthocyanin accumulation responses, but that in WT phyB2 is a very low abundance photoreceptor. However, quantification of PHY mRNA levels in tomato showed that PHYB2 levels vary between 33-50% of that of PHYB1 expression levels (Hauser *et al.*, 1998).

In Chapter 4 the different lines with *PHY*OE were crossed with each other and with *CRY*OE lines in order to create plants with two *PHY*OE genes or plants with *PHY*OE and *CRY*OE. Combining the 16-fold increase in phyA with a 32-fold increase in phyB1 or 200-fold increase in phyB2 had little effect. There was some effect of combining *PHYB1*OE with *PHYB2*OE, but the strongest effect was from combining *PHYB2*OE with *CRY2*OE (Chapter 4). This indicated that at the photoreceptor-level the light responses are mostly saturated.

Besides by the protein level, the activity of ectopically produced phytochromes may by limited by other factors such as assembly into phytochrome holoprotein. In our plants the ectopically produced phytochrome was assembled into functional phytochrome holoprotein, as indicated by the FR-reversible phenotype in the *PHY*OE lines (Chapter 3), the light dependent turnover of the ectopically produced phyA (Chapter 2), the complementation by the *PHYA*OE transgene of the *phyA* mutant phenotype and the complementation by the *PHYB2*OE transgene of the *phyB1phyB2* double mutant phenotype (Chapter 2). However, the action of phytochrome holoprotein may also by limited by activation or deactivation through phosphorylation and dephosphorylation or may be limited by the level of interacting proteins. These effects are discussed in Section 5.5.1 and 5.5.2.

5.4 Phytochromes mutants versus *PHY*OE studies

Although mutant studies are excellent tools to set a function for the phytochrome at natural conditions, these studies cannot reveal whether or how much a specific light response is limited by the endogenous levels of any of the photoreceptors. Our studies revealed that endogenous phyB2 levels are limiting, as *PHYB2*OE had a strong effect on the elongation and pigmentation responses (Chapter 2). Moreover, combining *PHYB2*OE with *PHYB1*OE had only a very small effect, but combining *PHYB2*OE with *CRY2*OE did result in an even stronger suppression of stem elongation and a further enhancement of pigmentation. The analysis of the effect of *PHY*OE thus complements previous studies using type specific phytochrome mutants by revealing the level of saturation in phyA, phyB1 and phyB2 signalling but also by revealing novel interactions occurring at elevated levels of *PHY* gene expression. For instance, although mutant analysis indicated that the phyA-dependent low fluence rate response component is dependent on the presence of either phyB1 or phyB2 (Weller *et al.*, 2000), an up to 32-fold increase of phyB1 level compared to WT resulted in an actual reduction of this response (Chapter 2)

suggesting a negative interaction between phyA and phyB1 at elevated levels of phyB1. From previous mutant studies (Weller *et al.*, 2000) it could be inferred that *PHYA*OE amplifies the response to low fluence rates. In contrast, in some lines the response to low fluence rates was actually reduced by *PHYA*OE (chapter 2). Similarly, based on these mutant studies *PHYB1*OE would be predicted to result in a large shift and *PHYB2*OE in only a small shift of the R-HIR to lower fluence rates (unless this response is already saturated at WT phyB1 and phyB2 levels). However, *PHYB1*OE results only in a small shift of the R-HIR, while *PHYB2*OE results in a large shift in the R-HIR (Chapter 2).

Also, single mutant studies in tomato showed that at the WT level, phyB1 contributes more strongly to de-etiolation than phyB2 under cR (van Tuinen *et al.*, 1995a; Weller *et al.*, 2000). In contrast, *PHYB1*OE under control of the *CaMV* double-35S promoter has only little effect, but *PHYB2*OE under control of the same *CaMV* double-35S promoter has a strong effect on de-etiolation (Chapter 2). The results show that conclusions drawn from mutant studies are not easily transferred to situations of elevated phytochrome levels.

5.5 Phytochrome signal transduction

5.5.1 Overlap in the control of light responses by different phytochromes

Recently many elements of the signalling cascade in the phytochrome-mediated signal transduction have been identified (reviewed in Quail, 2006 and 2007). A summary of some of the major branches of the cellular activities that drive photomorphogenesis, mostly derived from studies on *Arabidopsis,* is given in Figure 5.1. The figure shows that there is substantial overlap in the targets of the phyA and phyB signal transduction pathways, indicating that in many cases the action of these two types of photoreceptors should be seen in the context of interaction and/or co-action with the other photoreceptors.

Microarray analysis aimed to identify the genes targeted by phyA and phyB signalling in *Arabidopsis* seedlings showed that approximately 70% of the light regulated genes were both activated by cR (type II phy regulated) and cFR (type I phy regulated) (Tepperman *et al.*, 2001, 2004 and 2006). Of the light regulated genes 30% exhibit apparent phyB-dependency for the cR response (Tepperman *et al.*, 2004), while approximately 10% of these genes were regulated by phyA (Tepperman *et al.*, 2001). The overlap in phyA and phyB regulated response can in part be explained by shared interacting signal-transducing factors and shared

transcription factors targeted for regulation. For instance, following light-induced translocation to the nucleus, both phyA and phyB are proposed to interact specifically in their active Pfr form with PIF3, which induces rapidly responding transcription-factor genes, ultimately leading to control of the various aspects of photomorphogenesis. Some of the primary target genes, such as circadian clock-associated 1 (CCA1) and late elongated hypocotyl (LYH) are core of the circadian clock mechanism. Phytochrome effects on gene expression may thus be through direct transcriptional control and/or indirect through entrainment of the circadian clock, like activation of chlorophyll *a/b*-binding protein (*CAB*) gene expression. The PIF3 (and other members of bHLH) proteins were reported to bind to phyA and phyB in *Arabidopsis* with different affinity (Huq *et al.,* 2004), which may contribute to differential effects of *PHY*OE on branches of the phytochrome-signalling network.

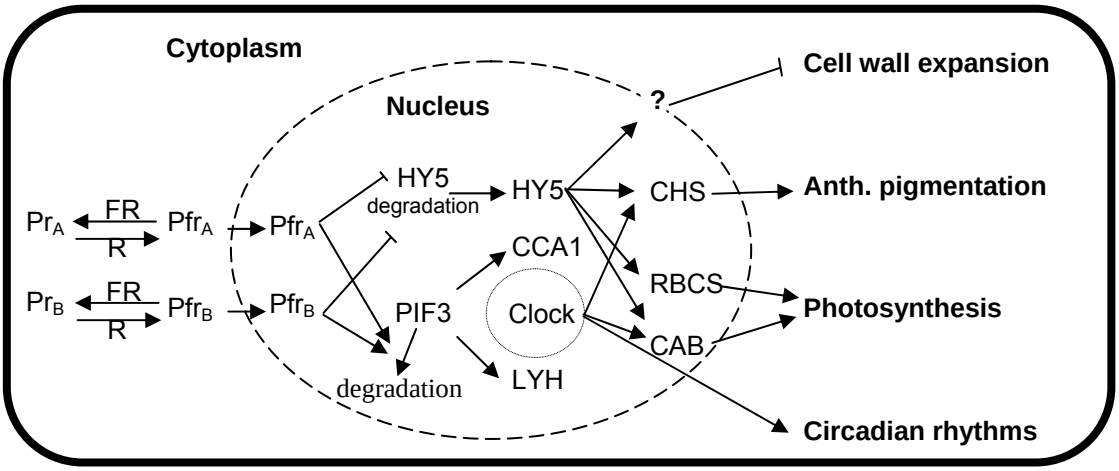


Figure 5.1. Simplified model of some of the major known branches in light-signalling pathways derived from current genetic and molecular studies. Following light-induced translocation to the nucleus, both phyA and phyB are proposed to interact specifically in their active Pfr form with nuclear components, resulting in enhanced degradation of bHLH transcription factors like PIF3 and increased stability of transcription factors like HY5. PIF-like transcription factors may act positively or negatively, depending on the light response (e.g. PIF3 enhances anthocyanin pigmentation but reduces chlorophyll pigmentation). Among the primary transcriptional targets are CCA1 and LYH, resulting in regulation of the circadian clock, which contributes to the pleiotropic effects of phytochrome action.

Single and double mutant studies of phyB1 and phyB2 in tomato have shown a redundancy in the function of these two phytochromes. While the mutant studies show a big effect of loss in phyB1 function and hardly any effect of loss in phyB2 function the overexpression results show the opposite: a big effect for *PHYB2*OE

and only a small effect for *PHYB1*OE. This offers great opportunities to study the sequence divergence between phyB1 and phyB2. Different phyB1/phyB2 chimeras could be constructed to identify the regions within these proteins where sequence divergence results in functional differences.

5.5.2. Phosphorylation in phytochrome mediated signalling

Both the Pr and the Pfr form of phytochrome can be phosphorylated at several sites by the intrinsic kinase activity of phytochrome protein (Lapko *et al.*, 1997). The de-phosphorylation of the Pfr form, mediated by specific phosphatases such as PAPP5 (type 5 protein phosphatase; Ryu *et al.*, 2005) and FyPP (protein phosphatase 2A) (Kim *et al.*, 2002), enhances plant photoresponsiveness by increasing phyA affinity for its downstream molecular partners by increasing phytochrome stability upon activation (Kim *et al.*, 2005; Rubio and Deng, 2005). Thus, the phosphorylation of one or more amino acid residues in the phytochrome could be a regulatory step for one or several aspects of phytochrome signalling. The modification of phytochrome activity by phosphorylation may be part of the elaborate mechanism for tuning the flux of light signalling to reach a certain homeostasis in light responses. Assuming that similar regulatory mechanisms operate in tomato, it is to be expected that initial phosphorylation of ectopically produced phytochrome protein is efficient, due to stable dimer formation. Consequently, the action of light-activated ectopically produced phytochrome (Pfr) in tomato may be limited by endogenous phosphatase activity. The enhanced responses evoked by *PHYB*OE suggest that WT PAPP5-like phosphatase levels in tomato are sufficient to de-phosphorylate the ectopically produced phytochrome protein (Husaineid *et al*., 2007; Chapter 2). However, the saturation in the suppression of hypocotyl elongation, which was observed in *PHYB2*OE lines (Chapter 4), could origin from limiting endogenous PAPP5-like activity. This would result in a sub-pool of phosphorylated Pfr, which is prevented to interact with downstream factors. Whether de-phosphorylation is limiting for *PHY*OE action could be tested by combining *PHYB2*OE with increased levels of PAPP5 overexpression, or by using mutant phyB2 forms in which the phosphorylation target sites have been modified.

Some of the members of phytochrome interacting factors in the basic helix-loop-helix (bHLH) protein family can bind to both phyA and phyB (PIF3: Kim *et al.*, 2000, 2003), while others are specific for certain types of phytochrome (PIF4 and phyB: Huq and Quail, 2002). These interacting factors act as a negative regulator for both R and FR responses (Kim *et al.*, 2000, 2003). It was recently demonstrated that the phytochrome-induced phosphorylation of PIF proteins might represent the

primary biochemical mechanism of phytochrome signaling, by targeting these negative regulators for degradation (Al-Sady *et al.*, 2006). The phytochrome-induced phosphorylation of these proteins seems to initiate rapid migration to nuclear speckles that may function as sites of ubiquitination and/or proteasomal degradation. Because in this case the action of phytochrome is through the destruction of a negative regulator, increasing the phytochrome levels may not lead to an actual increase in the response, but the response may be evoked at lower light intensities, as was shown to occur for *PHYB2*OE in our tomato plants.

5.6 Manipulation of photoreceptors

Analysis of plants expressing phytochrome transgenes has revealed the potential of genetic modification to alter the architecture of crop plants. In the last 20 years many attempts have been made using this approach. Table 5.1 lists the plant species that have been transformed with *PHY* transgenes and the transgenes that they have been transformed with. Studies in different plant species such as tomato (Boylan and Quail 1989), tobacco (Robson *et al*., 1996) and potato (Thiele *et al*, 1999) indicated that the performance of crop plants might be improved by altering specific phytochrome levels. The positive effect of specific phytochrome overexpression on yield in potato has now also been confirmed in the field (Boccalandro *et al.,* 2003). In tomato plants with oat *PHYA*OE, the anthocyanin accumulation was enhanced under WL (Boylan and Quail, 1989).

In previous studies, heterologous *PHYA*OE under the *35S* promoter appeared to suppress plant elongation under increased FR conditions, as reported for transgenic tobacco (McCormac *et al*., 1992b; Ballaré *et al*., 1994; Casal and Sánchez 1994a; Robson *et al*., 1996). In contrast we show that of homologous overexpression of *PHYA* (which is also under the *35S* promoter) in tomato has little effects on plant elongation and anthocyanin accumulation in response to FR (Chapter 2; Husaineid *et al*., 2007). This difference in effect of homologous and heterologous *PHYA*OE may be due to differences in stability of the phyA protein in a homologous or heterologous host plant (in tomato; Boylan and Quail 1989 and in tobacco; Robson *et al*., 1996). We demonstrated that tomato plants with tomato *PHYB1*OE and *PHYB2*OE enhanced anthocyanin accumulation under WL and cR. In these plants the elongation response was suppressed both at seedling and adult stage of development (Chapter 2, 3 and 4).

Table 5.1. Summary of phytochrome overexpression under CaMV 35S promoter.

***PHY* Source**	***PHY* Host**	**References**
tomato (*PHYA*, *PHYB1* and *PHYB2*)	tomato	Husaineid *et al*., 2007
tobacco (*PHYA*)	tobacco	Sharkey *et al*., 1991
tobacco (*PHYB*)	tobacco	Fernandez *et al*., 2005 and Zheng *et al*., 2006
potato (*PHYA*)	potato	Heyer *et al*., 1995, Yanovsky *et al*., 1998 and 2000
potato (*PHYB*)	potato	Jackson *et al*., 1996
***Arabidopsis* (*PHYA*)**	*Arabidopsis*	Casal *et al*., 1996
***Arabidopsis* (*PHYB*)**	*Arabidopsis*	Wagner *et al*., 1991, McCormac *et al*., 1993b, Hennig *et al*., 1999b and Sharrock *et al*., 2003
***Arabidopsis* (*PHYC*)**	*Arabidopsis*	Qin *et al*., 1997
***Arabidopsis* (*PHYD*)**	*Arabidopsis*	Sharrock *et al*., 2003
***Arabidopsis* (*PHYE*)**	*Arabidopsis*	Sharrock *et al*., 2003
oat (*PHYA*)	tomato	Boylan and Quail, 1989, McCormac *et al*., 1992a and Casal *et al*., 1995
oat (*PHYA*)	tobacco	Keller *et al*., 1989, Cherry *et al*., 1991, McCormac *et al*., 1991a,1991b, 1992b,1993a, 1993c, Ballaré *et al*., 1994, Casal and Sánchez 1994a 1994b, Casal *et al*., 1995, Jordan *et al*., 1995b, Robson *et al*., 1996, Rousseaux *et al*., 1997, Mazzella *et al*., 1997 and Halliday *et al*., 1997
rice (*PHYA*)	tobacco	Kay *et al*., 1989, Nagatani *et al*., 1991and Schäfer *et al*., 1994, Emmler *et al*., 1996
***Arabidopsis (PHYB*)**	tobacco	Halliday *et al*., 1997
oat (*PHYA*)	potato	Casal *et al*., 1996
***Arabidopsis* (*PHYB*)**	potato	Thiele *et al*., 1999, Boccalandro *et al*., 2003 and Schittenhelm *et al*., 2004 and *et al*., 2004
oat (*PHYA*)	*Arabidopsis*	Boylan and Quail, 1991, Boylan *et al*., 1994 and Whitelam *et al*., 1992
rice (*PHYA*)	*Arabidopsis*	Halliday *et al*., 1999
rice (*PHYB*)	*Arabidopsis*	Wagner *et al*., 1991and Casal *et al*., 2000
oat (*PHYA*)	*Aster* (*Asteraceae*)	Wallerstein *et al*., 2002
***Arabidopsis* (*PHYB*)**	*Aster* (*Asteraceae*)	Wallerstein *et al*., 2002
***Arabidopsis* (*PHYA*)**	horseradish	Saitou *et al*., 1999
tobacco (*PHYB*)	chrysanthemum	Zheng *et al*., 2001
oat (*PHYA*)	hybrid aspen	Olsen *et al*., 1997, Mølmann *et al*., 2005
oat (*PHYA*)	rice	Clough *et al*., 1995 and Casal *et al*., 1996
oat (*PHYA*)	wheat	Shlumukov *et al*., 2001 and Sineshchekov *et al*., 2001

5.6.1 Effects of *PHY*OE on shade avoidance responses

When plants grow in close proximity, the shade-avoidance strategy is evoked. This strategy is very important, enabling plants to compete with their neighbours for the photosynthetically available radiation. The important waveband necessary for proximity perception via phytochromes is the far-red spectral region (700-800 nm; Fig. 5.2a). The reduction in R:FR functions as an alarm to induce shade avoidance responses, which modify growth to increase capture of sunlight. This latter adaptation is achieved by enhanced internode and petiole extension growth and increased apical dominance.

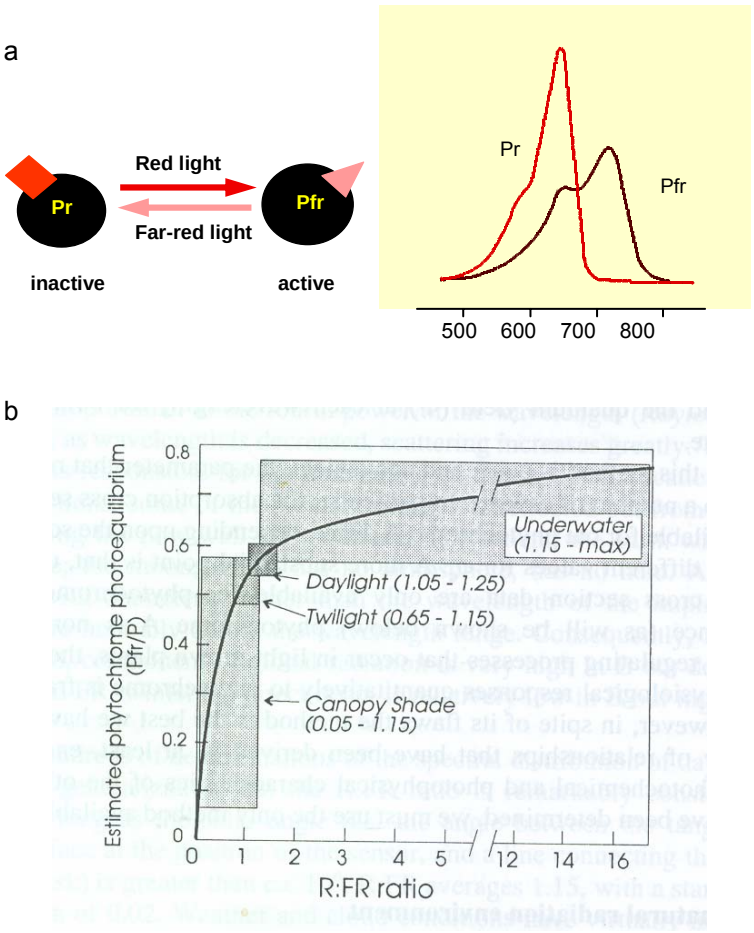


Figure 5.2. (a) Phytochrome photoconversion and absorption spectra of Pr and Pfr and (b) the relationship between R:FR and calculated Pfr/Ptotal. This relationship is a rectangular hyperbola in which the steepest part of the curve lies within the R:FR range found within vegetation canopies (Smith, 1994).

Phenotypically similar growth responses to those described for SAR can be observed under (1) combination of a high temperature during the light period and a lower temperature during the dark period (DIF; Langton, 1998) (2) low light intensity,

especially reduction in B resulting in enhancement of elongation known to be mainly mediated by cryptochromes (Vandenbussche *et al.*, 2005) or (3) when plants are submerged in water (Pierik *et al.*, 2005). In case of submergence in water and low light intensity, the resemblance in the morphological and biochemical response might be explained by separate signal-transduction cascades that both affect a common downstream component (Voesenek *et al.*, 2006). Submerged plants growing in water experience both low light intensities and reduced gas exchange (Voesenek *et al.*, 2006). In order to restore contact with the atmosphere, plants growing under water try to bring the leaf tips closer to the water surface. This avoidance response is similar to the situation in which plants try to bring leaves closer to the light which enhances plant performance and fitness (Schmitt *et al.*, 2003). However, the R:FR increases in a submerged environment because water specifically absorbs the longer wavelengths, suggesting that submergence-induced responses take place under different conditions as phytochrome-mediated SAR. Also, the study of Mommer *et al*. (2005) demonstrated that shade and submergence do not give the same response for the photosynthetic acclimation in the leaves.

Figure 5.2b shows the relationship between R:FR and calculated Pfr/Ptotal for a fixed stable pool of phytochrome (Smith, 1994). The nature of this relationship ensures that reduction in R:FR below a value of about 1.0 lead to the greatest reduction in the Pfr/Ptotal. In daylight the R:FR ratio is approximately 1.2. Within plant communities, this ratio can be reduced down to 0.09 (Smith, 1982), depending on proximity or plant density. In the *PHYB1*OE and *PHYB2*OE lines the total stable phytochrome (Ptot) pool has increased and consequently, for any given R:FR situation, the level of stable Pfr has increased compared to WT. The hypothetical relationship between Pfr pool size and growth (Smith, 2000) was used to calculate the shade avoidance index for different Ptot pool sizes (SAI; Chapter 3). The resulting graph shows that increasing the Ptot levels relative to WT will initially result in an increase of the SAI (Chapter 3, figure 3.9). Above a doubling of the WT Ptot pool size the SAI will actually decrease to zero, depending on where the suppression of elongation through phytochrome signalling reaches saturation. The hypothetical relationship between Ptot pool size and SAI shows that SAR is only fully suppressed when elongation is fully suppressed regardless of the R:FR light condition. Therefore, the plants with *PHYB1*OE or *PHYB2*OE still respond to shade, but both plants on the inside and outside of a population are shorter than WT plants that receive full sunlight (Chapter 3). The decrease in SAI in line A/1 could be explained by slightly different Ptot pool sizes in shade and non-shade, due to differential effects on phyA stability. The relative increase in the Ptot pool under

increased FR conditions leads to a conditional dwarfing, which in the case of homologous *PHYA*OE is milder than in the case of heterologous *PHYA*OE (Robson *et al*., 1996), due to intrinsic higher turnover of phyA in the light in a homologous host.

5.6.2 Effects of *PHY*OE on biomass partitioning

The shade avoidance responses may be negative in terms of crop productivity particularly because of the retarded leaf development, acceleration of flowering and increased biomass allocation towards stem instead of harvestable organs. The introduction of homologous *PHYAOE* in tomato had no differential effect on biomass allocation to leaf and stem (Chapter 3), although in line A1 the SAI was reduced compared to WT. In contrast, overexpression of oat *PHYA* in tobacco resulted in an increased leaf index (LDW:TDW) (Robson *et al*., 1996). As mentioned before, the difference in effect of homologous and heterologous *PHYA*OE may be due to differences in stability of the phyA protein in a homologous or heterologous host plant (in tomato; Boylan and Quail 1989 and in tobacco; Robson *et al*., 1996).

The *PHYB2*OE resulted in a reduced TDW in 8-week old tomato plants (75% of WT TDW, Chapter 3), indicating that besides potential beneficial effects on the SAR, the constitutive *PHYB2*OE may have negative pleiotropic effects. The reduced TDW in these plants could be the combined result of reduced leaf size and an less optimal positioning of leaves for photosynthesis (although this last response to R:FR was not quantified in our *PHY*OE lines). The effect of *PHYB2*OE in tomato on biomass accumulation differs from the effect of *Arabidopsis PHYB*OE in potato (Boccalandro *et al*., 2003; Robson *et al*., 1996; Thiele *et al*., 1999). In potato the *PHYB*OE resulted in an increased leaf, root and tuber yield.

Because leaf production of young tomato plants with a moderate *PHYB2*OE was reduced 20% compared to WT, while the production of stem biomass was reduced 40 % compared to WT, fruit biomass allocation in these plants still might show a relative increase. However, the fruit yield of these plants was approximately 50% lower than WT (Anke Van der Ploeg, unpublished data), indicating that only increasing plant density in greenhouses by more than 50% will increase tomato production per m^{-2} and potentially reduce gas consumption in greenhouse tomato cultivation.

5.6.3 Effects of *PHY*OE on carotenoid and anthocyanin

Light has shown to affect carotenoid and anthocyanin accumulation in a number of

plant species, including tomato. Alba *et al*., (2000) has shown that phytochrome-mediated light signal transduction was required for normal ripe fruit pigmentation, but did not affect other ripening characteristics. Using genetically modified tomato plants overexpressing either *PHYB1* or *PHYB2*, we have shown that *PHYB1*OE and *PHYB2*OE result in elevated anthocyanin levels in seedlings and adult plants (Chapter 2). Furthermore, the data in this thesis provides evidence for an interaction between phyB2 and cry2 that can enhance anthocyanin biosynthesis in vegetative parts of the plant (Chapter 4). Future experiments may provide evidence whether these manipulations also enhance pigmentation in fruits, which would demonstrate that manipulation of photoreceptors may be a useful approach for optimizing fruit pigmentation and associated nutritional quality. It has been shown that pigmentation in tomato fruits may also be enhanced by mutations in suppressors of light responses. For instance, the tomato high pigment mutants *hp1* and *hp2* have been characterized by darker green fruit at green fruit stage and higher carotenoid levels at ripe fruit stage (Kendrick *et al.*, 1997). It would therefore be of interest to see whether a combination of enhanced photoperception and reduced suppressor activity, using fruit specific promoters, may lead to even higher anthocyanin and carotenoid levels in tomato fruit. Attempts to manipulate fruit pigmentation through repression of the repressor DET1 using constitutive promoters, resulted in additional negative pleiotropic effects, while attempts using tomato fruit-specific promoters were unsuccessful (Davuluri *et al*., 2004).

5.6.3 Advantages and disadvantages of *PHY*OE in tomato

The Netherlands greenhouse production of tomato increased significantly as a result of higher production levels and in 2003 was almost twice as high as in 1980 (reviewed in Van der Ploeg and Heuvelink, 2006). However, the energy consumption to maintain this high production is covered mainly with natural gas, by which the horticulture sector strongly contributes to CO2 emission into the environment. In the Netherlands 50 to 60 m^3 gas m^{-2} are used annually for heating in greenhouses. To improve the environmental aspects associated with greenhouse horticulture, measures have to be taken, which reduce the environmental impact on one side and which keep crop production on a high level in terms of mass and quality on the other side. Several attempts have been made to increase the yield and efficiency in the production of crop plants using transgenic systems, based upon manipulation of light signalling in plants.

We investigated the potential effect of phytochrome overexpression on tomato growth and development, mainly to see whether plants could be grown at

higher density without loss of yield to biomass allocation to stem due to SAR. As discussed under 5.6.2, total dry weight production after 8 weeks growth and fruit yield were decreased substantially. For tomato the constitutive overexpression of homologous phytochrome genes therefore does not seem to be beneficial. Because many genes involved in photosynthesis are co-ordinately regulated by phytochromes, constitutive *PHY*OE may lead to sub-optimal regulation of several enzymes of carbon metabolism and to undesired alterations in development (e.g. chloroplast development), which may affect the productivity of the plant. For instance, although the number of tubers is increased in potatoes overexpressing *PHYB*, the tuber size is smaller (Thiele *et al*., 1999).

A second way overcoming the disadvantage of the shade-avoidance in dense populations may be by manipulating specific targets of SAR. For instance, ethylene has been implicated in SAR related responses (e.g. leaf senescence: Guo and Echer, 2004; leaf angle: Pierik *et al.*, 2004) and these responses could be manipulated through targeting ethylene production or the ethylene-sensing mechanism in plants. We have attempted to manipulate ethylene sensitivity in tomato plants using ectopic expression of a dominant negative form of the ethylene-receptor gene from Arabidopsis (*ETR1-1*). However, the resulting lines showed undesirable phenotypes such as enhanced disease susceptibility and deviating growth characteristics.

The effect of *PHY*OE on anthocyanin accumulation in vegetative parts of the plant may be used as a motive to also investigate the effect on anthocyanin and carotenoids in the fruits of these plants. Both anthocyanin and carotenoid derivatives as lycopene have anti-oxidant and anti-cancer activity. For instance, lycopene, the major carotenoid in tomato has been recently reported to suppress human prostate cancer cells (Limpens *et al*., 2006). Therefore manipulation of the anthocyanin and carotenoid levels in tomato fruit may greatly improve health related quality aspects of tomato fruits.

5.7 Concluding remarks and future work

The work presented in this thesis shows a functional divergence between the tomato phyA, phyB1 and phyB2, resulting in differential effects of *PHYA*OE, *PHYB1*OE and *PHYB2*OE. Although shade responses were altered in some of the transgenic lines, the results indicate that negative pleiotropic effects may overshadow the potentially beneficial effects of *PHY*OE on the shade avoidance

response. Most likely this is due to the constitutive activity of the *PHY*OE genes. However, the results also show that manipulation of specific traits is possible through photoreceptor manipulation, but that practical applications may depend on a more subtle approach, using tissue specific promoters or developmentally regulated promoters.

References

Ahmad, M. and Cashmore, A.R. 1993. HY4 gene of *Arabidopsis thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature*, **366**: 162-166.

Ahmad, M. and Cashmore, A.R. 1996. The *pef* mutants of *Arabidopsis* thaliana define lesions early in the phytochrome signalling pathway. *Plant J.*, **10**: 1103-1110.

Ahmad, M. and Cashmore, A.R. 1997. The blue-light receptor cryptochrome 1 shows functional dependence on phytochrome A or phytochrome B in *Arabidopsis* thaliana. *Plant J.*, **8**: 421-427.

Ahmad, M., Jarrillo, J.A., Smirnova, O. and Cashmore, A.R. 1998. The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome in vitro. *Mol. Cell*, **1**: 939-948.

Ahmad, M. 1999. Seeing the world in red and blue: insight into plant vision and photoreceptors. *Curr. Opin. Plant Biol.*, **2**: 230-235.

Alabadi, D., Gil, J., Blazquez, M. and Garcia-Martinez, J.L. 2004. Gibberellins repress photomorphogenesis in darkness. *Plant Physiol.*, **134**: 1050-1057.

Alba, R., Cordonnier-Pratt, M-M. and Pratt, L.H. 2000. Fruit-localized phytochromes regulate lycopene accumulation independently of ethylene production in tomato. *Plant Physiol.,* **123**: 363-370.

Al-Sady, B., Ni, W., Kircher, E., Schäfer, E. and Quail, H. 2006. Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. *Mol. Cell*, **23**: 439-446.

Aukerman, M.J., Hirschfeld, M., Wester, L., Weaver, M., Clack, T., Amasino, R.M. and Sharrock, R.A. 1997. A Deletion in the PHYD gene of the *Arabidopsis wassilewskija* ecotype defines a role for phytochrome D in red/far-red light sensing. *Plant Cell*, **9**: 1317-1326.

Ballaré, C.L., Scopel, A.L., Sánchez, R.A., Casal, J.J. and Ghersa, C.M. 1987. Early detection of neighbour plants by phytochrome perception of spectral changes in reflected sunlight. *Plant Cell Environ.*, **10**: 551-557.

Ballaré, C.L., Scopel, A.L. and Sánchez, R.A. 1990. Far-red radiation reflected from adjacent leaves: an early signal of competition in plant canopies. *Science*, **247**: 329-332.

Ballaré, C.L., Scope, A.L., Jordan, E.T. and Vierstra, R.D. 1994. Signalling among neighbouring plant, and developmental of size inequalities in plant populations. *Proc. Natl. Acad. Sci.*, **91**: 10094-10098.

Ballaré, C.L., Scopel, A.L. and Sánchez, R.A. 1997. Foraging for light: photosensory ecology and agricultural implications. *Plant Cell Environ.*, **20**: 820-825.

Ballesteros, M.L., Bolle, C., Lois, L.M., Moore, J.M., Vielle-Calzada, J.P., Grossniklaus, U. and Chua, N-M. 2001. LAF1, a MYB transcription activator for phytochrome A signalling. *Genes Dev.*, **15**: 2613-2625.

Bauer, D., Viczian, A., Kircher, S., A., Nobis, T., Nitschke, R., Kunkel, T., Panigrahi, K.C.S., Adam, E., Fejes, E., Schäfer, E. and Nagy, F. 2004. Constitutive photomorphogenesis 1 and multiple photoreceptors control degradation of phytochrome interacting factor 3, a transcription factor required for light signalling in Arabidopsis. *Plant Cell*, **15**: 2125-2130.

Baurle, I. and Dean, C. 2006. The timing of developmental transitions in plants. *Cell*, **125**: 655-664.

Beggs, C.J. and Wellmann, E. 1994. Photocontrol of flavonoid biosynthesis. In *Photomorphogenesis in Plant.* 2nd Ed. (Kendrick, R.E. and Kronenberg G.H.M. eds). pp. 733-750. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Behringer, F.J., Davies, P.J. and Reid, J.B. 1992. Phytochrome regulation of stem growth and indole-3-acetic acid levels in the *lv* and *Lv* genotypes of *Pisum*. *Photochem. Photobiol.*, **56**: 677-684.

Boccalandro, H.E., Ploschuk, E.L., Yanovsky, M.J., Sánchez, R.A., Gatz, C. and Casal, J.J. 2003. Increased phytochrome B alleviates density effects on tuber yield of field potato crops. *Plant Physiol.*, **133**: 1539-1546.

Bolle, C., Koncz, C. and Chua, N-M. 2000. PAT1, a new member of the GRAS family, is involved in phytochrome A signal transduction. *Genes Dev.*, **14**: 1269-1278.

Borthwick, H.A., Hendricks, S.B., Parker, M.W., Toole, E.H. and Tool, V.K. 1952. A reversible photoreaction controlling seed germination. *Proc. Natl. Acad. Sci.*, **38**: 662-666.

Bowler, C., Neuhaus, G., Yamagata, H. and Chua, N-M. 1994. Cyclic GMP and calcium mediate phytochrome phototransduction. *Cell*, **77**: 73-81.

Boylan, M.T. and Quail, P.H. 1989. Oat phytochrome is biologically active in transgenic tomatoes. *Plant Cell*, **1**: 765-773.

Boylan, M.T. and Quail, P.H. 1991. Phytochrome A overexpression inhibits hypocotyl elongation in transgenic *Arabidopsis*. *Proc. Natl. Acad. Sci.*, **88**: 10806-10810.

Boylan, M., Douglas, N. and Quail, P.H. 1994. Dominant negative suppression of *Arabidopsis* photoresponses by mutant phytochrome A sequences identifies spatially discrete regulatory domains in the photoreceptor. *Plant Cell*, **6**: 449-460.

Brautigam, C.A., Smith, B.S., Ma, Z., Palnitkar, M., Tomchick, D.R, Machius, M. and Deisenhofer, J. 2004. Structure of the photolyase-like domain of cryptochrome 1 from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci.*, **101**: 12142-12147.

Briggs, W.R. and Huala, E. 1999. Blue-light photoreceptors in higher plants. *Annu. Rev. Cell Dev. Biol.*, **15**: 33-62.

Briggs, W.R. and Christie, J.M. 2002. Phototropins 1 and 2: versatile plant blue-light receptors. *Trends in Plant Science*, **7**: 204-210.

Brown, B.A., Cloix, C., Jiang, G.H., Kaiserli, E., Herzyk, P., Kliebenstein, D.J. and Jenkins, G.I. 2005. A UV-B-specific signalling component orchestrates plant UV protection. *Proc. Natl. Acad. Sci.*, **102**: 18225-18230.

Buche, C., Poppe, C., Schäfer, E. and Kretsch, T. 2000. *eid1*: A new *Arabidopsis* mutant hypersensitive in phytochrome A-dependent high-irradiance responses. *Plant Cell*, **12**: 547-558.

Butler, W.L., Norris, K.H., Siegelman, H.W. and Hendricks, S.B. 1959. Detection, assay, and preliminary purification of the pigment controlling photoresponsive development of plants. *Proc. Natl. Acad. Sci.*, **45**: 1703-1708.

Butler, W.L. and Lane, W.S. 1965. Dark transformation of phytochrome *in vivo*. *Plant Physiol.*, **40**: 13-17.

Canton, F.R. and Quail, P.H. 1999. Both phyA and phyB mediate light-imposed repression of PHYA gene expression in *Arabidopsis*. *Plant Physiol.*, **121**: 1207-1215.

Casal, J.J., Sánchez, R.A. and Vierstra, R.D. 1994. Avena phytochrome A overexpressed in transgenic tobacco seedlings differentially affects red/far-red reversible and very-low-

fluence responses (cotyledon unfolding) during de-etiolation. *Planta*, **192**: 306-309.

Casal, J.J. and Sánchez, R.A. 1994a. Overexpression of oat phytochrome A gene differentially affects stem growth responses to red/far-red ratio signals characteristic of sparse and dense canopies. *Plant Cell Environ.*, **17**: 409-417.

Casal, J.J. and Sánchez, R.A. 1994b. Impaired stem-growth responses to blue-light irradiance in light-grown transgenic tobacco seedlings overexpression *Avena* phytochrome A. *Physiologia Plantarum*, **91**: 268-272.

Casal, J.J., Sánchez, R.A., Boylan, M.T., Vierstra, R.D. and Quail, P.H. 1995. Is the far-red-absorbing from of *Avena* phytochrome A that is present at the end of the able to sustain stem-growth inhibition during the night in transgenic tobacco and tomato seedling? *Planta*, **197**: 225-232.

Casal, J.J., Clough, R.C. and Vierstra, R.D. 1996. High-irradiance responses induced by far-red light in grass seedlings of the wild type of overexpressing phytochrome A. *Planta*, **200**: 132-137.

Casal, J.J. and Mazzella, M.A. 1998. Conditional synergism between cryptochrome 1 and phytochrome B is shown by the analysis of *phyA*, *phyB*, and *hy4* simple, double, and triple mutants in *Arabidopsis*. *Plant Physiol.*, **118**: 19-25.

Casal, J.J. 2000a. Phytochromes, Cryptochromes, Phototropin: Photoreceptor interaction in plants. *Photochem. Photobiol.*, **71**: 1-11.

Casal, J.J., Yanovsky, M.J. and Luppi, J.P. 2000b. Two photobiological pathways of phytochrome A activity, only one of which shows dominant negative suppression by phytochrome B. *Photochemistry and Photobiology* **71**: 481-486.

Casal, J.J., Luccioni, L.G., Oliverio, K.A. and Boccalandro, H.E. 2003. Light phytochrome signalling and photomorphogenesis in *Arabidopsis*. *Photochem. Photobiol.*, **2**: 625 - 636.

Casal, J.J. and Yanovsky, M.J. 2005. Regulation of gene expression by light. *Int. J. Dev. Biol.,* **49**: 501-511.

Casal, J.J. 2006. *The photoreceptor interaction network*. In *Photomorphogenesis in Plants and Bacteria*. 3rd Ed. (Schäfer, E. and Nagy, F., eds) pp. 407-437. Dordrecht, The Netherlands: Springer.

Cashmore, A.R., Jarillo, J.A., Wu, Y.J. and Liu, D. 1999. Cryptochromes: blue light receptors for plants and animals. *Science*, **284**: 760-765.

Cerdan, P. and Chory, J. 2003. Regulation of flowering time by light quality. *Nature*, **423**: 881-885.

Chattopadhyay, S., Lay-Hong, A., Puente, P., Xing-Wang, D. and Wei, N. 1998. *Arabidopsis* bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *Plant Cell*, **10**: 673-684.

Chen, M., Chory, J. and Fankhauser, C. 2004. Light signal transduction in higher plants. *Annu. Rev. of Genetics*, **38**: 87-117.

Chen, M., Tao, Y., Shaw, A. and Chory, J. 2005. Regulation of phytochrome B nuclear localization through light-dependent unmasking of nuclear-localization signals. *Curr. Biol.*, **15**: 637-642.

Cherry, J.R. Hershey, H.P. and Vierstra, R.D. 1991. Characterization of tobacco expressing functional oat phytochrome*. Plant Physiol.*, **96**: 775-785.

Choi, G., Yi, H., Lee, J., Kwon, Y-K., Soh, M.S., Shin, B., Luka, Z., Hahn, T-R. and Song, P-I. 1999. Phytochrome signalling is mediated through nucleoside diphosphate kinase 2*. Nature,* **401**: 610-613.

Christie, J.M., Swartz, T.E., Bogomolni, R.A. and Briggs, W.R. 2002. Phototropins LOV domains exhibit distinct roles in regulating photoreceptor function. *Plant J.*, **32**: 205-219.

Clack, T., Mathews, S. and Sharrock, R.A. 1994. The phytochrome apoprotein family in *Arabidopsis* is encoded by five genes- the sequences and expression of PHYD and PHYE. *Plant Mol. Biol.*, **25**: 413-427.

Clough, R.C., Casal, J.J., Jordan, E.T., Christou, P. and Vierstra, R.D. 1995. Expression of functional oat phytochrome A in transgenic rice. *Plant Physiol.*, **109**: 1039-1045.

Clough, R.C., Jordan, E.T., Lohman, K.N., Marita, J.M., Walker, J.M., Gatz, C. and Vierstra, R.D. 1999. Sequences within both the N-terminal domains phytochrome A are required for PER ubiquitination and degradation. *Plant J.*, **17**: 155-167.

Cluis, C.P., Mouchel, C.F. and Hardtke, C.S. 2004. The *Arabidopsis* transcription factor HY5 integrates light and hormone signalling pathways. *Plant J.*, **38**: 332-347.

Cordonnier-Pratt, M.-M., Greppin, H. and Pratt, L.H. 1986a. Phytochrome from *Avena* shoots characterized with a monoclonal antibody to phytochrome from etiolated *Pisum* shoots. *Biochemistry*, **25**: 7657-7666.

Cordonnier-Pratt, M.-M., Greppin, H. and Pratt, L.H. 1986b. Identification of a highly conserved domain on phytochrome from angiosperms to algae. *Plant Physiol.*, **80**: 982–987.

Cox, M.C.H., Benschop, J.J., Vreeburg, R.A.M., Wagemaker, C.A.M., Moritz, T., Peeters, A.J.M. and Voesenek, L.A.C.J. 2004. The roles of ethylene, auxin, abscisic acid, and gibberellin in the hyponastic growth of submerged *rumex palustris* petioles. *Plant Physiol.*, **136**: 2948-2960.

Davis, S.J., Bhoo, S.H., Durski, A.M., Walker, J.M. and Vierstra, R.D. 2001. The heme-oxygenase family required for phytochrome chromophore biosynthesis is necessary for photomorphogenesis in higher plants. *Plant Physiol.*, **126**: 665-669.

Davuluri, G.R., Tuinen, A., Mustilli, A.C., Manfredonia, A., Newman, R., Burgess, D., Brummell, D.A., King, S.R., Palys, J., Uhlig, J., Pennings, H.M.J. and Bowler, C. 2004. Manipulation of DET1 expression in tomato results in photomorphogenic phenotypes caused by post-transcriptional gene silencing. *Plant J.*, **40**: 344-354.

Devlin, P.F., Patel, S.R. and Whitelam, G.C. 1998. Phytochrome E influences internode elongation and flowering time in *Arabidopsis*. *Plant Cell*, **10**: 1479-1487.

Devlin, P.F., Robson, P.R.H., Patel, S.R., Goosey, L., Sharrock, R.A. and Whitelam, G.C. 1999. Phytochrome D acts in the shade-avoidance syndrome in *Arabidopsis* by controlling elongation growth and flowering time. *Plant Physiol.*, **119**: 909-916.

Devlin, P.F. and Kay, S.A. 2000. Cryptochromes are required for phytochrome signalling to the circadian clock but not for rhythmicity. *Plant Cell*, **12**: 2499-2510.

Devlin, P.F., Yanovsky, M.J. and Kay, S.A. 2003. A genomic analysis of the shade avoidance response in *Arabidopsis*. *Plant Physiol.*, **133**: 1617-1629.

Devlin, P. 2006. *Circadian regulation of photomorphogenesis*. In *Photomorphogenesis in Plants and Bacteria*. 3rd Ed. (Schäfer, E. and Nagy, F., eds) pp. 567-604. Dordrecht, The Netherlands: Springer.

Dodd, A.N., Salathia, N., Hall, A., Kevei, E., Toth, R., Nagy, F., Hibberd, J.M., Millar,

A.J. and Webb, A.A.R. 2005. Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science*, **309**: 630-633.

Doyle, M.R., Davis, S.J., Bastow, R.M., McWatters, H.G., Kozma-Bognar, L., Nagy, F., Millar, A.J. and Amasino, R.M. 2002. The ELF4 gene controls circadian rhythms and flowering time in *Arabidopsis* thaliana. *Nature*, **419**: 74-77.

Dudley, S.A. and Schmitt, J. 1996. Testing the adaptive plasticity hypothesis: Density-dependent selection on manipulated stem length in *Impatiens capensis*. *Am. Nat.*, **147**: 445-465.

Duek, P.D. and Fankhauser, C. 2003. HFR1, a putative bHLH transcription factor, mediates both phytochrome A and cryptochrome signalling. *Plant J.*, **34**: 827-836.

Duek, P.D., Elmer, M.V., van Oosten, V.R. and Fankhauser, C. 2004. The degradation of HFR1, a putative bHLH transcription factor involved in light signalling, is regulated by phosphorylation and requires COP1. *Curr. Opin. Plant Biol.*, **14**: 2296-2301.

Duek, P.D. and Fankhauser, C. 2005. bHLH class transcription factors take centre stage in phytochrome signalling. *Trends in Plant Science*, **10**: 51-54.

El-Din El-Assal, S., Alonso-Blanco, C., Peeters, A.J.M., Wagemaker, C., Weller, J.L. and Koornneef, M. 2003. The role of cryptochrome 2 in flowering in *Arabidopsis*. *Plant Physiol.*, **133**: 1504-1516.

Emmler, K., Kretsch, T. and Schäfer, E. 1996. Overexpression of rice phytochrome A in tobacco seedlings modifies responsiveness but not competence to phytochrome. *Planta,* **199**: 50-56.

Facella, P., Lopez, L., Chiaooetta, A., Bitonti, M.B., Giuliano, G. and Perrotta, G. 2006. CRY-DASH gene expression is under the control of the circadian clock machinery in tomato. *FEBS Letters,* **580**: 4618-4621.

Fairchild, C.D., Schumaker, M.A. and Quail, P.H. 2000. HFR1 encodes an atypical bHLH protein that acts in phytochrome A signal transduction. *Genes Dev.*, **14**: 2377-2391.

Fankhauser, C., Yeh, K.C., Lagarias, J.C., Zhang, H., Elich, T.D. and Chory, J. 1999. PKS1, a substrate phosphorelated by phytochrome that modulates light signalling in *Arabidopsis*. *Science*, **284**: 1539-1541.

Fankhauser, C. and Chory, J. 2000. RSF1, an *Arabidopsis* locus implicated in phytochrome A signalling. *Plant Physiol.*, **124**: 39-46.

Fankhauser, C. and Staiger, D. 2002. Photoreceptors in *Arabidopsis thaliana*: light perception, signal transduction and entrainment of the endogenous clock. *Planta*, **216**: 1-16.

Fernandez, A.P., Gil P., Valkai, I., Nagy, F. and Schäfer, E. 2005. Analysis of the function of the photoreceptorors phytochrome B and phytochrome D in *Nicotiana plumbaginifolia* and *Arabidopsis thaliana*. *Plant Cell Physiol.* **46**: 790-796.

Folta, M. and Spalding, E.P. 2001. Opposing roles of phytochrome A and phytochrome B in early cryptochrome-mediated growth inhibition. *Plant J.*, **28**: 333-340.

Foo, E., Ross, J.J, Davies, N.W., Reid, J.B. and Weller, J.L. 2006a. A role for ethylene in the phytochrome-mediated control of vegetative development. *Plant J.*, **46**: 911 - 921.

Foo, E., Platten, J.D., Weller, J.L. and Reid, J.B. 2006b. PhyA and cry1 act redundantly to regulate gibberellin levels during de-etiolation in blue light. *Physiologia Plantarum*, **127**: 149 - 156.

Franklin, K.A., Praekelt, U., Stoddart, W.M., Billingham, O.E., Halliday, K.J. and Whitelam, G.C. 2003a. Phytochromes B, D, and E act redundantly to control multiple physiological responses in *Arabidopsis*. *Plant Physiol.*, **131**: 1340-1346.

Franklin, K.A., Davis, S.J, Stoddart, W.M., Vierstra, R.D. and Whitelam, G.C. 2003b. Mutant analyses define multiple roles for phytochromes C in *Arabidopsis thaliana* photomorphogenesis. *Plant Cell*, **15**: 1981-1989.

Franklin, K.A. and Whitelam, G.C. 2004. Light signals, phytochromes and cross-talk with other environmental cues. *J. Exp. Bot.*, **55**: 271-276.

Franklin, K.A. and Whitelam, G.C. 2006. *The role of phytochromes in adult plants*. In *Photomorphogenesis in Plants and Bacteria*. 3rd Ed. (Schäfer, E. and Nagy, F., eds) pp. 475-497. Dordrecht, The Netherlands: Springer.

Garg, A.K., Sawers, R.J.H., Wang, H., Kim, J-K., Walker, J.M. Brutnell, T.P., Parthasarathy, M.V., Vierstra, R.D. and Wu, R.J. 2006. Light-regulated overexpression of an *Arabidopsis* phytochrome A gene in rice alters plant architecture and increases grain yield. *Planta*, **223**: 627 - 636.

Gilbert, I.R., Jarvis, P.G. and Smith, H. 2001. Proximity signal and shade avoidance differences between early and late successional trees. *Nature*, **411**: 792 - 795.

Giliberto, L., Perrotta, G., Pallara, P., Weller, J.L., Fraser, P.D., Bramley, P.M., Fiore, A., Tavazza, M. and Giuliano, G. 2005. Manipulation of the blue light photoreceptor cryptochrome 2 in tomato affects vegetative development, flowering time, and fruit antioxidant content. *Plant Physiol.*, **137**: 199-208.

Goosey, L., Palecanda, L. and Sharrock, R.A. 1997. Differential patterns of expression of the *Arabidopsis PHYB*, *PHYD* and *PHYE* phytochrome genes. *Plant Physiol.,* **115**: 959-969.

Guo, H., Yang, H., Mockler, T. and Lin, C. 1998. Regulation of flowering time by *Arabidopsis* photoreceptors. *Science,* **279**: 1360-1363.

Gou, H. and Duong, H. 1999. The *Arabidopsis* blue light receptor cryptochrome 2 is a nuclear protein regulated by a blue light-dependent post-transcriptional mechanism. *Plant J.*, **19**: 279-287.

Gou, H. and Ecker, J.P. 2004. The ethylene signalling pathway: new insights. *Curr. Opin. Plant Biol.*, **7**: 40-49

Hall, A., Kozma-Bognar, L., Toth, R., Nagy, F. and Millar, A.W.J. 2001. Conditional circadian regulation of phytochrome A gene expression. *Plant Physiol.*, **127**: 1808-1818.

Hall, A., Kozma-Bognar, L., Bastow, R.M., Nagy, F. and Millar, A.J. 2002. Distinct regulation of CAB and PHYB gene expression by similar circadian clocks. *Plant J.*, **32**: 529-537.

Halliday, K., Thomas, B. and Whitelam, G.C. 1997. Expression of heterologous phytochrome A, B and C in transgenic tobacco plants alters vegetative development and flowering time. *Plant J.*, **12**: 1079-1090.

Halliday, K.J., Bolle, C., Chua, N-M. and Whitelam, G.C. 1999. Overexpression of rice phytochrome A partially compliments phytochrome B deficiency in *Arabidopsis*. *Planta*, **207**: 401-409.

Hare, P.D., Moller, S.G., Huang, Li-F. and Chua, N-M. 2003. LAF3, a novel factor required for normal phytochrome A signalling. *Plant Physiol.*, **133**: 1592-1604.

Hauser, B.A., Cordonnier-Pratt, M.M., Daniel-Vedele, F. and Pratt, L.H. 1995. The phytochrome gene family in tomato includes a novel subfamily. *Plant Mol. Biol.*, **29**: 1143-

1155.

Hauser, B.A., Pratt, L.H. and Cordonnier-Pratt, M.M. 1997. Absolute quantification of five phytochrome transcripts in seedlings and mature plants of tomato (*Solanum lycopersicum* L.). *Planta*, **201**: 379-387.

Hauser, B.A., Cordonnier-Pratt, M.M. and Pratt, L.H. 1998. Temporal and photoregulated expression of five tomato phytochrome genes. *Plant J.*, **14**: 431-439.

Hennig, L., Funk, M., Whitelam, G.C. and Schäfer, E. 1999a. Functional interaction of cryptochrome 1 and phytochrome D. *Plant J.,* **20**: 289-294.

Hennig, L., Poppe, C., Unger, S. and Schäfer, E. 1999b. Control of hypocotyl elongation in *Arabidopsis thaliana* by photoreceptor interaction. *Planta,* **208**: 257-263.

Hennig, L., Buche, C. and Schäfer, E. 2000. Degradation of phytochrome A and the light irradiance response in *Arabidopsis*: a kinetic analysis. *Plant Cell Environ.*, **23**: 727-734.

Hershey, H.P., Colbert, J.T., Lissemore, J.L., Barker, R.F. and Quail, P.H. 1984. Molecular cloning of cDNA for Avena phytochrome. *Proc. Natl. Acad. Sci.*, **81**: 2332-2336.

Hershey, H.P., J.L., Barker, R.F., Idler, K.B., Murray, M.G. and Quail, P.H. 1987. Nucleotide sequence and characterization of a gene encoding the phytochrome polypeptide from Avena. *Gene,* **61**: 339-348.

Heyer, A.G., Mozley, D., Landschutze, V., Thomas, B. and Gatz, C. 1995. Function of phytochrome A in potato plants as revealed through the study of transgenic plants. *Plant Physiol*., **109**: 53-61.

Hiltbrunner, A., Viczian, A., Bury, E., Tscheuschler, A., Kircher, S., Toth, R., Honsberger, A., Nagy, F., Fankhauser, C. and Schäfer, E. 2005. Nuclear accumulation of the phytochrome A photoreceptor requires FHY1. *Curr. Opin. Plant Biol.*, **15**: 2125-2130.

Hisada, A., Hanzawa, H., Weller, J.L., Nagatani, A., Reid, J.B. and Furuya, M. 2000. Light-induced nuclear translocation of endogenous pea phytochrome A visualized by immunocytochemical procedures. *Plant Cell*, **12**: 1063-1078.

Hoecker, U., Xu, Y. and Quail, P.H. 1998. SPA1: a new genetic locus involved in phytochrome A-specific signal transduction. *Plant Cell*, **10**: 19-33.

Hsieh, H.L., Okamoto, H., Wang, M.L., Ang, L.H., Matsui, M., Goodman, H. and Deng, X.W. 2000. FIN219, an auxin-regulated gene, defines a link between phytochrome A and the downstream regulator COP1 in light control of *Arabidopsis* development. *Genes Dev.*, **14**: 1958-1970.

Huala, E., Oeller, P.W., Liscum, E., In-Seob, H., Larsen, E. and Briggs, W.R. 1997. *Arabidopsis* NPH1: A protein kinase with a putative redox-sensing domain. *Science*, **278**: 2120-2123.

Hudson, M.E., Lisch, D.R. and Quail, P.H. 2003. The FHY3 and FAR1 genes encode transposase-related proteins involved in regulation of gene expression by the phytochrome A-signalling pathway. *Plant J.*, **34**: 453-471.

Huq, E., Tepperman, J.M. and Quail, P.H. 2000. GIGANTEA is a nuclear protein involved in phytochrome signalling in *Arabidopsis*. *Proc. Natl. Acad. Sci.*, **97**: 9789-9794.

Huq, E. and Quail, P.H. 2002. PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signalling in *Arabidopsis*. *EMBO J.*, **21**: 2441-2450.

Huq, E., Al-Sady, B. and Quail, P.H. 2003. Nuclear translocation of the photoreceptor phytochrome B is necessary for its biological function in seedling photomorphogenesis.

Plant J., **35**: 660-664.

Huq, E., Al-Sady, B., Hudson, M., Kim, C., Apel, K. and Quail, P.H. 2004. Phytochrome-interacting factor 1 is a critical bHLH regulator of chlorophyll biosynthesis. *Science*, **305**: 1937-1941.

Husaineid, S.S.H, Kok, R.A, Schreuder, M.E.L., Hanumappa, M., Cordonnier-Pratt, M.M., Pratt, L.H., van der Plas, L.H.W. and van der Krol, A.R. (2007). Overexpression of homologous phytochrome genes in tomato: exploring the limits in photoperception. *J. Exp. Bot.*, **58**: 615-626.

Im, Y.J., Kim, J-Il, Shen, Y., Na, Y., Han, Y-J., Kim, S-H., Song, P-S. and Eom, S.H. 2004. Structural analysis of Arabidopsis thaliana nucleoside diphosphate kinase-2 for phytochrome-mediated light signalling. *J. Mol. Biol.,* **343**: 659-670.

Jackson, S.D., Heyer A., Dietze, J. and Prat, S. 1996. Phytochrome B mediates the photoperiodic control of tuber formation in potato. *Plant J.,* **9**: 159-166.

Jarillo, J.A., Capel, J., Tang, R-H., Yang, H-Q, Alonso, J.M., Ecker, J.R. and Cashmore, A.R. 2001. An Arabidopsis circadian clock component interacts with both CRY1 and phyB. *Science*, **410**: 487-490.

Jiao, Y., Ma, L., Strickland, E. and Deng, X.W. 2005. Conservation and divergence of light-regulated genome expression patterns during seedling development in rice and *Arabidopsis*. *Plant Cell*, **17**: 3239-3256.

Johnson, E., Bradley, M., Harberd, N.P. and Whitelam, G.C. 1994. Photoresponses of light-grown *phyA* mutants of *Arabidopsis*. Phytochrome A is required for the perception of daylength extensions. *Plant Physiol.*, **105**: 141-149.

Jones, A.M., Ecker, J.R. and Chen, J-G. 2003. A reevaluation of the role pf the heterotrimeric G proteins in coupling light responses in *Arabidopsis*. *Plant Physiol.*, **131**: 1623-1627.

Jordan, E.T., Hatfield, P.M., Hondred, D., Talon, M., Zeevaart, J.A.D. and Vierstra, R.D. 1995a. Phytochrome A overexpression in transgenic tobacco. *Plant Physiol.*, **107**: 797-805.

Jordan, E.T., Cherry, J.R., Welker, J.M. and Vierstra, R.D. 1995b. The amino-terminus of phytochrome A contains two distinct functional domains. *Plant J.*, **9**: 243-257.

Jordan, E.T., Marita, J.M., Clough, R.C. and Vierstra, R.D. 1997. Characterization of regions within the N-terminal 6-kilodalton domain of phytochrome A that modulate its biological activity. *Plant Physiol.*, **115**: 693-407.

Kaczorowski, K.A. and Quail, P.H. 2003. *Arabidopsis* PSEUDO-RESPONSE REGULATOR7 is a signalling intermediate in phytochrome-regulated seedling de-etiolation and phasing of the circadian clock. *Plant Cell*, **15**: 2654-2665.

Kagawa, T., Sakai, T., Suetsugu, N., Oikawa, K., Ishiguro, S., Kato, T., Tabata, S., Okada, K. and Wada, M. 2001. *Arabidopsis* NPL1: A phototropin homolog controlling the chloroplast high-light avoidance response. *Science*, **291**: 2138-2141.

Karniol, B., Wagner, J.R., Walker, J.M. and Vierstra, R.D. 2005. Phylogenetic analysis of the phytochrome superfamily reveals distinct microbial subfamilies of photoreceptors. *Biochem. J.*, **392**: 103-116.

Kasperbauer, M.J. 1992. Phytochrome regulation of morphogenesis in green plants: From the Beltsville spectrograph to coloured mulch in the field. *Photochem. Photobiol.*, **56**: 823-832.

Kasperbauer, M.J. and Hunt, P.G. 1992. Root size and shoot/root ratio as influenced by

light environment of the shoot. *J. of Plant Nutrition*, **15**: 685-697.

Kasperbauer, M.J. and Hunt, P.G. 1998. Far-red light affects photosynthate allocation and yield of tomato over red mulch. *Crop Sci*., **38**: 970-974.

Kasperbauer, M.J. and Loughrin, J.H. 2004. Crop ecology, management & quality. *Crop Sci*., **44**: 2123-2126.

Kay, S.A., Nagatani, A., Keith, B., Deak, M., Furuya, M. and Chua, N-H. 1989. Rice phytochrome is biologically active in transgenic tobacco. *Plant Cell*, **1**: 775-782

Kay, S.A. 1997. Circadian rhythms: PAS, present, and future: clues to the origins of circadian clocks. *Science*, **276**: 753-754

Keller, J.M., Shanklin, J., Vierstra, R.D. and Hershey, H.P. 1989. Expression of a functional monocotyledonous phytochrome in transgenic tobacco. *EMBO J.*, **8**: 1005-1012.

Kendrick, R.E. and Kronenberg, G.H.M. 1994. *Photomorphogenesis in plants,* 2nd Ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kendrick, R.E., Kerckhoffs, L.H.J., Van Tuinen, A. and Koornneef, M. 1997. Photomorphogenic mutants of tomato. *Plant Cell Environ.*, **20**: 746-751.

Kerckhoffs, L.H.J., Schreuder, M.E.L., van Tuinen, A., Koornneef, M. and Kendrick, R.E. 1997. Phytochrome control of anthocyanin biosynthesis in tomato seedlings: analysis using photomorphogenic mutants. *Photochem. Photobiol.*, **65**: 374-381.

Kerckhoffs, L.H.J., Kelmenson, P.M., Schreuder, M.E.L., Kendrick, C.I., Kendrick, R.E., Hanhart, C.J., Koornneef, M., Pratt, L.H. and Cordonnier-Pratt, M.-M. 1999. Characterization of the gene encoding the apoprotein of phytochrome B2 in tomato and identification of molecular lesions in two mutant alleles. *Mol. Gen. Genet.*, **261**: 901-907.

Khanna, R., Kikis, E.A. and Quail, P.H. 2003. EARLY FLOWERING 4 functions in phytochrome B-regulated seedling de-etiolation. *Plant Physiol.*, **133**: 1530-1538.

Khanna, R., Huq, E., Kikis, E.A., Al-Sady, B., Lanzatella, C. and Quail, P.H. 2004. A novel molecular recognition motif necessary for targeting photoactivated phytochrome signalling to specific basic helix-loop-helix transcription factors. *Plant Cell*, **16**: 3033-3044.

Kim, B.C., Tennessen, D.J. and Last, R.L. 1998. UV-B-induced photomorphogenesis in *Arabidopsis* thaliana. *Plant J.*, **15**: 667-674.

Kim, L., Kircher, S., Toth, R., Adam, E., Schäfer, E. and Nagy, F. 2000. Light-induced nuclear import of phytochrome-A:GFP fusion proteins is differentially regulated in transgenic tobacco and *Arabidopsis*. *Plant J.*, **22**: 125-133.

Kim, D.-H., Kang, J.-G., Yang, S.-S., Chung, K.-S., Song, P-S. and Park, C.-M. 2002. A phytochrome-associated protein phosphatase 2A modulates light signals in flowering time control in *Arabidopsis*. *Plant Cell*, **14**: 3043-3056.

Kim, J., Yi, H., Choi, G., Shin, B., Song, P-S. and Choi, G. 2003. Functional characterization of phytochrome interacting factor 3 in phytochrome-mediated light signal transduction. *Plant Cell*, **15**: 2399-2407.

Kim, J-Il., Park, J-E., Zarate, X. and Song, P-S. 2005. Phytochrome phosphorylation and plant light signalling. *Photochem. Photobiol.*, **4**: 681-687.

Kim, Y.M., Woo, J.C., Song, P.S. and Soh, M.S. 2002. HER1, a phytochrome A-signalling component, acts in a separate pathway from HY5, downstream of COP1 in *Arabidopsis thaliana*. *Plant J.*, **30**: 711-719.

Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M. and Shimazaki, K. 2001.

phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature*, **414**: 656-660.

Kircher, S., Kozma-Bognar, L., Kim, L., Adam, E., Harter, K., Schäfer, E. and Nagy, F. 1999. Light quality-dependent nuclear import of the plant photoreceptors phytochrome A and B. *Plant Cell*, **11**: 1445-1456.

Kircher, S., Gil, P., Kozma-Bognar, L., Fejes, E., Speth, V., Husselstein-Muller, T., Bauer, D., Adam, E., Schäfer, E. and Nagy, F. 2002. Nucleocytoplasmic partitioning of the plant photoreceptors phytochrome A, B, C, D, and E Is regulated differentially by light and exhibits a diurnal rhythm. *Plant Cell*, **14**: 1541-1555.

Kleine, T., Lockhart, P. and Batschauer, A. 2003. An *Arabidopsis* protein closely related to synchronize cryptochrome is targeted to organelles. *Plant J.*, **35**: 93-103.

Kleiner, O., Kircher, S., Harter, K. and Batschauer, A. 1999. Nuclear localization of the *Arabidopsis* blue light receptor cryptochrome 2. *Plant J.*, **19**: 289-296.

Koornneef, M., Rolff, E. and Spruit, C.J.P. 1980. Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana* L. Heynh. Z. *Pflanzenphysiol.*, **100**: 147-160.

Koornneef, M., Cone, J.W., Dekens, R.G., O'Herne-Robert, E.G., Spruit, C.J.P. and Kendrick, R.E. 1985. Photomorphogenesis response of long hypocotyl mutants of tomato. *Plant Physiol.*, **120**: 153-165.

Kong, S-G., Lee, D-S., Kwak, S-N., Kim, J-K., Sohn, J-K. and Kim, I-S. 2004. Characterization of sunlight-grown transgenic rice plants expressing *Arabidopsis* phytochrome A. *Mol. Breeding.*, **14**: 35-45.

Kunkel, T., Neuhaus, G., Batschauer, A., Chua, N.-H. and Schäfer, E. 1996. Functional analysis of yeast-derived phytochrome A and B phycocyanobilin adducts. *Plant J.,***10**: 625-636.

Kuno, N. and Furuya, M. 2000. Phytochrome regulation of nuclear gene expression in plants. *Cell Develop. Biol.*, **11**: 485-493.

Langton, F.A. 1998. Regulation of stem extension by temperature. In: *Genetic and environmental manipulation of horticultural crops*. (Cockshull, K.E., Gray, D., Seymour, G.B. and Thomas, B., eds) pp. 191-203. Oxford: CABI Publishing.

Lariguet, P., Boccalandro, H.E., Alonso, J.M., Ecker, J.R., Chory, J., Casal, J. and Fankhauser, C. 2003. A growth regulatory loop that provides homeostasis to phytochrome A signalling. *Plant Cell*, **15**: 2966-2978.

Lapko, V.N., Jiang, X-Y., Smith, D.L. and Song, P-S. 1997. Post-translational modification of oat phytochrome A: Phosphorylation of a specific serine in a multiple serine cluster. *Biochemistry*, **36**: 10595–10599

Lariguet, P. and Fankhauser, C. 2004. Hypocotyl growth orientation in blue light is determined by phytochrome A inhibition of gravitropism and phototropin promotion of phototropism. *Plant J.*, **40**: 826-834.

Lazarova, G.I., Kerckhoffs, L.H.J., Brandstadter, J., Matsui, M., Kendrick, R.E., Cordonnier-Pratt, M.-M. and Pratt, L.H. 1998a. Molecular analysis of PHYA in wild type and phytochrome A deficient mutants of tomato. *Plant J.*, **14**: 653-662.

Lazarova, G.I., Kubota, T., Frances, S., Peters, J.L., Hughes, M.J.G., Brandstadter, J., Szell, M., Matsui, M., Kendrick, R.E., Cordonnier-Pratt, M.-M. and Pratt, L.H. 1998b. Characterization of tomato PHYB1 and identification of molecular defects in four mutant alleles. *Plant Mol. Biol.*, **38**: 1137-1146.

Levin, I., Frankel, P., Gilboa, N., Tanny, S. and Lalazar, A. 2003. The tomato dark green

mutation is a novel allele of the tomato homolog of the DEETIOLATED1 gene. *Theor. Appl. Genet.*, **106**: 454-460.

Libenson, S., Rodriguez, V., Pereira, M.L., Sánchez, R.A. and Casal, J.J. 2002. Low red to far-red ratios reaching the stem reduce grain yield in sunflower. *Crop Sci.*, **42**: 1180-1185.

Lieberman, H., Segev, O., Gilboa, N., Lalazar, A. and Levin, I. 2004. The tomato homolog of the gene encoding UV-damaged DNA binding protein 1 (DDB1) underlined as the gene that causes the high pigment-1 mutant phenotype. *Theor. Appl. Genet.*, **108**: 1574-1581.

Limpens, J., Schroder, F.H., de Ridder, C.M.A., Bolder, C.A., Wildhagen, M.F., Obermuller-Jevic, U.C., Kramer, K. and van Weerden, W.M. 2006. Combined lycopene and vitamin E treatment suppresses the growth of PC-346C human prostate cancer cells in nude mice. *J. Nutr.*, **136**: 1287-1293.

Lin, C. Yang, H. Guo, H., Mockler, T., Chen, J. and Cashmore, R. 1998. Enhancement of blue-light sensitivity of Arabidopsis seedlings by a blue light receptor cryptochrome 2. *Proc. Natl. Acad. Sci.*, **95**: 2686-2690.

Lin, C. and Shalitin, D. 2003. Cryptochrome structure and signal transduction. *Annu. Rev. Plant Biol.*, **54**: 469-496.

Lissemore, J.L., Colbert, J.T. and Quail, P.H. 1987. Cloning of cDNA for phytochrome from etiolated Cucurbita and coordinate photoregulation of the abundance of two distinct phytochrome transcripts. *Plant Mol. Biol.*, **8**: 485-496.

Liu, X.L., Covington, M.F., Fankhauser, C., Chory, J. and Wagner, D.R. 2001. ELF3 encodes a circadian clock-regulated nuclear protein that functions in an *Arabidopsis* PHYB signal transduction pathway. *Plant Cell*, **13**: 1293-1304.

Liu, Y., Roof, S., Ye, Z., Barry, C., van Tuinen, A., Vrebalov, J., Bowler, C. and Giovannoni, J. 2004. Manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato. *Proc. Natl. Acad. Sci.*, **101**: 9897-9902.

López-Juez, E., Kobayashi, M., Sakurai, A., Kamiya, Y. and Kendrick, R.E. 1995. Phytochrome, gibberellins, and hypocotyl growth. A study using the cucumber (*Cucumis sativus* L.) *long hypocotyl* mutant. *Plant Physiol.*, **107**: 131-140.

Ma, L., Li, J., Qu, L., Hager, J., Chen, Z., Zhao, H. and Deng, X.W. 2001. Light control of *Arabidopsis* development entails coordinated regulation of genome expression and cellular pathways. *Plant Cell*, **13**: 2589-2607.

Ma, L., Chen, Z., Liu, X., Jiao, Y., Su, N., Li, L., Wang, X., Cao, M., Sun, N. and Zhang, X. 2005. A microarray analysis of the rice transcriptome and its comparison to *Arabidopsis*. *Genome Res.,* **15**: 1274-1283.

Maloof, J.N., Borevitz, J.O., Dabi, T., Lutes, J., Nehring, R.B., Redfern, J.L., Trainer, G.T., Wilson, J.M., Asami, T., Berry, C.C., Weigel, D. and Chory, J. 2001. Natural variation in light sensitivity of *Arabidopsis*. *Nature Genetics,* **29**: 441-446.

Mancinelli, A.L. 1990. Interaction between light quality and light quantity in the photoregulation of anthocyanin production. *Plant Physiol.*, **92**: 1191-1195.

Mancinelli, A.L., Rossi, F. and Moroni, A. 1991. Cryptochrome, phytochrome and anthocyanin production. *Plant Physiol.*, **96**: 1079-1085.

Mancinelli, A.L. 1994. The physiology of phytochrome action. In *Photomorphogenesis in Plants.* Kendrick RE, Kronenberg GHM (eds), Kluwer Academic Publishers, Dordrecht, pp

211-269.

Martínez-García, J.F., Monte, E. and Quail, P.H. 1999. A simple, rapid and quantitative method for preparing *Arabidopsis* protein extracts for immunoblot analysis. *Plant J.*, **20**: 251-257.

Martínez-García, J.F., Huq, E. and Quail, P.H. 2000. Direct targeting of light signals to a promoter element-bound transcription factor. *Science*, **288**: 859-863.

Más, P., Devlin, P.F., Panda, S. and Kay, S.A. 2000. Functional interaction of phytochrome B and cryptochrome 2. *Nature*, **408**: 207-211.

Más, P., Alabadi, D., Yanovsky, M.J., Oyama, T. and Kay, S.A. 2003a. Dual role of TOC1 in the control of circadian and photomorphogenic responses in *Arabidopsis*. *Plant Cell*, **15**: 223-236.

Más, P., Woe-Yeon, K., Somers, D.E. and Kay, S.A. 2003b. Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. *Nature*, **426**: 567-570.

Mateos, J.L., Luppi, J.P., Ogorodnikova, O.B., Sineshchekov, V.A., Yanovsky, M.J. Braslavsky, S.E., Wolfgang, G. and Casal, J.J. 2006. Functional and biochemical analysis of the N-terminal domain of phytochrome A. *J. Biol. Chem.*, **281**: 34421-34429.

Mathews, S. and Sharrock, R.A. 1997. Phytochrome gene diversity. *Plant Cell Environ.*, **20**: 666-671.

Matsushita, T., Mochizuki, N. and Nagatani, A. 2003. Dimers of the N-terminal domain of phytochrome B are functional in the nucleus. *Nature*, **424**: 571-574.

Mazzella, M.A., Magliano, T.M.A. and Casal, J.J. 1997. Dual effect of phytochrome A on hypocotyl growth under continuous red light. *Plant Cell Environ.*, **20**: 261-267.

Mazzella, M.A. and Casal, J.J. 2001. Interactive signalling by phytochrome and cryptochrome generates de-etiolation homeostasis in *Arabidopsis thaliana*. *Plant Cell Environ.*, **24**: 155-162.

Mazzella, M.A., Arana, M.V., Staneloni, R.J., Perelman, S., Rodriguez, M.J., Muschietti, J., Cerdan, P.D., Chen, K., Sánchez, R.A., Zhu, T., Chory, J. and Casal, J.J. 2005. Phytochrome control of the *Arabidopsis* transcription anticipates seedling exposure to light. *Plant Cell*, **17**: 2507-2516.

McCormick, S. 1991. Transformation of tomato with *Agrobacterium tumefaciens*. In *Plant Tissue Culture Manual*, K. Lindsey, ed (Dordrecht, the Netherlands: Kluwer Academic Publishers), pp. 1-9.

McCormac, A.C., Smith, H. and Whitelam, G.C. 1991a. Photoregulation of germination in seed of transgenic lines of tobacco and Arabidopsis which express a introduced cDNA encoding phytochrome A or phytochrome B. *Planta*, **191**: 386-393.

McCormac, A.C., Cherry J.R, Hershey H.P. Vierstra R.D. and Smith, H. 1991b. Photoresponses of transgenic tobacco plants expressing an oat phytochrome gene. *Planta*, **185**: 162-170.

McCormac, A.C., Whitelam, G.C., Boylan, M.T., Quail, P.H. and Smith, H. 1992a. Contrasting responses of etiolated and light-adapted seedlings to red: far-red ratio: A comparison of wild type, mutant and transgenic plants has revealed differential functions of members of the phytochrome family. *Plant Physiol.*, **140**: 707-714.

McCormac, A.C., Smith, H. and Whitelam, G.C. 1992b. Light-grown plants of transgenic tobacco expressing an introduced oat phytochrome A gene under the control of a constitutive viral promoter exhibit persistent growth inhibition by far-red light. *Planta*, **188**,

173-181.

McCormac, A.C., Wagner, D., Boylan, M.T., Quail, P.H., Smith, H. and Whitelam, G.C. 1993a. Photoresponses of transgenic *Arabidopsis* seedling expressing introduced phytochrome B-encoding cDNA: evidence that phytochrome A and phytochrome B have distinct photoregulatory functions. *Plant J.*, **4**: 19-27.

McCormac, A.C., Smith, H. and Whitelam, G.C. 1993b. Photoregulation of germination in seed of transgenic lines of tobacco and Arabidopsis which express an introduced cDNA encoding phytochrome A or phytochrome B. *Planta*, **191**: 386-393.

McCormac, A.C., Smith, H. and Whitelam, G.C. 1993c. Expression of oat-phyA-cDNA in a suspension cell culture of transgenic tobacco: A signal-cell system for the study of phytochrome function. *J. Exp. Bot.,* **44**:1095-1103.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N-H. and Kay, S.A.1995. Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. *Science*, **267**: 1161-1163.

Millar, A.J. 2003. A suite of photoreceptors entrains the plant circadian clock. *J Biol Rhythms*, **18**: 217-226.

Mockler, T.C., Guo, H., Yang, H., Duong, H. and Lin, C. 1999. Antagonistic actions of *Arabidopsis* cryptochromes and phytochrome B in the regulation of floral induction. *Development*, **126**: 2073-2082.

Møller, S.G., Ingles, P.J. and Whitelam, G.C. 2002. The cell biology of phytochrome signalling. *New Phytol.*, **154**: 553-590.

Mølmann, J.A, Asante, D.K.A., Jensen, J.B., Krane, M.N., Ernstsen, A., Junttila, O. and Olsen, J.E. 2005. Low night temperature and inhibition of gibberellin biosynthesis override phytochrome action and induce bud set and cold acclimation, but not dormancy in *PHYA* overexpression and wild-type of hybrid aspen. *Plant Cell Environ.*, **28**: 1579-1588.

Mommer, L., Pons, T.L. and Visser, E.J.W. 2005. Photosynthetic consequences of phenotypic plasticity in response to submergence: *Rumex palustris* as a case study. *J. Exp. Bot.,* **57**: 283-290.

Monte, E., Alonso, J.M., Ecker, J.R., Zhang, Y., Li, X., Young, J., Austin-Phillips, S. and Quail, P.H. 2003. Isolation and characterization of *phyC* mutants in *Arabidopsis* reveals complex crosstalk between phytochrome signalling pathways. *Plant Cell*, **15**: 1962-1980.

Monte, E., Tepperman, J.M., Al-Sady, B., Kaczorowski, K.A., Alonso, J.M., Ecker, J.R., Li, X., Zhang, Y. and Quail, P.H. 2004. The phytochrome-interacting transcription factor, PIF3, acts early, selectively, and positively in light-induced chloroplast development. *Proc. Natl. Acad. Sci.,* **101**: 16091-16098.

Montgomery, B.L. and Lagarias, J.C. 2002. Phytochrome ancestry: sensors of bilins and light. *Trends in Plant Science*, **7**: 357-366.

Mustilli, A.C., Fenzi, F., Ciliento, R., Alfano, F. and Bowler, C. 1999. Phenotype of the tomato *high pigment-2* mutant is caused by a mutation in the tomato homolog of *DEETIOLATED1*. *Plant Cell*, **11**: 145-157.

Nagatani, A., Kay, S.A., Deak, M., Chua, N-H. and Furuya, M. 1991. Rice type I phytochrome regulate hypocotyl elongation in transgenic tobacco seedlings. *Proc. Natl. Acad. Sci..*, **88**: 5207-5211.

Nagatani, A., Reed, J.W. and Chory, J. 1993. Isolation and initial characterization of *Arabidopsis* mutants that are deficient in phytochrome A. *Plant Physiol.*, **102**: 269-277.

Nagatani, A. 2004. Light -regulated nuclear localization of phytochrome *Curr. Opin. Plant Biol.*, **7**: 708-711.

Nagy, F. and Schäfer, E. 2002. Phytochromes control photomorphogenesis by differentially regulated, interacting signalling pathways in higher plants. *Annu. Rev. Plant Biol.*, **53**: 329-355.

Neff, M.M. and Chory, J. 1998. Genetic interactions between phytochrome A, phytochrome B and cryptochrome 1 during *Arabidopsis* development. *Plant Physiol.*, **118**: 27-36.

Neff, M.M., Fankhauser, C. and Chory, J. 2000. Light: an indicator of time and place. *Genes Dev.*, **14**: 257-271.

Neuhaus, G., Bowler, C., Kern, R. and Chua, N.-H. 1993. Calcium/Calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell*, **73**: 937-952.

Ni, M., Tepperman, J.M. and Quail, P.H. 1998. PIF3, a phytochrome-interacting factor necessary for normal photoinduced signal transduction, is a novel basic helix-loop-helix protein. *Cell*, **95**: 657-667.

Ninu, L., Ahmad, M., Miarelli, C., Cashmore, A.R. and Giuliano, G. 1999. Cryptochrome 1 controls tomato development in response to blue light. *Plant J.*, **18**: 551-556.

Oh, E., Kim, J., Park, E., Kim, J-Il, Kang, C. and Choi, G. 2004. PIL5, a phytochrome-interacting basic helix-loop-helix protein, is a key regulator of seed germination in *Arabidopsis thaliana*. *Plant Cell*, **16**: 3045-3058.

Oh, E., Yamaguchi, S., Kamiya, Y., Bae, G. Chung, W-I. and Choi, G. 2006. Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in *Arabidopsis thaliana*. *Plant J.,* **47**: 124-139.

Oka, Y., Matsushita, T., Mochizuki, N., Suzuki, T., Tokutomi, S. and Nagatani, A. 2004. Functional analysis of a 450-Amino acid N-terminal fragment of phytochrome Bin *Arabidopsis*. *Plant Cell*, **16**: 2104-2116.

Okamoto, H., Matsui, M. and Deng, X.W. 2001. Overexpression of the heterotrimeric G-protein a-subunit enhances phytochrome-mediated inhibition of hypocotyl elongation in *Arabidopsis*. *Plant Cell*, **13**: 1639-1652.

Olsen, J.E., Junttila, O., Nilsen, J., Eriksson, M.E., Martinussen, I., Olsson, O., Sandberg, G. and Moritz, T. 1997. Ectopic expression of oat phytochrome A in hybrid aspen changes critical daylength for growth and prevents cold acclimatization. *Plant J.*, **12**: 1339-1350.

Onodera, A., Kong, S-G, Doi, M., Shimazaki, K-I., Christie, J., Mochizuki, N. and Nagatani, A. 2005. Phototropin from *Chlamydomonas reinhrdtii* is functional in *Arabidopsis thaliana*. *Plant Cell Physiol.*, **46**: 367-374

Osterlund, M.T., Hardtke, C.S., Wei, N. and Deng, X.W. 2000. Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*. *Nature*, **405**: 462-466.

Park, E., Kim, J., Lee, Y., Shin, J., Oh, E., Chung, W.-I., Liu, J.R. and Choi, G. 2004. Degradation of phytochrome interacting factor 3 in phytochrome-mediated light signalling. *Plant Physiol.*, **45**: 968-975.

Parks, B.M. and Quail, P.H. 1993. *hy8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. *Plant Cell*, **5**: 39-48.

Penfield, S., Josse, E.M., Kannangara, R., Gilday, A.D., Halliday, K.J. and Graham, I.A. 2005. Cold and light control seed germination through the bHLH transcription factor

SPATULA. *Curr. Opin. Plant Biol.*, **15**: 1998-2006.

Perrotta, G., Ninu, L., Flamma, F., Weller, J.L., Kendrick, R.E., Nebuloso, E. and Giuliano, G. 2000. Tomato contains homologues of *Arabidopsis* cryptochromes 1 and 2. *Plant Mol. Biol.*, **42**: 765-773.

Perrotta, G., Yahoubyan, G., Nebuloso, E., Renzi, L. and Giuliano, G. 2001. Tomato and barley contain duplicated copies of cryptochrome 1. *Plant Cell Environ.*, **24**: 991-998.

Peters, J.L., Van Tuinen, A., Adamse, P., Kendrick, R.E. and Koornneef, M. 1989. High pigment mutant of tomato exhibit high sensitivity for phytochrome action. *Plant Physiol.*, **134**: 661-666.

Peters, J.L., Schreuder, M.E.L., Verduin, S.J.W. and Kendrick, R.E. 1992. Physiological characterization of a high-pigment mutant of tomato. *Photochem. Photobiol.*, **56**: 75-82.

Pierik, R., Visser, E.J.W., Dekroon, H. and Voesenek, L.A.C.J. 2003. Ethylene is required in tobacco to successfully compete with proximate neighbours. *Plant Cell Environ.*, **26**: 1229-1234.

Pierik, R., Cuppens, M.L.C., Voesenek, L.A.C.J. and Visser, E.J.W. 2004. Interactions between ethylene and gibberellins in phytochrome-mediated shade avoidance responses in tobacco. *Plant Physiol.*, **136**: 2928-2936.

Pierik, R., Millenaar, F.F., Peeters, A.J.M. and Voesenek, L.A.C.J. 2005. New perspectives in flooding research: the use of shade avoidance and *Arabidopsis thaliana*. *Annu. Rev. of Botany*. **96**: 533-540.

Poppe, C., Sweere, U., Drumm-Herrel, H. and Schäfer, E. 1998. The blue light receptor cryptochrome 1 can act independently of phytochrome A and B in *Arabidopsis* thaliana. *Plant J.*, **16**: 465-471.

Pratt, T.H. 1995. Phytochromes: differential properties, expression patterns and molecular evolution. *Photochem. Photobiol.*, **61**: 10-21.

Qin, M., Kuhn, R., Moran, S. and Quail, P.H. 1997. Overexpressed phytochrome C has similar photosensory specificity to phytochrome B but a distinctive capacity to enhance primary leaf expansion. *Plant J.*, **12**: 1163-1172.

Quail, P.H. 1994. Phytochrome genes and their expression. In *Photomorphogenesis in plants*, 2nd Ed. (RE Kendrick, GHM Kronenberg, eds), pp 71-104. Kluwer Academic Publishers, Dordrecht.

Quail, P.H. 1997. An emerging molecular map of the phytochromes. *Plant Cell Environ.*, **20**: 657-665.

Quail, P.H. 2002a. Photosensory perception and signalling in plant cells: new paradigms? *Curr. Opin. Plant Biol.*, **14**: 180-188.

Quail, P.H. 2002b. Phytochrome photosensory perception and signal transduction. *Rev. Mol. Cell Biol.*, **3**: 85-93.

Quail, P.H. 2006. Phytochrome signal transduction network. In *Photomorphogenesis in Plants and Bacteria.* 3rd Ed. (Schäfer, E. and Nagy, F., eds) pp. 335-356. Dordrecht, The Netherlands: Springer.

Quail, P.H. 2007. Phytochrome-regulated gene expression. *J. Integr. Plant Biol.*, **49**: 11-21.

Reed, J.W., Nagatani, A., Elich, T.D., Fagan, M. and Chory, J. 1994. Phytochrome A and phytochrome B have overlapping but distinct functions in *Arabidopsis* development. *Plant Physiol.*, **104**: 1139-1149.

Robson, P.R.H., McCormac, A.C., Irvine, A.S. and Smith, H. 1996. Genetic engineering of harvest index in tobacco through overexpression of a phytochrome gene. *Nature Biotechnol.*, **14**: 995-998.

Robson, P.R.H. and Smith, H. 1997. Fundamental and biotechnological application of phytochrome transgenes. *Plant Cell Environ.*, **20**: 831-839.

Roig-Villanova, I., Bou, J., Sorin, C., Devlin, P.F. and Martinez-Garcia, J.F. 2006. Identification of primary target genes of phytochrome signalling: early transcriptional control during shade avoidance responses in *Arabidopsis*. *Plant Physiol.*, **141**: 85-96.

Rousseaux, M.C., Ballaré, C.L., Jordan, E.T. and Vierstra, R.D.1997. Directed overexpression of *PHYA* locally suppresses stem elongation and leaf senescence responses to far-red radiation. *Plant Cell Environ.*, **20**: 1551-1558.

Rubio, V. and Deng, X.W. 2005. Phy Tunes: phosphorylation status and phytochrome-mediated signalling. *Cell*, **120**: 290-292.

Ryu, J.S., Kim, J.-I., Kunkel, T., Kim, B.C., Cho, D.S., Hong, S.H., Kim, S.-H., Fernandez, A.P., Kim, Y. and Alonso, J.M. 2005. Phytochrome-specific type 5 phosphatase controls light signal flux by enhancing phytochrome stability and affinity for a signal transducer. *Cell*, **120**: 395-406.

Saibo, N.J.M., Vriezen, W.H., Beemster, G.T.S. and Van der Straeten, D. 2003. Growth and stomata development of *Arabidopsis* hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. *Plant J.*, **33**: 989-1000.

Saitou, T., Tokutomi, S., Harada, H. and Kamada H. 1999. Overexpression of phytochrome A enhances the light-induced formation of adventitious shoot on horseradish hairy roots. *Plant Cell*, **18**: 754-758.

Sakai, T., Kagawa, T., Kasahara, M., Swartz, T.E., Christie, J.M., Briggs, W.R., Wada, M. and Okada, K. 2001. *Arabidopsis* nph1 and npl1: Blue light receptors that mediate both phototropism and chloroplast relocation. *Proc. Natl. Acad. Sci.*, **98**: 6969-6974.

Sakamoto, K. and Nagatani, A. 1996. Nuclear localization activity of phytochrome B. *Plant J.*, **10**: 859-868.

Salome, P.A. and Mcclung, C.R. 2005. What makes the *Arabidopsis* clock tick on time? A review on entrainment. *Plant Cell Environ.*, **28**: 21-38.

Salter, M.G., Franklin, K.A. and Whitelam, G.C. 2003. Gating of the rapid shade-avoidance response by the circadian clock in plants. *Nature*, **426**: 680 - 683.

Sancar, A. 2003. Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem. Rev.*, **103**: 2203-2237.

Schäfer, E., Emmler, K. and Chua, N-M. 1994. Rice phytochrome A controls apical hook opening after a single light pulse in transgenic tobacco seedlings. *Plant Physiol.*, **6**: 935-940.

Schäfer, E. and Nagy, F. 2006. *Photomorphogenesis in Plants and Bacteria*. 3rd Ed. Dordrecht, The Netherlands: Springer.

Schardl, C.L., Byrd, A.D., Benzion, G., Altschuler, M., Hildebrand, D.F. and Hunt, A. G. 1987. Design and construction of a versatile system for the expression of foreign genes in plants. *Gene*, **61**: 1-11.

Schittenhelm, S., Menge-Hartmann, U. and Oldenburg, E. 2004. Photosynthesis, carbohydrate metabolism, and yield of phytochrome B-overexpression potatoes under different light regimes. *Crop Sci.*, **44**: 131-143.

Schmitt, J., McCormac, A.C. and Smith, H. 1995. A test of the adaptive plasticity hypothesis using transgenic and mutant plants disabled in phytochrome-mediated elongation responses to neighbours. *Am. Nat.*, **146**: 937-953.

Schmitt, J., Stinchcombe, J.R., Heschel, M.S. and Huber, H. 2003. The adaptive evolution of plasticity: phytochrome-mediated shade avoidance responses. *Integr. Comp. Biol.*, **43**: 459-469.

Schneider-Poetsch, H.A.W., Kolukisaoglu, Ü., Clapham, D.H., Hughes, J. and DLamparter, T. 1998. Non-angiosperm phytochromes and the evolution of vascular plants. *Plant Physiol.*, **102**: 612-622.

Seeley, K.A., Byrne, D.H. and Meagher, R.B. 1982. Red light-independent instability of oat phytochrome mRNA *in vivo*. *Plant Cell*, **4**: 29-38.

Seo, H.S., Watanabe, E., Tokutomi, S., Nagatani, A. and Chua, N-H. 2004. Photoreceptor ubiquitination by COP1 E3 ligase desensitizes phytochrome A signalling. *Genes Dev.,* **18**: 617-622.

Seo, M., Hanada, A., Kuwashara, A., Endo, A., Okamoto, M., Yamaushi, Y., North, H., Marion-Poll, A., Sun, T-P., Koshiba, T., Yamaguchi, S. and Nambara, E. 2006. Regulation of hormone metabolism in *Arabidopsis* seeds: phytochrome regulation of abscisic acid metabolism and abscisic acid regulation of gibberellin metabolism. *Plant J.*, **48**: 354-366.

Sessa, G., Carabelli, M., Sassi, M., Ciolfi, A., Possenti, M., Mittempergher, F., Becker, J., Morelli, G. and Rubert, I. 2005. A dynamic balance between gene activation and repression regulates the shade avoidance response in *Arabidopsis. Genes Dev.*, **19**: 2811-2815.

Sharkey, T.H., Vassey, T.L., Vanderveer, P.J. and Vierstra, R.D. 1991. Patterns of expression and normalized levels of the five *Arabidopsis* phytochromes. *Plant Physiol.*, **130**: 442-456.

Sharrock, R.A., Clack, T. and Goosey, L. 2001. *The differential activities of phytochromes B and D are determined by a central domain*. Abstract of 19th annual symposium Current Topics in Plant Biochemistry, Physiology and Molecular Biology in Missouri, USA.

Sharrock, R.A. and Clack, T. 2002. Patterns of expression and normalized levels of the five *Arabidopsis* phytochromes. *Plant Physiol.*, **130**: 442-456.

Sharrock, R.A., Clack, T. and Goosey, L. 2003. Signalling activities among the *Arabidopsis* phyB/D/E-type phytochromes: a major role for the central region of the apoprotein. *Plant J.*, **34**: 317-326.

Sharrock, R.A. and Clack, T. 2004. Heterodimerization of type II phytochromes in *Arabidopsis*. *Proc. Natl. Acad. Sci.*, **101**: 11500-11505.

Sharrock, R.A. and Mathews, S. 2006. Phytochrome genes in higher plants: structure, expression and evolution. In *Photomorphogenesis in Plants and Bacteria*. 3rd Ed. (Schäfer, E. and Nagy, F., eds) pp. 99-129. Dordrecht, The Netherlands: Springer.

Shen, H., Moon, J. and Huq, E. 2005. PIF1 is regulated by light-mediated degradation through the ubiquitin-26S proteasome pathway to optimize photomorphogenesis of seedling in *Arabidopsis*. *Plant J.*, **44**: 1023-1035.

Shen, Y., Kim, J-II. and Song, P-S. 2005. NDPK2 as a transducer in the phytochrome-mediated light signalling. *J. Biol. Chem.*, **280**: 5740-5740.

Shlumukov, L., Barro, F., Barcelo, P., Lazzari, P. and Smith, H. 2001. Establishments of

far-red high irradiance responses in wheat transgenic expression of an oat phytochrome A gene. *Plant Cell Environ.*, **24**: 703-712.

Sineshchekov, V., Koppel, L., Shlumukov, L., Barro, F., Barcelo, P., Lazzari, P. and Smith, H. 2001. Fluorescence and photochemical properties of phytochrome in wild-type wheat and a transgenic line overexpressing an oat phytochrome A (PHYA) gene: functional implications. *Plant Cell Environ.*, **24**: 1289-1297.

Smith, H. and Holmes, M.G. 1977. The function of phytochrome in the natural environment. III. Measurement and calculation of phytochrome photoequilibrium. *Photochem. Photobiol.*, **25**: 547-550.

Smith, H. 1982. Light quality, photoperception, and plant strategy. *Annu. Rev. Plant Physiol.*, **33**: 481-518.

Smith, H. 1994. Sensing the light environment: the function of the phytochrome family. In *Photomorphogenesis in plants*, 2nd Ed. (RE Kendrick, GHM Kronenberg, eds), pp 377-416. Kluwer Academic Publishers, Dordrecht.

Smith, H. and Whitelam, G.C. 1997. The shade avoidance syndrome: multiple responses mediated by multiple phytochromes. *Plant Cell Environ.*, **20**: 840-844.

Smith, H. 1998. Engineering phytochrome genes to improve crop performance. In: *Genetic and environmental manipulation of horticultural crops*. (Cockshull, K.E., Gray, D., Seymour, G.B. and Thomas, B., eds) pp. 175-190. Oxford: CABI Publishing.

Smith, H. 2000. Phytochromes and light signal perception by plants - an emerging synthesis. *Nature*, **407**: 585-591.

Soh, M.-S., Kim, Y.-M., Han, S.-J. and Song, P-S. 2000. REP1, a basic helix-loop-helix protein, is required for a branch pathway of phytochrome A signalling in *Arabidopsis*. *Plant Cell*, **12**: 2061-2074.

Somers, D.E. and Quail, P.H. 1995. Temporal and spatial expression patterns of PHYA and PHYB genes in *Arabidopsis*. *Plant J.*, **7**: 413-427.

Somers, D.E. Devlin, P.F and Kay, S.A. 1998. Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science*, **282**: 1488-1490.

Srinivas, A., Behera, R.K., Kagawa, T., Wada, M. and Sharma, R. 2004. High pigment1 mutation negatively regulates phototropic signal transduction in tomato seedlings. *Plant Physiol.*, **134**: 790-800.

Staiger, D., Allenbach, L., Salathia, N., Fiechter, V., Davis, S.J., Millar, A.J., Chory, J. and Fankhauser, C. 2003. The *Arabidopsis* SRR1 gene mediates phyB signalling and is required for normal circadian clock function. *Genes Dev.*, **17**: 256-268.

Sullivan, J.A., Shirasu, K. and Deng, X.W. 2003. The diverse roles of the ubiquitin and 26S proteasome in the life of plants. *Nature Rev. Gen.,* **4**: 948-958.

Tepperman, J.M., Zhu, T.O., Chang, S.H., Wang, X. and Quail, P.H. 2001. Multiple transcription-factor genes are early targets of phytochrome A signalling. *Proc. Natl. Acad. Sci.*, **98**: 9437-9442.

Tepperman, J.M., Hudson, M.E., Khanna, R., Zhu, T.O., Chang, S.H., Wang, X. and Quail, P.H. 2004. Expression profiling of *phyB* mutant demonstrates substantial contribution of other phytochromes to red-light-regulated gene expression during seedling de-etiolation. *Plant J.*, **38**: 725-739.

Tepperman, J.M., Hwang, Y-S. and Quail, P.H. 2006. phyA dominates in transduction of red-light to rapidly responding genes at the initiation of *Arabidopsis* seedling de-etiolation.

Plant J., **48**: 728-742.

Thiele, A., Herold, M., Lenk, I., Quail, P.H. and Gatz, C. 1999. Heterologous expression of *Arabidopsis* phytochrome B in transgenic potato influences photosynthetic performance and tuber development. *Plant Physiol.*, **120**: 73-81.

Thummler, F., Schuster, H. and Bonenberger, J. 1992. Expression of oat phyA cDNA in the moss *Ceratodon purpureus*. *Photochem. Photobiol.*, **56**: 771-776.

Toth, R., Kevei, E., Hall, A., Millar, A.J., Nagy, F. and Kozma-Bognar, L. 2001. Circadian Clock-Regulated Expression of Phytochrome and Cryptochrome Genes in *Arabidopsis*. *Plant Physiol.*, **127**: 1607-1616.

Ulm, R., Baumann, A., Oravecz, A., Mate, Z., Adam, E. and Oakeley, E. 2004. Genome-wide analysis of gene expression reveals function of the bZIP transcription factor HY5 in the UV-B response of *Arabidopsis*. *Proc. Natl. Acad. Sci.*, **101**: 1397-1402.

Ulm, R. 2006. UV-B perception and signalling in higher plants. In *Photomorphogenesis in Plants and Bacteria*. 3rd Ed. (Schäfer, E. and Nagy, F., eds) pp. 279-304. Dordrecht, The Netherlands: Springer.

Vandenbussche, F., Vriezen, W.H., Smalle, J., Harren, F.J. and van der Straeten, D. 2003. Ethylene and auxin control the *Arabidopsis* response to decreased light intensity. *Plant Physiol.*, **133**: 517-527.

Vandenbussche, F., Pierik, R., Millenaar, F.F., Voesenek, L.A. and Van Der Straeten, D. 2005. Reaching out of the shade. *Curr. Opin. Plant Biol.*, **8**: 462-468.

Van der Ploeg, A. and Heuvelink, E. 2006. Influence of sub-optimal temperature on tomato growth and yield: a review. *J. of Horticultural Science & Biochem.*, **80**: 652-659.

van Tuinen, A., Kerckhoffs, L.H.J., Nagatani, A., Kendrick, R.E. and Koornneef, M. 1995a. Far-red light-insensitive, phytochrome A-deficient mutants of tomato. *Mol. Gen. Genet.*, **246**: 133-141.

van Tuinen, A., Kerckhoffs, L.H.J., Nagatani, A., Kendrick, R.E. and Koornneef, M. 1995b. A temporarily red light-insensitive mutant of tomato lacks a light-stable B-like phytochrome. *Plant Physiol.*, **108**: 939-947.

Vierstra, R.D. and Quail, P.H. 1983. Purification and initial characterization of 124-Kilodalton phytochrome from *Avena*. *Biochemistry*, **22**: 2498-2505.

Vierstra, R.D., Cordonnier-Pratt, M.-M. and Quail, P.H. 1984. Native phytochrome: Immunoblot analysis of relative molecular mass and in vitro proteolytic degradation for several species. *Planta*, **160**: 521-528.

Voesenek, L.A.C.J., Colmer, T.D., Pierik, R., Millenaar, F.F. and Peeters, A.J.M. 2006. How plants cope with complete submergence. *New Phytologist,* **170**: 213-226.

Wade, H.K., Bibikova, T.N., Valentine, W.J. and Jenkins, G.I. 2001. Interaction within a network of phytochrome, cryptochrome and UV-B phototransduction pathways regulate chalcone synthase gene expression in *Arabidopsis* leaf tissue. *Plant J.,* **25**: 675-685.

Wagner, D., Tepperman, J.M. and Quail, P.H. 1991. Overexpression of phytochrome B induces a short hypocotyl phenotype in transgenic *Arabidopsis*. *Plant Cell,* **3**:1275-1288.

Wagner, D.R., Fairchild, C.D., Kuhn, R.M. and Quail, P.H. 1996. Chromophore-bearing NH_2-terminal domains of phytochromes A and B determine their photosensory specificity and differential light lability. *Proc. Natl. Acad. Sci.*, **93**: 4011-4015.

Wagner, J.R., Brunzelle, J.S., Forest, K.T. and Vierstra, R.D. 2005. A light-sensing knot

revealed by the structure of the Chromophore-binding domain of phytochromes. *Nature*, **438**: 325-331.

Wallerstein, I., Wallerstein, I., Libman, D., Mchinic, B. and Whitelam, G.C. 2002. Modification in *Aster* response to long-day conditions caused by overexpression of phytochrome A and B. *Plant Science*, **163**: 439-447.

Wang, H. Ma, L.G., Li, L.M., Zhao, H.Y. and Deng, X.W. 2001. Direct interaction of *Arabidopsis* cryptochromes with COP1 in light control development. *Science*, **294**: 154-158.

Wang, H. and Deng, X.W. 2004. Phytochrome signalling mechanism. *Am. Soc. Plant Biol.*, **the *Arabidopsis* book**: 1-28.

Ward, J.M., Cufr, C.A., Denzel, M.A. and Neff, M.M. 2005. The Dof transcription factor OBP3 modulates phytochrome and cryptochrome signalling in *Arabidopsis*. *Plant Cell*, **17**: 475-485.

Weller, J.L., Ross, J.J. and Reid, J.B. 1994. Gibberellins and phytochrome regulation of stem elongation in pea. *Planta*, **192**: 489-496.

Weller, J.L., Schreuder, M.E.L., Smith, H., Koornneef, M. and Kendrick, R.E. 2000. Physiological interactions of phytochromes A, B1 and B2 in the control of development in tomato. *Plant J.*, **24**: 345-356.

Weller, J.L., Perrotta, G., Schreuder, M.E.L., van Tuinen, A., Koornneef, M., Giuliano, G. and Kendrick, R.E. 2001. Genetic dissection of blue-light sensing in tomato using mutants deficient in cryptochrome 1 and phytochromes A, B1 and B2. *Plant J.*, **25**: 427-440.

Whippo, C.W. and Hangarter, R.P. 2003. Second positive phototropism results from coordinated co-action of the phototropins and cryptochromes. *Plant Physiol.*, **132**: 1499-1507.

Whitelam, G.C., McCormac, A.C., Boylan, M.T. and Quail, P.H. 1992. Photoresponses of *Arabidopsis* seedlings expressing an introduced oat *phyA* cDNA: persistence of etiolated plant type responses in light-grown plants. *Photochem. Photobiol.*, **56**: 617-621.

Whitelam, G.C., Johnson, E., Peng, J., Carol, P., Anderson, M.L., Cowl, J.S. and Harberd, N.P. 1993. Phytochrome A null mutants of *Arabidopsis* display a wild-type phenotype in white light. *Plant Cell*, **5**: 757-768.

Yamaguchi, R., Nakamura, M., Mochizuki, N., Kay, S.A. and Nagatani, A. 1999. Light-dependent translocation of phytochrome B-GFP fusion protein to the nucleus in transgenic *Arabidopsis*. *J. Cell Biol,* **145**: 437-445.

Yang, J., Lin, R., Sullivan, J., Hoecker, U., Liu, B., Xu, L., Deng, X.W. and Wang, H. 2005. Light regulates COP1-mediated degradation of HER1, a transcription factor essential for light signalling in *Arabidopsis*. *Plant Cell*, **17**: 804-821.

Yang, J. and Haiyang, W. 2006. The central coiled-coil domain and carboxyl-terminal WD-repeat domain of *Arabidopsis* SPA1 are responsible for mediating repression of light signalling. *Plant J.*, **47**: 564-476.

Yanovsky, M.J., Alconada-Magliano, T.M., Mazzella, M.A., Gatz, C., Thomas. B. and Casal, J.J. 1998. Phytochrome A affects stem growth, anthocyanin synthesis, sucrose-phosphate-synthase activity and neighbour detection in sunlight-grown potato. *Planta*, **205**: 235-241.

Yanovsky, M.J, Izaguirre, M., Wagmaister, J.A., Gatz, C, Jackson, S.D., Thomas, B. and Casal, J.J. 2000. Phytochrome A resets the circadian clock and delays tuber formation under long days in potato. *Plant J.*, **23**:225-232.

Ye, K.C. and Lagarias, J.C. 1998. Eukaryotic phytochromes: Light regulated serine/threonine protein kinases with histidine kinase ancestry. *Proc. Natl. Acad. Sci.,* **95**: 13976-13981.

Yi, C. and Deng, X.W. 2005. COP1: from plant photomorphogenesis to mammalian tumorigenesis. *Trends in Cell Biol*., **15**: 618-625.

Zheng, Z-L., Yang, Z., Jang, J-C. and Metzger, J.D. 2001. Modification of plant architecture in chrysanthemum by ectopic expression of tobacco phytochrome B1 gene. *J. Amer. Soc. Hort. Sci.*, **126**: 19-26.

Zheng, Z-L, Yang, Z., Jang, J-C. and Metzger, J.D. 2006. Phytochromes A and B1 have distinct functions in the photoperiod control of flowering in the obligate long-day plant *Nicotiana sylvestris*. *Plant Cell Environ.*, **29**: 1673-1685.

Zimmermann, S., Baumann, A., Jackel, K., Marbach, I., Engelberg, D., and Frohnmeyer, H. 1999. UV-responsive genes of *Arabidopsis* revealed by similarity to the Gcn4-mediated UV response in yeast. *J.Biol. Chem.*, **274**: 17017-17024.

Summary

In this thesis we investigated the effects of elevated levels of phyA, phyB1 and phyB2 on the shade avoidance response by overexpression of tomato phytochrome genes in tomato. We used a transgenic assay based upon overexpression of the tomato *PHYA, PHYB1* and *PHYB2* under control of the constitutive double-*35S* promoter (*PHY*OE) to determine whether a shift in the response to shade can be obtained which could be beneficial for plant cultivation at high densities. In addition, the *PHY*OE plants enhance our insight in phytochrome function and saturation.

In Chapter 1 an overview is given of the importance of light signalling in plant growth and development. Light quality, quantity, direction and duration are monitored in plants by a series of photoreceptors, each covering a specific range of spectral information. The best characterized photoreceptors are phytochromes which specifically are activated by red light (R) and inactivated by far-red light (FR). Phytochromes control different aspects of photomorphogenesis, such as seed germination, hypocotyl elongation, anthocyanin accumulation and shade avoidance responses (SAR). The phytochrome holoproteins contain a polypeptide component and a covalently attached light-absorbing chromophore unit. Phytochromes exist in two forms: the inactive R-absorbing form (Pr) and the active FR-absorbing form (Pfr). These two forms of the phytochrome are interconvertible by R and FR, respectively. Within the phytochrome protein family two phytochrome types are distinguished: type 1 which is abundant in dark (D)-grown seedlings in the Pr form, but which, due to the light labile form of Pfr, decreases rapidly in the light (phyA) and type 2, which is low abundant but more light stable than Pfr and therefore present both in D-grown seedlings and in light-grown seedlings (phyB-E).

Within higher density populations taller plants have a greater probability of keeping some leaves in sunlight above those of competing plants, and such shade-induced elongation would favour survival among other plants. This natural adaptation phenomenon is called SAR and is considered of great advantage to plant survival in dense populations. Tomato plants show a strong response to natural shade, which is characterized by an enhanced stem elongation, reduced leaf size and reduced anthocyanin accumulation. However, this adaptive strategy is a great disadvantage for tomato growers, since SAR favours a higher assimilate allocation to the stem, leading to less assimilate available for the fruit and resulting in a lower harvest index. Therefore, a suppression of SAR could lead to increased yield of tomato plants when grown at higher density.

In Chapter 2, 3 and 4 we describe the characterization of the plants we obtained with *PHYA*OE, *PHYB1*OE and *PHYB2*OE. A number of phytochrome-regulated responses were measured under different light conditions (WL, cR, cFR, B, natural light (greenhouse) or EODFR) to characterize the effect of *PHY*OE on hypocotyl length and/or stem length and on anthocyanin accumulation.

In Chapter 2 the expression levels in the *PHY*OE lines were characterized. Western blot analysis shows an up to 16-fold, 32-fold and 200-fold increase in PHYA, PHYB1 and PHYB2 levels in transgenic *PHYA*OE, *PHYB1*OE and *PHYB2*OE tomato lines, respectively (Chapter 2). Results show that the ectopically produced phytochrome was assembled into functional phytochrome holoprotein, as indicated by the light dependent turnover of the ectopically produced phyA, complementation by the *PHYA*OE transgene of the *phyA* mutant phenotype and complementation by the *PHYB2*OE transgene of the *phyB1phyB2* double mutant phenotype (Chapter 2). Despite an up to 16-fold increase of PHYA in *PHYA*OE lines, only a mild suppression of hypocotyl elongation and enhanced anthocyanin accumulation was observed in these lines under WL and cFR. The results suggest that WT phyA signalling under cFR is already near saturation. At very high levels of *PHYA*OE a threshold dependent negative effect was observed for stem elongation and anthocyanin accumulation (Chapter 2). The *PHYB1*OE and *PHYB2*OE resulted in a similar level of the total phytochrome pool, but the effect of *PHYB2*OE on plant growth and anthocyanin accumulation studied under WL and cR was shown to be stronger than that of *PHYB1*OE (Chapter 2). In Chapter 2 we also studied the fluence-rates response relationship for anthocyanin accumulation in the *PHY*OE lines during a 24 h R or FR treatment of 4-day-old D-grown seedlings. The results revealed that *PHYB2*OE exhibited a strong amplification of the anthocyanin accumulation under cR (680 nm). In contrast, *PHYB1*OE exhibited only little amplification of the anthocyanin accumulation under the same conditions. Mutant analysis has indicated that the phyA-dependent low fluence rate response component depends on the presence of phyB2. However, the increase of phyB1 in the B1/4 line results in an actual reduction of this response (Chapter 2), suggesting a negative interaction between phyA and phyB1 at elevated levels of phyB1. From phytochrome mutant studies it could be inferred that *PHYA*OE amplifies the response to low fluence rates of R and that the response to higher fluence rates of R are amplified by *PHYB1*OE and less so by *PHYB2*OE. However, our results show that *PHYA*OE actually lowers the response at low fluence rates of R, while *PHYB2*OE has a bigger effect on the R-HIR than *PHYB1*OE (Chapter 2).

In Chapter 3 we continued the characterization of the *PHY*OE lines, first by

demonstrating that the phenotype induced by *PHY*OE is R/FR reversible and second by testing growth responses in a dense population of plants to quantify the effect on SAR. The effect of *PHY*OE on stem length and anthocyanin accumulation was shown to be largely reversed by a 20 min FR treatment at the end of the photoperiod. These experiments imply that the phenotypes in the transgenic lines are phytochrome induced. Although our 20 min of FR treatment converts a large proportion of Pfr to Pr, the remaining Pfr^{phyB2} from *PHYB2*OE is predicted to be still more than 5-fold higher than Pfr^{phyB2} levels from endogenous *PHYB2* expression in plants without the FR treatment. However, apparently these remaining elevated levels of Pfr^{phyB2} after FR treatment are not effective in suppression of hypocotyl elongation or anthocyanin accumulation (Chapter 2), suggesting that the other activated phytochromes may be required for the full effect of *PHYB2*OE.

Plant populations for selected *PHYA*OE, *PHYB1*OE and *PHYB2*OE lines were grown at a density of 100 plants m^{-2} and plant growth parameters were determined for plants inside and plants at the outer edge of each population. Although transgenic tomato plants were 20-40% shorter than WT plants, the SAR in the centre of the population was similar and in some cases even stronger compared to the WT (Chapter 3). We calculated the shade avoidance index (SAI) for a given Ptot pool size, assuming a R:FR of 0.6 for shaded plants and R:FR of 1.0 for non shaded plants. The results show that a small increase in Ptot pool size resulted in an increase in SAI, while only at high Ptot values (which cause near-saturation of the elongation response) the SAI is reduced to zero (Chapter 3).

In Chapter 4 we tested the possible limitation of the *PHY*OE effect due to other endogenous photoreceptor levels, by combining different *PHY*OE genes, or by combining different *PHY*OE genes with *CRY1*OE or *CRY2*OE. Of the plants with different combinations of *PHY*OE, only the combination of *PHYB1*OE/*PHYB2*OE resulted in a small additional suppression of stem elongation. In contrast no significant increase in anthocyanin accumulation for the same combination was observed, indicating different levels of saturation of these two different responses. Combining *CRY1*OE or *CRY2*OE with *PHY*OE in most cases had little additional effect. The results show that the effect of *PHYB2*OE on hypocotyl elongation and anthocyanin accumulation was significantly enhanced by *CRY2*OE under WL. In contrast, for plants grown under B, the effect of double hemizygous, B2/9C2, on hypocotyl elongation was less pronounced than in the hemizygous C2WT, indicating that under this light condition *PHYB2*OE and *CRY2*OE act antagonistically. In addition to manipulation of the SAR, these results therefore demonstrate that manipulation of photoreceptors may be a useful approach for

optimizing pigmentation. When applied in fruits, this may enhance nutritional quality.

In Chapter 5 the general conclusions and points of discussion are presented. We showed that young tomato *PHYB2*OE plants with a moderate overexpression level grown for 8 weeks in the greenhouse were 40% shorter than WT, but that leaf production in these plants was reduced only 20%. Although it might still be possible from these results, that *PHY*OE plants allocate more biomass to fruits, subsequent experiments with greenhouse-grown plants showed a substantial reduction of fruit biomass production in the *PHYB2*OE lines. We conclude that: (1) constitutive overexpression of phytochrome genes may not be suitable for manipulation of SAR in tomato; (2) the results from phytochrome mutant studies are not always predictive for *PHY*OE results and (3) besides effect on shade avoidance *PHY*OE may also be used to manipulate anthocyanin levels in tomato to enhance fruit quality. However, both for manipulation of SAR and manipulation of pigmentation level in fruits, the use of tissue specific promoters may be required to prevent unwanted effects of *PHY*OE on root, leaf and fruit development.

Samenvatting

In dit proefschrift worden de effecten van verhoogde niveaus van phyA, phyB1 en phyB2 op de 'shade avoidance response' (schaduw-vermijdings-reactie: SAR) onderzocht door tomaat fytochroomgenen in tomaat tot overexpressie te brengen. Bepaald werd of de overexpressie van tomaat *PHYA*, *PHYB1* en *PHYB2*, onder controle van de dubbele 35S-promoter (*PHY*OE), een verschuiving in de SAR kon bewerkstelligen die mogelijk van nut zou kunnen zijn bij de teelt van planten onder hoge dichtheid. Bovendien kunnen de planten met *PHY*OE ons inzicht verschaffen in de functie en verzadigingsniveaus van de signalering van de individuele fytochromen.

In hoofdstuk 1 wordt een overzicht gegeven van het belang van lichtsignalering in planten bij groei en ontwikkeling. Belichtingskwaliteit, -kwantiteit, -richting en -duur worden door de plant waargenomen door middel van een serie fotoreceptoren die elk een specifiek gebied van het lichtspectrum kunnen detecteren. De meest uitvoerig gekarakteriseerde fotoreceptoren zijn de Fytochromen, die specifiek geactiveerd worden door rood licht (R) en geïnactiveerd worden door ver-rood (far red: FR) licht. Fytochromen bepalen verschillende aspecten van de fotomorfogenese, zoals zaadkieming, hypocotyl-strekking, anthocyaan-ophoping en de SAR. Het fytochroom holo-eiwit bevat een polypeptide-component en een covalent gebonden lichtabsorberende chromofoor-unit. Fytochromen komen voor in twee vormen: de inactieve R-absorberende vorm (Pr) en de actieve FR-absorberende vorm (Pfr). Deze twee vormen kunnen in elkaar overgaan door R en FR belichting respectievelijk. Binnen de familie van fytochroomeiwitten kunnen twee types onderscheiden worden: type 1 (phyA) is ruim aanwezig in de Pr vorm in donker-gegroeide zaailingen, maar wordt door licht omgezet in een instabiele Pfr vorm, zodat in licht de hoeveelheid van type 1 fytochroom sterk afneemt. Type 2 fytochromen (phyB-E) zijn laag abundant maar stabiel in het licht en daarom aanwezig in zowel donker- als licht-gegroeide zaailingen.

Binnen een dichte plantenpopulatie hebben langere planten een grotere kans om hun bladeren in het zonlicht te houden dan kleinere planten in de populatie. Een bladschaduw geïnduceerde strekking (één van de aspecten van SAR) zou daarmee van voordeel zijn voor overleving van een plant binnen een populatie. Tomaat vertoont een sterke reactie op schaduw, welke wordt gekarakteriseerd door stengelstrekking, gereduceerd bladoppervlak en een kleinere hoeveelheid anthocyanen. Echter, deze aanpassingsstrategie is nadelig voor telers van tomaat,

aangezien de SAR tot een verhoogd assimilaat transport naar de stengel leidt, waardoor minder assimilaten beschikbaar zijn voor de vrucht, resulterend in een verlaagde oogstindex. Een onderdrukking van SAR in tomaat zou daarom de productie van tomatenplanten, geteeld onder hoge dichtheid, kunnen verhogen. Geprobeerd werd om de SAR gerelateerde respons in tomaat te onderdrukken door middel van overexpressie van specifieke lichtreceptoren.

In hoofdstuk 2, 3 en 4 worden de planten gekarakteriseerd die zijn verkregen met *PHYA*OE, *PHYB1*OE en *PHYB2*OE. Een aantal fytochroom gerelateerde processen werden gemeten onder verschillende licht condities (WL, cR, cFR, B, natuurlijk licht in kassen en bij FR belichting aan het einde van de dagperiode). Vooral de hypocotyl- en/of stengellengte en de ophoping van anthocyanen in zaailingen of blad werden gekwantificeerd.

In hoofdstuk 2 werden de expressieniveaus in de *PHY*OE lijnen bepaald. Western blot analyse laat maximaal een 16-voudige toename in PHYA, 32-voudige toename in PHYB1 en 200-voudige toename in PHYB2 niveaus zien, respectievelijk in de transgene *PHYA*OE, *PHYB1*OE en *PHYB2*OE lijnen. Het ectopisch geproduceerde fytochroom werd geassembleerd tot functioneel holo-eiwit, zoals bleek uit de licht gevoelige afbraak van ectopisch geproduceerd phyA in de *PHYA*OE lijnen, de complementatie van de *phyA* mutant door *PHYA*OE en de complementatie van de phyB1phyB2 dubbel mutant door *PHYB2*OE. Ondanks de 16-voudige verhoging van het PHYA werd slechts een mild fenotype voor hypocotyl-strekking en anthocyaan-ophoping in de *PHYA*OE planten onder WL of cFR waargenomen. De resultaten suggereren dat WT phyA-signalering onder cFR al verzadigd is. Bij heel hoge niveaus van *PHYA*OE werd een drempelwaarde-afhankelijk negatief effect op strekking en anthocyaan-ophoping waargenomen (hoofdstuk 2). De *PHYB1*OE en *PHYB2*OE resulteerden in een vergelijkbare toename van de totale hoeveelheid fytochroom (Ptot), maar het effect van *PHYB2*OE op plantengroei en anthocyaan-ophoping onder WL en cR was sterker dan het effect van *PHYB1*OE (hoofdstuk 2). In hoofdstuk 2 wordt ook de relatie tussen anthocyaan-pigmentatie en lichtintensiteit beschreven. Vier dagen oude zaailingen werden 24 uur met verschillende intensiteiten van cR of cFR belicht. De resultaten laten een sterke amplificatie van de licht respons onder cR in de *PHYB2*OE lijn zien, in tegenstelling tot de *PHYB1*OE planten, die slechts een kleine verhoging van het anthocyaan-niveau onder dezelfde condities lieten zien. Uit fytochroom mutant-studies is gebleken dat phyA voornamelijk de respons bij lage lichtintensiteit regelt, terwijl de respons op hoge lichtintensiteit (HIR) door phyB1 en phyB2 wordt bepaald. Echter, een toename in phyA leidt tot een vermindering in de

hoeveelheid anthocyaan bij lage lichtintensiteit. De phyA-afhankelijke respons op lage lichtintensiteit is afhankelijk van phyB1. Een toename in phyB1 in lijn B1/4 resulteert echter eveneens in een verminderde respons bij lage lichtintensiteit. Dit duidt op een mogelijke negatieve interactie tussen phyA en verhoogde hoeveelheid phyB1. Bovendien, heeft een toename in phyB2 een groter effect op de HIR dan een toename in phyB1 (hoofdstuk 2).

In hoofdstuk 3 wordt de analyse van de *PHY*OE lijnen vervolgd door eerst te laten zien dat het fenotype dat wordt geïnduceerd door *PHY*OE R/FR omkeerbaar is en ten tweede, door de groei van deze planten binnen een dichte populatie planten te testen en daarmee het effect van *PHY*OE op de SAR te kwantificeren. Het effect van *PHY*OE op stengelstrekking en anthocyaan-ophoping was grotendeels omkeerbaar door een 20 min FR puls aan het einde van de dag. Deze experimenten suggereren dat de fenotypes van de transgene planten worden geïnduceerd door actief fytochroom. Hoewel de 20 min FR behandeling het grootste deel van Pfr omzet in Pr, zou toch de overgebleven hoeveelheid Pfr^{phyB2} in de *PHYB2*OE-planten meer dan het 5-voudige moeten zijn van de Pfr^{phyB2} hoeveelheid in WT zonder FR-behandeling. Echter, deze hoeveelheid Pfr^{phyB2} in B2/9 was niet effectief in het onderdrukken van de strekking of de ophoping van anthocyaan (hoofdstuk 2), wat er op duidt dat de overige actieve fytochromen misschien nodig zijn voor het volle effect van *PHYB2*OE.

Plantpopulaties van geselecteerde *PHYA*OE, *PHYB1*OE en *PHYB2*OE lijnen werden opgegroeid bij een dichtheid van 100 planten m^{-2} en de groeiparameters werden bepaald voor planten aan de buitenkant en aan de binnenkant van de populaties. Hoewel de transgene tomatenplanten tot 20-40% korter waren dan WT planten, was de SAR in het centrum van de populatie in het algemeen vergelijkbaar of zelfs sterker dan in WT (hoofdstuk 3). De shade avoidance index (SAI) werd berekend voor verschillende hoeveelheden Ptot, met de aanname van een R:FR van 0.6 voor schaduw- en 1.0 voor niet-schaduw-planten. De resultaten laten zien dat een kleine toename in de hoeveelheid Ptot resulteert in een toename van SAR terwijl alleen bij een zeer hoge hoeveelheid Ptot (waarbij verzadiging van de onderdrukking van de strekkingsrespons optreedt) de SAI terugloopt naar nul (hoofdstuk 3).

In hoofdstuk 4 werd getest of de effecten van *PHY*OE worden beperkt door de expressieniveaus van andere lichtreceptor-genen, door verschillende *PHY*OE-genen, *PHY*OE- en *CRY1*OE- of *PHY*OE- en *CRY2*OE-genen met elkaar te combineren d.m.v. kruisingen. Van de planten met verschillende combinaties van *PHY*OE gaf alleen de combinatie *PHYB1*OE met *PHYB2*OE een klein additioneel

effect op de stengelstrekking, terwijl deze combinatie geen effect had op de ophoping van anthocyanen. Dit duidt op verschil in verzadigingsniveaus voor de fytochroom geïnduceerde strekkings- en pigmentatie-respons. De combinatie *CRY1*OE of *CRY2*OE met *PHY*OE had in de meeste gevallen geen effect. Een combinatie van *PHYB2*OE met *CRY2*OE resulteerde onder WL wel in nog kortere planten en nog hogere anthocyaan-niveaus dan in elk van de ouderplanten. Onder B licht was het effect op de stengelstrekking in de dubbel hemizygoot B2/9C2 minder sterk dan in de hemizygoot C2WT, wat er op duidt dat onder B *PHYB2*OE en *CRY2*OE antagonistisch op elkaar werken. Naast een veranderde SAR laat de manipulatie van fotoreceptorgenen zien dat hiermee mogelijk pigmentatie van planten kan worden vergroot, wat bevorderlijk kan zijn voor de voedingswaarde van gewassen.

In hoofdstuk 5 worden de algemene conclusies en discussiepunten gepresenteerd. Het bleek dat 8-weken oude tomatenplanten met *PHYB2*OE tot 40% korter zijn dat WT planten, en dat de blad biomassaproductie in deze planten 20% lager is dan in WT. Hoewel deze resultaten nog steeds de mogelijkheid open laten dat tomaten met *PHY*OE een toename in vruchtbiomassa vertonen, hebben vervolgexperimenten met kas-gegroeide planten laten zien dat planten met *PHYB2*OE een aanzienlijke reductie in vruchtbiomassa hebben t.o.v. WT. We concluderen dat: (1) een constitutieve expressie van fytochroomgenen niet geschikt is voor een productieve manipulatie van SAR in tomaat. (2) dat de resultaten van fytochroom mutant-studies niet altijd indicatief zijn voor *PHY*OE-resultaten en (3) behalve voor manipulatie van de SAR zou de overexpressie van fotoreceptor-genen gebruikt kunnen worden voor het verhogen van anthocyaan-niveaus in tomaat om zo de gezondheidsbevorderende kwaliteit te verhogen. Echter, voor zowel de manipulatie van SAR als van de vruchtpigmentatie zal het nodig zijn om weefselspecifieke promotoren te gebruiken om ongewilde pleiotrope effecten in wortel en blad te vermijnden.

Publications

Husaineid, S.H., Kok, R.A, Schreuder, M.E.L., Hanumappa, M., Cordonnier-Pratt, M.M., Pratt, L.H., van der Plas, L.H.W. and van der Krol, A.R. 2007. Overexpression of homologous phytochrome genes in tomato: exploring the limits in photoperception. *J. of Experimental Botany*. **58**: 615-626.

Anke van der Ploeg, **Husaineid, S.H.**, Schreuder, L.H., van der Plas, L.H.W., van der Krol, A.R. and Ep Heuvelink. 2007. Overexpression of homologous phytochrome genes in tomato: characterization of shade-avoidance responses and greenhouse grown plants. *J. of Experimental Botany*. (in preparation).

International symposia and congresses

Husaineid, S.H., van der Krol, A.R. and Kendrick, R.E. 2001. *Overexpression of tomato phytochrome genes in tomato*. Poster and abstract, Plant Photobiology meeting, University of Missouri, Columbia, USA.

Husaineid, S.H., Kok, R.A. van der Krol, A.R. and Kendrick, R.E. 2003. *Effects of tomato phytochrome transgenes in tomato, petunia and Arabidopsis*. Poster and abstract, 7th International Congress of Plant Molecular Biology, Barcelona, Spain.

Husaineid, S.H., van der Krol, A.R. and Kendrick, R.E. 2003. *Overexpression of tomato phytochrome genes in tomato*. Poster and abstract, Plant Photobiology meeting, Phylipps-University of Marburg, Marburg, Germany.

Vroemen, C. W., Guhl, K., **Husaineid, S.H.** and Mordhorst, A.P. 2003. *Enhancer trapping of genes involved in suspensor. Shoot meristem and boundary formation in Arabidopsis*. Poster and abstract, Embryogenesis and development regulation in plants, Turin, Italy. 2003- pp. 77- 81.

Presentations

Husaineid, S.H. 2004**.** *Manipulation of shade avoidance response in tomato*.
Oral presentation at the meeting on Experimental Plant Sciences, Lunteren, The Netherlands.
Husaineid, S.H. 2004**.** *Manipulation of shade avoidance response in tomato*.
Oral presentation at the Theme I Symposium of EPS, Leiden University, Leiden, The Netherlands.

CURRICULUM VITAE

Said Saleh Hindi Husaineid was born in Joret Al Shamah, Bethlehem, West-Bank, on September 21st 1962. After finishing highschool in September 1980 he started the undergraduate programme at the Department of Biology, Birzeit University, finishing with a BSc degree in Biology and Biochemistry in May 1985. From 1999 to 2001 he obtained a fellowship from the Netherlands Fellowship Programme (NFP) to follow the international Master of Science programme in Biotechnology at Wageningen University, the Netherlands. In his MSc thesis he worked with Prof. Dr. Sacco de Vries and Dr. Casper Vroemen where he used molecular techniques to study the expression of *Arabidopsis* genes involved in the plant pattern formation at the embryo stage leading to identification and partial cloning of the cDNA of one of these genes. In February 2001 he started his Ph.D. at Wageningen University and Research Centre, the Netherlands**.** In the laboratory of Plant Physiology, department of Plant Sciences he worked with the group of Prof. Dr. Linus H.W. van der Plas, Dr. Sander van der Krol and Dr. Richard E. Kendrick in a project entitled: 'Energy efficiency of greenhouse crops'. Using molecular and physiological characterization, he carried out experiments on overexpression of tomato phytochrome genes in tomato.

This research is part of the research program ‘’Rassen onder glas met minder gas’’, i.e. aiming at breeding more energy-efficient greenhouse crops. This program is financially supported by the Dutch Horticultural Product Board (Productschap Tuinbouw), the Dutch Organisation for Energy and Environment (NOVEM), the Department of Agricultural Research (DLO), the ministry of Agriculture, Nature and Food Quality (LNV) and several private breeding companies.

وذلك لأنه معلوم التفاعل بين جينات وبروتينات أنواع مختلفة من مستقبلات الضوء . ولتحقيق هذا الغرض تم تلقيح أزهار من *PHY*OE كمصدر للبويضة مع *PHY*OE أخر كمصدر لحبة اللقاح بالإضافة الى استخدام كربتوكروم معدل جينيا في نبات البندورة (*CRY*OE) كمصدر لحبات اللقاح . عند دراسة تأثير التلقيح الخلطي بين *PHY*OE / *PHY*OE على تثبيط استطالة النبات تبين أن ناتج تلقيح *PHYB1*OE / *PHYB2*OE فقط أدت لإضافة بسيطة على التثبيط مقارنة مع الأبوين و في المقابل لم تتأثر صفة تحفيز إنتاج صبغة الانثوسيانين في أي من تلقيحات *PHY*OE أما في حالة التلقيح بين *PHY*OE و *CRY*OE فقد دلت النتائج انه من بين مجموعات التلقيح المختلفة فقط *PHYB2*OE / *CRY2*OE أظهر زيادة في تثبيط الاستطالة مقارنة مع الأبوين عند نمو النباتات في الدفيئات حيث الإضاءة الطبيعية ، في حين لم يظهر تأثير في حجرات الأشعة الزرقاء ؛ من نتائج البحث في الفصل الرابع استنبطنا أنه إضافة على إمكانية توظيف الفايتوكروم للتحكم بظاهرة تجنب الظل فإنه يمكننا أن نوظف الفايتوكروم و الكربتوكروم كوسيلة للتحكم بالكميات المثلى من صبغة الانثوسيانين في ثمار نبات البندورة وان هذا التوظيف لو استغل بشكل محدد قد يساهم في زيادة القيمة الغذائية لثمار البندورة .

يستعرض الفصل الخامس من هذه الرسالة النتائج العامة ويربط الاستخلاصات في فصول الرسالة مع بعضها البعض ، من ذلك بيَّنا أن نبات البندورة المعدل وراثيا لإنتاج كميات أكبر من فايتوكروم *PHYB2*OE يثبط استطالة النبتة الى 40 % مقارنة مع النبات غير المعدل ولكن هذا النقص في الطول يقابله فقط 20 % نقص في إنتاج الأوراق وعلى الرغم من أن ذلك يقودنا لاحتمالية إعادة توزيع الطاقة في النبات بحيث تنتج نسبة اكبر من الثمار كما هو الحال في الأوراق إلا أن نتائج تجارب أجريت في مجموعات بحث أخرى استخدمت نفس النباتات دلت على أن هذه النباتات تنتج ثمار بكميات اقل مقارنة مع إنتاج النباتات غير المعدلة وراثيا .

الاستنتاجات

1. التعديل الوراثي في جينات الفايتوكروم قد لا يكون الوسيلة المناسبة لزيادة التحكم في ظاهرة تجنب الظل في نبات البندورة .
2. النتائج المستخلصة من طفرات في جينات الفايتوكروم ليست دائما منبئة بتأثير تعديل الفايتوكروم وراثيا .
3. بالإضافة لتأثير *PHY*OE على ظاهرة تجنب الظل يمكن توظيف *PHY*OE أيضا في التحكم بكمية مثلى من صبغة الأنثوسيانين اللازمة لرفع القيمة الغذائية لثمار البندورة .

وننوه هنا انه في كلتا الحالتين : التحكم في ظاهرة تجنب الظل والتحكم في كمية صبغة الأنثوسيانين قد يكون من الأنسب استخدام تقنية التعديل الوراثي في كمية الفايتوكروم وبشكل محدد في خلايا أعضاء معينة من نبات البندورة لتجنب التأثير الجانبي من *PHY*OE على نمو وتطور كل من الجذور والأوراق والثمار في نبات البندورة .

PHYB2 في

نباتات من *PHYB2*OE كان لها تأثير كبير على كل من استطالة النبات حيث ثبطتها وعلى كمية صبغة الأنثوسيانين حيث حفزتها عند إكثار النباتات في حجرات من الأشعة الحمراء وفي الدفيئات في المقابل كان تأثير زيادة PHYBI في نباتات *PHYB1*OE قليل على نفس الصفات في نفس الظروف .

وفي الفصل الثاني درسنا أيضا العلاقة بين كمية صبغة الانثوسيانين المتكونة في النباتات المعدَّلة وراثياً مع درجات متفاوتة من الأشعة فوق الحمراء والأشعة الحمراء عند تعريض النبات لمدة 24 ساعة متواصلة من هذه الأشعة . أظهرت نتائج الدراسة أنه في حالة نباتات *PHYB2*OE تتضاعف كميات صبغة الانثوسيانين مع زيادة درجة الأشعة الحمراء وفي حالة نباتات *PHYB1*OE فإن التضاعف في صبغة الانثوسيانين قليل وبمقارنة هذه النتائج مع ما هو معروف عن تأثير كل من PHYB1 و PHYB2 بكمياتها الطبيعية وتحت نفس الظروف فإن الدراسة الحالية أظهرت انعكاس التأثير في دور كل من *PHYB1*OE و *PHYB2*OE في حالة التعديل الوراثي الذي ينتج نسبيا كميات أكبر من بروتين PHYB2 مقارنة مع كميته الطبيعية .

في الفصل الثالث من هذه الرسالة درسنا تأثير تعريض النباتات المعدَّلة وراثيا لمدة 20 دقيقة بالأشعة فوق الحمراء عند نهاية فترة الإضاءة ، أظهرت الدراسة أن التثبيط في الاستطالة وتحفيز إنتاج صبغة الانثوسيانين قد اختفى تماما عند تعريض النباتات مدة 20 دقيقة من الأشعة فوق الحمراء . هذا التأثير الواضح للأشعة فوق الحمراء على الصفات الناتجة في النباتات المعدَّلة وراثيا يشير الى أن هذه الصفات إنما هي ناتجة عن التعديل الجيني في الفايتوكرومات وحيث أن النبات الذي ينمو في تجمعات كثيفة يتعرض لكمية أكبر من الأشعة فوق الحمراء لان أوراق النبات القريب منها تمتص الأشعة الحمراء وينعكس عنها نسبة عالية من الأشعة فوق الحمراء فقد قمنا بدراسة تأثير التعديل الجيني في النباتات عند إكثارها في تجمعات كثيفة بينت النتائج المدرجة في الفصل الثالث أنه عند إكثار النباتات المعدَّلة جينيا بكثافة كبيرة (100 نبتة / م2) فإن النباتات النامية في مركز التجمع والتي تتعرض لنسبة أعلى من الأشعة فوق الحمراء تكون أطول (انظر صورة الغلاف) من النباتات النامية على أطراف التجمع وبحساب نسبة الاستطالة في كل من النباتات المعدَّلة جينيا تبين أنه كلما زادت كمية الفايتوكروم في النبتة وخصوصا في حالة *PHYB2*OE فان نسبة الاستطالة في نباتات مركز التجمع تزداد ولكن عند حد معين تنعدم الاستطالة بسبب الإشباع التام للتثبيط الناتج عن الكميات الكبيرة من الفايتوكروم .

في الفصل الرابع من هذه الرسالة بحثنا احتمال محدودية تأثير التعديل الجيني في بعض الفايتوكرومات بعدم توفر كميات مناسبة من الفايتوكرومات الأخرى في نفس العائلة أو إمكانية عدم توفر كميات مناسبة من الكربتوكرومات (نوع أخر من مستقبلات الضوء يرمز لها *CRY*

phyA و phyB1 و phyB2 و phyE و phyF وتقسم هذه البروتينات الى قسمين ، القسم الأول ويضم phyA ويتميز بكثرة وجوده في خلايا النبات النامية في الظلام ولكنه غير ثابت حيث ينخفض تركيزه بشكل كبير في خلايا النبات الذي ينمو بوجود الضوء ، أما القسم الثاني فهو بقية الفايتوكرومات وتتميز بقلة تركيزها في خلايا النبات ولكنها ثابتة نسبياً ولا يتأثر تركيزها أو إنتاجها في الخلايا بوجود الضوء .

نبذة عن ظاهرة تجنب الظل وتأثيرها على الإنتاج :

إن قدرة النبات على تحديد مدى اقتراب نبات أخر إليه والتي تزودها بها بروتينات الفايتوكروم ، تجعله قادرا على تجنب الوقوع في ظل هذه النباتات محفزاً عنده ظاهرة تجنب الظل . تتمثل هذه الظاهرة فسيولوجيا في مجموعة من الصفات أهمها سرعة استطالة الساق وانخفاض كبير في إنتاج صبغة الأنثوسيانين ، أثناء هذه الظاهرة يوظف النبات معظم طاقاته في بناء خلايا الساق على حساب خلايا الأعضاء الأخرى كالأوراق والثمار في الوقت الذي تكون فيه هذه الظاهرة إيجابية بالنسبة لنوع النبات من حيث مساهمتها في المنافسة على البقاء فإنها تكون سلبية للمزارع لأن إنتاج النبتة من الثمار يقل لذلك فان تثبيط هذه الظاهرة متوقع منه أن يؤدي الى زيادة المحصول الزراعي ، وبالنسبة لنبات البندورة المستخدم في هذا البحث فانه يعتبر من أنواع النبات التي تتنافس بقوة على الضوء في المجتمعات الكثيفة لذلك فإن ظاهرة تجنب الظل فيها شديدة .

في الفصل الثاني والثالث والرابع من هذه الرسالة استخدمنا نباتات بندورة معدَّلة وراثيا لاحتوائها على جينات الفايتوكروم المنقولة لها بعد تعديل جيني يسمح بحثّها على مضاعفة إنتاج البروتينات المرتبطة بها وقد رمزنا لها *PHYA*OE و *PHYB1*OE و *PHYB2*OE. في هذا البحث تم تحليل نتائج تأثير زيادة بروتينات الفايتوكروم على كل من استطالة النبات وكمية صبغة الأنثوسيانين المتكونة وذلك بعد إكثار النباتات في حجرات صممت خصيصاً للإضاءة إما بالأشعة فوق الحمراء أو الحمراء أو الزرقاء وكذلك في الدفيئات حيث الإضاءة طبيعية ، إضافةً لذلك فقد بحثنا تأثر إضافة أشعة فوق الحمراء لمدة 20 دقيقة في نهاية فترة الإضاءة وذلك خلال الأسابيع الثلاثة الأولى من فترة نمو النباتات ويذكر أننا استخدمنا نباتات غير معدَّلة وراثيا وأخرى فيها طفرات في جينات الفايتوكرومات الثلاثة السابقة كوسيلة للمقارنة .

في الفصل الثاني من هذه الرسالة بيّنا أن مستوى بروتينات PHYA و PHYB1 و PHYB2 قد ارتفع الى 16 و 32 و 200 على التوالي مقارنة مع المستوى الطبيعي . أظهرت النتائج أن البروتينات الناتجة فعّالة ومشابهة في صفاتها للبروتينات الطبيعية وقادرة على القيام بدورها في طفرات من الجينات المقابلة لها وقد بينت النتائج في الفصل الثاني أنه بالرغم من تضاعف PHYA الى 16 ضعف فان تأثير ذلك قليل على كل من استطالة

الخلاصة

ساعد التقدم في علم الوراثة الجزيئية في استخدام معلومات المورثات (الجينات) للتحكم ببعض الصفات الوراثية في أنواع مختلفة من الكائنات الحية . فيما يتعلق بالنبات ، فقد اعتمد العلماء على تقنيات علمية حديثة في الوراثة الجزيئية مكنت من فصل الجينات وتحديد الصفات المرتبطة بها ومن ثم هندستها وراثياً بهدف التحكم في كمية البروتينات التي تنتجها وذلك بعد إعادتها إلى خلايا النبات .

في هذه الرسالة قمنا بالتحقق من تأثير مضاعفة كمية الفايتوكروم (مستقبل ضوئي) على صفة تجنب الظل في نبات البندورة ، استخدمنا لهذا الغرض التقنيات الحديثة في علم الوراثة الجزيئية لفصل الجينات الثلاثة والتي رمزنا إليها بالرمز *PHYA* و *PHYB1* و *PHYB2* عرفت هذه الجينات مسبقا بأنها تحدد كمية بروتينات الفايتوكروم والتي رمزنا لها بالرمز phyA و phyB1 و phyB2 بعد فصل الجينات الثلاثة تم تعديلها جينيا بهدف حثها على مضاعفة إنتاج البروتين المرتبط بها عند نقلها لخلايا نباتات البندورة .

إن الهدف الرئيس للبحث المقدم في هذه الرسالة هو محاولة تحديد ما إذا كان التعديل الوراثي في جينات الفايتوكروم والمتوقع منه تعديل ظاهرة تجنب الظل سيؤدي إلى تحسين الإنتاج في نبات البندورة عند إكثارها بكثافة أكبر في الدفيئات ، إضافة لذلك تهدف هذه الدراسة لمحاولة زيادة المعلومات الوراثية والفسيولوجية المتوفرة عن هذه الفايتوكرومات في نمو نبات البندورة وفي مراحل تطورها المختلفة .

يستعرض الفصل الأول من هذه الرسالة أهمية الضوء كمصدر معلومات والتأثير الكبيرلذلك على نمو النبات وتطوره ، علاوة على كونه مصدر الطاقة المستخدم في عملية التمثيل الضوئي حيث تعتمد خلايا النبات على أكثر من نوع من المستقبلات الضوئية التي تزودها بمعلومات عن كمية الضوء ونوعيته واتجاهه وطول فترة وصوله إلى النبات . يعتبر الفايتوكروم من البروتينات الأكثر تشخيصا ودراسة بين هذه المستقبلات الضوئية ، يسمح التركيب الجزيئي للفايتوكروم بامتصاص الأشعة الحمراء من الطيف الضوئي مؤديا ذلك إلى تحول بروتين الفايتوكروم من الشكل غير الفعال إلى الشكل الفعال، فيما يسمح التركيب الفعال للفايتوكروم بامتصاص الأشعة فوق الحمراء في الضوء مؤديا ذلك إلى إبطال مفعول بروتين الفايتوكروم ، إن هذه الظاهرة الفريدة في تحول بروتين الفايتوكروم من الشكل غير الفعال إلى الشكل الفعال وبالعكس اعتمادا على امتصاص الأشعة الحمراء أو الأشعة فوق الحمراء تعطي الفايتوكروم قدرة على قياس نسبة الأشعة الحمراء الى الأشعة فوق الحمراء في الضوء الواصل الى النبتة وحيث أن هذه النسبة تقل في تجمعات النبات الكثيفة نظرا لان أوراق النبات تمتص الأشعة الحمراء فان بروتين الفايتوكروم يزود النبتة بمعلومات دقيقة عن مدى قربها من نباتات أخرى . تحتوي خلايا نبات البندورة على

الإهداء

اهدي هذه الرسالة إلى كل من رسم خطوة للأمام في طريق حياتي .....

إلى نبع العطاء والحنان ..... والدتي الحبيبة

إلى روح والدي وأخي ووالدة زوجتي

إلى شركائي في الحياة:
زوجتي عدوية وأولادي: أسامة ومحمود وأماني وأحمد وحمزة

وإلى إخوتي وأخواتي

بسم الله الرحمن الرحيم

تعديل وراثي في صفة تجنب الظل:
هندسة جينية في نبات البندورة لحث جينات الفايتوكروم
على مضاعفة إنتاج بروتين الفايتوكروم

رسالة دكتوراه مقدمة من:

سعيد صالح هندي "حسين عيد"

جامعة فاخننجن – قسم علوم النبات
مختبرات فسيولوجيا النبات

فاخننجن - هولندا
نيسان /2007

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Said S.H. Husaineid
Date: 23 April 2007
Group: Laporatory of Plant Physiology, Wageninge University

1) Start-up phase	date
► **First presentation of your project**	
Genetic modification of tomato plants, Laboratory of Plant Physiology, Wageningen University	18 December 2001
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Internal course on radiology (molecular biology), Larenstein, Velp, The Netherlands	13-27 February 2002
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Utrecht University, Utrecht, The Netherlands	04 June 2004
► **EPS theme symposia**	
Theme I Symposium 'Developmental Biology of Plants', Leiden University, Leiden, The Netherlands	25 February 2004
► **NWO Lunteren days and other National Platforms**	
Annual meeting of energy efficient program, Plant Breeding, Wageningen University	2001/2002/2003/2004
Meeting on Experimental Plant Sciences, Lunteren, The Netherlands	26-27 March 2001
Meeting on Experimental Plant Sciences, Lunteren, The Netherlands	15-16 April 2002
Meeting on Experimental Plant Sciences, Lunteren, The Netherlands	07-08 April 2003
Meeting on Experimental Plant Sciences, Lunteren, The Netherlands	05-06 April 2004
► **Seminars (series), workshops and symposia**	
Mini-symposium, Utrecht University, Utrecht, The Netherlands	01 October 2003
Seminar series (Frontiers in plant development 2001), Wageningen University	2001
► **Seminar plus**	
► **International symposia and congresses**	
Plant Photobiology meeting, University of Missouri, Columbia, USA	30 May-02 June 2001
7th International Congress of Plant Molecular Biology, Barcelona, Spain	23-28 June 2003
Plant Photobiology meeting, Phylipps-University of Marburg, Marburg, Germany	02-06 September 2003
► **Presentations**	
Oral Presentation (4x): Annual meeting of energy efficient research program	2001/2002/2003/2004
Poster Presentation (4x) at ALW Lunteren	2001/2002/2003/2004
Poster at Plant Photobiology, University of Missouri, Columbia, USA	30 May-02 June 2001
Oral Presentation at the EPS study day, Wageningen University, Wageningen, The Netherlands	02 February 2003
Poster at International Congress of Plant Molecular Biology, Barcelona, Spain	23-28 June 2003
Poster at Plant Photobiology, Phylipps-University of Marburg, Marburg, Germany	02-06 September 2003
Oral presentation at the Theme I Symposium of EPS, Leiden University, Leiden, The Netherlands	25 February 2004
Oral presentation at the meeting on Experimental Plant Sciences, Lunteren, The Netherlands	06 April 2004
► **IAB interview**	
Utrecht University, Utrecht, The Netherlands	04 June 2004
Subtotal Scientific Exposure	*17.6 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
EPS Summerschool (Environmental signalling), Utrecht University, Utrecht, The Netherlands	27-29 Agust, 2001
► **Journal club**	
Literature study of Photobiology group, Laboratory of Plant Physiology, Wageningen	2001-2006
► **Individual research training**	
Subtotal In-Depth Studies	*3.9 credits**

4) Personal development	date
► **Skill training courses**	
Dutch language course, ROC, Wageningen, The Netherlands	2003
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**26,5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*