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MASTER'S THESIS

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Abstract

Protein phosphatase 2A (PP2A) is a serine/threonine-specific phosphatase that regulates diverse signaling events in plants. PP2A consist of three subunits, a catalytic subunit (C), a scaffolding subunit (A), and a regulatory subunit (B). The catalytic subunit C occurs in association with the scaffolding subunit A to make the core enzyme. The A subunit works as a scaffold and is essential for recruiting the regulatory subunit B to form the ABC trimer. Previous studies have shown that a PP2A mutant behaves like a CRY (Cryptochrome) overexpressor/strong signaling mutant with respect to blue light and hypocotyl length. This led to the hypothesis that B´α is part of a PP2A complex that dephosphorylates CRY1. Increased phosphorylation of CRY1 will in theory result in more efficient or longer lasting repression of COP1 (Constitutively photomorphogenic 1), which again gives higher levels of CO (Constans). CO directly binds to and activates FT (Flowering locus T) which is an activator of flowering.

Hypocotyl length of B´α mutants was compared to WT (Wild type) by sowing seeds in ½ MS (Murashige-Skoog) media and exposing them to blue light. Shorter hypocotyls of the B´α mutant in blue light were confirmed in 8 out of 9 experiments.

The flowering phenotype of B´α mutants were investigated by comparing them to WT in 8 h, 12 h, and 16 h day lengths. The flowering experiments with the B´α mutants revealed a "weak" phenotype of early flowering in both short and long days. Though the B´α mutants showed earlier flowering in all experiments, statistical significance was only observed in 12 h and 16 h light when compared to WT.

The possible protein-protein interaction between B´α and CRY1 were analyzed by bimolecular fluorescence complementation (BiFC) assay. B´α and CRY1 were tagged with N- or C-terminal fragments of the fluorescent protein Venus and transiently expressed in *Arabidopsis* mesophyll protoplasts. Fluorescent signals were observed both in combinations of CRY1/ B´α and "empty" vectors, and thereby questioning the specificity of the BiFC assay. However fluorescent signals showing dots/speckles were observed in the combination of CRY1/ B´α and not in control experiments, which indicates protein-protein interaction between CRY1 and B´α.

The flowering and hypocotyl experiments indicate that B´α is involved in the dephosphorylation of CRY1, hence strengthening the working hypothesis. However, more experiments should be done in the future before any firm conclusions are drawn.

Abbreviations

BiFC	Bimolecular fluorescence complementation
CaMV	Cauliflower mosaic virus
CDF1	Cycling dof factor
CO	Constans
COP1	Constitutively photomorphogenic1
CRY1	Cryptochrome1
CRY2	Cryptochrome2
DNF	Day neutral flowering
DSP	Dual specificity phosphatase
FAD	Flavin adenine dinucleotide
FKF1	Flavin-binding Kelch repeat
FT	Flowering locus T
GI	Gigantea
HEAT	Huntingtin elongation A subunit Tor
HY5	Long hypocotyl 5
LB	Luria-Bertani
MS	Murashige-Skoog
MTHF	Methyltetrahydrofolate
NR	Nitrate reductase
PEG	Polyethylene glycol
PP2A	Protein phosphatase 2A
PPM	Metal ion-dependent protein phosphatase
PPP	Phosphoprotein phosphatase
PTP	Phosphotyrosine phosphatase

1 Introduction

1.1 Protein phosphorylation and dephosphorylation

Post translational modifications of proteins as phosphorylation and dephosphorylation often serves as an "on-and-off" switch in the regulation of cellular activities. The *Arabidopsis* genome encodes over 1000 protein kinases and 150 protein phosphatases that catalyze reversible phosphorylation. For optimal regulation in a cell, a balance of kinases and phosphatases is necessary (Kerk et al. 2008; Luan 2003; Meimoun et al. 2007).

Protein kinases transfers phosphate groups from adenosine triphosphate (ATP) to a protein. Many of the molecules in a signal transduction pathway are kinases, and they often act on other protein kinases in the pathway. Protein phosphatases can rapidly remove phosphate groups (dephosphorylation) and thereby inactivating the protein kinases. Phosphatases can also make kinases available for reuse or provide the mechanism for turning off the signal transduction pathway (Campbell et al. 2008).

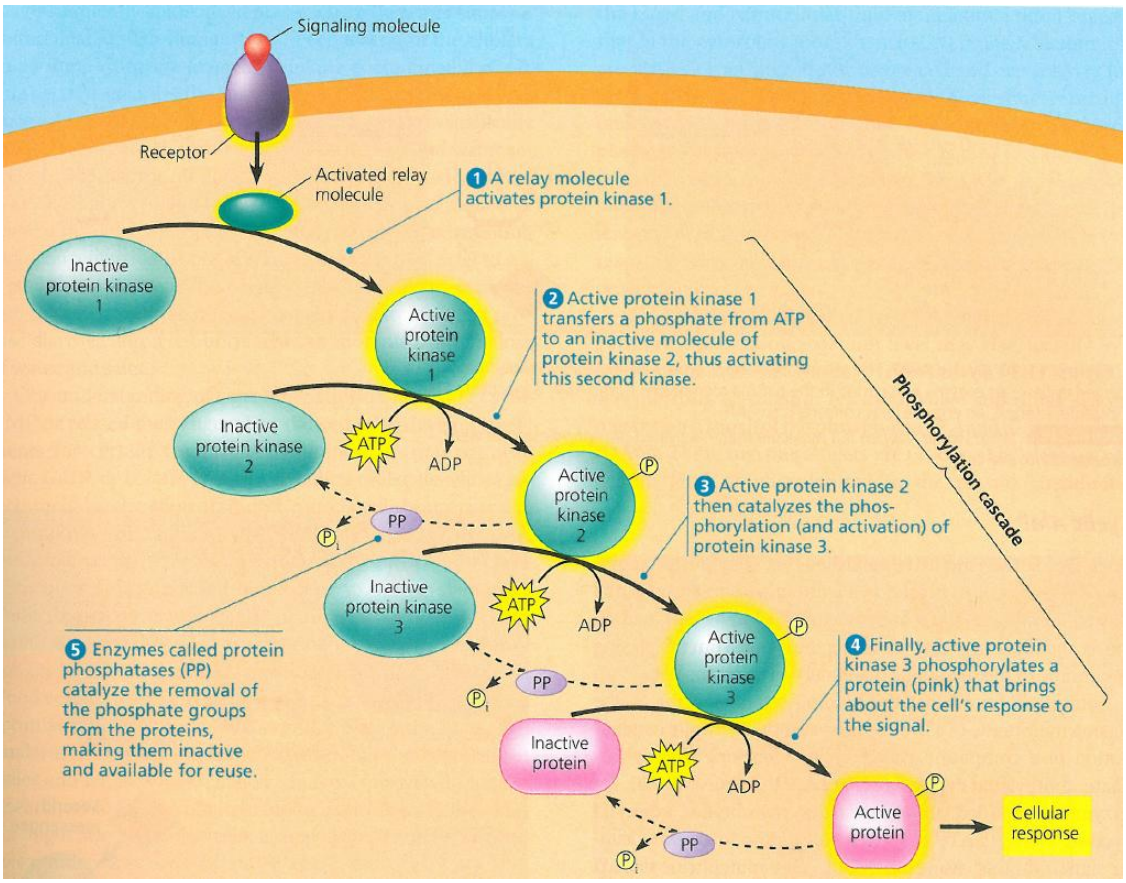


Figure 1.1. Example of a phosphorylation cascade, from signal to cellular response. A series of different molecules in a pathway are phosphorylated in turn, each molecule adding a phosphate group to the next in line. In this example, phosphorylation activates each molecule, dephosphorylation returns it to its inactive form. The figure is obtained from Campbell et al. 2008.

1.2 Protein phosphatases

Protein phosphorylation plays an important role in regulating many cellular processes in eukaryotes by modulating the conformation, activity, localization and stability of substrate proteins. According to their substrate specificity, protein phosphatases are classified into families of Ser/Thr (serine/threonine) specific phosphoprotein phosphatases (PPP), metal ion-dependent protein phosphatase (PPM), phosphotyrosine phosphatase (PTP) and dual specificity phosphatase (DSP) (Farkas et al. 2007; Hunter 1995). Protein phosphatase 2A (PP2A) which is an important subject in this present study, is a group of protein phosphatases found in eukaryotes. PP2A is a part of the 26 genes encoding catalytic subunits of the PPPs in *Arabidopsis*. (Figure 1.2) (Farkas et al. 2007).

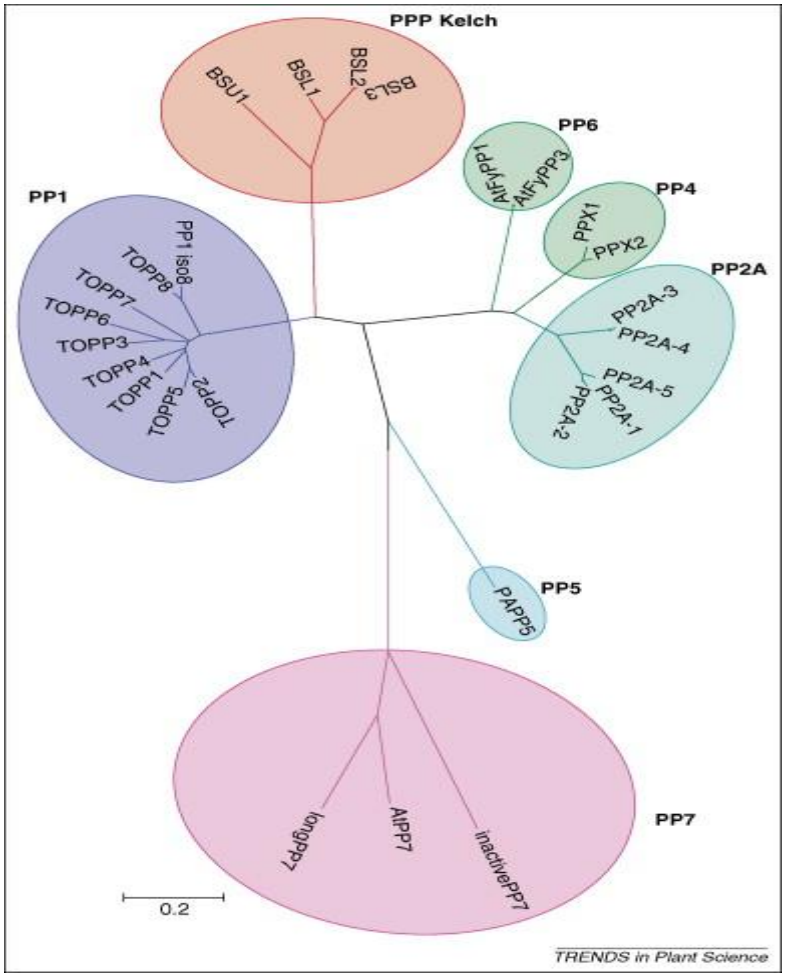


Figure 1.2. Family tree of *Arabidopsis thaliana* phosphoprotein phosphatase (PPP) catalytic subunits (Farkas et al. 2007).

1.2.1 Protein phosphatase 2A

Protein phosphatase 2A (PP2A) is a serine/threonine-specific phosphatase (Farkas et al. 2007; Matre et al. 2009). PP2A regulates diverse signaling events in plants and consist of a catalytic subunit (C), a scaffolding subunit (A), and a regulatory subunit (B) (Jonassen et al. 2011; Trotta et al. 2011). PP2As are heterotrimeric enzymes consisting of the C, A and B subunits. In *Arabidopsis* there are 5, 3 and 18 of these subunits, respectively (Heidari et al. 2011).

The catalytic subunit C (36 kDa) occur either in association with the scaffolding subunit A (65 kDa) to make the core enzyme, or the core enzyme associates with the third subunit B to form the heterotrimeric complex (Farkas et al. 2007; Janssens and Goris 2001).

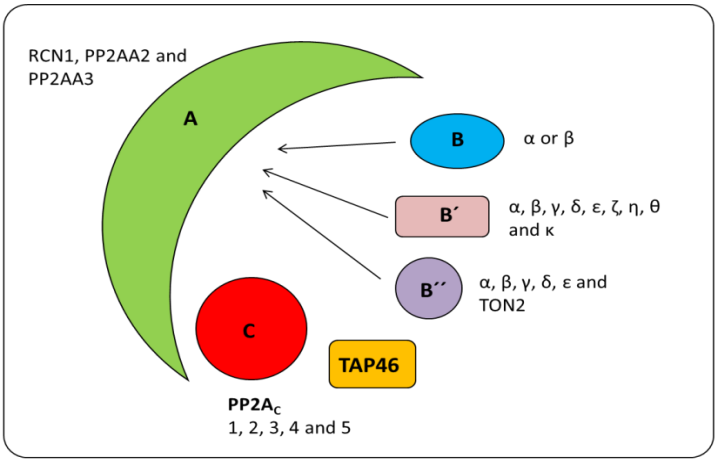


Figure 1.3. Schematic structure of PP2A in *Arabidopsis*. A: scaffolding subunit, C: catalytic subunit and B (including three subfamily B, B´ and B´´): variable regulatory subunits. C subunits are encoded by five genes (PP2AC-1, 2, 3, 4 and 5); A subunits are encoded by three genes (RCN1, PP2AA2, PP2AA3); the B subunits are encoded by two related genes (α and β), the B´ subunits are encoded by nine genes (α, β, γ, δ, ε, ζ, η, θ and κ); the B´´ subunits are encoded by six genes (α, β, γ, δ, ε and TON2) and TAP46 is an unrelated regulatory subunit. Figure 1.3 is based on Janssens and Goris, 2001 and Farkas et al. 2007.

1.2.2 Scaffolding subunits

The A subunits of PP2A works as scaffolds and are composed of tandem HEAT (Huntingtin Elongation A subunit Tor) repeats that form a hook-like architecture that is essential for recruiting one of the many regulatory B subunits to form the ABC trimer (Farkas et al. 2007; van den Ham et al. 2003). The catalytic subunit binds to helices of the HEAT motif through repeat 11 to 15 (Janssens and Goris 2001; Ruediger et al. 1994).

1.2.3 Regulatory subunits

The B regulatory subunits of PP2A are very diverse, and can be classified as a member of the subunit families B, B` and B`` (Terol et al. 2002). In *Arabidopsis*, B subunits are encoded from two genes (α and β), B´ subunits are encoded from nine genes (α, β, γ, δ, ε, ζ, η, θ and κ) and B´´ subunits are encoded from six genes (α, β, γ, δ, ε and TON2) (Farkas et al. 2007; Figure 1.1).

Bα and Bβ have shown to interact with NR (Nitrate Reductase) in bimolecular fluorescence complementation assays and yeast two hybrid assays. A double mutant of Bα × Bβ, appeared to be lethal indicating that the function of these B subunits cannot be replaced by other regulatory subunits (Heidari et al. 2011).

The five B`` subunits encoded in *Arabidopsis* carries putative calcium-binding EF-hand motifs. The TON2 B`` subunit shows similarity to the human B`` subunit PR72 and mutation in TON2 causes abnormalities in microtubule assembly (Farkas et al. 2007).

The B´ subunits can be divided into at least three subfamilies of regulatory subunits in plants, α, η and κ (Terol et al. 2002). B´α which is the main objective in this present study, along with B´β was shown to interact with BZR1, a transcription factor of the brassinosteroid signaling pathway (Tang et al. 2011). Recently, the regulatory subunit B´ γ has been shown to have a specific role as a negative regulator of immune reactions in *Arabidopsis*. Knock-down B´γ mutants show imbalanced antioxidant metabolism and premature disintegration of chloroplasts upon ageing (Trotta et al. 2011).

1.2.4 Catalytic subunits

The PP2A catalytic C subunit is highly conserved among eukaryotes. The activity, specificity and subcellular localization of the C subunits depend on the association of this subunit with different A and B regulatory subunits (Ikehara et al. 2007; Terol et al. 2002). In *Arabidopsis* five genes code for the C subunit and their structure have remained remarkably constant throughout evolution (Farkas et al. 2007; Janssens and Goris 2001).

Recent studies investigating a loss of function mutation of the PP2A C subunit, suggests that PP2A is a negative regulator of the ABA (abscisic acid) pathway, and that silencing of PP2A C subunits activates plant defense responses and localized cell death (He et al. 2004; Pernas et al. 2007).

1.3 Flowering

Plants undergo major physiological change in the transition from vegetative growth to reproductive development, and these mechanisms which regulate the transition to flowering in plants have been the subject of intense physiological study for many years. Five genetically defined pathways have been identified that control flowering and they are influenced by the environment in which the plant is grown as well as the developmental state of the plant (Amasino 1996; Srikanth and Schmid 2011).

1.3.1 Flowering time regulation

Day length is an important factor for flowering, and the quality of the light also plays a significant role in the transition to flowering. Plants detect fluctuations in environmental conditions such as temperature, and some genes are required to regulate flowering time in response to these changes (Coupland 1995; Srikanth and Schmid 2011).

The circadian clock makes it possible for plants to discriminate between long and short days, and then respond to day length by flowering. In *Arabidopsis* there are three photoreceptors which perceive the light signals that regulate the photoperiodic flowering, phytochromes, cryptochromes and phototropins. The phytochromes (phyA and phyB) are red and far red light photoreceptors. PhyB is necessary for early morning repression of CO (Constans), and phyA is a repressor of phyB. Cryptochromes (CRY1 and CRY2) perceive blue/UV-A light, and when active, they bind to COP1 (Constitutively photomorphogenic 1) and represses its function. COP1 marks CO for degradation, hence inhibiting the activation of FT (Flowering locus T) (Putterill et al. 2004; Srikanth and Schmid 2011; Sullivan and Deng 2003). Recent

studies have shown that a CRY1 mutation can lead to hypersensitivity to blue light and early flowering under short day conditions, and a missense mutation of CRY2 shows late flowering (Exner et al. 2010; Shalitin et al. 2003).

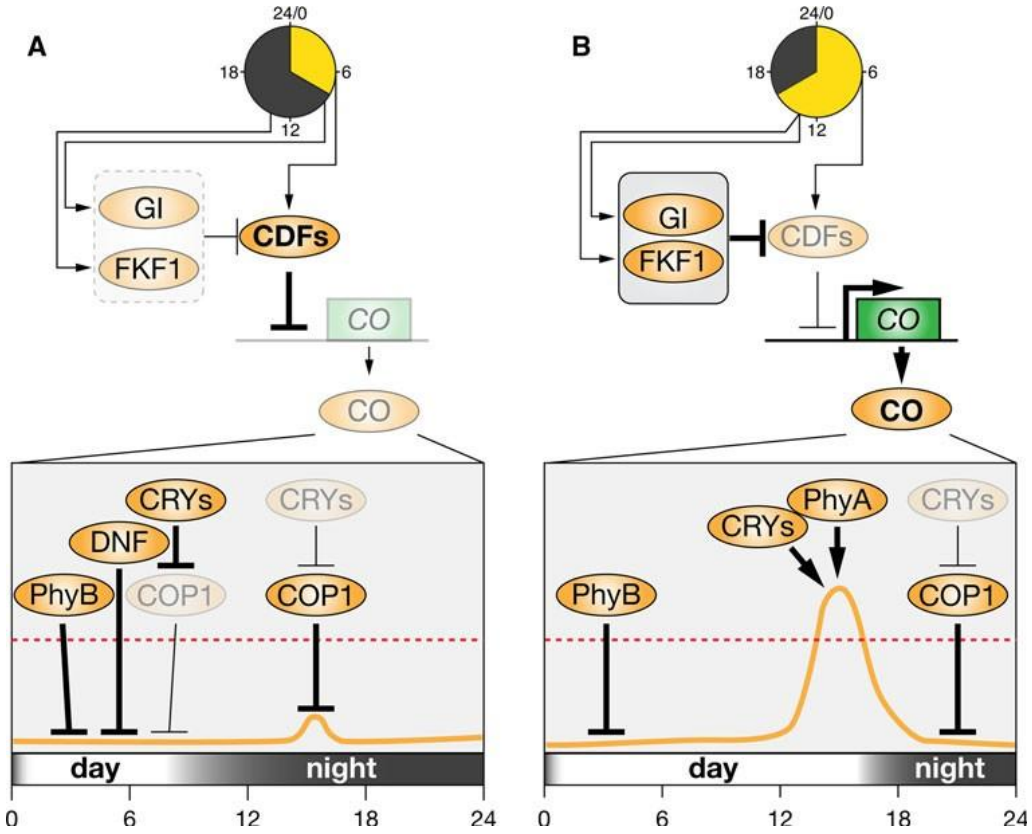


Figure 1.4 A, B. Regulation of CO at a transcriptional and protein level. CO is regulated by the circadian clock and binds to FT (Flowering locus T) which is the activator of flowering. **A:** In short days, GI (Gigantea) and FKF1 (Flavin-binding Kelch repeat) are not able to efficiently repress CDF1 (Cycling Dof factor), a transcriptional inhibitor of CO. CO protein levels are very low to start with in SD (short days) as indicated by the graph. PhyB (phytochrome b) plays an important role for maintaining the low level of CO in the early hours of the day. DNF (Day Neutral Flowering) is important for maintaining low levels of CO between 4 and 7 h after dawn. Active CRY (cryptochrome) protein represses COP1 (Constitutively photomorphogenic 1), a ubiquitin ligase that marks CO for degradation. In the dark, the inactive CRY is no longer able to repress COP1 resulting in almost no CO protein being present. **B:** In long days, both FKF1 and GI peak at approximately 13 h after dawn, resulting in active repression of CDF1, and thereby CO transcription. The protein levels of CO are regulated by phyB in the early morning hours, while active CRY and phyA repress phyB during the rest of the day. Active CRY protein binds to and inhibits transport of COP1 into the nucleus and thereby preventing the ubiquitinating of the CO protein. Genes are represented in green, proteins in orange. Dull colors represent inactive genes/proteins while bold colors indicate active genes/proteins. Dashed box shows weak complex formation, and the grey box shows efficient complex formation. The clock is a 24 h clock. The graph represents expression of CO protein through the day (SD/LD, short day/long day), with the day length represented on the x-axis (Srikanth and Schmid 2011).

1.4 Cryptochromes

Cryptochromes were the first blue light-activated photoreceptors to be characterized and they are found throughout the biological kingdom including plants, animals and humans. The cryptochromes show significant similarity to photolyases, a class of light-dependent DNA repair enzymes. Unlike photolyases, most cryptochromes do not efficiently repair DNA and

instead mediate blue light-dependent developmental, growth, and/or circadian responses (Burney et al. 2009; Smith et al. 2010; Zeugner et al. 2005). Based on sequence similarities, cryptochromes can be classified into three groups: plant cryptochromes, animal cryptochromes and cryptochrome-DASH proteins (named DASH because they are found in *Drosophila*, *Arabidopsis*, *Synechocystis* and *Homo sapiens*) (Smith et al. 2010).

Cryptochromes are flavoproteins with two chromophores that sense blue and UV-A light in plants (Lin and Todo 2005). The two chromophores are flavin adenine dinucleotide (FAD) and pterin methyltetrahydrofolate (MTHF). The mechanism by which blue light triggers photolyase activity is not fully understood, but cryptochromes probably act in a similar way compared to the repair of thymidine dimers in DNA by photolyases. MTHF absorbs blue light and transfers excitation energy to the FAD. Electron transfer from FAD to either a signaling partner protein or to an amino acid residue in the cryptochrome protein itself is then presumed to activate signal transduction (Smith et al. 2010).

The *Arabidopsis* cryptochrome family has three members, CRY1, CRY2 and CRY3 (CRY-DASH). The cryptochrome photoreceptors control deetiolation, entrain the circadian clock, and they are involved in the flowering time control (Exner et al. 2010). CRY1 and CRY2 are closely related, but CRY3 shows less sequence similarity to the other CRYs and its function have not yet been defined (Smith et al. 2010).

1.4.1 Cryptochrome 1

The CRY1 photoreceptor promotes anthocyanin accumulation and suppression of hypocotyl elongation, and synchronizes the circadian clock to the day-night cycle. In the flowering time control, CRY1 together with CRY2 plays a major role by inhibiting the activity of COP1. Recent studies have shown that a mutation in CRY1 leads to hypersensitive CRY1 protein which results in elevated expression of CO and FT leading to very early flowering under SD conditions (Exner et al. 2010; Smith et al. 2010) (Part 1.3.1 and Figure 1.4).

1.4.2 Cryptochrome 2

CRY2 has a smaller role than CRY1 in suppressing hypocotyl elongation in blue light and is important only in very low intensity blue light where maximal photoreceptor sensitivity is required. CRY2 is important in promoting the transition from vegetative growth to flowering, and mutations that weaken CRY2 function delay the transition to flowering (Smith et al. 2010). Recent studies suggest that CRY2 may play a more essential role in the acceleration of flowering under continuous light than in SDs or LDs (Nefissi et al. 2011).

1.5 Bimolecular fluorescence complementation

The specificity of intracellular signaling and developmental patterning in biological systems relies on selective interactions between different proteins in specific cellular compartments. The bimolecular fluorescence complementation (BiFC) has emerged as a powerful technique for the visualization of protein interactions and modifications in living cells (Kerppola 2009; Waadt et al. 2008). The technique is based on the facilitated association of complementary

fragments of a fluorescent protein that are fused to interaction partners. Complex formation by the interaction partners will put the fluorescent protein fragments in proximity to each other, which can facilitate their association (Kerppola 2009).

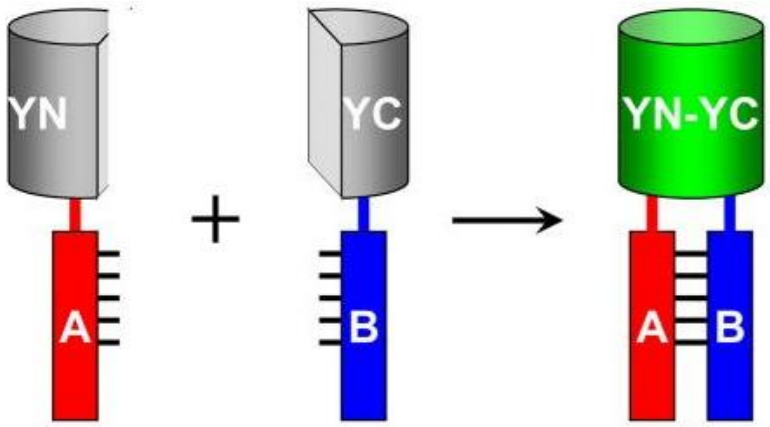


Figure 1.5. Principle of BiFC analysis. The red and blue rectangles represent interaction partners (A and B). They are fused together with a N and C terminal fragment of a fluorescent protein, represented by silver half cylinders (YN and YC). If the proteins interact with each other, they can facilitate association of the fluorescent proteins to produce a fluorescent complex (green cylinder YN-YC). The figure is obtained from Kerppola 2009.

1.6 Hypocotyls

The hypocotyls of the model plant *Arabidopsis thaliana* have been studied extensively through the years. The hypocotyl is the embryonic stem that forms the connection between the two cotyledons and the seedling root. There are approximately 20 epidermal cells in an *Arabidopsis* hypocotyl, and most of them are formed in the embryo. Only a few cell divisions occur in this organ after germination, and the hypocotyl grows mainly through longitudinal cell expansion. The hypocotyl is strongly influenced by external and internal factors, such as light, gravity, temperature and hormones (Vandenbussche et al. 2005).

In the dark the hypocotyls are etiolated and the cotyledons remain closed forming an apical hook. The absence of light will reduce the root growth, and the hypocotyl will elongate strongly (Gendreau et al. 1997).

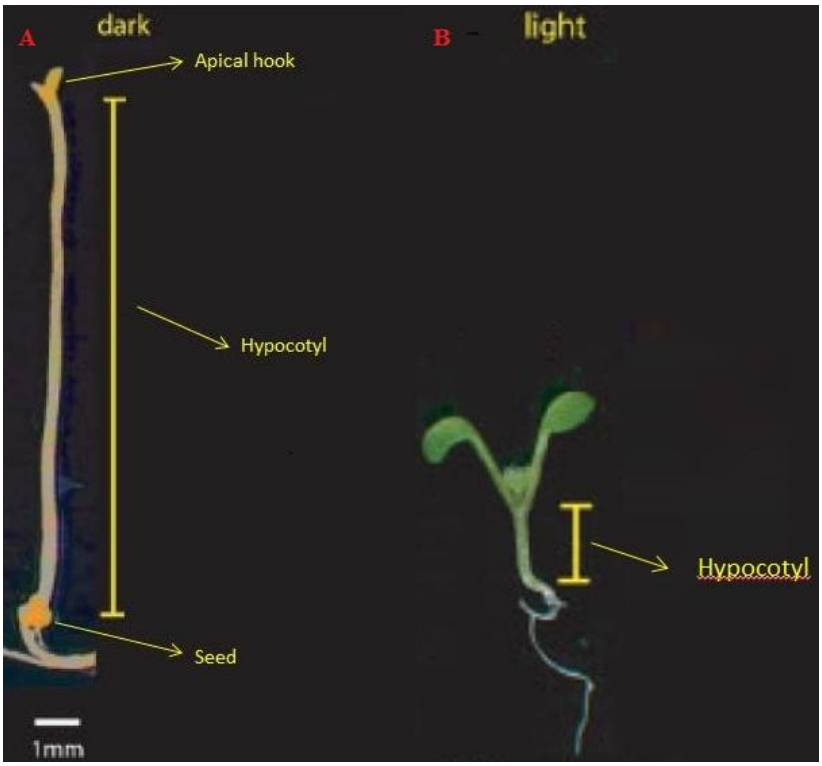


Figure 1.6 A and B. Hypcotyl growth in dark and light. **A:** Upon germination in the dark, the hypocotyl is etiolated resulting in elongated hypocotyl. The cotyledons remain closed, forming an apical hook. **B:** Germination in light induces deetiolation resulting in greening of the hypocotyls. Leaves develop and the hypocotyl growth is strongly inhibited. The figure is obtained from Vandenbussche et al. 2005.

In the light deetiolation is induced and the cotyledons open and expand. Leaves develop and the photosynthetic apparatus is installed. In the presence of light the hypocotyl growth is strongly inhibited. COP1 is known as a negative regulator of photomorphogenesis, and is promoting the degradation of HY5 (Long hypocotyl 5). HY5 acts as a positive regulator of photomorphogenesis and binds to promoters of genes involved in photosynthesis and pigment synthesis. Studies have shown that HY5 mutants have elongated hypocotyls in all wavelengths of light (Holtan et al. 2011; Jonassen et al. 2008).

Three families of photoreceptor that influence hypocotyl elongation have been identified in *Arabidopsis*: The blue/UV-A light receptors cryptochromes, red/far red receptors phytochromes and the blue/UV-A light phototropins. The cryptochromes CRY1 and CRY2 are important during deetiolation and suppression of hypocotyl elongation under blue light. CRY1 plays the prevalent role in response to high light intensities and CRY2 is most important in response to low light irradiance. The phytochromes are important for suppression of hypocotyl elongation in red and far red light. The phototropins together with the hormone auxin are responsible for the bending of hypocotyls in light directional growth (Exner et al. 2010; Fankhauser and Casal 2004; Vandenbussche et al. 2005).

1.7 Objectives of the present study

The hypothesis in the present study is that B`α is a part of a PP2A complex that dephosphorylates CRY1. Introductory experiments have shown that a PP2A mutant behaves

like a CRY overexpressor/strong signaling mutant with respect to blue light (Jonassen et al. unpublished). In this mutant the B subunit B`α is inactivated by a T-DNA insertion mutation, which in theory would lead to increased phosphorylation status of CRY1.

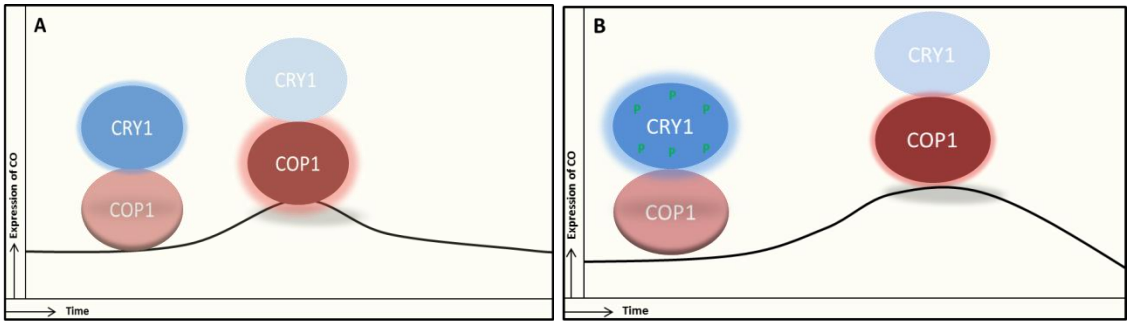


Figure 1.8 A, B. Expression of CO at protein level. **A:** Active CRY1 will repress COP1 which inhibits COP1 to mark CO for degradation. After a given time when CRY1 is no longer active, it will no longer be able to repress COP1 leading to degradation of CO. **B:** More phosphorylated CRY1 protein represses COP1 longer or more efficiently, resulting in a higher level of expressed CO protein. Bold colors represent active proteins, while dull colors represent inactive proteins. P represents an increased phosphorylation status. The graph represents the expression of CO protein through time.

Increased phosphorylation of CRY1 will in theory result in more efficient or longer lasting repression of COP1. Repressed COP1 would lead to enhanced expression of the transcription factor HY5 and many factors promoting photomorphogenesis including short hypocotyls (Jonassen et al. 2008). Furthermore repression of COP1 would lead to higher levels of CO protein. CO directly binds to and activates FT which is an activator of flowering. This suggests that an increase in phosphorylation of CRY1 will lead to earlier flowering and shorter hypocotyls in blue light. The main objectives of the present study were:

1. Confirm that a B`α mutant has short hypocotyls in blue light by sowing seeds in a Petri dish with ½ MS media and expose them to blue light.

2. Investigate if mutation in B`α leads to earlier flowering in different day lengths.

3. Investigate if CRY1 and B`α interact in situ in BiFC assays.

2 Materials and methods

2.1 Materials

2.1.1 Kits

GeneElute™ HP Plasmid Midiprep Kit	(Sigma-Aldrich (St. Louis, MO, USA)
GeneElute™ Plasmid Miniprep Kit	(Sigma-Aldrich (St. Louis, MO, USA)
GeneElute™ Gel Extraction Kit	(Sigma-Aldrich (St. Louis, MO, USA)
GeneElute™ PCR Clean-Up Kit	(Sigma-Aldrich (St. Louis, MO, USA)
GeneElute™ Plant Genomic DNA Miniprep Kit	(Sigma-Aldrich (St. Louis, MO, USA)

2.1.2 Enzymes

HotStarTaq Plus DNA polymerase	(Qiagen (Hilden, Germany)
Pwo SuperYield DNA Polymerase	(Roche Applied Science (Mannheim, Germany)
Restriction enzymes	(New England BioLabs (Ipswich, MA, USA)
T4 DNA Ligase (1U/µl)	(Invitrogen (Carlsbad, CA, USA)

2.1.3 Plant material

In all the experiments in this project *Arabidopsis thaliana* ecotype Columbia was used as wild type plant. The *Arabidopsis* T-DNA mutant lines used in the experiments in this project are listed in table 2.1.

Table 2.1. *Arabidopsis* mutant lines

Gene ID/name	**SALK-line**
AT5G03470 B`α	SALK_077700
AT5G03470 B`α	SALK_077486
AT5G03470 B`α	SALK_052612

Seeds were sown on soil watered with complete Hoagland solution (Hoagland and Arnon 1950), stored in the dark at 4°C for 2-3 days and moved to a controlled plant room. The light/dark cycles in the plant rooms were 16 h light and 8 h dark, 12 h light and 12 h dark and 8 h light and 16 h dark. The plants were placed in the different plant rooms depending on the purpose of the experiment.

Hoagland solution:

KH_2PO_4	1 mM
KNO_3	5 mM
$Ca(NO_3)_2{:}4H_2O$	5 mM
$MgSO_4{:}7H_2O$	2 mM
Fe-EDTA	1 µM
H_3BO_3	46.23 µM
$MnCl_2{:}4H_2O$	9.2 µM
$CuSO_4{:}5H_2O$	0.36 µM
$ZnSO_4{:}7H_2O$	0.77 µM
$Na_2MoO_4{:}H_2O$	0.12 µM

For hypocotyl length experiments seeds were sown on plates with MS medium without sucrose. The plates were stored in the dark at 4°C. After 2-3 days the plates were moved to the light for 2 h at room temperature and then stored in the dark at 4°C overnight. The plates were then placed in either the dark or blue light for 2-4 days.

1/2 MS medium:

Stock solutions:

A: KNO_3	95 g/l
B: NH_4NO_3	120 g/l
C: $MgSO_4{:}7H_2O$	37 g/l
D: KH_2PO_4	17 g/l
E: $CaCl_2{:}2H_2O$	44 g/l

Minor 1:

$ZnSO_4:7H_2O$	0.92 g/l
H_3BO_3	0.62 g/l
$MnSO_4:4H_2O$	2.23 g/l

Minor 2:

$Na_2MoO_42H_2O$	0.025 g/l
$CuSO_4:5H_2O$	0.003 g/l
$CoCl_2:6H_2O$	0.003 g/l
KI	0.083 g/l

½ MS ÷sucrose:

A: 10 ml

B: 6.5 ml

C: 5 ml

D: 10 ml

E: 5 ml

Minor 1: 5 ml

Minor 2: 5 ml

Fe/EDTA: 25 ml

2.1.4 Oligonucleotide primers

For amplification of cDNAs by polymerase chain reaction (PCR), a pair of gene specific oligonucleotide primers with the desired restriction endonuclease sites were designed and ordered from Invitrogen.

Table 2.2. Oligonucleotide primers used in this study. f=forward, r. s=reverse stop and r. ns=reverse nonstop.

Name	**Sequence (5`→3`)**
Primers for cloning into BiFC vectors	
Cry1ct f	AACACTAGTATGGCAATAGAGAACGGATCCGA
Cry1 r. s	TCGCTCGAGTTACCCGGTTTGTGAAAGCCGTC
Cry1 r. ns	TCGCTCGAGCCCGGTTTGTGAAAGCCGTCTCC
B`α f	AACACTAGTATGTTTAAGAAGATCATGAAAGG
B`α r. s	TCGCTCGAGCTAAGAAGTGATCATAGGATCT
B`α r. ns	TCGCTCGAGAGAAGTGATCATAGGATCTTCT
Primers for sequencing	
Kudla-35S f	CCCACTATCCTTCGCAAGAC
Kudla-NosT r	GACCGGCAACAGGATTCAAT
Primers for genotyping	
B`α SALK_077486 f	CAAGCAAAGCCAAAATCATTG
B`α SALK_077486 r	ACCACGAGATGCGTGATTTAC
B`α SALK_077700 f	TCGTGTCTTTGGTTCTGATTTG
B`α SALK_077700 r	AAGGGCCTGTGAACCATAAAC
B`α SALK_052612 f	TTCCACTGAATCCCTCATACG
B`α SALK_052612 r	CTACTGAGGCATCGAGCAATC
Left border primer of T-DNA	ATTTTGCCGATTTCGGAAC

2.2 Methods

2.2.1 DNA isolation

Prior to the DNA isolation, leaves from *Arabidopsis thaliana*; wild type and mutant plants were harvested by cutting leaves with a scalpel and wrapping them in aluminum foil and storing the samples in liquid nitrogen. The DNA was isolated by using GeneElute^{TM} Plant Genomic DNA Miniprep Kit (Sigma-Aldrich (St. Louis, MO, USA).

The plant tissue was ground in liquid nitrogen, transferred to a tube and 350 µl of lysis solution (part A) and 50 µl lysis solution (part B) were added. The tube was then inverted and a vortexer was used to mix the plant tissue. The mixture was then incubated at 65°C for 10 min with an occasional invertion of the tube during the incubation. After the incubation 130 µl precipitation solution was added and the sample was incubated on ice for 5 min and then centrifuged for 5 min at 13000 rpm. The supernatant was transferred to a filtration column and centrifuged for 1 min at 13000 rpm.

A binding column was prepared by adding 500 µl column preparation solution to the column in a collection tube, centrifuging it for 1 min and then discard the flow-through. 700 µl binding solution was added to the flow-through liquid from the filtration column. The binding solution and flow-through liquid was mixed by inverting the tube a couple of times before 700 µl of the mixture was transferred to the prepared binding column. The binding column was

then centrifuged for 1 min at 13000 rpm. The flow-through liquid was discarded and the binding column was transferred to a new collection tube. 500 µl wash solution was added to the column and centrifuged for 1 min at 13000 rpm before the column was transferred to a new collection tube. A second 500 µl of wash solution was added to the column and centrifugation is this time set to 3 min at 13000 rpm. Before elution the column is transferred to a new collection tube and the elution solution is pre warmed to 65°C. After adding the pre warmed elution solution the column was centrifuged for 1 min at 13000 rpm.

The DNA from the plants was isolated for genotyping. The genotyping was done by applying PCR and gel electrophoresis techniques.

2.2.2 Isolation of plasmid DNA

Plasmid DNA was isolated from an overnight recombinant *E. coli* culture using GeneElute™ Plasmid Miniprep Kit (Sigma-Aldrich). 1-5 ml of an overnight culture was centrifuged at 13000 rpm for 1 min. The supernatant was discarded and the pellet was completely resuspended with 200 µl of Resuspension Solution. The resuspended cells were then lysed by adding 200 µl of the Lysis Solution followed by an immediate inversion of the tube to mix the contents. The lysis reaction was stopped before exceeding 5 min by adding 350 µl of the Neutralization Solution. The mixture was mixed by gentle inversion of the tube. The cell debris was then centrifuged at 13000 rpm for 10 min.

A binding column was prepared by adding 500 µl of the Column Preparation Solution to a GenElute Miniprep Binding Column in a collection tube and centrifuging it at 13000 rpm for 1 min. The flow-through liquid was discarded.

The cleared lysate was added to the prepared binding column and centrifuged at 13000 rpm for 1 min. The flow-through was discarded. The column was then washed by adding 750 µl of the Wash Solution and centrifuged at 13000 rpm for 1 min. The flow-through was discarded and the column was centrifuged again to remove any excess ethanol. The DNA was eluted by transferring the column to a fresh collection tube and adding 50 µl of elution solution.

2.2.3 PCR

Polymerase Chain Reaction (PCR) was used throughout the experiment for genotyping, colony and gene cloning PCR. All the DNA amplifications were done using the thermo cycler GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA, USA.

Two DNA polymerases were used in the PCRs through the experiments, Pwo SuperYield DNA Polymerase and HotStar Taq Plus DNA Polymerase. Pwo polymerase was used for gene cloning while Taq polymerase was used for colony PCR and genotyping. Components of the two reactions are listed in table 2.3 and 2.4. Table 2.3 shows the components needed for gene cloning PCR. Table 2.4 shows the components needed for the colony PCR.

Table 2.3. Reaction composition for gene cloning PCR

Component	**Volume, µl**
Sterile double distilled H_2O	17.0
10x Pwo SuperYield PCR buffer with Mg^{2+}	2.5
2.5 mM dNTP	2.0
25 mM $MgCl_2$	1.5
Forward primer	2.0
Reverse primer	2.0
Template DNA	1.5
Pwo SuperYield DNA Polymerase	0.25

In colony PCR, a small amount of an *E. coli* colony was added to sterile double distilled water, and 1 µl of this water was added to the reaction mixture.

Table 2.4. Reaction composition for genotyping and colony PCR

Component	**Volume, µl**
Sterile double distilled H_2O	9.4
10x PCR buffer	2.5
2.5 mM dNTP	2.0
25 mM $MgCl_2$	2.0
Forward primer	1.5
Reverse primer	1.5
Template DNA	1.0
HotStar Taq Plus DNA Polymerase	0.1

2.2.4 Agarose gel electrophoresis

Gel electrophoresis is a technique used to separate molecules, this is done by leading electricity through a gel. How far the molecule migrates in the gel depends on the size of the molecule. The DNA is transferred to small wells at the negative charged end of the gel. The size of the sample is determined by comparing it to a ladder containing DNA fragments with known sizes (Sletten and Lillo 2010). Gel electrophoresis was used to determine the size of DNA after PCR, restriction endonuclease digestions or purifications.

Before loading the sample Crystal 5x DNA Loading Buffer Blue (Bioline) and the fluorescent nucleic acid dye GelRed™ (Biotium) were added to the sample. Electrophoresis was carried out at 90 V in a Power Pac Basic electrophoresis chamber (Bio-Rad) using 1x TAE buffer as running buffer. Following electrophoretic separation, bands containing DNA were visualized with UV-light using BioDoc-It™ Imaging system (UVP, Upland, CA, USA). A DNA ladder, HyperLadder™ I (Bioline) was loaded next to the samples as a DNA size marker.

2.2.5 Bimolecular fluorescence complementation cloning

2.2.5.1 BiFC vectors

Bimolecular fluorescence complementation (BiFC) technique was used to investigate the possible interaction between the proteins B`α and CRY1. The vectors were obtained from University of Münster (Münster, Germany). The BiFC vectors used in this present study are listed in table 2.5.

Table 2.5. BiFC vectors

Nr	Name	Vector	Fluorophore	MCS	Bacterial selection
7	pVYCE(R)	pUC19	$Venus_{C155}$	C of tag	Ampicillin
9	pVYNE(R)	pUC19	$Venus_{N143}$	C of tag	Ampicillin

The BiFC vectors used in this present study are based on the fluorescent protein Venus, and they encode either the N-terminal (pVYNE(R), Figure 2.1) or the C-terminal (pVYCE(R), Figure 2.1) amino acids of the Venus protein. These vectors enable the protein of interest to be fused in the multiple cloning site which is at the C-terminus of the tag. The BiFC cloning cassette is under controlled by the cauliflower mosaic virus (CaMV) 35S promoter and the terminator of the Nos gene (NosT).

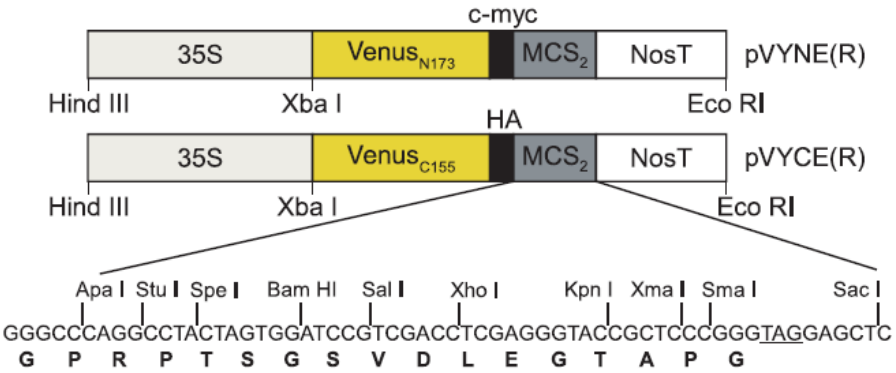


Figure 2.1. Schematic representation of BiFC vectors. pVYNE(R) and pVYCE(R). c-myc, c-myc epitope tag; HA, hemagglutinin epitope tag; 35S, 35S promoter of the cauliflower mosaic virus (CaMV) ; VenusN173, N-terminal fragment of Venus containing amino acids 1–173; VenusC155, C-terminal fragment of Venus containing amino acids 156–239; NosT, terminator of the Nos gene. Restriction sites in the expression cassettes and multiple cloning sites (MCS) are indicated (Waadt et al. 2008).

2.2.5.2 Generation of BiFC constructs

The BiFC constructs were generated by amplifying cDNAs using gene specific primers flanked with SpeI and XhoI restriction site for forward and reverse primers respectively. The PCR products would then contain the restriction sites corresponding to the restriction sites in the BiFC vectors.

Full length cDNA clone of the *Arabidopsis* CRY1 with the gene ID AT4G08920 and stock number U12079 was obtained from Arabidopsis Biological Research Center (ABRC) (Ohio State University, OH, USA).

B`α was amplified from a pWEN18 vector obtained from Polina Matre (Matre 2009).

2.2.5.3 Restriction endonuclease digestion of DNA

Vectors and inserts were prepared for directional cloning by digesting them with restriction endonucleases. Restriction sites introduced to the 5` and 3` ends of the cDNAs by PCR and corresponding multiple cloning sites in the vectors were digested with the same restriction endonucleases. About 1 µg of DNA (PCR product or vector) was mixed with 3 µl NEBuffer 4, 3 µl BSA and 1 µl of each restriction enzyme (XhoI and SpeI). The double digestion was carried out at 37 °C for 3 h.

2.2.5.4 Ligation

The vectors and inserts were cut with the same restriction enzymes which results in complementary cohesive ends. These ends were joined together by T4 DNA Ligase (Invitrogen). The amount of vector and insert used in the ligation was calculated by:

$$3\times\frac{amount\,of\ vector}{size\,of\ vector}=\frac{amount\,of\ insert}{size\,of\ insert}$$

A ligation reaction contained 5 µl 5x Ligase Reaction Buffer, 1 µl T4 DNA Ligase, vector, insert and water to an amount of 20 µl. The ligation mix was gently mixed, left in room temperature for 1 h and left at 4 °C overnight.

2.2.5.5 Transformation of competent *E. coli* cells

Competent cells of *E. coli* JM109 were thawed on ice. 10 µl of the ligation mix was added to 50 µl of the competent cells and mixed gently by pipetting up and down. After incubation on ice for 15 min, the cells were given a heat shock of 42 °C for 90 s. The cells were then left on ice for 10 min. 200 µl of LB medium was then added and the cells were incubated in the shaker at 37 °C for 1 h. The cells were then spread on an agar plate with ampicillin. The plates were incubated at 37 °C overnight.

2.2.6 Isolation of *Arabidopsis* mesophyll protoplasts

The protocol for protoplast isolation and transfection are based on and slightly modified from a method described by (Sheen 2001; Yoo et al. 2007). Ten to 15 leaves of *Arabidopsis thaliana* ecotype Columbia were cut into 0.5 to 1.0 mm thin stripes and put in 10 ml mannitol for 1 h. The mannitol was then substituted with 12 ml of enzyme solution, and the mixture was incubated in the dark overnight. The mixture was then filtered in a nylon mesh into a small falcon tube and centrifuged at 100 g for 5 min. The supernatant was discarded and the pellet was re-suspended in 3 ml of W5-solution before counting the cells under the

microscope using a hemacytometer. The mixture was incubated on ice for 1 h before it was centrifuged at 100 g for 5 min and re-suspended in 2.5 ml of MMg-solution.

Table 2.5. Protoplast isolation and transfection solutions

Solution	Content	Volume	Sterilization
Enzyme solution	150 mg Cellulase (Sigma) 50 mg Pectinase (Sigma) 25 ml 0.8 M Mannitol 1 ml 1 M KCl 10 ml 100 mM MES pH 5.7 13.5 ml dH_2O Dissolve completely (for 45-60 min) at room temperature, and then add: 0.5 ml 1M $CaCl_2$ 50 mg BSA	50 ml	Sterile filter
W5 solution	7.7 ml 5 M NaCl 31.25 ml 1 M $CaCl_2$ 1.25 ml 1 M KCl 5 ml 100 mM MES pH 5.7 204.8 ml dH_2O 5 mM glucose	250 ml	Autoclave
MMg solution	50 ml 0.8 M Mannitol 1.5 ml 1 M $MgCl_2$ 4 ml 100 mM MES pH 5.7	100 ml	Autoclave
PEG solution (40%)	4 g PEG 4000 (Merck) 3 ml dH_2O 2.5 ml 0.8 M Mannitol 1 ml 1M $CaCl_2$	10 ml	No sterilization

2.2.7 DNA-PEG transfection

Plasmid DNA (6 µg) was mixed gently with 200 µl protoplast mix in a small petri dish, and 220 µl PEG-solution was added by carefully dripping it into the plate. The mixture was then incubated for 20-30 min before adding 880 µl W5-solution in the same manner as the PEG-solution. The mixture was transferred to a small falcon tube and centrifuged at 100 g for 1 min, and re-suspended in 1 ml of W5-solution. The protoplasts were incubated in the dark or blue light for 6-48 h before the expression of proteins were examined.

3 Results

3.1 Flowering

To investigate the flowering phenotype of the T-DNA single mutant lines of B´α, flowering in 8, 12 and 16 h day lengths were investigated with WT as a comparison. To assure that early flowering is not caused by increased growth, two growth parameters, number of leaves and diameter of the rosettes were measured until the plants started shooting.

3.1.1 Flowering in 8 h day light

In 8 h day light, flowering time of WT and the T-DNA single mutant line (SALK_077700) of B´α were investigated.

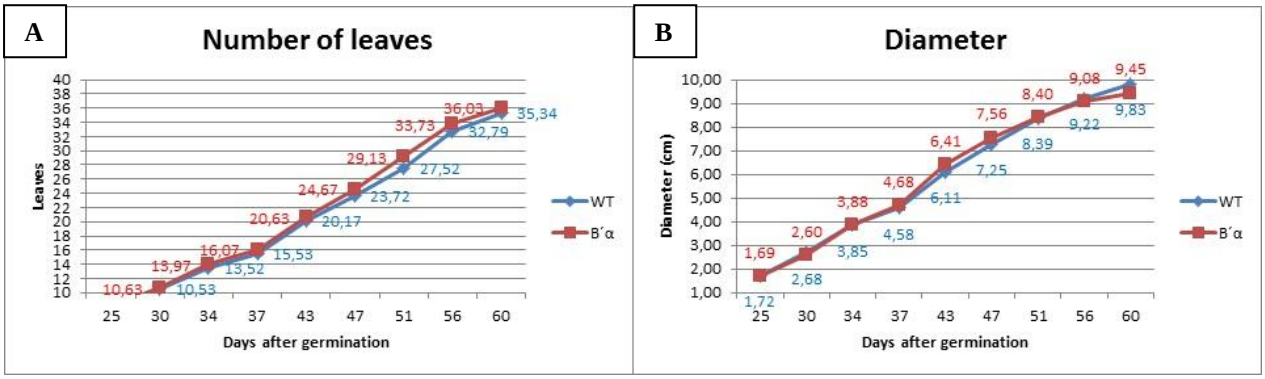


Figure 3.1 A, B. Plant growth in 8 h light. A: Average number of leaves at specified days after germination. B: Average diameter at certain days after germination. n_{WT}=29 $n_{B`\alpha}$=30.

In 8 h day light the WT and the B´α mutant plants showed a similar growth rate in means of number of leaves and diameter. As figure 3.1 shows, the number of leaves and diameter is almost identical through the measurement period.

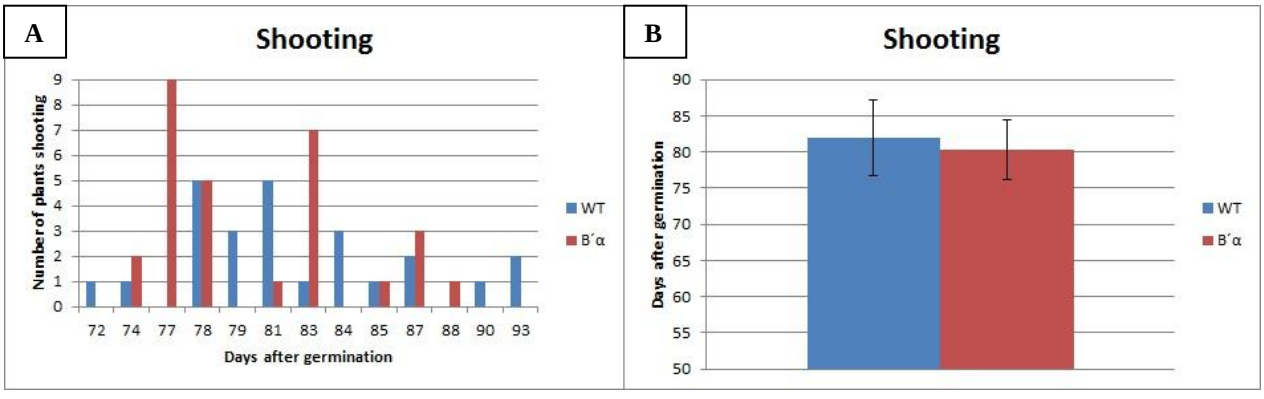


Figure 3.2 A, B. Shooting plants in 8 h light. A: Number of shooting plants at specified days after germination. B: Average day of shooting for WT and B´α. The lines in graph B represents SD. n_{WT}=29 $n_{B`\alpha}$=30.

No significant differences in shooting were observed between the WT and B´α in 8 h light. The first WT plant started shooting at day 72 and the first B´α plant started shooting at day 74 (Figure 3.2 A). Figure 3.2 B shows the average day of shooting in 8 h light, the WT plants

average day of shooting was day 82 after germination and the B´α plants average day of shooting was day 80 after germination.

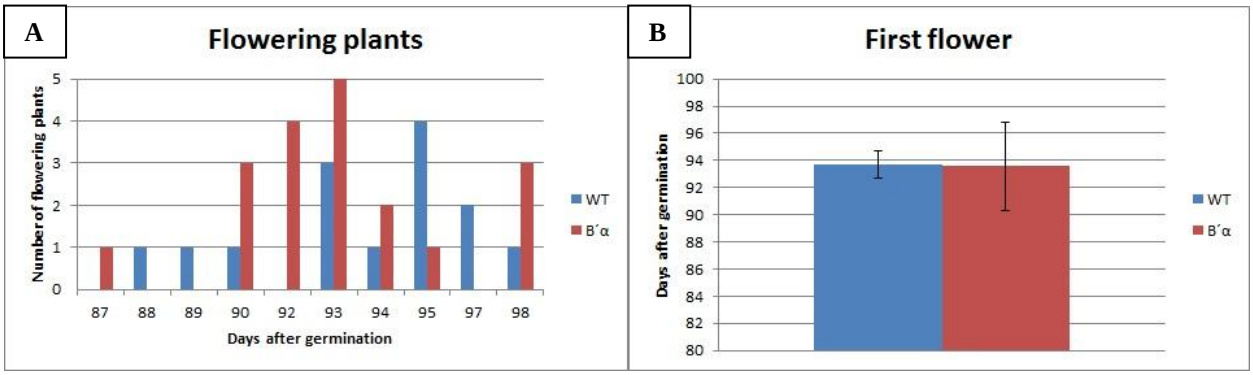


Figure 3.3 A, B. Flowering plants in 8 h light. A: Number of flowering plants at specified days after germination. B: Average day of flowering for WT and B´α. The lines in graph B represents SD. n_{WT}=29 $n_{B`\alpha}$=30.

In 8 h light there were no significant differences in flowering between the WT and the B´α plants. The first B´α plant flowered at day 87 while the first WT plant flowered at day 88 (Figure 3.3 A). The average day of the first observed flower in 8 h light was 93.5 for B´α and 93.7 for the WT (Figure 3.3 B).

3.1.2 Flowering in 12 h day light

In 12 h day light, flowering time of WT and the T-DNA single mutant line (SALK_077700) of B´α were investigated.

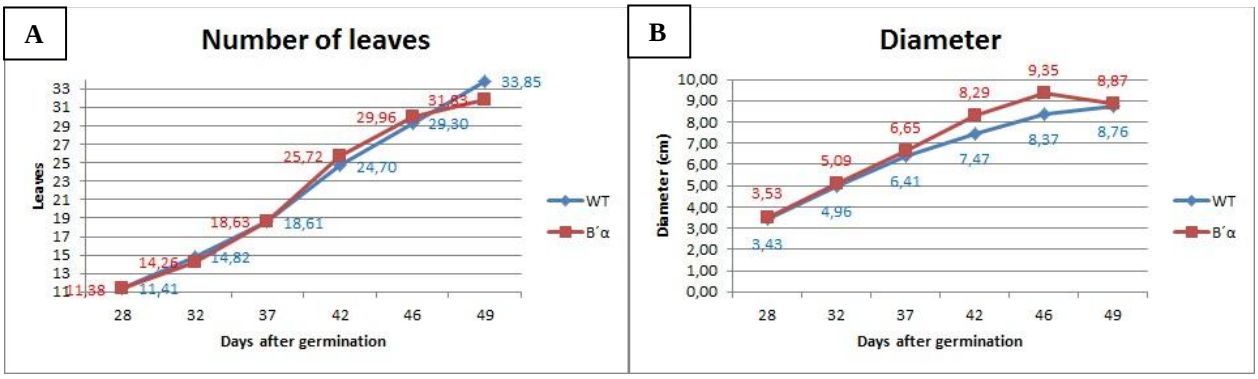


Figure 3.4 A. B. Plant growth in 12 h light. A: Average number of leaves at certain days after germination. B: Average diameter at certain days after germination. n_{WT}=27 $n_{B`\alpha}$=25.

In 12 h light the WT and B´α plants showed a similar growth rate in means of number of leaves. Figure 3.4 shows that the number of leaves is almost identical and the diameter does not differ much through the measurement period.

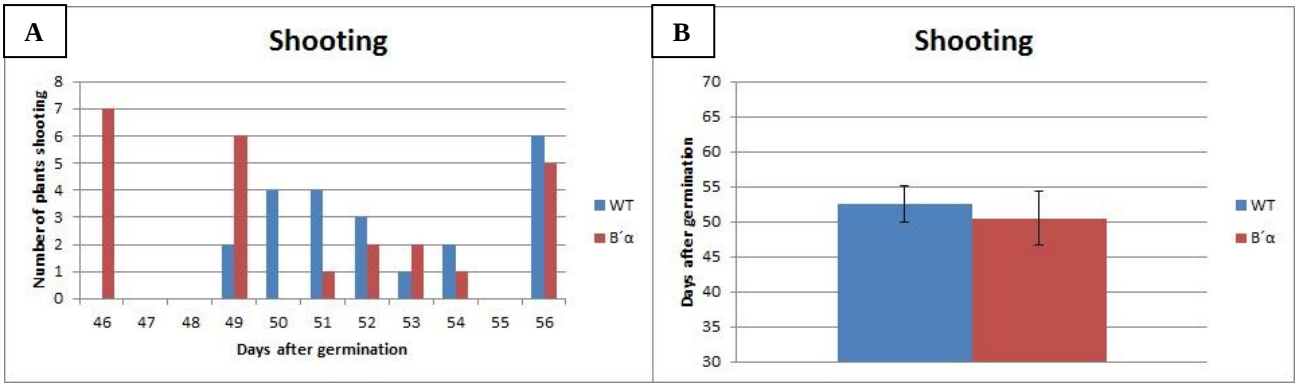


Figure 3.5 A, B. Shooting plants in 12 h light. A: Number of shooting plants at specific days after germination. B: Average day of shooting for WT and B´α. The lines in graph B represents SD. n_{WT}=27 $n_{B`\alpha}$=25.

A significant difference in means of shooting between WT and B´α was observed in 12 h light. As figure 3.5 A shows, the B´α plants started shooting at day 46. At this day, seven B´α plants (28%) had a stem length of 0.5 cm. At day 49, there were made observations of two, (7.4%) of the WT plants shooting, while thirteen (52%) B´α plants were shooting at this day.

Figure 3.5 B shows the average day of shooting in 12 h light. The average day of shooting for the WT and B´α plants was 52 and 50 days after germination respectively.

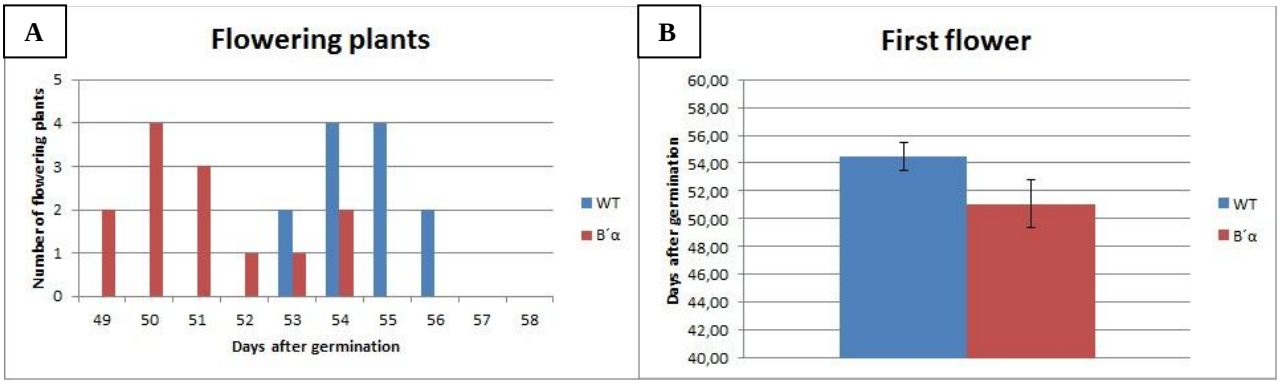


Figure 3.6 A, B. Flowering plants in 12 h light. A: Number of flowering plants at specific days after germination. B: Average day of flowering for WT and B´α. The lines in graph B represents SD. n_{WT}=27 $n_{B`\alpha}$=25.

A significant difference in flowering between WT and B´α was observed in 12 h light. The first flower of the WT and B´α plants was observed at day 53 and 49 respectively. When two (7.4%) WT plants flowered at day 53, ten (40%) of the B´α plants had already produced their first flower (Figure 3.6 A). The average day of the first flower in 12 h light was day 54 for the WT plants and day 51 for the B´α plants (Figure 3.6 B).

3.1.3 Flowering in 16 h day light

In 16 h day light, flowering time of WT and the T-DNA single mutant lines (SALK_077700 and SALK_077486) of B´α were investigated.

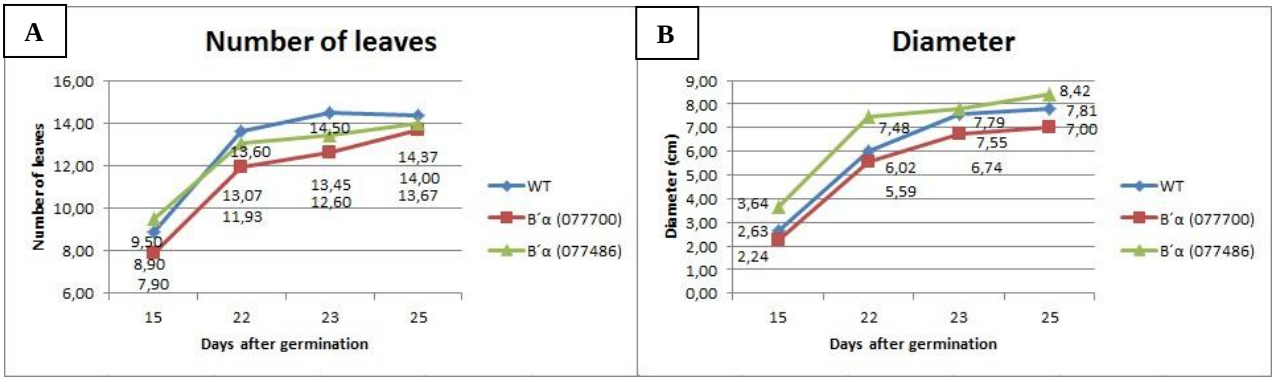


Figure 3.7 A, B. Plant growth in 16 h light. A: Average number of leaves at certain days after germination. B: Average diameter at certain days after germination. n_{WT}=27 $n_{B`\alpha(SALK\_077700)}$=22 $n_{B`\alpha(SALK\_077486)}$=29.

In 16 h light the WT and B´α plants showed a similar growth rate in means of number of leaves. Figure 3.7 A shows that the average number of leaves is almost identical and do not differ with more than 1.9 leaves through the measurement period. As figure 3.7 B shows, the difference in diameter between the plants are slightly different.

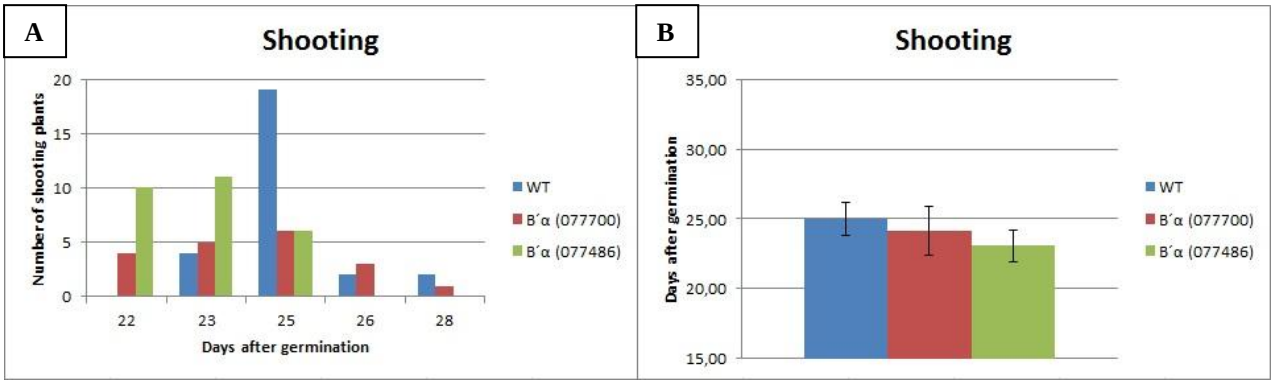


Figure 3.8 A, B. Shooting plants in 16 h light. A: Number of shooting plants at certain days after germination. B: Average day of shooting for WT, B´α (SALK_077700) and B´α (SALK_077486). The lines in graph B represents SD. n_{WT}=27 $n_{B`\alpha(SALK\_077700)}$=22 $n_{B`\alpha(SALK\_077486)}$=29.

A significant difference in means of shooting between WT and B´α (SALK_077486) was observed in 16 h light. As figure 3.8 A shows, ten (34.5%) of the B´α (SALK_077486) plants started shooting at day 22. When four (14.8%) of the WT plants started shooting at day 23, 21 (72.4%) of the B´α (SALK_077486) plants were shooting at this day.

Figure 3.8 B shows the average day of shooting in 16 h light. The average day of shooting for WT, B´α (SALK_077700) and B´α (SALK_077486) was 25.00, 24.16 and 23.07 respectively.

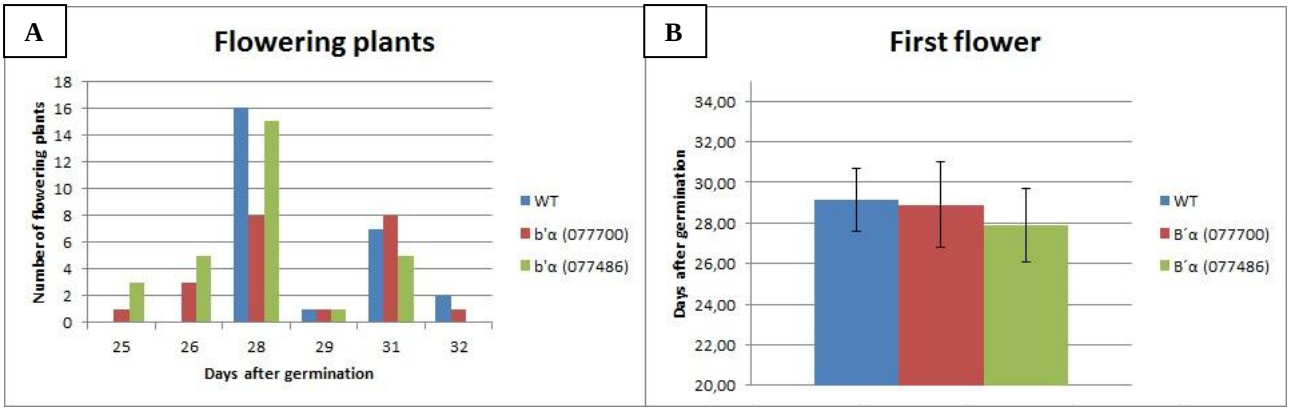


Figure 3.9 A, B. Flowering plants in 16 h light. A: Number of flowering plants at a certain day after germination. B: Average day of flowering for WT, B´α (SALK_077700) and B´α (SALK_077486). The lines in graph B represents SD. n_{WT}=27 $n_{B`\alpha(SALK\_077700)}$=22 $n_{B`\alpha(SALK\_077486)}$=29.

A significant difference in flowering between WT and B´α (SALK_077486) was observed in 16 h light. The first flower of WT and B´α (SALK_077486) was observed at day 28 and 25 respectively. 8 (27.5%) of the B´α (SALK_077486) plants produced their first flower before the first WT flower was observed (figure 3.8 A). The average day of the first flower in 16 h light for WT, B´α (SALK_077700) and B´α (SALK_077486) was 29.15, 28.91 and 27.90 respectively (Figure 3.9 B).

3.1.4 Flowering phenotype of *Arabidopsis* B´α mutants

Statistical significance in flowering between the WT and the B´α mutant plants was calculated by using Mann Whitney U-test. This test will compare two data sets and tell if the difference in the two data sets is significantly different from each other. The Mann Whitney test was performed by comparing the days each plant produced its first flower.

Figure 3.10 A, B. Early flowering phenotype of B´α mutant in 12 h light. A: WT. B: B´α (SALK_077700). Picture was taken at day 51 after germination.

In 12 h light there a significant difference in flowering was observed. The first B´α (SALK_077700) flower appeared 3.42 days before the first WT flower. As figure 3.10 shows, there are several B´α (SALK_077700) plants flowering at day 51 after germination while no WT plants are flowering at this day.

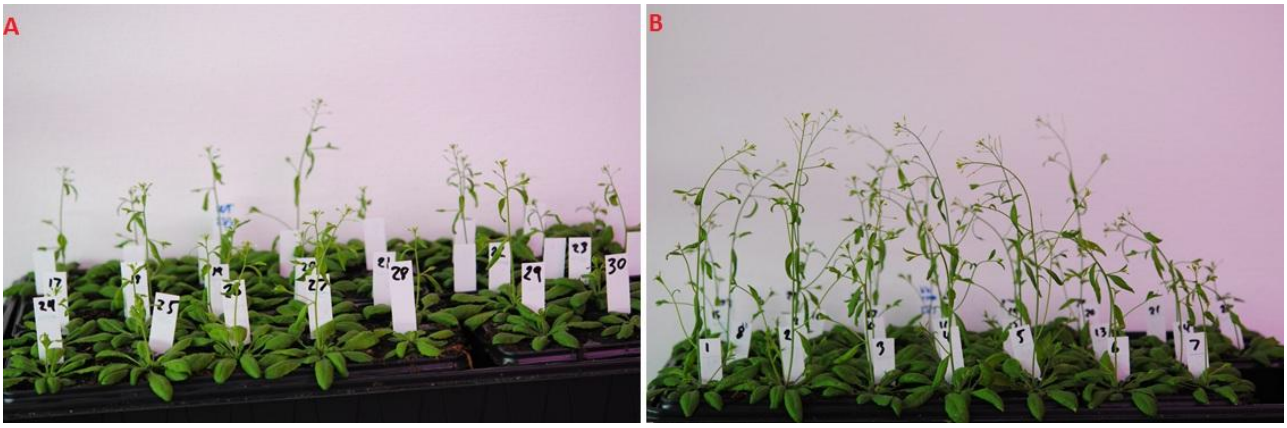


Figure 3.11 A, B. Early flowering phenotype of B´α mutant in 16 h light. A: WT. B: B´α (SALK_077486). Picture was taken at day 29 after germination.

A significant difference in flowering between WT and B´α (SALK_077486) was observed in 16 h light. The first B´α (SALK_077486) flower appeared 1.25 days before the first WT flower. At day 29 after germination, 82.7% of the B´α (SALK_077486) plants were flowering while 66.6% of the WT plants were flowering at this day (Figure 3.11).

Table 3.1. Average day of shooting and flowering for WT and the B´α plants in different day lengths. Green numbers represents statistically significant difference compared to WT. Red numbers represent statistically insignificant difference compared to WT.

Day length ⇨	**8 h**		**12 h**		**16 h**	
Plants ⇩	**Shooting**	**Flowering**	**Shooting**	**Flowering**	**Shooting**	**Flowering**
WT	81.92±5.22	93.71±3.00	52.50±2.56	54.50±1.00	25.00±1.18	29.15±1.57
B´α (SALK_077700)	80.24±4.10	93.57±3.26	50.46±3.82	51.08±1.71	24.16±1.74	28.91±2.09
B´α (SALK_077486)					23.07±1.14	27.90±1.82

In all the flowering experiments in the different day lengths, the B´α mutants show earlier shooting and flowering. Statistically significant differences between WT and B´α mutants were only observed in 12 and 16 h light (Table 3.1). The B´α (SALK_077486) was not available or not yet received during the experiments in 8 and 12 h light.

3.2 Bimolecular fluorescence complementation analysis

3.2.1 Generation of BiFC constructs

To investigate a possible protein-protein interaction between CRY1 and B´α, constructs of BiFC-tagged versions of the two proteins were generated. The C terminal part of CRY1 was amplified from the full length cDNA of the *Arabidopsis* CRY1 which was obtained from Arabidopsis Biological Research Center (ABRC) at Ohio State University. B´α was amplified from a pWEN18 vector obtained from Polina Matre (Matre 2009). The BiFC vectors (described in section 2.2.5.1 in Materials and methods) were obtained from the University of Münster.

The cDNAs were amplified using gene specific primers flanked with SpeI and XhoI restriction site for forward and reverse primers respectively. The resulting PCR products would then contain additional DNA sequences corresponding to the unique SpeI and XhoI restriction endonuclease sites in the multiple cloning sites of the BiFC vectors. The PCR products were analyzed by agarose gel electrophoresis to verify successful amplification (Figure 3.12). The sizes of the PCR products were correct (theoretical sizes: CRY1 c-terminal part 546 bp and B´α 1488 bp.

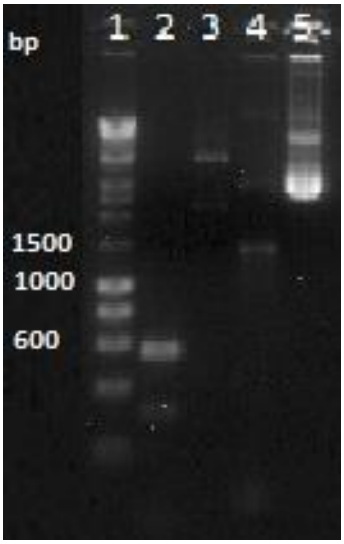


Figure 3.12. PCR products for CRY1 c-terminal part and B´α. 1: Hyperladder I. 2: PCR product of CRY1 c-terminal part. 3: CRY1 c-terminal part in plasmid. 4: PCR product of B´α. 5: B´α in plasmid.

The PCR products and BiFC vectors were purified using GenElute PCR Clean-Up Kit and GeneElute Gel Extraction Kit. The purified PCR products and BiFC vectors were then double digested with the same restriction endonucleases before ligation. After ligation, the recombinant plasmids were transformed into competent cells of *E. coli* for replication. A colony PCR using vector and gene specific primers was performed followed by analysis by agarose gel electrophoresis to screen for positive transformants among the *E. coli* colonies.

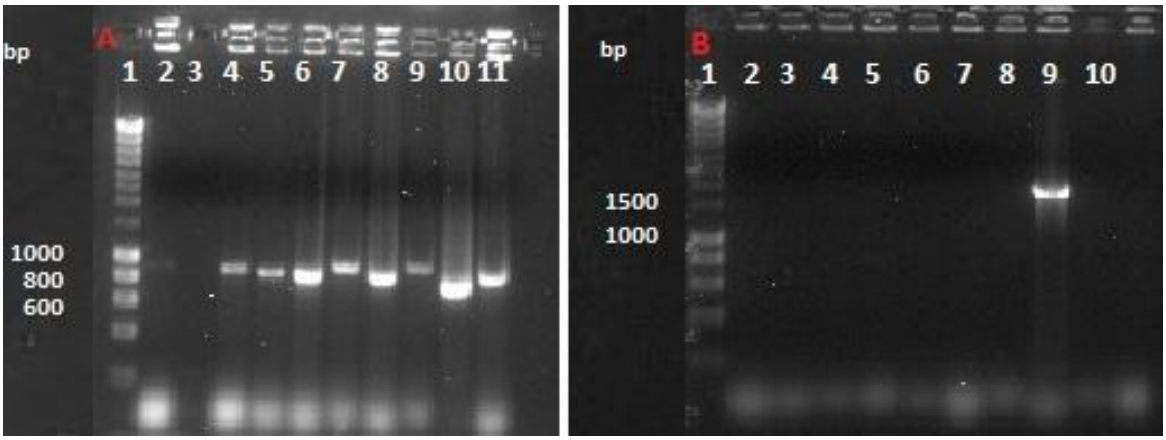


Figure 3.13 A,B. Colony PCR with vector specific primer Kudla-35Sf and the gene specific reverse primer. A: Colony PCR of pVYCE(R)-CRY1 c-terminal part. Well 4,5,6,7,8,9,10 and 11 show positive colonies containing CRY1 c-terminal part inserted in the vector. B: Colony PCR of pVYNE(R)-B´α. Well 9 show positive colony containing B´α inserted in the vector.

The plasmids was isolated using GeneElute Plasmid HP Midiprep Kit to have high amounts of plasmids for the upcoming BiFC assays. To verify that the insert was present in the BiFC vector,

3.2.2 Visualization of protein-protein interactions

After generation of the BiFC constructs, the combination of pVYCE(R)-CRY1 c-terminal part/PVYNE(R)-B´α was co-transformed into *Arabidopsis* mesophyll protoplast to express the fusion genes. The combination of pVYCE(R)-pVYNE(R) was also co-transformed as a negative control.

Fluorescence microscopy analysis of pVYCE(R)-CRY1 c-terminal part combined with pVYNE(R)-B´α revealed fluorescence which indicates protein-protein interaction. Two different fluorescent signals in the combination of pVYCE(R)-CRY1 c-terminal part and pVYNE(R)-B´α were detected (Figure 3.14 A and B). The signal showing dots/speckles (Figure 3.14 A), was detected in a few number of cells. The recurring signal in most transformed protoplast is shown in figure 3.14 B.

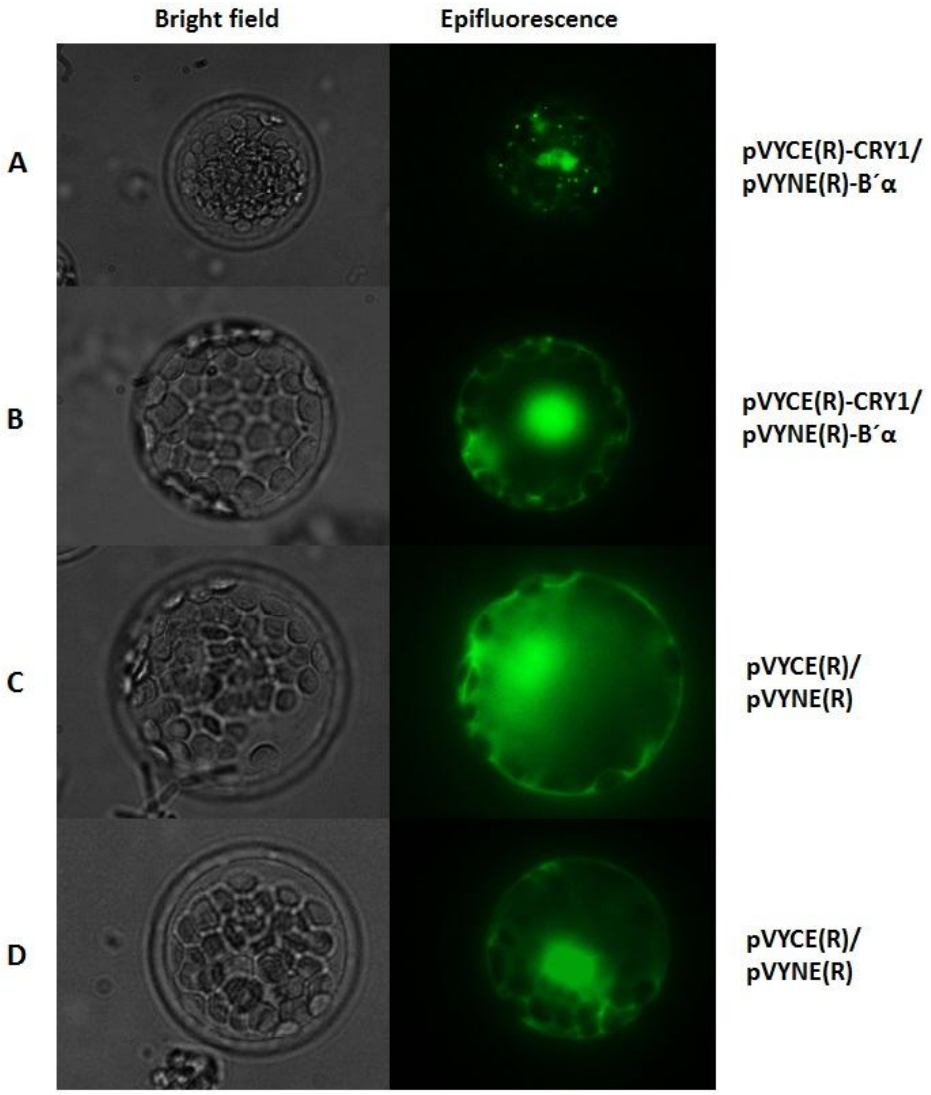
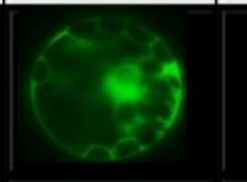
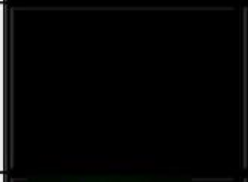
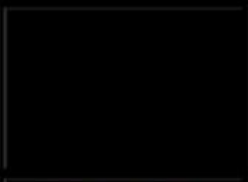
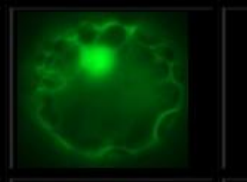
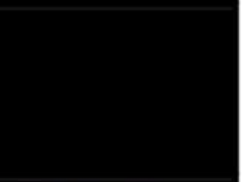
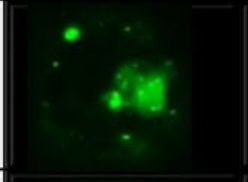
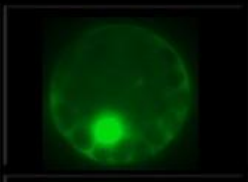
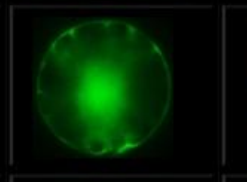
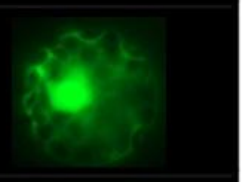
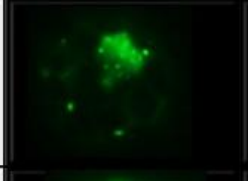
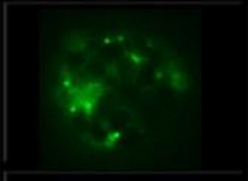
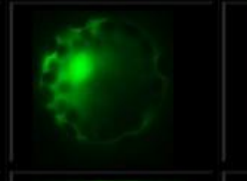
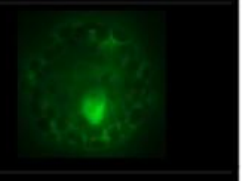
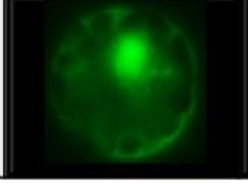
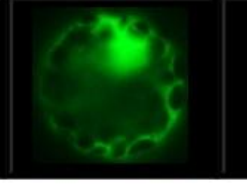


Figure 3.14. Interaction of CRY1 c-terminal part with B´α examined in *Arabidopsis* mesophyll protoplasts. **(A)** co-expression of CRY1 c-terminal part and B´α. **(B)** co-expression of CRY1 c-terminal part and B´α. **(C)** co-expression of empty vectors pVYCE(R) and pVYNE(R). **(D)** co-expression of empty vectors pVYCE(R) and pVYNE(R).

The negative control consisting of the empty vectors pVYCE(R)/pVYNE(R), revealed more fluorescent protoplast throughout all experiments. These signals (shown in figure 3.14 C and D) are similar or very much alike the signals detected in the samples of pVYCE(R)-CRY1 c-terminal part/PVYNE(R)-B´α, with the exception of the signals showing dots/speckles. No experiments revealed the fluorescent signal showing dots/speckles in the negative control, indicating that this particular signal could be the "real" signal for protein-protein interaction between CRY1 and B´α.

Table 3.2. Fluorescent signals observed when the transformed *Arabidopsis* mesophyll protoplasts are incubated in dark and blue light for 6 to 48 h.

Incubation ➡		Dark	Blue light	Dark	Blue light
Hours ⬇	Sample ➡	CRY1/B´α	CRY1/B´α	pVYCE(R)/ pVYNE(R)	pVYCE(R)/ pVYNE(R)
6					
12					
24					
36					
48					

Co-transformation of the empty vectors pVYCE(R)/pVYNE(R) revealed fluorescent signals in every sample incubated in the dark. When incubated in blue light, fluorescence was detected in the samples incubated for 24 and 36 h. Co-transformation of pVYCE(R)-CRY1 c-terminal part/PVYNE(R)-B´α revealed fluorescent signals when incubated in the dark for 24, 36 and 48 h, and after 24 and 36 h when incubated in blue light. The most recurring signal observed in the samples of pVYCE(R)-CRY1 c-terminal part/PVYNE(R)-B´α was very much alike or identical to the signals observed in the negative controls. The fluorescent signals showing dots/speckles was only observed in the samples of pVYCE(R)-CRY1 c-terminal part/PVYNE(R)-B´α incubated in the dark for 24 and 36 h, and when incubated in blue light for 36 h (Table 3.2).

The BiFC experiments were successfully repeated ten times, but the most frequent parameters used for incubation were darkness for 24-36 h where most transformed protoplast was observed. Table 3.2 gives a representative picture of the fluorescent signals observed in the experiments.

3.3 Hypocotyl length experiments

To investigate the hypothesis that an increased phosphorylation status of CRY1 would lead to shorter hypocotyls in blue light, seeds of B´α T-DNA single mutant lines (SALK_077700) or (SALK_077486) were sown together with WT on plates with ½ MS without sucrose.

To assure that the difference in growth was caused by the light and not by difference in germination, the seeds were stored at 4ºC in the dark. After 2-3 days the seeds were moved to the light for 2 h at room temperature, and then stored in the dark at 4ºC overnight. The seeds were then placed in blue light for 2-4 days before measuring the hypocotyl length. This treatment induces simultaneous germination, but differences in the germination between B´α and WT were observed throughout the experiments. To account for the difference in germination, seeds of WT and B´α were put in the dark as a control to the blue light experiment.

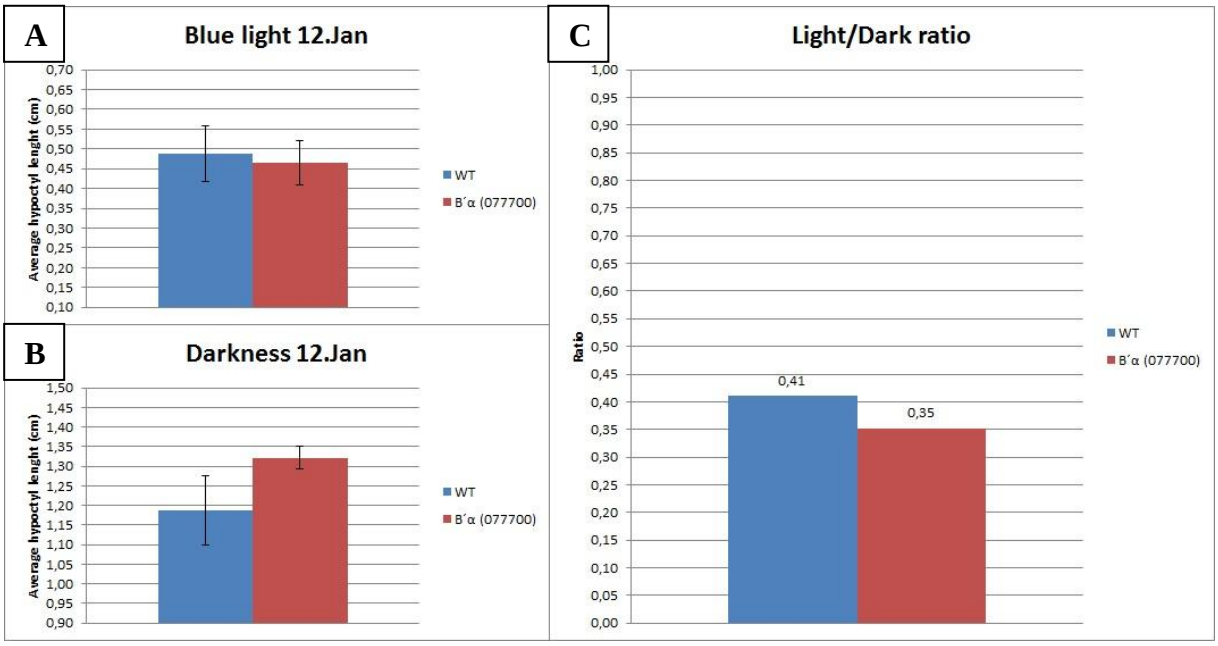


Figure 3.15 A, B and C. Hypocotyl length experiment 12 Jan. for B´α (SALK_077700). **A and B:** Average hypocotyl length from 3 plates in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077700). The lines in graph A and B represent the SE for the average hypocotyl length from the 3 plates.

In the hypocotyl experiment done 12 Jan, the B´α (SALK_077700) hypocotyls were marginally shorter than the WT hypocotyls (Figure 3.15 A). By comparing the hypocotyls in blue light with the growth in darkness, a blue light effect on the B´α (SALK_077700) hypocotyls was observed. As figure 3.5 C shows, the length of the B´α (SALK_077700) hypocotyls were 35% in blue light compared with the growth in darkness. The WT hypocotyls had a length of 41% in blue light compared with the growth in darkness.

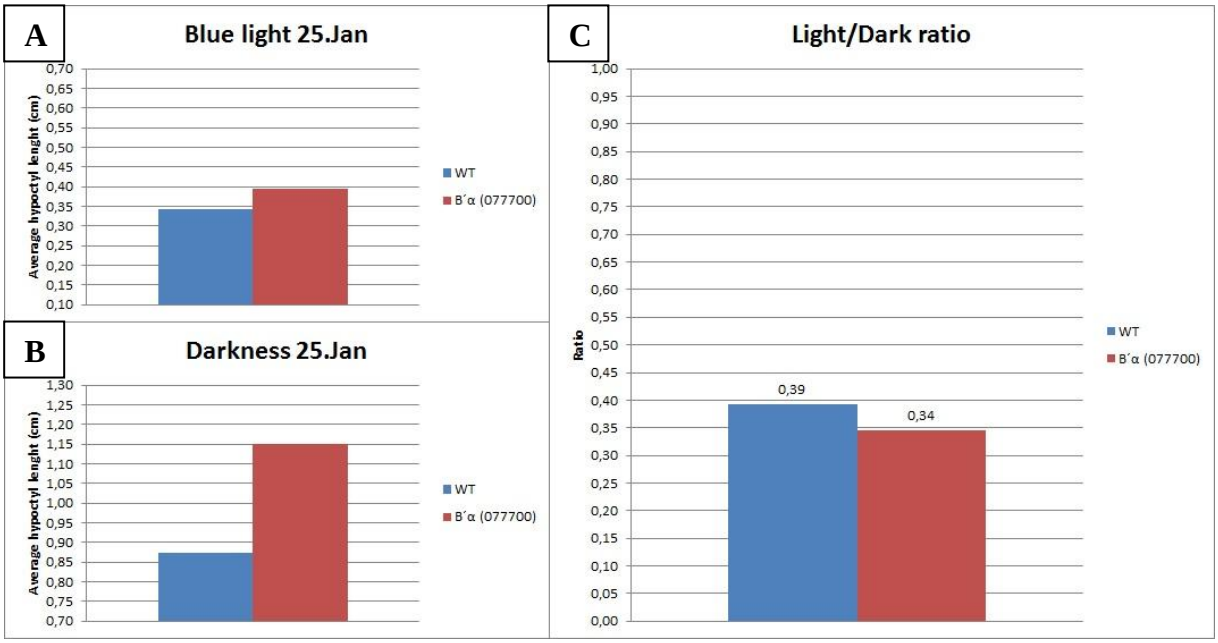


Figure 3.16 A, B and C. Hypocotyl length experiment 25 Jan. for B´α (SALK_077700). **A and B:** Hypocotyl length from 1 plate in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077700).

Hypocotyl experiment from 25 Jan. showed that B´α (SALK_077700) hypocotyls were longer than WT in blue light (Figure 3.16 A). The hypocotyls of B´α (SALK_077700) were also longer in the dark compared with WT, which shows that the B´α (SALK_077700) hypocotyls were 5% shorter than WT when the growth in blue light and dark is compared (Figure 3.16 B and C).

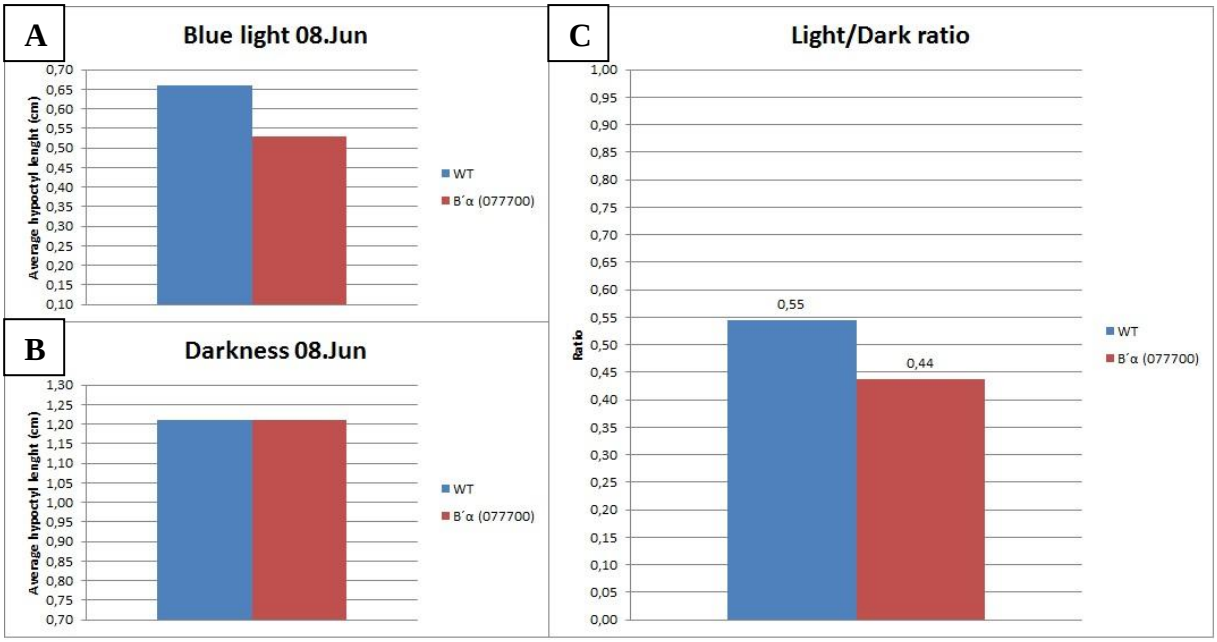


Figure 3.17 A, B and C. Hypocotyl length experiment 08 Jun. for B´α (SALK_077700). **A and B:** Hypocotyl length from 1 plate in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077700).

Hypocotyl length experiment from 08 Jun. revealed shorter B´α (SALK_077700) hypocotyls than WT in blue light. The length of the hypocotyls in dark were identical, resulting in 11 % shorter B´α (SALK_077700) hypocotyls when the growth in blue light and darkness is compared (Figure 3.17).

All hypocotyl length experiments with B´α (SALK_077700) revealed a shortening of the hypocotyls in blue light compared to WT. The highest light/dark ratio difference between B´α (SALK_077700) and WT was observed in the experiment done 08 Jun, where the WT ratio was 0.11 higher than B´α (SALK_077700). The lowest light/dark ratio difference between B´α (SALK_077700) and WT was observed in the experiment done 25 Jan, where the WT ratio was 0.5 higher than B´α (SALK_077700). The average light/dark ratio for B´α (SALK_077700) and WT was 0.38±0.03 and 0.45±0.05 respectively (Table 3.3).

Table 3.3. Light/Dark ratios for average hypocotyl length in blue light and darkness for B´α (SALK_077700) and WT.

Light/Dark ratio:	**B´α (SALK_077700)**	**WT**
12 Jan.	0.35	0.41
25 Jan.	0.34	0.39
08 Jun.	0.44	0.55
Average:	0.38	0.45
SE:	0.03	0.05

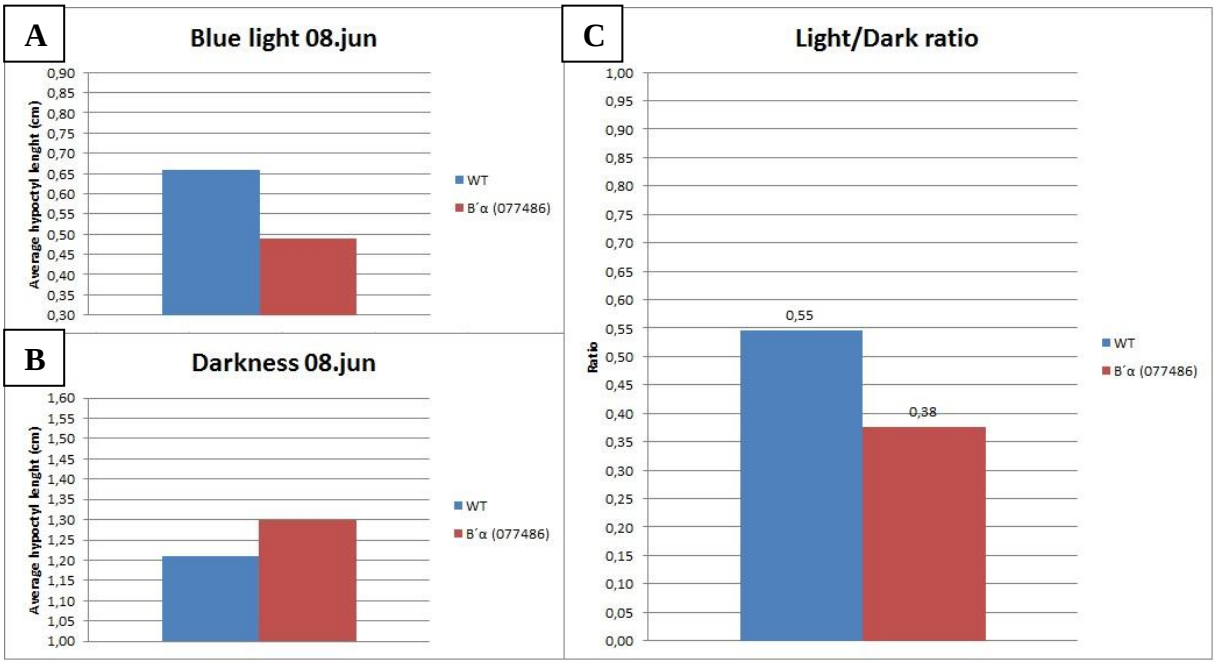


Figure 3.18 A, B and C. Hypocotyl length experiment 08 Jun. for B´α (SALK_077486). **A and B:** Hypocotyl length from 1 plate in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077486).

In the hypocotyl experiment done 08 Jun, the hypocotyl length for the B´α (SALK_077486) was investigated. In blue light, the B´α (SALK_077486) hypocotyls were shorter compared with WT, but longer in the dark (Figure 3.18 A and B). The ratio of light/dark for WT and

B´α (SALK_077486) showed that B´α (SALK_077486) was 17% shorter compared to WT in blue light (Figure 3.18 C).

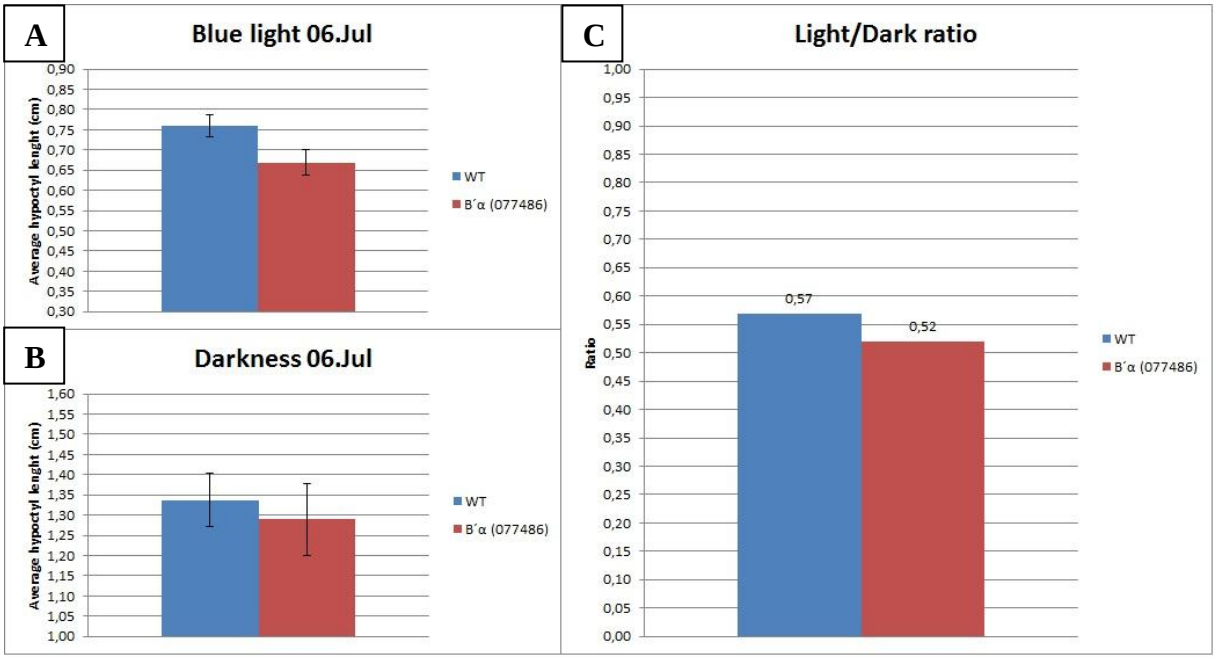


Figure 3.19 A, B and C. Hypocotyl length experiment 06 Jul. for B´α (SALK_077486). **A and B:** Average hypocotyl length from 3 plates in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077486). The lines in graph A and B represent the SE for the average hypocotyl length from the 3 plates.

Hypocotyl experiment from 06 Jul. revealed shorter B´α (SALK_077486) hypocotyls both in blue light and darkness (Figure 3.19 A and B). The light/dark ratio for WT and B´α (SALK_077486) was 0.57 and 0.52 respectively (Figure 3.19 C).

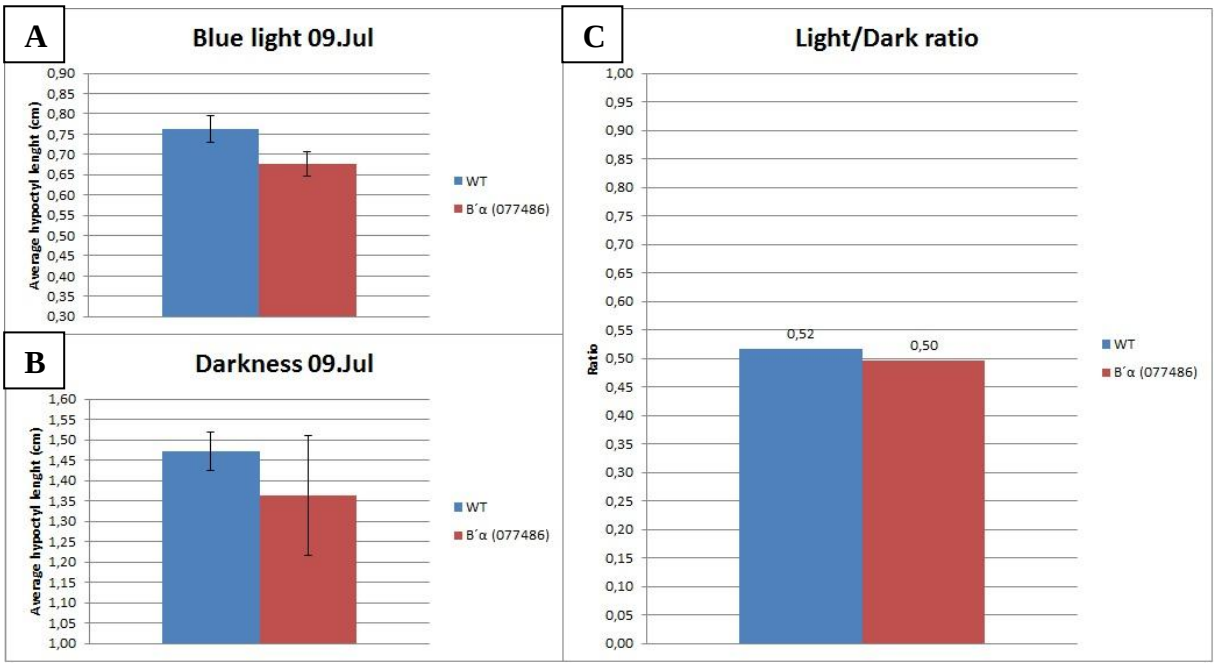


Figure 3.20 A, B and C. Hypocotyl length experiment 09 Jul. for B´α (SALK_077486). **A and B:** Average hypocotyl length from 3 plates in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077486). The lines in graph A and B represent the SE for the average hypocotyl length from the 3 plates.

In the hypocotyl experiment done 09 Jul. B´α (SALK_077486) hypocotyls were shorter in blue light and in the dark which gives a light/dark ratio of 0.52 for WT and 0.50 for B´α (SALK_077486) (Figure 3.20).

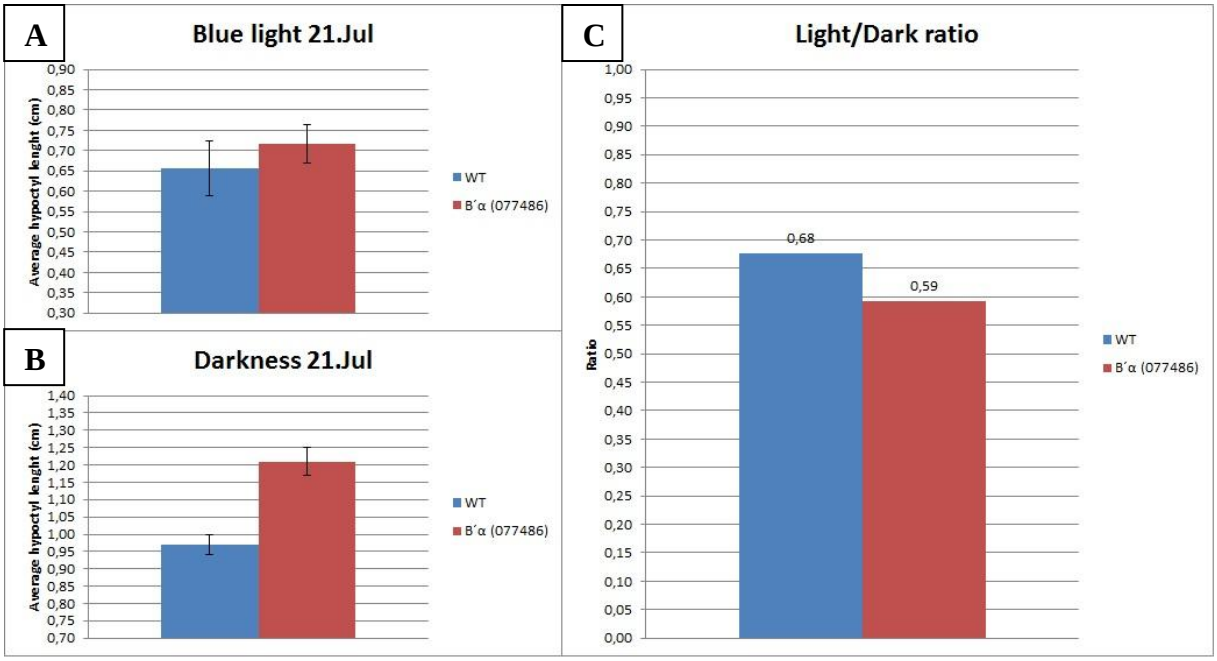


Figure 3.21 A, B and C. Hypocotyl length experiment 09 Jul. for B´α (SALK_077486). **A and B:** Average hypocotyl length from 3 plates in blue light and 2 plates in darkness. **C:** Ratio of light/dark for WT and B´α (SALK_077486). The lines in graph A and B represent the SE for the average hypocotyl length from the 3 plates in blue light and the 2 plates in darkness.

The hypocotyl experiment done 21 Jul. showed longer B´α (SALK_077486) hypocotyls in both blue light and darkness (Figure 3.21 A and B). Even though the WT showed shorter hypocotyls in blue light, the light/dark ratio shows that the B´α (SALK_077486) hypocotyls were 9% shorter compared with WT.

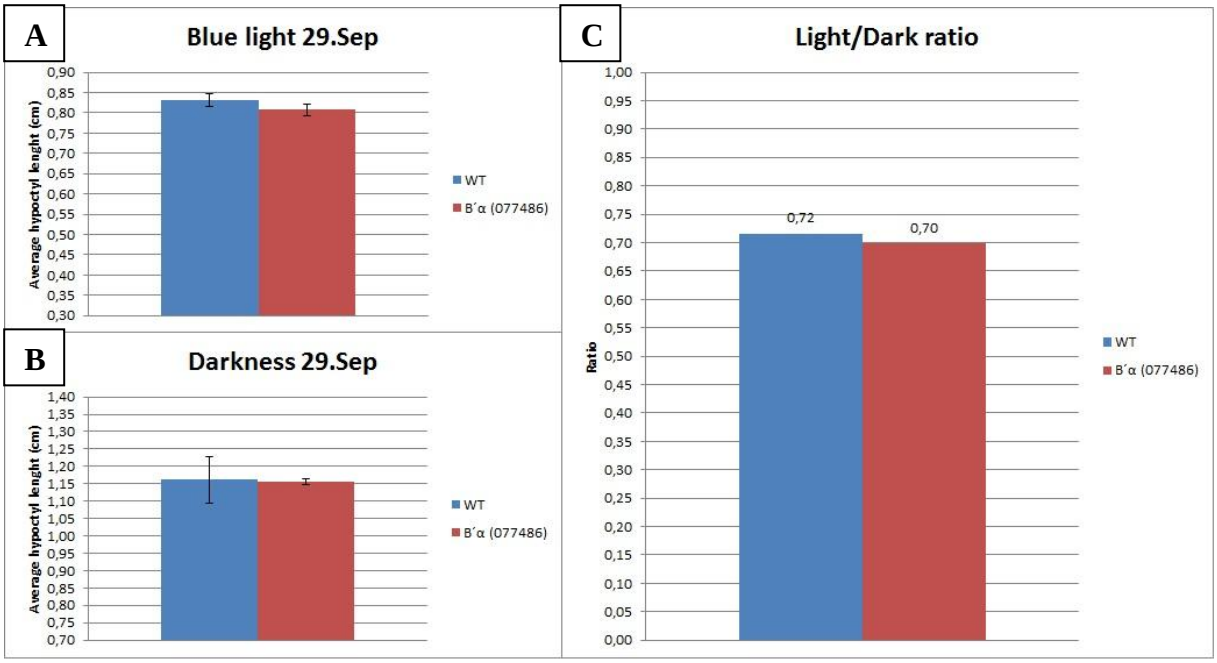


Figure 3.22 A, B and C. Hypocotyl length experiment 29 Sep. for B´α (SALK_077486). **A and B:** Average hypocotyl length from 3 plates in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077486). The lines in graph A and B represent the SE for the average hypocotyl length from the 3 plates.

Hypocotyl experiments done 29 Sep. showed that B´α (SALK_077486) hypocotyls were marginally shorter in blue light and darkness (Figure 3.22 A and B), which gave a light/dark ratio of 0.72 for WT and 0.70 for B´α (SALK_077486) (Figure 3.22 C).

The hypocotyl experiment done 11 Oct. showed longer B´α (SALK_077486) hypocotyls in both blue light and darkness (Figure 3.23 A and B). The experiment that was done at this day was the only experiment showing longer B´α (SALK_077486) hypocotyls when the light/dark ratio was calculated. The light/dark ratio for WT was 0.56 and 0.58 for B´α (SALK_077486) (Figure 3.23).

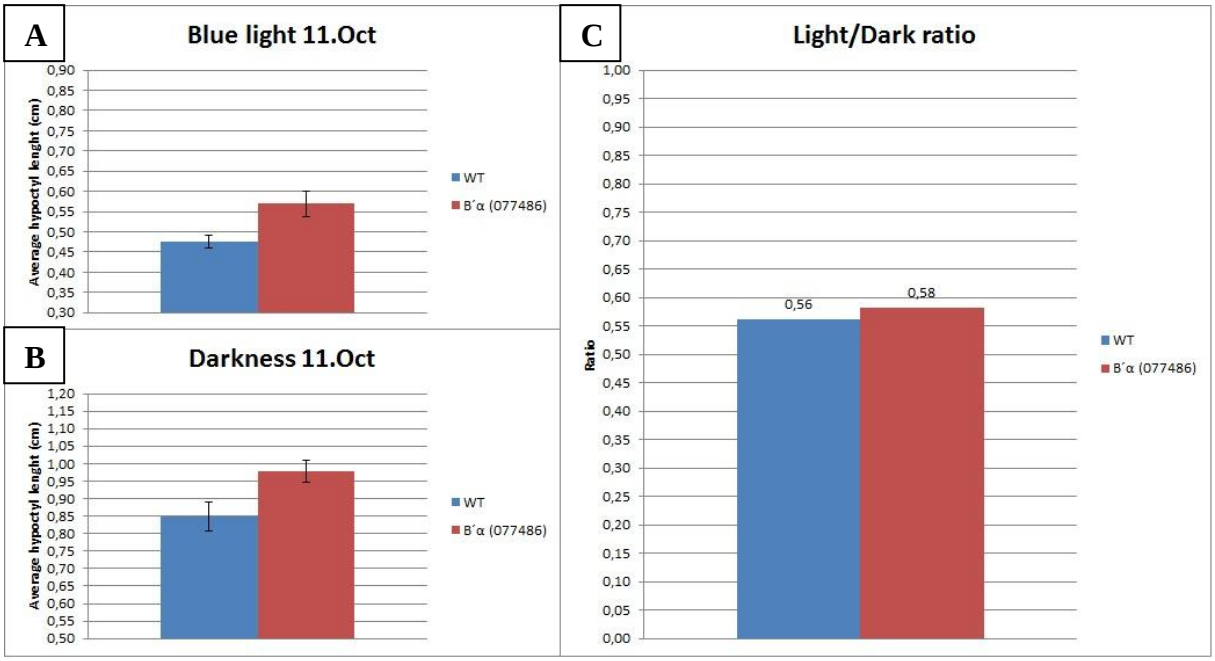


Figure 3.23 A, B and C. Hypocotyl length experiment 11 Oct. for B´α (SALK_077486). **A and B:** Average hypocotyl length from 3 plates in blue light and darkness respectively. **C:** Ratio of light/dark for WT and B´α (SALK_077486). The lines in graph A and B represent the SE for the average hypocotyl length from the 3 plates.

All the hypocotyl length experiments except the last one from 11 Oct. showed a lower light/dark ratio for B´α (SALK_077486) compared with WT in blue light, i.e. shorter hypocotyls. The highest light/dark ratio difference between B´α (SALK_077486) and WT was observed in the experiment done 08 Jun. where the B´α (SALK_077486) ratio was 0.17 lower than WT. The lowest difference in light/dark ratio was observed at 09 Jul, 29 Sep. and 11 Oct. where the B´α (SALK_077486) ratio was 0.02 lower and 0.02 higher at 11 Oct (See Table 3.4).

Table 3.4. Light/Dark ratios for average hypocotyl length in blue light and darkness for B´α (SALK_077486) and WT.

Light/Dark ratio:	**B´α (SALK_077486)**	**WT**
08.Jun.	0.38	0.55
06.Jul.	0.52	0.57
09.Jul.	0.50	0.52
21.Jul.	0.59	0.68
29.Sep.	0.70	0.72
11.Oct.	0.58	0.56
Average:	0.54	0.56
SE:	0.04	0.03

When the data from the hypocotyl experiments for B´α (SALK_077700) and B´α (SALK_077486) are combined, the B´α hypocotyls in blue light compared with WT in 8 out of 9 experiments. The average light/dark ratio in the hypocotyl experiments with B´α (SALK_077700) was 0.38±0.03 for B´α (SALK_077700) and 0.45±0.05 for WT (Table 3.3).

The average light/dark ratio in the hypocotyl experiments with B´α (SALK_077486) was 0.54±0.04 for B´α (SALK_077486) and 0.56±0.03 for WT (Table 3.4).

4 Discussion and outlook

4.1 Flowering

Recent studies have shown that a CRY1 mutation can lead to hypersensitivity to blue light and early flowering under short day conditions (Exner et al. 2010). The hypothesis of the present study was that B`α is a part of a PP2A complex that dephosphorylate CRY1, and that a PP2A mutant behaves like a CRY overexpressor/strong signaling mutant with respect to blue light and hypocotyl elongation (Jonassen et al. unpublished). Increased phosphorylation of CRY1 will in theory result in more efficient or longer lasting repression of COP1, which again gives higher levels of CO (See part 1.7 Figure 1.8). CO directly binds to and activates FT which is an activator of flowering.

The flowering experiments revealed significant earlier flowering when the B´α mutant and WT were compared in 12 h and 16 h light. In fact all experiments showed earlier flowering for the B`α mutant, but the results were not statistically significant in all experiments when compared with WT. The biggest difference in flowering was observed in 12 h light where the average day of flowering for B`α (SALK_077700) was 51.08±1.71 and 54.50±1.00 for WT (See part 3.14 table 3.1). Flowering experiments in 8 h and 16 h light for B`α (SALK_077700) showed statistical insignificant differences in flowering compared with WT, indicating that the day length have an influence on the B`α mutants. Seeds for B`α (SALK_077486) was not yet obtained when the experiments in 8 h and 12 h light was performed. The B`α (SALK_077486) showed significant earlier flowering in 16 h light compared with WT, and experiments of this SALK-line in 8 h and 12 h light should be done in future studies to see if the difference would be more clear in shorter day-lengths. The flowering experiments were very time consuming, especially in short days where flowering can occur as late as 3 months after germination. There was no time for more experiments in short day lengths in the present study, and this should be repeated in the future before any firm conclusions are drawn concerning the flowering phenotype of B´α mutants.

The B`α mutation was not lethal, and showed no impairment compared to WT which indicates that the function of B`α can be replaced by other subunits such as B`β. B`β is a close homolog to B`α, and is 96% similar and 85% identical. Recent studies have shown that a PP2A-B`αβ double mutant leads to a phenotype of dwarfism and low fertility. The double mutant developed numerous flowers, and showed both early and late flowering. Single mutants of these two genes looked normal compared with WT (Jonassen et al. 2011). The B´α mutants in the present study showed a “weak” phenotype of early flowering, which indicates the possibility of other subunits replacing the function of B´α in flowering.

In all the flowering experiments, some plants were removed due to problems with flies. Counter measures were made by watering the plants with a substance to exterminate the larvae in the soil, but it did not solve the problem when flies came from other plants in the plant room.

4.2 Bimolecular fluorescence complementation analysis

The bimolecular fluorescence complementation (BiFC) has emerged as a powerful technique for the visualization of protein interactions and modifications in living cells (Waadt et al. 2008). In the present study, the transient expression system of *Arabidopsis* mesophyll protoplast was used. The transformation efficiency of *Arabidopsis* mesophyll protoplasts can reach 90%. Transformation in the present BiFC study was done by using polyethylene glycol (PEG) and epifluorescence was analyzed after 6-48 h.

Fluorescence was detected in the combination of CRY1 c-terminal part/ B`α in protoplasts incubated for 24 to 48 h. However, fluorescence was also observed in all the negative control experiments with the "empty" vectors. Fluorescence in the negative controls was detected in a high number of cells in the experiments. In the samples where the protoplasts was incubated for 6 and 12 h, fluorescence was only detected in the negative controls which indicates that self-assembly of the fluorescent protein fragments can easily occur.

A fluorescent signal showing dots/speckles was only observed in samples of CRY1/ B`α (See part 3.2.2 Table 3.2). These signals were observed in samples incubated in the dark for 24 and 36 h, and in blue light for 36 h. The fluorescent signal showing dots/speckles was first discovered when the samples were incubated in blue light, but were later observed in higher frequency in the samples incubated in the dark.

The BiFC vectors used in the present study are based on the fluorescent protein Venus. Venus generates a high fluorescence intensity, but it has been reported to result in a higher background signal (Waadt et al. 2008). The background signals were observed in all negative controls in the present study, questioning the specificity of BiFC analysis. Even though dots/speckled like signals were observed only in the co-expression of CRY/ B`α, it is difficult to draw any conclusions because the number of fluorescent signals in the negative controls was so high.

Other techniques that would be interesting to do in future experiments to lessen the problems with background signal is Försters resonance energy transfer (FRET) which is a widely used method for the analysis of protein interaction. FRET is based upon the transfer of energy from an excited donor fluorophor to a close acceptor fluorophor, resulting in enhanced emission of the acceptor. The FRET technique has a high sensitivity, but due to low FRET efficiencies of fluorescent proteins and complexity of fluorescence emissions of donor and acceptor, it is a challenging method for studying protein-protein interactions (Banning et al. 2010; Song et al. 2011).

4.3 Hypocotyl length

The hypocotyl is the embryonic stem that forms the connection between the two cotyledons and the seedling root. There are approximately 20 cells in an *Arabidopsis* hypocotyl, and the growth is caused mainly through longitudinal cell expansion. The hypocotyl is strongly influenced by external and internal factors, such as light, gravity, temperature and hormones (Vandenbussche et al. 2005).

CRY is important in suppression of hypocotyl suppression in blue light, and recent studies have shown that a CRY1 mutant is hypersensitive toward blue light in hypocotyl growth inhibition (Exner et al. 2010). The hypothesis of the present study was that B`α is a part of a PP2A complex that dephosphorylate CRY1, and that a PP2A mutant behaves like a CRY overexpressor/strong signaling mutant with respect to blue light and hypocotyl elongation (Jonassen et al. unpublished). Increased phosphorylation of CRY1 will in theory result in more efficient or longer repression of COP1 which is not only important in the flowering pathway. Repression of COP1 would in theory lead to enhanced expression of the transcription factor HY5 and many factors promoting short hypocotyls.

One problem that was faced in some of the blue light hypocotyl experiments was phototropism. Phototropism is controlled by the phototropin blue light photoreceptors (Fankhauser and Casal 2004). In the experiments where phototropism was observed, it raises the question whether the hypocotyl elongation is caused by CRY1 or phototropin.

In 8 out 9 experiments, the mutant B`α showed shorter hypocotyls in blue light. The difference between B`α (SALK_077700) and WT hypocotyls was larger than in the experiments where B`α (SALK_077486) and WT were compared. When all the hypocotyl results are combined, the results show that mutations in B`α will lead to shorter hypocotyls in blue light.

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Appendix

B´α (077700) hypocotyls:

Blue light:

	Plate 1		Plate 2	
12 Jan.				
Length (cm):	B´α	WT	B´α	WT
	0,15	0,30	0,50	0,60
	0,50	0,40	0,50	0,50
	0,25	0,40	0,60	0,50
	0,50	0,40	0,40	0,60
	0,20	0,40	0,50	0,50
	0,50	0,50	0,60	0,50
	0,60	0,30	0,60	0,65
	0,50	0,50	0,25	0,65
	0,50	0,50	0,60	0,60
	0,40	0,50	0,60	0,45
		0,40	0,60	0,55
		0,40	0,50	0,60
Average:	0,41	0,42	0,52	0,56
SD:	0,1542	0,0718	0,1076	0,0669

Dark:

	Plate 1		Plate 2	
12 Jan.				
Length (cm):	B´α	WT	B´α	WT
	1,40	1,10	1,30	1,10
	1,50	1,30	1,30	1,30
	1,40	1,30	1,30	1,30
	1,40	1,30	1,50	1,50
	1,50	1,30	1,00	1,00
	1,20	1,00	1,30	1,30
	1,50	1,20	1,30	1,30
	1,10	1,00	1,40	1,40
	1,50	0,90	1,00	1,00
	1,00	1,20	1,30	1,30
	1,35	0,40	1,30	1,30
		1,20	1,50	1,50
Average:	1,35	1,10	1,29	1,28
SD:	0,1746	0,2594	0,1564	0,1658

Blue light:

	Plate	
25 Jan.	1	
Length (cm):	B´α	WT
	0,30	0,30
	0,40	0,25
	0,50	0,30
	0,30	0,40
	0,45	0,50
	0,40	0,50
	0,40	0,30
	0,40	0,30
	0,35	0,35
	0,40	0,30
	0,40	0,30
	0,45	0,30
Average:	0,40	0,34
SD:	0,0582	0,0821

Dark:

	Plate	
25 Jan.	1	
Length (cm):	B´α	WT
	1,00	0,80
	1,20	0,70
	1,20	1,10
	1,00	0,90
	0,90	0,90
	1,20	0,80
	1,20	1,00
	1,25	0,70
	1,30	1,00
	1,40	0,60
	1,00	1,10
Average:	1,15	0,87
SD:	0,1533	0,1679

Blue light:

	Plate	
08 Jun.	1	
Length (cm):	B´α	WT
	0,55	0,55

	0,60	0,70
	0,55	0,70
	0,45	0,55
	0,60	0,80
	0,50	0,65
	0,40	0,60
	0,55	0,60
	0,50	0,70
	0,55	0,70
Average:	0,53	0,66
SD:	0,0635	0,0798

Dark:

	Plate	
08 Jun.	1	
Length (cm):	B´α	WT
	1,20	1,20
	1,30	1,20
	1,30	1,20
	1,20	1,10
	1,05	1,40
	1,20	1,30
	1,10	1,20
	1,00	1,20
	1,40	1,30
	1,30	1,00
Average:	1,21	1,21
SD:	0,1257	0,1101

B´α (077486) hypocotyls:

Blue light:

	Plate	
08 Jun.	1	
Length (cm):	B´α	WT
	0,60	0,55
	0,40	0,70
	0,40	0,70
	0,60	0,55
	0,55	0,80
	0,45	0,65
	0,60	0,60

	0,30	0,60
	0,40	0,70
	0,60	0,70
Average:	0,49	0,66
SD:	0,1125	0,0798

Dark:

	Plate	
08 Jun.	1	
Length (cm):	B´α	WT
	1,20	1,20
	1,50	1,20
	1,30	1,20
	1,20	1,10
	1,40	1,40
	1,10	1,30
	1,10	1,20
	1,40	1,20
	1,40	1,30
	1,40	1,00
Average:	1,30	1,21
SD:	0,1414	0,1101

Blue light:

	Plate					
06 Jul.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	0,86	0,92	0,65	0,36	0,57	0,69
	0,50	0,73	0,84	0,69	0,73	0,76
	0,51	0,62	0,68	0,81	0,67	0,61
	0,86	0,85	0,80	0,43	0,68	0,80
	0,70	1,07	0,77	0,76	0,52	0,84
	0,79	1,10	0,64	0,85	0,62	0,72
	0,64	0,84	0,86	0,85	0,45	0,65
	0,52	0,81	0,81	0,72	0,85	0,81
	0,76	0,85	0,50	0,78	0,40	0,64
	1,05	0,77	0,64	1,03	0,59	0,89
	0,75	0,79	0,67	0,63	0,49	0,38
	0,59	0,63	0,61	0,80	0,57	0,80
		0,86	0,64	0,98	0,87	0,61
		0,72	0,60	0,63	0,46	0,98
		0,64	0,66	0,84		

				0,69		
Average:	0,71	0,81	0,69	0,74	0,61	0,73
SD:	0,17	0,14	0,10	0,18	0,14	0,15

Dark:

	Plate					
06 Jul.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	1,51	1,56	1,35	1,29	1,06	1,45
	1,55	1,48	1,35	1,47	0,89	1,24
	1,44	1,51	1,36	1,54	0,96	1,24
	1,62	1,37	1,05	1,41	1,31	1,19
	1,30	1,38	1,24	1,51	1,23	1,45
	1,54	1,79	1,42	1,39	1,01	1,19
	1,08	1,39	1,39	1,27	1,15	0,88
	1,41	1,26	1,41	1,46	1,09	1,32
	1,73	1,45	1,21	1,23	1,08	0,83
	1,48	1,39	1,51	1,27	1,14	0,93
	1,08	1,31	1,38	1,57	1,22	1,37
	1,48	1,28	1,31	1,39	1,18	1,38
	1,15		1,09	1,44	1,22	1,17
			1,65	1,23	1,09	1,23
				1,13		1,30
Average:	1,41	1,43	1,34	1,37	1,12	1,21
SD:	0,2039	0,1458	0,1561	0,1314	0,1141	0,1938

Blue light:

	Plate					
09 Jul.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	0,89	0,95	0,67	0,38	0,52	0,71
	0,47	0,70	0,81	0,67	0,72	0,69
	0,51	0,58	0,71	0,82	0,68	0,80
	0,83	0,86	0,83	0,40	0,74	0,57
	0,70	1,08	0,78	0,73	0,50	0,85
	0,77	1,13	0,61	0,84	0,63	0,74
	0,60	0,94	0,86	0,87	0,53	0,68
	0,51	0,80	0,84	0,64	0,88	0,83
	0,75	0,83	0,51	0,79	0,40	0,65
	1,04	0,73	0,62	1,04	0,58	0,85
	0,76	0,77	0,66	0,70	0,45	0,41
	0,61	0,81	0,60	0,82	0,57	0,81
	0,67	0,76	0,66	0,96	0,90	0,57

	0,87	0,65	0,67	0,60	0,51	0,95
			0,72	0,81		
				0,69		
Average:	0,71	0,83	0,70	0,73	0,61	0,72
SD:	0,1638	0,1548	0,1013	0,1774	0,1526	0,1419

Dark:

	Plate					
09 Jul.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	1,73	1,50	1,42	1,55	1,18	1,63
	1,81	1,65	1,40	1,85	1,15	1,41
	1,74	1,57	1,39	1,66	1,28	1,35
	1,53	1,50	1,03	1,55	1,23	1,58
	1,71	1,43	1,27	1,71	0,96	1,34
	1,40	1,48	1,05	1,50	1,15	1,20
	1,69	1,56	1,47	1,37	1,06	1,48
	1,14	1,35	1,49	1,54	1,17	1,15
	1,52	1,36	1,57	1,24	1,32	1,45
	1,90	1,74	1,22	1,41	1,25	1,32
	1,57	1,53	1,67	1,62	0,83	1,43
	1,25	1,48	1,50	1,62	1,11	1,43
	1,56		1,40	1,54		
	1,24		1,29	1,55		
			1,71	1,27		
				1,20		
Average:	1,56	1,51	1,39	1,51	1,14	1,40
SD:	0,2279	0,1121	0,1829	0,1489	0,1404	0,1393

Blue light:

	Plate					
21 Jul.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	0,61	0,65	0,85	0,84	0,70	0,82
	0,73	0,68	0,58	1,00	0,51	0,59
	0,70	0,57	0,64	0,57	0,75	0,65
	0,59	0,59	0,89	0,81	0,92	0,69
	0,75	0,44	0,79	0,71	0,76	0,62
	0,62	0,49	0,69	0,70	0,52	0,60
	0,62	0,49	0,45	0,72	0,59	0,55
	0,62	0,51	0,47	0,67	0,86	0,63

	0,79	0,44	0,78	0,80	0,90	0,72
	0,35	0,42	0,53	0,63	0,92	0,92
	0,47	0,54	0,70	0,90	0,82	0,82
	0,79	0,55	0,70	0,54	0,93	0,59
	0,82	0,54	0,62	0,81	0,83	0,59
	0,67	0,47	0,69	0,89	1,42	0,77
		0,57	0,93		0,76	
Average:	0,65	0,53	0,69	0,76	0,81	0,68
SD:	0,1294	0,0755	0,1445	0,1313	0,2179	0,1113

Dark:

	Plate			
21 Jul.	1		Plate 2	
Length (cm):	B´α	WT	B´α	WT
	1,15	0,95	1,48	1,04
	1,16	1,09	1,10	1,13
	1,10	0,82	1,37	0,91
	1,01	0,81	1,39	0,99
	1,20	0,95	1,23	0,94
	1,21	0,97	1,37	0,83
	1,19	0,95	1,15	0,82
	1,19	0,99	1,29	0,94
	1,20	0,91	1,16	1,05
	1,11		1,23	1,16
	1,31		1,18	1,10
	1,23		1,23	1,03
	0,99		1,09	1,01
	1,33		1,30	
Average:	1,17	0,94	1,26	1,00
SD:	0,0961	0,0854	0,1168	0,1057

Blue light:

	Plate					
29 Sep.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	0,68	1,06	0,95	0,96	0,71	0,77
	0,98	0,57	1,01	0,79	0,74	0,87

	0,69	0,51	0,88	0,78	0,75	0,66
	0,84	0,88	0,62	0,80	0,79	0,94
	0,83	0,80	0,72	0,89	0,72	0,83
	0,89	0,84		0,99	0,92	0,73
	0,81	0,91		0,75	0,80	
	0,70	0,96			0,85	
		0,85				
		0,98				
		0,92				
Average:	0,80	0,84	0,83	0,85	0,78	0,80
SD:	0,1085	0,1656	0,1611	0,0960	0,0741	0,1008

	Plate			
29 Sep.	1		Plate 2	
Length (cm):	B´α	WT	B´α	WT
	1,17	1,24	1,15	1,19
	0,94	1,23	1,24	1,15
	1,21	1,33	1,27	0,98
	0,99	1,30	1,34	0,88
	1,30	1,30	1,08	1,02
	1,22	1,29	1,07	1,09
	1,03	1,27	1,15	
	1,29		1,09	
Average:	1,14	1,28	1,17	1,05
SD:	0,1388	0,0343	0,0987	0,1165

Blue light:

	Plate					
11 Oct.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	0,69	0,35	0,65	0,53	0,59	0,51
	0,54	0,42	0,73	0,45	0,48	0,45
	0,64	0,54	0,42	0,34	0,45	0,42
	0,55	0,42	0,52	0,31	0,59	0,31
	0,68	0,42	0,56	0,63	0,44	0,36
	0,41	0,34	0,61	0,46	0,47	0,43
	0,47	0,47	0,69	0,62	0,53	0,43
	0,72	0,27	0,61	0,65	0,60	0,65
	0,52	0,69	0,61	0,64	0,65	0,50
	0,69	0,61	0,59	0,43	0,46	0,49
	0,76	0,43	0,78	0,72	0,48	
	0,57	0,61	0,76	0,27	0,43	
	0,38		0,58	0,54	0,46	

			0,48			
			0,70			
			0,77			
			0,49			
			0,57			
			0,51			
Average:	0,59	0,46	0,61	0,51	0,51	0,46
SD:	0,1211	0,1252	0,1048	0,1419	0,0750	0,0927

Dark:

	Plate					
11 Oct.	1		Plate 2		Plate 3	
Length (cm):	B´α	WT	B´α	WT	B´α	WT
	0,87	0,76	0,72	0,75	0,85	1,09
	0,97	1,01	0,81	0,82	0,98	0,89
	1,14	0,78	1,33	0,93	1,02	0,57
	0,88	0,79	0,70	0,88	1,07	0,88
	1,06	0,90	0,56	0,88	1,09	0,82
	1,06	1,04	1,12	0,88	1,09	1,08
	1,23	0,89	0,99	0,65	0,96	0,83
	0,85	0,87	1,10	0,50	1,14	0,80
	1,17	0,64	0,93	0,60	0,75	0,97
	1,33	0,81	0,95		1,03	0,93
	1,09	0,98			1,02	0,84
	0,98	1,14			0,81	0,97
	0,94	0,83			0,97	
	0,87	1,08			1,17	
					0,87	
					0,92	
Average:	1,03	0,89	0,92	0,77	0,98	0,89
SD:	0,1476	0,1395	0,2316	0,1512	0,1193	0,1374

B´α (077700)

Average 12 Jan.	**B´α**		**WT**	
	Light	**Dark**	**Light**	**Dark**
Average:	0,47	1,32	0,49	1,19
SD	0,08	0,04	0,10	0,12
SE	0,06	0,03	0,07	0,09
Average 25 Jan.	B´α		WT	
	Light	Dark	Light	Dark
Average:	0,40	1,15	0,34	0,87
SD				

Average 08 Jun.	B´α		WT	
	Light	Dark	Light	Dark
	0,53	1,21	0,66	1,21

Light/dark ratio:	**B´α**	**WT**
12 Jan.	0,31	0,35
	0,39	0,47
25 Jan.	0,35	0,39
08 Jun.	0,55	0,61
	0,54	0,55
	0,47	0,55
Average:	0,46	0,49
SD:	0,09	0,10

B´α (077486)

Average 08.jun	B´α		WT	
	Light	Dark	Light	Dark
Average:	0,49	1,3	0,66	1,21
SD				
Average 06.jul	B´α		WT	
	Light	Dark	Light	Dark
Average:	0,67	1,29	0,76	1,34
SD	0,06	0,15	0,05	0,11
SE	0,03	0,09	0,03	0,07
Average 09.jul	B´α		WT	
	Light	Dark	Light	Dark
Average:	0,68	1,36	0,76	1,47
SD	0,05	0,21	0,06	0,07
SE	0,03	0,15	0,03	0,05
Average 21.jul	B´α		WT	
	Light	Dark	Light	Dark
Average:	0,72	1,21	0,66	0,97
SD	0,08	0,06	0,12	0,04
SE	0,05	0,04	0,07	0,03
Average 29.sep	B´α		WT	
	Light	Dark	Light	Dark
Average:	0,81	1,16	0,83	1,16
SD	0,03	0,01	0,03	0,11
SE	0,01	0,01	0,02	0,07
Average 11.okt	B´α		WT	
	Light	Dark	Light	Dark

Average:	0,57	0,98	0,48	0,85
SD	0,05	0,06	0,03	0,07
SE	0,03	0,03	0,02	0,04

Light/Dark ratio:	**B´α**	**WT**
08.jun	0,38	0,55
06.jul	0,52	0,57
09.jul	0,50	0,52
21.jul	0,59	0,68
29.sep	0,70	0,72
11.okt	0,58	0,56
Average:	0,54	0,60
SD:	0,11	0,08