

Master thesis in biological chemistry

Title: *Phenotype identification, cloning and expression of B55 subunit mutants of protein phosphatase 2A in Arabidopsis model plant*

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Abbreviations

Amp: Ampicillin

ATP: Adenosine triphosphate

Bp: nucleotide base pair

Bp: Base pairs

BSA: bovine serum albumin

Ca²⁺ : calcium ion

C-terminus: carboxyl-terminus

ddH₂O: double distilled water

DH5α: Douglas Hanahan bacterial strain 5α

DNA: deoxyribonucleic acid

dNTPs: deoxyribonucleotide triphosphates

dsDNA: Double stranded deoxyribonucleic acid

DTT: dithiothreitol

DTT: dithiothreitol

E. coli: *Escherichia coli*

EDTA: ethylenediaminetetraacetic acid

GST: glutathione S-transferase

HRP: horseradish peroxidase

IgG: immunoglobulin gamma

IPTG: Isopropyl β-D-1-thiogalactopyranoside

kDa: kiloDalton

kDa: kiloDalton

LB: Luria-Bertani broth

MCS: Molecular Cloning Site

N-terminus: amino-terminus

OD: optical density

PBS: Phosphotase Buffered Saline

PBST: Phosphotase Buffered Saline Tween

PCR: Polymerase Chain Reaction

pH: inverse log of hydrogen ion concentration

pH: inverse log of hydrogen ion concentration

PP2A: Protein phosphatase 2A

RNA: ribonucleic acid

Rpm: Revolutions per minute

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

ssDNA: Single stranded deoxyribonucleic acid

Tris: Tris hydroxymethyl aminomethane

Tween-20: polyoxyethylene-sorbitan monolaurate

UV: ultraviolet

Abstract

Plant photoreceptors sense light and regulate certain downstream genes to alter morphological features. In Arabidopsis, these are three classes of photoreceptors: far/red light absorbing phytochromes, UV/blue light absorbing cryptochromes and phototropins. Reversible protein phosphorylation is a major signaling mechanism in eukaryotic cellular function. Recent research suggests that reversible phosphorylation of photoreceptors is a vital regulatory mechanism in light signal transduction pathway. Protein phosphatase has been proposed to play an important role in cellular function both plants and animals. The B subunits of PP2A play a crucial role in localization and substrate specificity of PP2A in mammals. It has also been proposed that B subunits have the same role in plants. The Arabidopsis seedlings were planted on half MS medium containing 1% sucrose and without sucrose in different light conditions to observe the phenotypic appearance. To investigate a possible dephosphorylation in the signaling pathways from phytochromes and cryptochromes by PP2A in the nucleus, phenotypic identification of the Arabidopsis single B55 mutant seedlings were first investigated in this study. Subcloning and expression of B55 and B β recombinant proteins were then conducted to probe the target proteins with antibody by Western blot analysis. The primary purpose of this study is then to identify the phenotypic observations (particularly the hypocotyl elongation) of B55 subunit mutants in Arabidopsis seedlings and compare with that of wild type (Col). For further study of role of PP2A in de-etiolation, the genes for B55 subunit and B β in Arabidopsis as a model organism had to be subcloned into expression plasmid vector, PGEX-6p-1. The expression plasmid vector was transformed into *E. coli*, DE3, for protein expression under IPTG induction. The proteins were co-purified with GST by using Gluthathione-agarose beads and eluted with elution buffer. The purified proteins were detected with secondary antibody conjugated with Horse Radish peroxidase (HRP). Some problems were encountered for optimization of the proteins during purification and Western blot detection.

Key words: Photoreceptors Protein phosphatase Gibberellin



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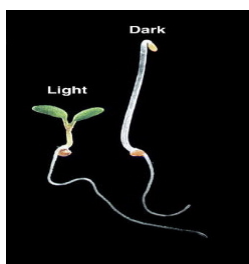
1 Introduction

Being sessile in nature, plants have developed complex mechanisms to constantly optimize their growth and development in response to environmental changes such as light, humidity, salinity and temperature. Light is one of the most important environmental stimuli for plant growth and development. In addition to providing as a source of energy for plant life, light acts as a central role in regulating plant development such as seed germination, seedling photomorphogenesis, phototropism, stomata and chloroplast movement, shade avoidance, circadian rhythms and flowering time (Wang and Deng, 2003; Jiao et al., 2007).

Plants can perceive and respond to changes in direction, duration, quantity, and spectral quality of light environment and modify their growth accordingly (Nemhauser and Chory, 2002). Recent studies have focused on the mechanisms of detecting light signals by the photoreceptors and transducing these signals into phenotypic effect (Fairchild and Quail, 1998, Smith, and Lin, 2000).

1.1 De-etiolation (Photomorphogenesis)

Among the responses to light induction, de-etiolation is one of the most important phenomena in plant growth and development (Deng, 1994). When dark-grown seedlings are exposed to light, they undergo a developmental process called photomorphogenesis. De-etiolation involves inhibition of hypocotyls elongation, opened apical hook, expanded cotyledons, pigment (chlorophyll and anthocyanin) synthesis and flower induction. In contrast, seedlings grown in darkness during germination show skotomorphogenesis in which hypocotyls elongate, cotyledons remain closed and have apical hooks until the seedlings get light or die (Deng, 1994).



Haiyang Wang and Xing Wang Deng ©2002 American Society of Plant Biologists

Figure 1: De-etiolated and etiolated *Arabidopsis* seedlings grown in light and dark respectively.

1.2 Photoreceptors

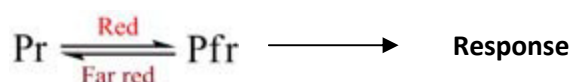
The light perceived by plants is processed in various photomorphogenic responses through the action of photoreceptors. Three major classes of photoreceptors have been identified in the model plant, *Arabidopsis thaliana* in combination of physiological, biochemical and molecular genetic studies: the red (R) and far-red (FR) light perceiving phytochromes (phy) (Quail, 2002b), Blue light perceiving cryptochromes (cry) during de-etiolation and flower induction (Lin, 2002) and the UV-B perceiving phototropins during phototropism, stomatal opening and chloroplast movement (Briggs and Christie, 2002). Each of which is able to detect light of particular wavelengths. These classes of photoreceptors sense and transduce light signals to regulate the downstream target genes contributing to the photomorphogenic development (Nemhauser and Chory, 2002).

1.2.1 Plant Phytochromes

Pytochromes are the most widely studied among the plant photoreceptors (Quail, 1991; Furuya 1993). There are five phytochrome genes (PhyA – PhyE) in the model plant species, *Arabidopsis thaliana* and they are encoded by five phytochrome genes. They are categorized into two groups according to their stability in light (Shamrock and Quail, 1989). Type I, light labile phytochrome, is highly abundant in dark-grown seedlings. PHYA is a light-labile phytochrome and its level drops once exposed to light (Quail et al 1995). Type II, light stable phytochrome, is predominantly abundant in light-grown seedlings. PHYB to PHYE are light stable phytochromes and present at relatively similar levels in dark-grown and light-grown seedlings (Clack et al., 1994).

1.2.2 Photoreversibility of phytochromes

Phytochromes exist in two distinct photo-reversible forms: the red absorbing form (Pr) which is synthesized in dark-grown (etiolated) seedlings and the far-red absorbing form (Pfr) which is the biological active form of phytochrome. Pfr is physiological active form of phytochrome because phenotype of phytochrome-deficient mutants is observed in light-grown seedlings. Irradiation of Pfr with far-red light converts it back to Pr (Quail, 2002). The photo-reversibility of the most distinct property of phytochrome and may be expressed as follows:



On the basis of radiation energy light they perceive for light response, phytochromes are categorized into different classes. These include low fluence response (LFRs) which responds to R/FR reversibility, very low fluence responses (VLFRs) which are not sensitive to a broad spectrum of light and are irreversible and high irradiance responses (HIRs) which require prolonged illumination (Casal, Davis, et al. 2002). PHYA is responsible for the VLFR and FR-HIR) and PHYB is responsible for the LFR and R-HIR during photomorphogenesis (Nagy, Schafer, 2002)

1.2.3 Phytochrome structure

Phytochromes are soluble dimers composed of monomers of ~125 kDa each (Fankhauser, Staiger, 2002). Each monomer of the phytochrome molecule consists of two major domains separated by a flexible hinge region: The N-terminal photosensory domain and the C-terminal regulatory domain. The N-terminal domain comprises four subdomains: P1 (N-terminal extension, NTE), P2, P3 (bilin lyase domain, BLD), and P4 (Montgomery BL, Lagarias JC. 2002). The P3 bears the chromophore and it is where the tetrapyrrole chromophore covalently attaches to the protein through a thioether linkage to a cysteine residue mediating light perception. The C-terminal domain contains two subdomains: a PAS-related domain (PRD) containing two PAS domains (Bolle C, Koncz C, Chua NH. 2000). The regulatory C-terminal domain contains the site where the two monomers associate with each other to form dimers and it also regulates and plays a major role in light-induced nuclear translocation (Quail, 2000).

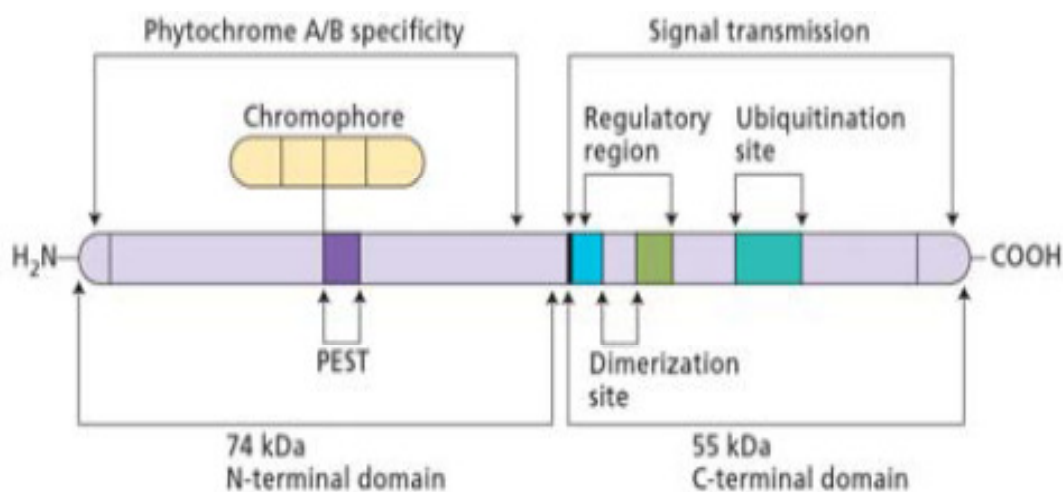
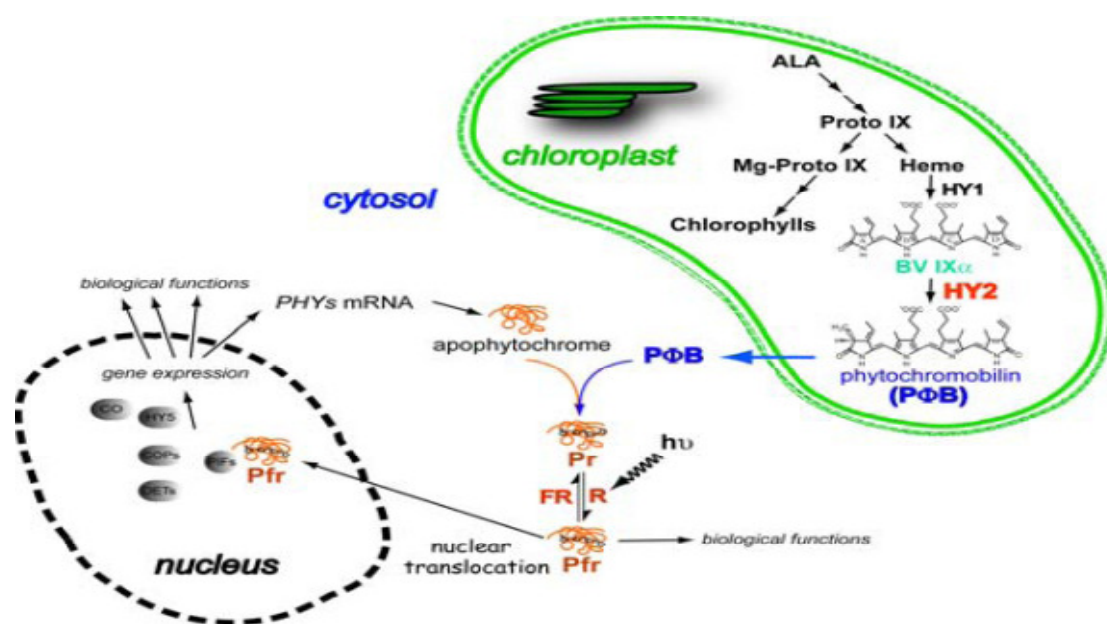


Figure 2: illustration of the two domains of the phytochromes, the N-terminal domain and the C-terminal domain. Plant physiology, Fourth edition, figure 17, part 1 © 2006 Sinauer Association

The chromophore is synthesized inside plastids and is leaked into the cytosol where it is attached to the apoprotein to form a holoprotein. As the chromophore absorbs the light, there is a conformational change of the apoprotein due to isomerization of chromophore from *cis* to *trans* of the double bond between carbon 15 and 16. This results in an exposure of nuclear localization site for localization of active phytochrome form into the nucleus (Song, 1999).



http://ipmb.sinica.edu.tw/faculty_show_e.php?sid=10&catid=4

Figure 3: Illustration of apophytochrome and phytylgeranylgeranyl pyrophosphate synthesis in the cytosol and plastid respectively. After binding, the photo-activated phytochromes in light are translocated into the nucleus to trigger the genes for photomorphogenesis.

1.2.4 Phytochrome localization

All five phytochromes in *Arabidopsis* are present in the cytoplasm in the dark and are transported into the nucleus in light-dependent manner (Kircher S, Gil P, Kozma-Bognar L, FejesE, Speth V, et al. 2002). Each phytochrome requires a light quality to be imported into the nucleus. PhyA is transported to the nucleus in far-red light while the other five phytochromes accumulate in the nucleus in the red light. PHY A migrates to the nucleus much faster than the other five phytochromes (Kircher S, Gil P, Kozma-Bognar L, FejesE, Speth V, et al. 2002). Two proteins in *Arabidopsis*, **FAR-RED ELONGATED HYPOCOTYLS 1 (FHY1)** and **FHY1 LIKE (FHL)** are required to facilitate the PHYA to be transported into the nucleus to trigger phyA signaling to light response

(Genoud et al., 2008). Two transposase-derived transcription factors, **FAR-RED ELONGATED HYPOCOTYLS 3 (FHY3)** and **FAR-RED IMPAIRED RESPONSE1 (FAR1)** regulate the expression of **FHY1** and **FHL** and the expression of **FHY3** and **FAR1** is negatively regulated by phyA. PhyB has nuclear localization site (NLS) at the C-terminal domain for nuclear translocation (Chen et al., 2005)

1.2.5 Downstream signaling components

Current research of photomorphogenesis focuses on downstream signaling of photoreceptors and majority of our understanding is derived from de-etiolation in *Arabidopsis* seedlings (Quail, 2002). **COP/DET/FUS** genes are necessary for the repression of photomorphogenesis in darkness. Mutation in these genes produces seedlings showing morphological features of light-grown seedlings when grown in darkness (Wang and Deng, 2002). This suggests that they encode proteins acting as repressors of photomorphogenesis. **Constitutive COP, DET and FUS** proteins function as a negative regulator of photomorphogenesis and exhibit light grown phenotype (short hypocotyls and open cotyledons) in the darkness (Sullivan et al., 2003; Yi and Deng, 2005). COP1 is a E3 ubiquitin ligase that shows nuclear accumulation in darkness and targets light regulatory transcriptions such as **HY5 (LONG HYPOCOTYL 5)** for degradation and allows accumulation of others that promote etiolated growth. HY5 is positive regulator of photomorphogenesis by binding the promoters of genes whose expression is controlled by light and mutants in HY5 show etiolated seedlings in light. (Osterlund et al., 2000).

Phytochromes and cryptochromes promote accumulation of HY5 under specific light conditions targeting the light-induced genes and reducing the nuclear abundance of COP1 causing it to migrate to the cytosol. The product of HY5 gene integrates signals from multiple photoreceptors to mediate hypocotyls inhibition in response to blue, red and far-red light. These light conditions cooperate in abolishing the activity of COP1 as a negative regulator.

Protein-protein interactions are required for many signal transduction mechanisms. A number of **PHYTOCHROME-INTERACTION PROTEINS (PIFs)** involved in the signal transduction have been identified and studied. Recent studies on the function of PIF3 showed that PIF3 negatively regulates PHY-mediated inhibition of hypocotyl

elongation and cotyledon opening (Kim et al., 2003). Photo-activated form of phytochrome (Pfr) imported into the nucleus interacts with downstream signal transducers such as PIF3 resulting in phosphorylation of PIF and degradation. COP1 positively regulates PIF3 levels and targets phytochromes for degradation.

HY5 is a vital transcription factor for photomorphogenesis that is regulated by multiple photoreceptors. HY5 also physically interacts with FHY3 and FAR1 and negatively regulates their activity (Li et al., 2010).

1.3 Cryptochromes

Cryptochromes are blue light photoreceptors that regulate various responses in plant, mainly de-etiolation of seedlings, circadian clock and photoperiod of flower induction (Lin and Shallitin, 2003). In Arabidopsis, there are two major blue photoreceptors: cry1 and cry2 which inhibit primarily hypocotyls elongation in blue light and regulation of flowering time respectively. They also regulate other light responses in Arabidopsis (Koomneef et al., 1980; Ahmed and Cashmore, 1993; El-Assal et al., 2001). Cry1 plays an important role in responding to the high light intensities which makes it remain stable in the light. On the contrary, cry2 is crucial in response to a low light irradiance and it is a light labile in nature (Lin, 2002). Cry3 is present in Arabidopsis, but its function has been described so far (Kleine et al., 2003). Cry1 is translocated from the nucleus to the cytosol on light activation while Cry2 is a predominantly localized in the nucleus in both light and dark (Yang et al., 2000)

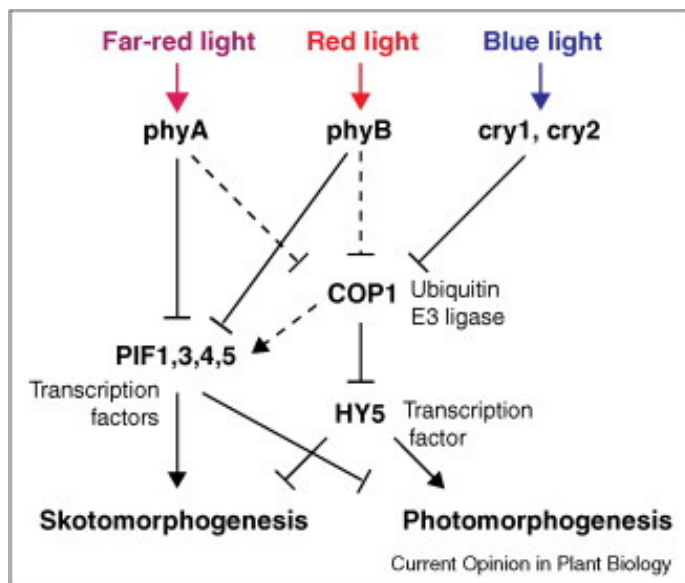
Cry apoprotein contains two domains: the N-terminal photolyase-homologous region (PHR) domain and C-terminal cryptochrome C-terminal extension (CCE) domain. PHR is the chromophore binding domain of crys that binds non-covalently to the **chromophore flavin adenine dinucleotide (FAD)** (Lin et al., 1995).

1.3.1 Photoactivation of CRY

Arabidopsis cry1 and cry2 undergo phosphorylation in etiolated seedlings exposed to blue light and the cry phosphorylation is required for its photoactivation. Under the blue light exposure, the cry1 is photoactivated which leads to the exit of COP1 from the nucleus to the cytosol as a result of structural modification of COP1. HY5 level in the nucleus is then increased due to dephosphorylation, increasing its biological

activity. More HY5 is then available to bind to G-box motifs and promote transcription of genes for photomorphogenesis (Shallitin, 2003).

Recently it was shown that cry1 interacts with **SUPPRESSOR OF PHYTOCHORME A (SPA1)** in a blue light dependent manner. This interaction can suppress the SPA1-COP1 interaction and HY5 degradation, thus promoting photomorphogenesis (Liu et al., 2011; Lian et al., 2011).



Sun Lau and Xing Wang Deng, 2010

Figure 4: A simplified model of the light signaling pathway. Photoreceptors act to repress two main the light signaling branches (COP1-HY5 and PIFs) under the light.

1.4 Phototropins

Phototropins are the most recently described blue-light absorbing plant photoreceptors which mediate responses phototropism, stomatal opening and chloroplast movements (Briggs and Christie, 2002). Arabidopsis contains two phototropins referred to as phot1 and phot2. Mutants lacking phot1, phot2 or both photoreceptors showed that they have overlapping function in controlling in various photoresponses (Christie, J.M, 2007). Both phytotropins are equally important in mediating the phototropism, but phot1 acting as the predominant photosensor (Sakai, T, 2001).

1.5 Effect of sugar on hypocotyls elongation

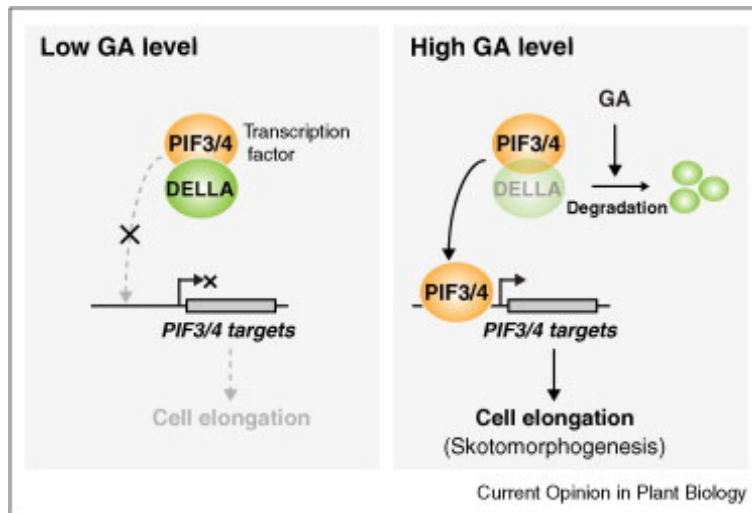
Sugar such as sucrose not only does it provides carbon and energy metabolism to the plant, but also has a vital signaling function during the plant growth and development. It is well documented that the effect of sucrose on plant life cycle is dependent on the presence of light (Zhang et al., 2010). Hypocotyl elongation of *Arabidopsis* seedlings is suppressed in the presence of light, but promoted in the dark. GA plays a vital role in dark-induced hypocotyls elongation and is an important component of the light signaling pathway controlling hypocotyls growth (Alabadi et al., 2004). PIF transcriptional factors act as positive regulator of hypocotyls elongation and play an important role in sucrose-induced hypocotyls elongation. Sucrose up-regulate the transcript level of PIFs in the dark and it is dependent on the presence of GAs (Zhongjuan et al., 2011).

1.6 Effect of gibberellins on light signaling pathways

Endogenous factors such as hormones also control de-etiolation of seedlings. Seedlings defective in either gibberellins (GA) or brassinosteroid signaling are unable to fully suppress photomorphogenesis after germination in darkness. The GA and light signaling pathway interact in the regulation from etiolation to de-etiolation by regulating the activity of the HY5 and PIF light signaling elements (David Alabadi, 2007).

In darkness, high level of COP1 and GA signaling represses negatively the photomorphogenesis caused by low level of HY5 where GA pathway enhances the activity of the PIF transcription factors to promote positively etiolated growth (David Alabadi et al., 2008). High GA levels result in low DELLA accumulation under these conditions. Low concentration of DELLA proteins permits the activity of the PIF transcription factors (Vriezen et al., 2004).

DELLA proteins stabilize partially inhibit PIF activity and promote HY5 accumulation in light. GA levels decrease during light-induced de-etiolation and stabilization of DELLA protein alleviates the negative effect of GA signaling on photomorphogenesis (Achard et al., 2007). Upon illumination, the COP1 is activated which results in instability of PIF activity and accumulation of HY5.



Sun lau and Xing Wang Deng, 2010

Figure 5: At low GA, DELLA proteins present high level and repress GA signaling. DELLAs interact with the PIF3 and PIF4 preventing from being expressed. At high GA level, DELLA are degraded and PIFs are released.

1.7 Reversible phosphorylation

Reversible phosphorylation regulates numerous cellular functions of the eukaryotes through signal transduction mechanism and is catalyzed by protein kinase and protein phosphatase (Hunter T., 1995). Recently, it has been proved that the protein phosphorylation is involved in almost all signaling pathways. The addition to or removal of a phosphate group from a protein has a huge impact on the protein structure, thus affect the functional properties of the protein (Johnson and Reilly, 1996).

In an early study, it was shown that the protein phosphorylation is regulated by light (Datta, Chen and Roux, 1985). Later, it was demonstrated that the red (R), far-red (FR) and blue light control the phosphorylation of some proteins both in the nucleus and in the cytoplasm proposing that there are some kind of protein kinase and protein phosphatases involved in light signal transduction pathways (Malec, Yahalom and Chamovitz, 2002).

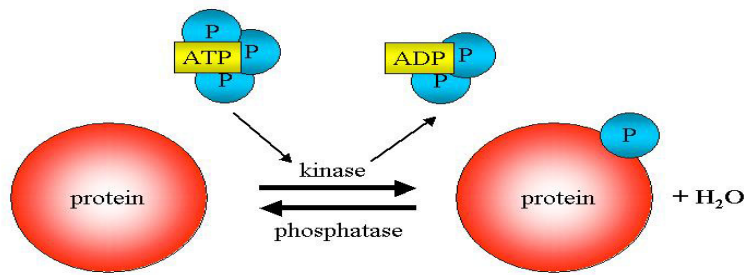


Figure 6: Illustration of reversible phosphorylation involving in post-translational modification of proteins. Analysis of the Structure and Function of Protein Phosphatase 2A, Basel, 2004

1.7.1 Phytochrome phosphorylation/dephosphorylation

Reversible phosphorylation of the photoreceptor proteins is a key light signaling mechanism in phytochrome. Recent studies suggest that the phosphorylation and dephosphorylation of phytochrome be a key regulatory mechanism in its light-signaling pathway.

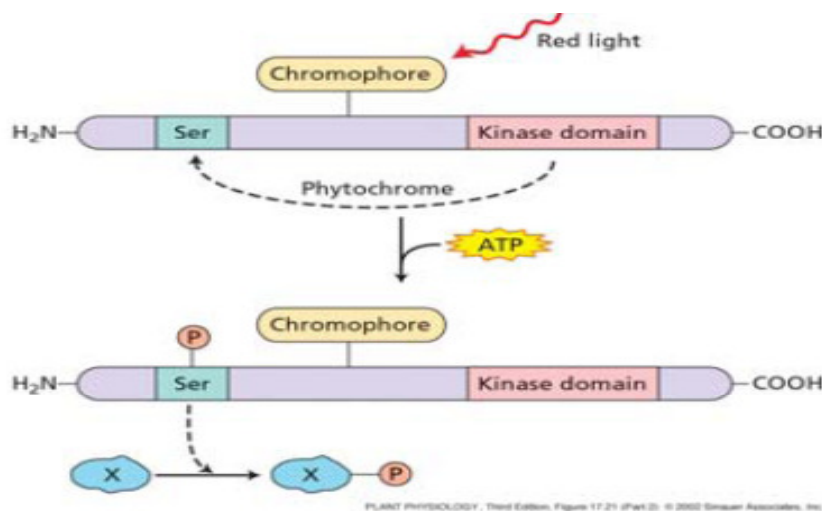


Figure 7: Illustration of how the phytochromes are phosphorylated under light. Plant physiology, Fourth edition, figure 17, part 1 © 2006 Sinauer Association

Phytochromes are phosphoproteins and there are two phosphorylation sites: Ser 8 in the NTE region that is phosphorylated in both the Pr and Pfr forms and Ser 599 in the hinge region that is phosphorylated in the Pfr form in vivo (Lapko et al., 1999). Since they are phosphoproteins and kinases, they can be phosphorylated by autophosphorylation and by other protein kinases (Han et al., 2010).

There are protein kinases and phosphatases responsible for phosphorylating and dephosphorylating phytochromes. So far there is no report of a protein kinase that can specifically phosphorylate phytochromes. There are also reports of protein phosphatases that dephosphorylate phytochromes. In *Arabidopsis*, serine/threonine specific protein phosphatase 2A interacts and dephosphorylates phy A (Kim, et al., 2002).

1.7.2 Crptochrome phosphorylation/dephosphorylaion

Phosphorylation plays an important role in cryptochrome activation in response to blue light irradiation (Shalitin et al., 2002). It was found that serine residues are the major phosphorylation sites of CRY1 (Bouly et al., 2003). It has been assumed that protein dephosphorylation has an equally important regulatory function (Luan, 1998).

In this study, we aim at investigating the role of PP2A in signaling pathways from phytochromes and cryptochromes in the nucleus by making phenotypic observations of *Arabidopsis* seedlings of wt (col) and two mutants seedlings for PP2A (B55 α and B55 β).

1.8 Protein phosphatase 2A

Protein phosphatase 2A is a serine/threonine specific phosphatase that plays an important role in many diverse eukaryotic cellular functions (Millward et al., 1999). Its structure consists of three subunits: a 36-38 kDa catalytic subunit (C), a 65 kDa scaffolding subunit (A) and a 48-78 kDa regulatory subunit (B). The regulatory B subunit plays a vital role in conveying substrate specificity and cellular localization of PP2A in mammals (Eichhorn et al., 2009). It is also believed that the different B subunits have such function in plants (Matre et al. 2009). B subunits are classified into at least three distinct groups in plants, B (55), B' and B'', based on molecular weight and domains (Farkaset et al., 2007). Only two members in *Arabidopsis*, B55 α (At1g51690) and B55 β (At1g17720) belong to B55 family. The B' family is divided into several subfamilies B' α (At5g03470) and B' β (At3g09880).

PP2A can act on phytochrome signaling pathways in different ways: It can directly dephosphorylate phytochromes or it can inactivate the kinases phosphorylating the phytochromes or act in the signaling pathway downstream of phytochromes. The aim

of this study is to observe whether the mutated B55 subunit of PP2A alter the phenotype of the seedlings grown in different light conditions.

Although dephosphorylation is significant as kinases in plants, much less research has been performed on phosphatases. The importance of PP2A is extensively studied in animals and fungal cells, but less is done in plant cells. PP2A is one of the most abundant types of serine/threonine phosphatases in all eukaryotic cells, comprising 1% of the total protein content in mammalian cells (Lin et al., 1998). Some mutants in *Arabidopsis* have been investigated and so far few mutants with phenotypes have been identified. It is therefore important to identify more mutant of PP2A which can provide additional function of PP2A in plant.

From there the roles of PP2A function in *Arabidopsis* plant would give us a hint for further examining its function by cloning the genes of appropriate expression vector to produce the protein in *E-coli* bacteria.

1.9 Subcloning

Subcloning is a molecular technique which is used for transferring a gene of interest into a target vector for studying the functionality of the protein expressed by the target gene. PGEX-6p-1 vector contains GST tag at the upstream of the multiple cloning site of the vector. When the gene is inserted into the desired site of the vector, the DNA polymerase lands the lac promoter before the GST gene and transcribes the GST with the gene of interest inserted into the restriction site of the vector. The GST protein can be later used for purification of the protein by binding Glutathione-Sepharose 4B beads and the protein can be eluted with the washing buffer to obtain the desired protein with GST tag.

2.0 Purpose of this study/Research objectives

The first aim is to observe the physiological response of *Arabidopsis* single mutant seedlings of B55 α and B55 β of PP2A in different light conditions and contrast with the wild type responses to deduce the function of the protein phosphatase 2A in cellular level in different light conditions in *Arabidopsis* plant. From there, we can determine the roles of PP2A B subunits in *Arabidopsis* plant.

The second aim was to subclone B55 and B β target genes from B55 construct into the destination vector of PGEX-6p-1 for its functionality. It is also essential to learn how to copy a particular gene of interest from an organism and insert into a plasmid vector.

The third aim was to test antibodies made against a short peptide of B55 α , B55 β and B β expressed in the host vector.

2 Materials and methods

2.1 Plant material

The *Arabidopsis* seeds of wild type (Columbia) and two single mutant seeds of Salk line of B55 α (Salk_062514), B55 β (Salk_095004) were obtained from European Arabidopsis Stock Centre (NASC, Nottingham, UK, session et al 2002). The single mutant seeds were grown in different monochromatic lights to observe the effect of lights on the hypocotyls growth, and then compared with the wild type.

2.1.1 Growth chamber

To obtain the desired monochromatic wavelength of red, far-red and blue light for *Arabidopsis* plant growth, a selective light filter was set up in the growth chamber for emitting the monochromatic light for blue, red and far-red. Seedlings grown for 16 hrs light and 8 hrs dark, separate growth room was used for the growth of the plants.

For plants in darkness, aluminum foil was wrapped on the plates before putting them in the growth chamber. The temperature for growth chamber is maintained around 22C⁰ for all light treatments.

2.1.2 Preparation of MS medium

The following solutions were first made for preparation of MS medium

KNO ₃	95g/l
NH ₄ NO ₃	120g/l
MgSO ₄ .7H ₂ O	37g/l
KH ₂ PO ₄	17g/l
CaCl ₂ .2H ₂ O	44g/l

Fe/EDTA for 1L

Na ₂ .EDTA	0.373g
FeSO ₄ .7H ₂ O	0.278g

Minor I for 1 L

ZnSO ₄ ·7H ₂ O	0.920g
H ₃ BO ₃	0.620
MnSO ₄ ·4H ₂ O	2.230g

Minor II for 1 L

Na ₂ MoO ₄ ·2H ₂ O	0.025g
CuSO ₄ ·5H ₂ O	0.003g
CoCl ₂ ·6H ₂ O	0.003g
KI	0.083g

Preparation of 1 liter of MS medium

KNO ₃	20ml
NH ₄ NO ₃	13ml
MgSO ₄ ·7H ₂ O	10
KH ₂ PO ₄	20
CaCl ₂ ·2H ₂ O	10
Minor I	10ml
Minor II	10ml
Fe/EDTA	50ml
Sucrose	30g

The PH was adjusted to 5.8 before addition of agar. The medium was autoclaved for 20 min at 110°C.

2.1.3 Preparation of half strength medium

Two half strength media for growing the seedlings of *Arabidopsis* were prepared from the prepared MS medium of which one is 1% sucrose while the other is lacking sucrose.

2.1.4 Surface seed sterilization and sowing

1 drop of Tween was added into 1% (w/v) Ca-hypochlorite solution. It was shaken and waited until it settled. 1ml of the prepared solution was pipetted into 9ml of 96% ethanol. The *Arabidopsis* seeds were filled into 1.5ml Eppendorf tubes and 1ml of the ethanol/Ca-hypochlorite solution was added and the tubes were gently inverted. The seeds were allowed to stand in the solution for 5 min. the supernatant was sucked off. The seeds were finally rinsed with 1ml of 95% ethanol twice to remove any residual hypochlorite. They were left to overnight on bench.

The surface sterilized seeds of Columbia wild type and mutants of B55 α and B55 β were sown on half strength MS medium containing 1% sucrose and without sucrose. Plates were sealed with surgical tape and incubated/cold-treated in the dark for 3 days at 4C⁰ to break dormancy and improve synchronous seed germination. The seed germination were then induced by exposing the plates for 1hr light treatment and then returned back to complete darkness for 23hrs at 22C⁰. Finally the plates were transferred into appropriate light treatments for 6 days before the phenotypic observations. For dark-grown condition, the plates were sealed with several layers of aluminum foil in the same growth chamber. Plates were positioned vertically in each step so that the seedlings could grow along the surface of the solidified medium.

2.1.5 Measurement of hypocotyls and root lengths

The seedlings were carefully harvested and placed on a black sheet for taking picture with normal camera and measured the hypocotyls lengths. The phenotypic observations of the seedlings were performed before harvesting while they were on the plates. The hypocotyls lengths were measured against a ruler using mm scale. The measured hypocotyls length was between the root-hypocotyl transition and the apical basis.

2.1.6 Statistical analysis

The means hypocotyl lengths of seedlings were calculated from the measured lengths of the 5 sample seedlings. For calculating the mean, three independent experiments were performed with 5 seedlings for each variety of plants. From there, the standard deviation and standard error were calculated for data analysis.

2.2 Subcloning

Subcloning is the transfer of gene of interest from one parental vector to destination vector for protein expression and thus studies its functionality. In this experiment, the genes of B55 α , B55 β and B β were previously cloned into the parental vector of PWEN and they were then subcloned into the expression vector of PGEX-6p-1 by using subcloning technique (Smith and Corcoron, 1990).

2.2.1 Cloning vector

The cloning vector, PGEX-6p-1, contains multiple cloning sites where the foreign DNA is to be inserted, antibiotic resistance gene and origin of replication. In this experiment, our genes of interest have been subcloned into PGEX-6p-1 vector since it has the essential elements for protein production (Sambrook and Russell, 2001).

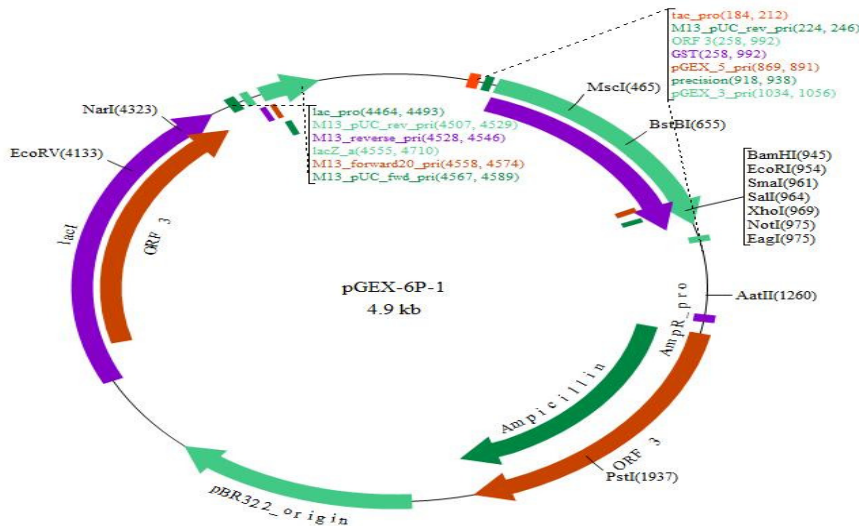


Figure 8: PGEX-6p-1 bearing the essential elements for subcloning. <http://www.gelifesciences.com>

Bioinformatics tools of (<http://tools.neb.com/NEBcutter2/index.php>) and (<http://www.arabidopsis.org>) were used in order to design the primers for gene amplification. The restriction enzymes that cut the vector, but not our gene of interest, were searched in database. Each restriction enzyme cleaves the recognition sequence of plasmid DNA at specific site. The following restriction enzymes were used for primer design for each gene.

Table 1 : Restriction enzymes used for primer design with their cleavage sites

Target DNA	Primers	Restriction enzymes	Restriction sequence	Cleavage site
Bα	Forward primer	BamHI	\downarrow 5'GGATCC 3'CCTAGG \uparrow	5'---G GATCC---3' 3'---CCTAG G---5'
	Reverse primer	NotI	\downarrow 5'GCGGCCGC 3'CGCCGGCG \uparrow	5'---GC GGCCGC---3' 3'---CGCCGG CG---5'
Bβ	Forward primer	BamHI	\downarrow 5'GGATCC 3'CCTAGG \uparrow	5'---G GATCC---3' 3'---CCTAG G---5'
	Reverse primer	NotI	\downarrow 5'GCGGCCGC 3'CGCCGGCG \uparrow	5'---GC GGCCGC---3' 3'---CGCCGG CG---5'
B`β	Forward primer	ECoRI	\downarrow 5'GAATTC 3'CTTAAG \uparrow	5'---G AATTC---3' 3'---CTTAA G---5'
	Reverse primer	XhoI	\downarrow 5'CTCGAG 3'GAGCTC \uparrow	5'---C TCGAG---3' 3'---GAGCT C---5'

2.2.2 Primer design

First, the primers for each gene of B55 α , B55 β and B` β were designed with the above restriction enzymes to amplify the genes. These restriction endonuclease sites were used for subcloning the CDS of genes into the destination vector. The 3` section of primers should be complementary to the DNA template (the primers were designed in such a way that they should be complementary to the 3` strand of the DNA template). In order to ensure that the gene is inserted into the vector, each primer were incorporated two different restrictions sites so that the gene could be cloned into destination vector in the right orientation (directional cloning). The designed primers were then used in PCR reaction to amplify the target gene and the PCR

product was purified before insertion into the destination vector (Nelson and Cox, 2002).

2.2.2.1 Forward primers

For the forward primer, the first 21 nucleotides of the CDS of the genes were chosen and restriction sites were introduced to the 5`end of the forward primers; that is upstream of the start codon. Extra nucleotides were added at the 5`end of the primer for structural support.

2.2.2.2 Reverse primers

For the reverse primer, the last 21 nucleotides of the CDS of the genes were selected and reversed. The bases were then sequenced from 5`to 3`direction and the restriction sites were incorporated at the 5`end of the primers. Additional bases were added for support.

Table 2: Primer sequence used for the gene amplification

Gene of interest	Primers	Sequence of the primers
B55 α	Forward primer	5`- ATC GGA TCC ATG AAC GGT GGT GAT GAG GTC - 3`
	Reverse primer	5`- AAT TGC GGC CGC TTA AGC ATA GTA CAT GTA - 3`
B55 β	Forward primer	5`- ATC GGA TCC ATG AAC GGT GGT GAC GAT GCC - 3`
	Reverse primer	5` - AAT TGC GGC CGC AAC TCA TGC ATA GTACAT GTACAA - 3`
B` β	Forward primer	5`- ATG AAT TCA TGT TTA AGA AAA TCA TGA AAG - 3`
	Reverse primer	5` - ATC TCG AGC TAG GAA GTG ATC ATA TGATC - 3`

2.2.3 Bacterial culture for extraction of plasmid DNA

LB agar and broth were prepared and autoclaved for use. 25 μ l of ampicillin was pipetted at the centre of the plates. The 25ml LB agar medium was poured into the plate and mixed well by shaking. It was waited until the solidification. Around 100 μ l of *Escherichia coli* (DH5 α) carrying the blue script vector with the genes of PWEN 25-51690-16, PWEN 18-177720-37 and PWEN 18-9880-2 were poured to the agar plates by streak method. The plates were then incubated at 37C⁰ for overnight. Plates were inverted lids downward.

3-4 of bacterial colonies from overnight culture were picked with a sterile loop and put in a tube containing 5ml of LB medium and 5 μ l of ampicilin. It was placed in a shaking incubator at 37°C for overnight.

2.2.4 Plasmid isolation from blue script vector

For isolation of bacterial culture, GenElute™ plasmid Miniprep Kit from Sigma was used. The liquid culture from the shaking incubator was centrifuged for 1 min. The supernatant was discarded and the pellet was worked according the protocol as follows.

The pellet was resuspended in 200 μ l of resuspension solution to remove RNA molecules. It was pipetted up and down for mixing well. 200 μ l of lysis solution containing NaOH was used. SDS was added to rupture cell membrane while the alkaline denatures chromosomal DNA and proteins. The solution became clear after 5 min. the covalently closed circular plasmid DNA is not denatured. 350 μ l of binding solution was then added and mixed gently by inverting 6 times to neutralize pH. With this solution, the chromosomal DNA precipitates with the proteins-SDS complex. The solution was spin for 10 min and transferred to binding column in a collection tube previously added 500 μ l column preparation solution with spinning. The flow-through was discarded and washed with 500 μ l and 700 μ l washing buffer twice. The solution was spin for 1 min and transferred to a new column. 50 μ l of elution solution was added and spin for 1 min. It was stored at -20C^o.

2.2.5 Agarose gel electrophoresis

Agarose gel electrophoresis is commonly used to separate and verify the size of the amplified DNA sequences. DNA is negatively charged due to negative charges of the phosphate groups in the backbone of the nucleic acids. Nucleic acids migrate toward the positive pole of the electric field through the gel with the shorter DNA molecules move faster than the longer DNA molecules since they slip through the pores more easily. The size of the PCR product can be determined by comparison with known sizes of the DNA ladder run alongside the PCR products.

Agarose is a linear polysaccharide that forms pores upon dissolving in hot water and cooling down again. The pore size depends on the concentration of the agarose. DNA was visualized by UV light using a gel red

0.4g of agarose powder was melted in 1x TAE buffer to make 0.8% (w/v) agarose gel for the separation of DNA molecules. The agarose gel was run for 45 min at 90V. The isolated plasmids were run on 0.8% gel to check its size whether the plasmids were successfully isolated.

Table 3: Reagents used for running the agarose gel

Item	Amount
Ladder	1,5 µl
Loading buffer	1,5 µl
BioRed gel	1,5 µl
DNA Sample	3,0 µl

2.2.6 Amplification of insert by PCR

Polymerase chain reaction (PCR) is used for amplification of target DNA. The reaction proceeds in three steps in each cycle with three distinct different temperatures

1. Denaturation step: the double stranded DNA is separated due to breakdown of hydrogen between the bases when the temperature is increased to 90C⁰ resulting in two separate single strands.
2. Annealing step: the primers anneal to the complementary sequences when the temperature is lowered to 60C⁰.
3. Elongation step: The polymerase enzyme adds nucleotide to the 3` of the primer by synthesizing a new complementary strand from 5` to 3`.

For obtaining a successful quantity of target DNA, 35 cycles were run in PCR (Reece, 2004)

The following solutions were used to run the PCR during gene amplification.

Table 4: The components used in the PCR mixture

Component	Volume
dH ₂ O	34.5 µl
10x conc.with Mg ²⁺	5 µl
dNTPs, 10nM	4 µl
Forward primer (30µM)	0.5 µl
Reverse primer (30µM)	0.5 µl
Taq DNA polymerase	0.5 µl
DNA template	5 µl
Total volume	50µl

Table 5: PCR program

Step	Cycle	Time	Target temperature
Initial denaturation		5min	95C ⁰
Denaturation	35 repeats	30sec	95C ⁰
Annealing		30sec	60C ⁰
Extension		2min:30sec	72C ⁰
Delay		5 min	72C ⁰
Cooling		infinite	4C ⁰

2.2.7 Purification of PCR product

The PCR product was run on 0.8% agarose gel and then the expected size band was excised under UV light for purification from the contaminants like the left primers, nucleotides, salt and polymerases that might affect the digestion and ligation steps. The GenElute Gel extraction Kit was followed.

An eppendorf tube was weighed and the DNA fragment of gene of interest was cut out from the gel with a clean razor blade under UV light and placed into the tube. The tube with the gel slice was weighed and then calculated the weight of the gel slice by finding the difference. 3 gel volume of the gel solubilization solution to the gel slice was added, that is 200mg of agarose gel in 600µl of gel solubilization solution. The

mixture was then incubated at 60C⁰ for 10 min until the gel is completely dissolved. For binding column preparation, the binding column was placed into 2ml collection tube. 500µl of the column preparation solution was added to the binding column. It was then centrifuged for 1min. the flow-through was discarded. The color of the mixture was yellow in the gel solubilization solution so there was no need to add Sodium Acetate. It was then preceded to the next step.

1 gel volume of the isopropanol was added and then the mixture was homogenized (200mg agarose gel in 200µl of isopropanol). The mixture was loaded into the binding column and centrifuged for 1min. The flow-through was discarded and the collection tube was assembled back. The binding column was added to wash solution and centrifuged for 1min. The flow-through was discarded and the binding column was centrifuged again for 1min without adding any additional wash solution. The binding column was transferred to a new collection column labeled with sample and 50µl of elution solution was added to the centre of the membrane. It was incubated for 1min and centrifuged for 1min. The sample was stored -20C⁰.

2.2.8 DNA concentration measurements

The concentration of DNA in each necessary step was determined to get an overview of the right amount of using in the next step. The concentration of 1µl of DNA sample was determined by NanoDrop instrument and measured absorption at 260 nm.

2.2.9 Digestion of PCR product and vector

Digestion enzymes cleave the DNA at specific base sequence so that the DNA fragment of the gene is inserted into the cut plasmid with the same restriction enzyme. In this experiment, the purified PCR product and the destination vector were digested with the same restriction enzymes to make complementary annealing sites prior to ligation. The circular DNA of the plasmid will be cut open at the recognition site by appropriate restriction enzymes and the PCR product will be cut where the restriction enzymes incorporated during the primer design.

Table 6: Reagents used in digestion of plasmid and vector

Items/reagents	Amount
Deionized water	3,5 ul
Reaction buffer	1,0 ul
Plasmid miniprep DNA	3,5 ul
BSA 10X	1,0 ul
Enzyme1	0,5 ul
Enzyme2	0,5 ul
Total	10 ul

The content of the tube were mixed well and incubated for 4hrs at 37C⁰. The digestion was then heat inactivated at 80C⁰ for 20min. The solution was run on a gel to check the digested product with different sizes. The digested product of the genes and vector were purified by GenElute PCR Clean up Kit.

2.2.10 Ligation of insert into vector

The linearized vector and digested PCR product were joined together by T4 DNA ligase after digestion. After the concentration measurement of both digested DNA plasmid and PCR product, a vector to insert ratio of 1:3 were used in the ligation reaction. The T4 DNA ligase catalyses the formation of phosphodiester bond between the 5`phosphate group and 3` hydroxyl group. The following solutions were used in the ligation mixture.

Table 7: Reagents used in ligation of plasmid with the vector

Items/reagents	Amount
Deionized water	3.5 ul
Reaction buffer	1.0 ul
Plasmid miniprep DNA	3.5 ul
BSA 10X	1.0 ul
Enzyme1	0.5 ul
Enzyme2	0.5 ul
Total	10 ul

2.2.11 Preparation of competent cell

Calcium ions treatment of *E. coli* cells and some other divalent cations makes these cells competent and allow taking up DNA from the environment (Glover and Hames, 1995). The cells that can take up the foreign DNA are called competent. The ligation reaction of PGEX-6p-1 with DNA target was introduced into competent cells of DH5 α bacteria. Only those bacteria acquired the plasmid with the resistant gene for antibiotics (ampicillin in this experiment) will grow in the culture.

A single colony of *E. coli* DH5 α picked up from the LB agar plate overnight at 37°C was transferred to 5ml LB medium in a sterile tube. The culture was incubated at 37°C for overnight in shaking incubator. 1ml of overnight culture was transferred to 250 ml-Erlenmeyer flask containing 100ml LB medium and cultured until the optical density reaches 0.4-0.5. The culture was centrifuged for 10 min at 4000 rpm after cooling on ice for 15 min. The pellet was gently re-suspended in 10 ml ice-cold 0.1 M $MgCl_2$. The cells were centrifuged for 10 min at 4000 rpm at 4°C and the pellet was gently re-suspended in 10 ml ice-cold 0.1M $CaCl_2$. The suspension was incubated on ice for 30 min and centrifuged as described before. The pellet cells were re-suspended in 1 ml MOPs glycerol. Aliquot of 100 μ l were transferred to Eppendorf tubes and stored -70°C until use.

2.2.12 Transformation

The recombinant DNA is introduced into the competent bacteria of *E. coli* (DH5 α) and the transformed bacteria are grown in LB agar medium overnight to test positive colonies.

The competent cells were thawed on ice for 15 min. The plasmid DNA was added into the competent cells and mixed well by pipeting several times. The mixture was incubated for 15 min on ice. 90 second of heat shock was performed at 42°C to promote DNA entry into the cells and then incubated for 5 min on ice. 300 μ l of LB medium was added and put in shaking at 37°C for 1 hour. 200 μ l of the cultured bacteria were added onto LB agar with ampicillin by streak method and incubated overnight at 37°C.

2. 2.13 Colony screening

The colonies were tested by colony PCR to check whether the plasmid contains the gene of interest. This involves lysing the bacteria and amplifying the gene of interest in the plasmid. The plasmids were then isolated from the positive colonies and purified for introducing the competent cells of Rosette (DE3) pLysS strain for protein production.

The presence of target genes of the purified plasmids were screened with gene specific primers and vector specific primers in PCR reaction and then run on a agarose gel to confirm the band size of the target genes in comparison with the known size of the ladder.

Table 8: Standard PCR to test the positive colonies

Component	Amount
Mg	1.5 μ l
Taq buffer	2.5 μ l
dNTP mixture	2 μ l for each nucleotide
Plasmid DNA	2 μ l
Forward primer	1.5 μ l
Reverse primer	1.5 μ l
Taq polymerase	0.1 μ l

2.3 Recombinant protein expression and purification

Glutathione S-transferase (GST) is a protein of 26kDa integrated into expression vector at N-terminus of the recombinant protein for aiding the protein production and purification. The expression of GST fusion protein by the PGEX-6p-1 vector is under control of the tac promoter which is induced by IPTG. LacI protein represses the tac promoter and the addition of IPTG inactivates the LacI repressor. The GST fusion protein can later be purified with glutathione sapharose since GST has a strong affinity for glutathione. In this experiment, glutathione agarose beads were used for isolating the GST fusion protein from the lysate.

Although many strains of *E. coli* can be used for expression with PGEX-6p-1 vector, Rosette (DE3) strain is suitable and used in this experiment. Rosetta carries a PLysS gene which encodes lysozyme for inhibiting the T7 RNA polymerase before induction of IPTG. This leads to suppress the basal level expression of the target protein. It is also able to express the fusion protein in a soluble form.

2.3.1 PCR test and production of protein

1 μ l of plasmid DNA with the target genes isolated from the positive colonies were introduced into 50 μ l of competent cells of the bacteria, Rosette (DE3) pLysS. Bacteria have some special characteristics for protein production such as it lacks the protease that could potentially degrade any fusion protein. The transformed bacteria were streaked overnight on a LB agar plate containing 100 μ g/ml ampicillin and chloramphenicol 34 μ g/ml at 37°C and then positive colony tests were carried out to check whether our target gene is in the transformed bacteria. A single positive colony of the transformed bacteria was picked up by a sterile loop and cultured in 5 ml LB medium in the presence of 5 μ l ampicillin and chloamphenicol in an overnight. 2% glucose was added to the LB medium to suppress the basal expression of the target protein.

1 ml overnight culture was transferred to pre-warmed 100 ml 2xTY medium at 37°C with ampicillin and chloramphenicol. 2% glucose was added to the 2xTY medium. The cell culture was incubated on a shaking incubator (250rpm) for 2 hrs at 37°C until OD₆₀₀ reached between 0.5 and 0.7. Spectrophotometer was used to measure the OD value.

2x TY medium (2X TY + 2% glucose) with ampicillin:

4 g Bacto Trypton

2.5 g Yeast Extract

1.25 g NaCl

dH₂O to 250 ml

Adjust pH to 7.0 with NaOH

Autoclave

2.3.2 Pre-expression control

1 ml of the cultured cells were removed and centrifuged for 10 min at 4°C. The supernatant were discarded while the pellet was re-suspended in 1x SDS protein loading buffer. The mixture was heated up to 95°C to denature proteins to reduce the disulfide bonds. It was then centrifuged at room temperature to settle contaminants at the bottom. Around 30 µl of the supernatant were loaded on the protein gel.

2.3.3 Protein induction

50 µl of 0.1mM IPTG was added to the culture of 100ml 2xTY at exponential growth phase to induce the expression of the fusion protein. The culture was then incubated on shaker at room temperature for 3 hrs. The culture was transferred to centrifuge tubes and then allowed to cool on ice for 10 min. The cell culture was centrifuged for 10 min at 4°C in 4000 rpm. The supernatant were discarded and the pellet was re-suspended with 4ml of cold lysis buffer (50mM Tris, pH 8, 100mM NaCl, 1mM EDTA). The mixture was centrifuged and the supernatant were removed. 40µl of 10mg/ml lysozyme, 20µl of 1M DTT and 4ml of lysis buffer were prepared and re-suspended with the pellet by repeating pipetting. A sample was taken to run the gel. The lysate was incubated for 15 min before storing -70°C until purification.

Lysis buffer:(50 mM Tris-HCl, pH 8, 1 mM EDTA, 0,1 M NaCl)

2.5 ml 1 M Tris (pH 8.0)

2.5 ml 2 M NaCl

0.1 ml 0.5 M EDTA (pH 8.0)

44. 9 ml dH₂O

Both pre-expression and protein induction with IPTG were run on SDS-PAGE gel to visualize the bands of fusion protein size on the protein gel.

2.3.4 Protein purification

The target proteins fused with GST tag were purified with glutathione agarose beads and then run on SDS-PAGE (10% mini-protein tax precast gel) for analysis.

The lysate were de-frosted on ice for 1 hour. 1ml of lysis buffer was transferred to an Eppendorf tube and sonicated for 30 seconds to rupture the cell wall. 400 μ of 10% Triton X-100 was added to lysed buffer in an Eppendorf tube and mixed well. The lysate and sonicated sample were centrifuged for 10 min at 4°C at 16000 rpm. Supernatants were transferred to new tubes to run the gel. 20 μ l of the supernatant were run on the protein gel.

150 μ l glutathione agarose beads and 1.5 ml supernatant of lysate were mixed in an eppendorf and rotated for 1 hour at 4°C. The mixture was spin for 1 min to make beads settle at the bottom. Supernatant was carefully removed by pipette. The supernatant was repeatedly washed with 1 ml PBS for 1 min at room temperature several times. The final volume was made 70 μ l. 20 μ l of the sample was kept for gel run. 50 μ l of elution buffer (10mM GSH in 50mM Tris-HCl, pH 8.0) was added to the washed supernatant of ratio of 1:1. The eluted fusion protein was stored at -70°C. 20 μ l were kept to run a gel

1xPBS: (10 mM phosphate-buffered saline):

140 mM NaCl, 7.7 mM Na₂HPO₄, 2.3 mM NaH₂PO₄ (pH 7.3)

Adjust pH to 7.3

100 ml of the PBS is transferred to a new tube/flask.

The rest is used to make PBST.

2.4 SDS PAGE and Western blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDSPAGE) is widely used for qualitative analysis of proteins. SDS is an ionic detergent and applies negative charges the protein by destroying the complex structure of the proteins. The proteins can then be separated according to their size using gel electrophoresis. Heating promotes the protein to be denatured and bound to the SDS. The negative charge of SDS-protein complex moves to the anode in the electric field. The small sized molecules run faster while the large sized molecules run slower. After staining,

different bands of protein molecules will be analyzed by comparing with the standard marker of known protein sizes.

In case of running western blot, the proteins in the samples were first separated by SDS-PAGE (10% mini-protein tax precast gel) according to their sizes and then transferred to a nitrocellulose membrane and the target protein is then detected by using antibodies.

2.4.1 SDS-PAGE protein analysis

20µl of samples in each step were taken and added to 20µl 2 x SDS gel loading buffer (Laemmli buffer) to denature proteins (Laemmli, 1970). The samples were heated for 10 min at 95°C and centrifuged for 1 min at room temperature. Supernatants were loaded on the gel to analyse the proteins by SDS-PAGE in gel electrophoresis system. Acrylamide gel was used in a discontinuous buffer with constant voltage of 200V. The gel was stopped when the dye reaches at the bottom of the gel. Proteins in polyacrylamide gel can be detected by Coomassie Blue stain under agitation at room temperature for 1 hour and then visualized by washing with de-staining solution of Coomassie Blue De-stain in several hours.

Electrophoresis buffer for SDS-PAGE

3 g Tris-base

15 g glycine

1 g SDS

dH₂O to 1 liter

1x Laemmli SDS-PAGE running buffer

100 ml 10x Tris-Glycine-SDS (TGS) running buffer

900 ml dH₂O

2.4.2 Western blot

Western blot (also known as immunoblot) is a technique used for transferring proteins from a gel to a membrane to be detected by antibodies specific to the target protein (Sambrook et al (1989)). The proteins are first separated by electrophoresis and the separated proteins on the gel are transferred to a nitrocellulose membrane. Once the proteins are bound to the membrane, antibodies can be used for detecting

the presence of the target protein in the sample of mixture of proteins (Duden *et al.*, 1991).

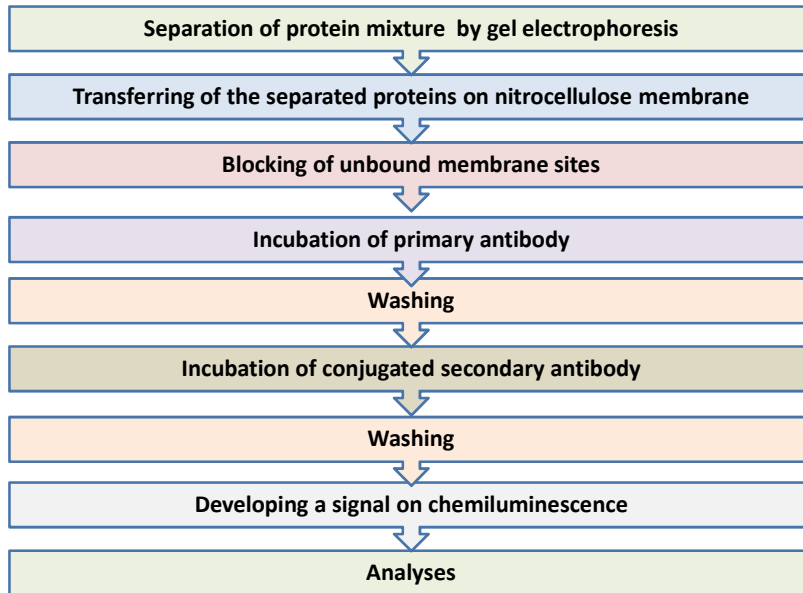


Figure 9: Basic immunological detection procedure

After the SDS-PAGE was finished, western blot was performed by transferring the protein on the gel to nitrocellulose membrane according to the below standard procedure.

In semi-wet transfer was used by assembling the gel/membrane/filter paper sandwich on electrode plate and the plate was put in the western blot machine for running to transfer the proteins into the nitrocellulose membrane on the side of the positive electrode and the gel on the side of the negative electrode and subjected to an electric field. Since SDS-treated proteins bear a negative charge and will migrate to the positive electrode under the electric current, they bind to the membrane. The electroblotting was done for 3 min with a voltage of 200V.

Transfer buffer for western blot

3.03 g Tris-base

14.4 g glycine

200 ml methanol

Mix up to 1L with dH₂O, adjust pH to 8.3

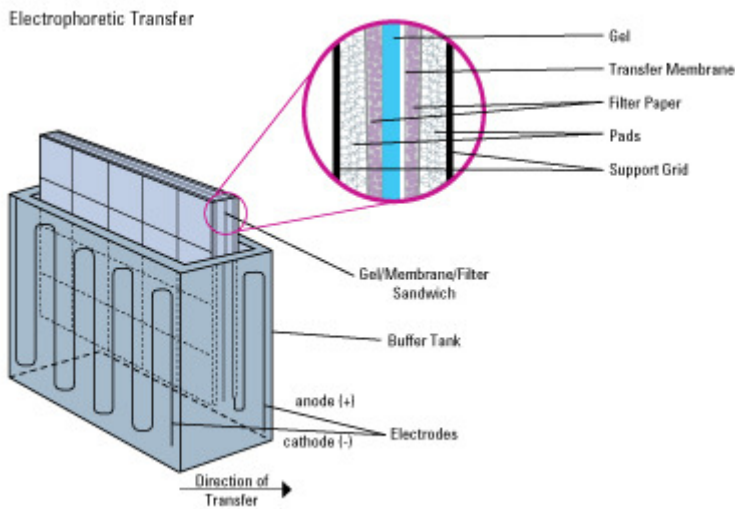


Figure 10: Electrophoretic transfer

The success of the transfer could be confirmed by visualization of proteins on membrane by Ponceau Red before proceeding to the next steps, but it has been done in this experiment.

2.4.3 Blocking

In order to reduce the non-specific protein interactions between the membrane and antibodies, membrane blocking is first performed after blotting. It is a very important step in detecting the target protein in following phases. The membrane can be immersed in Bovine serum albumin or non-fat dry milk (used in this experiment) to prevent or reduce unspecific binding of antibodies to the surface of the membrane during subsequent steps and improve sensitivity.

After the protein transfer was finished and membrane was separated from the sandwich, the membrane was blocked by incubating in 5% (w/v) non-fat dry milk prepared in 1x PBS plus 0.1% (v/v) Tween 20 in 1x PBS for 1 hour in gentle shaking at room temperature. Sufficient volume was added to keep the blot fully covered during shaking. The membrane was washed with PBST for 5 min and repeated the washing 3 times.

PBS-Tween (PBST) or Wash Buffer:

0.1% v/v Tween-20 in 1x PBS or

Add 0.5 g Tween-20 into 500 ml 1X PBS

Blocking/Incubation Buffer (PBST):

5% (w/v) non-fat dry milk and 0.1 % (v/v) Tween-20 in 1x PBS

20 ml PBST

1 g non-fat dried milk

2.4.4 Antibodies

Indirect detection method was used in this experiment. Primary antibody detects and binds to the target protein. This is followed by a labeled secondary antibody directed against the primary antibody. Secondary antibody has a horse radish peroxidase enzyme that is mostly used as labels for protein detection. The enzyme converts luminol to a light releasing substance. The light is then detected on film.

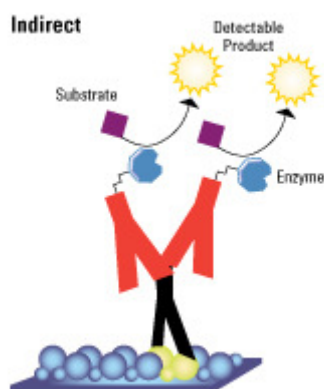


Figure 11: Indirect detection of primary antibody followed by secondary antibody conjugated to horseradish peroxidase

After blocking and washing, the membrane was immersed primary antibody of IgG molecule (rabbit) for 1 hour with gentle agitation in sufficient volume at room temperature at dilution of 1:5000 of primary antibody to blocking buffer. The primary antibody was chosen to specifically bind only the protein of interest to detect its antigen. It was washed with PBST for 15 min first to remove unattached antibody and repeated the washing 3 times at 5 min under continuous gentle shaking at RT. The blot was incubated with secondary goat anti-rabbit protein G conjugated to horseradish peroxidase (HRP) for 2 hour at room temperature with gentle agitation in sufficient volume at dilution of 1:1000 at secondary antibody to blocking buffer. The blot was washed with PBST three times for 5 min at room temperature.

Amershan ECL Plustm Western blotting detection Reagent was used for detecting the HRP conjugated antibodies on the membrane (Amersham Pharmacia). Equal amount of detection 1 and detection 2 was mixed and pipetted on the membrane with the protein side up and incubated for 1 min at RT. The detection reagent was drained out and the membrane was placed in plastic bag removing the air bubbles.

The blot was exposed to an x-ray film in dark room under red safe light and the film was developed to visualize where our target protein on the gel is with appropriate size molecular weight in the following procedure.

A sheet of Hyperfilm ECL was placed on the top of the membrane in a film cassette and closed by exposing for 1 min. It was done in a dark room. The film was developed and then washed with water. The film was incubated in fixer solution for a couple of minutes and washed with water again. It was dried and observed the bands on the membrane.

2.5 Isolation Total protein from Arabidopsis fresh leaves

Tissue samples taken from leaf tissue or any other source show a complex structure and require a high mechanical disruption in order to release the proteins. The following procedure was followed to extract protein and solubilize the proteins to determine protein concentration and detect the protein by western blot analysis.

For isolation of protein for 2D gel, 0.2 g of Arabidopsis fresh leaves of wild type (col) seedlings were finely powdered in liquid nitrogen using a pre-cooled mortar and pestle. The powder was transferred to 2ml sterile tube that was pre-cooled as well. 1.8 ml of 10% TCA in acetone containing 0.07% (v/v) 2-mercaptoethanol was added to the sample powder in the tube and mixed well. The mixture was incubated in -20C⁰ for 1 hour for protein precipitation. The mixture was then centrifuged at 4C⁰ for 15 min at 10000 rpm and the supernatant was removed. The pellet was re-suspended with acetone containing 0.07% 2-merphatoethanol and incubated -20C⁰ for 1hour. The pellet was centrifuged as before and washed twice with cold acetone containing 2-merphatoethanol for 30 min. The pellet was air-dried at room temperature and weighed. The dried pellet was stored at -20C⁰ until use.

Weight of samples

Sample 1: 0.1134 g

Sample: 0.1126 g

Preparation of TCA-acetone

14 μ l of 2-mercaptoethanol in 20 ml acetone was prepared first

500 μ l of 10% TCA was added to 4.5 ml of the above prepared solution

2.5.1 Protein determination

It is important to know the concentration of total protein before starting the Western blot to ensure that the sample is in proper range of detection. There are various methods for determining the protein concentration. In this experiment, Bio Rad Bradford assay was used for protein determination. The protein concentration is first measured by comparing the target sample to standard curve of known concentration.

The following procedure was followed for making the standard curve of known concentration.

First 10mg/ml concentration of Bovine serum albumin (BSA) standard was prepared. BSA dilution ranging from 0 to 10 mg was made from the prepared BSA standard in 2D buffer. Standard curve was constructed using a protein of known concentration. The absorbance reading was taken at 595 nm. From there, we can determine the protein of unknown concentration by plotting against the standard curve.

For measuring the concentration of the sample, 600 μ l 2D buffers were added to the air-dried pellet in the tube to solubilize the protein samples. 1:4 dilution of the dye reagent was prepared by adding 250 μ l of Bio Rad and 750 μ l of distilled water and mixed well. 5 μ l of the sample was mixed with 20 μ l of 0.12 M HCl. 875 μ l of prepared Bio Rad reagent was added to each sample. After 5 min the absorbance of the sample was measured at 595 nm in spectrophotometer.

3 Result

Reversible phosphorylation provides mechanisms to alter the function of a variety of protein signals. Protein phosphorylation and dephosphorylation is involved in light signal pathways. Protein phosphatase 2A (PP2A) is a protein phosphatase that plays a key role in many cellular functions through dephosphorylation (Johnson and Reilly, 1996). B subunits of Arabidopsis PP2A have been proposed in playing a crucial role in substrate specificity and cellular localization (Matre et al. 2009).

3.1 Plant material

In this study, we look how the mutated subunits of B55 α and B55 β of *Arabidopsis* seedlings of PP2A affect the plant phenotype, specially the hypocotyls elongation, in comparison with the wild type.

3.1.1 Seedlings grown in darkness

In darkness, phytochromes are inactive P_f form in the cytosol and cryptochromes are inactive binding to COP1 in the nucleus. This results in high level of repressors for photomorphogenesis such as PIF and COP1 in the nucleus. COP1 targets photomorphogenesis promoting transcription factors, such as HY5, for degradation. High level of GA contributes to the repression of photomorphogenesis in the dark in combination of COP1. The level of DELLA proteins are lowered as they are targeted for degradation and PIFs are active and regulate genes expression for skotomorphogenesis. Sucrose promotes the transcript levels of PIFs in a GA dependent manner in darkness.

No hypocotyls inhibition was observed in all dark-grown *Arabidopsis* seedlings on sucrose medium and in absence of sucrose. (Figure: 12 and 13).

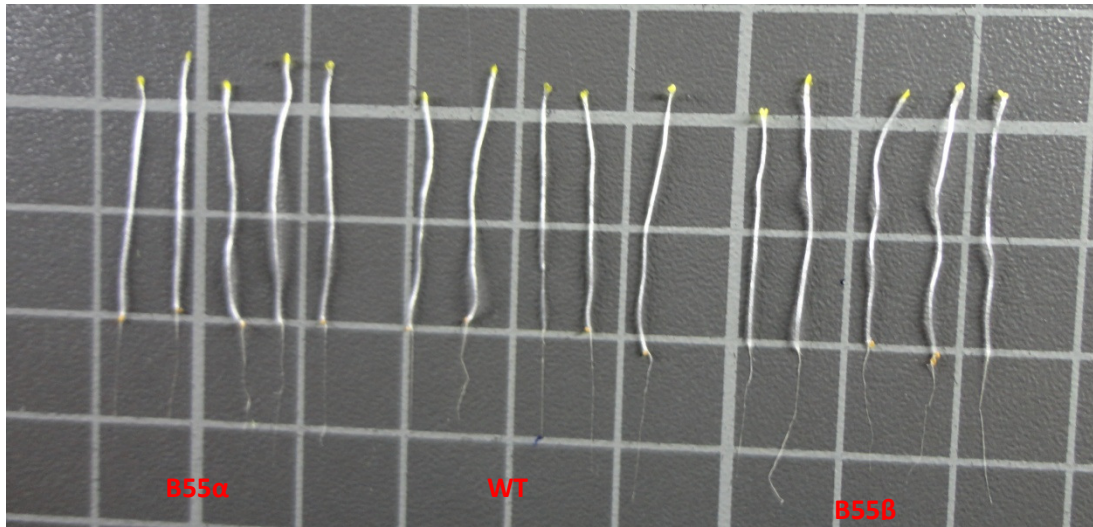


Figure 12: The hypocotyls elongation of B55 α and B55 β mutants of *Arabidopsis* seedlings grown in dark for 6 days on 1% sucrose are compared with the wild type (Col). Seedlings are etiolated with longer hypocotyls and roots. Cotyledons are unopened. One square is 10mmx10mm

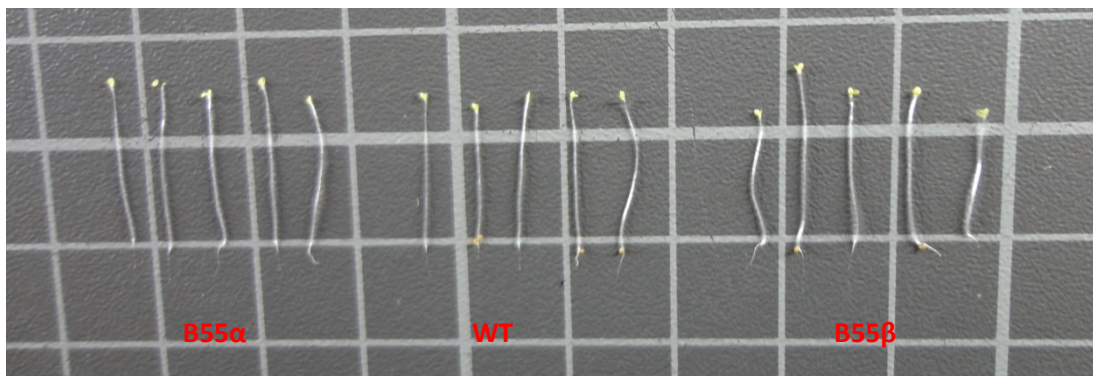


Figure 13: The hypocotyl elongation of B55 α and B55 β mutants of *Arabidopsis* seedlings grown in dark for 6 days in absence of sucrose are compared with the wild type (Col). Seedlings showed an etiolated phenotype with long hypocotyls and short roots. Cotyledons are unopened.

3.1.2 Seedlings grown in white light

Under white light treatment, since the white light contains all the monochromatic lights, the phyA and phyB are photo-activated and transported to the nucleus, and the cry1 is also photophosphorylated in the nucleus. The photo-activated phyA and phyB binds to the PIFs on the G-box by phosphorylating and subsequently degrade them. The removal of COP1 from the nucleus is involved in the light –regulated photomorphogenesis.

There is no activity of GA in the white light as there is a high level of DELLA proteins promoting the inhibition of hypocotyls growth. Since there are no PIFs and COP1

activities in the nucleus, the sucrose has no substantial effect on the hypocotyls elongation. *Arabidopsis* seedlings treated with white light have shown inhibition of hypocotyls elongation in both sucrose and without sucrose (Figure: 14 and 15).

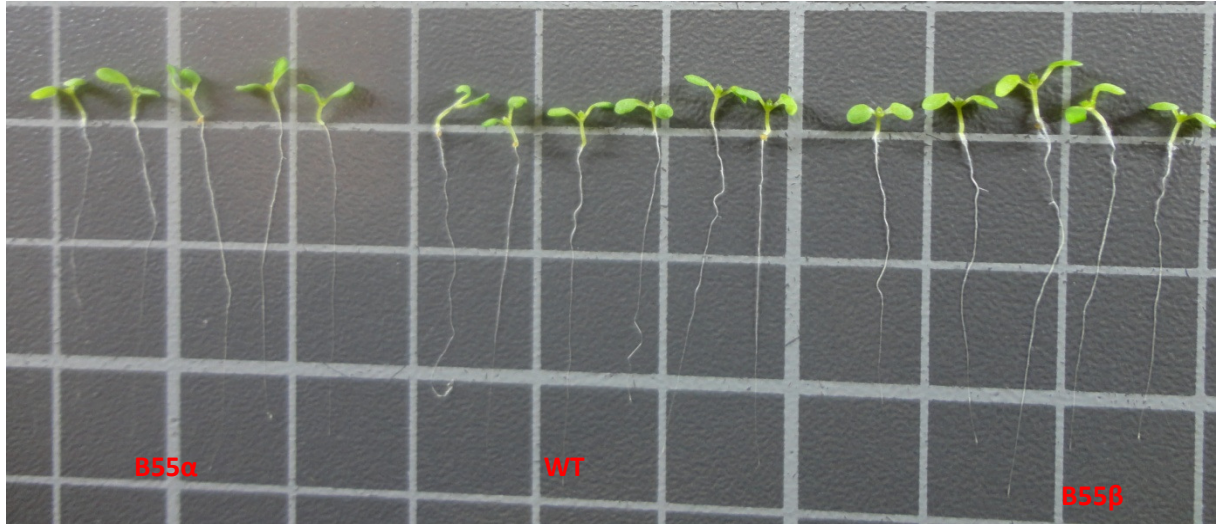


Figure 14: Hypocotyl elongation of *Arabidopsis* mutant seedlings of B55α and B55β are compared with the wild type (Col). Seedlings were grown for 6 days in white light (16hrs light and 8 hrs dark) on 1% sucrose medium. Seedlings showed short hypocotyls and long roots with opened cotyledons. One square is 10mmx10mm

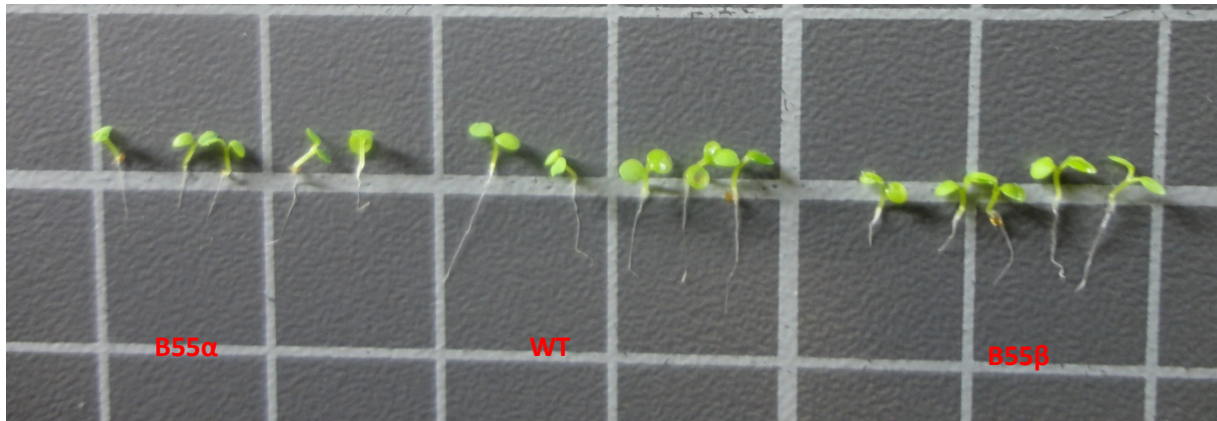


Figure 15: Hypocotyl elongation of *Arabidopsis* mutant seedlings of B55α and B55β are compared with the wild type (Col). Seedlings were grown for 6 days in white light (16hrs light and 8 hrs dark) in the absence of sucrose. Seedlings showed short hypocotyls and roots with opened cotyledons. One square is 10mmx10mm

3.1.3 Seedlings grown in blue light

Upon blue light illumination, cry1 is photoactivated and phosphorylated resulting in the exit of COP1 from the nucleus into the cytoplasm. HY5 will be free from the COP1 and promotes the genes for photomorphogenesis through dephosphorylation. The

hypocotyls growth of *Arabidopsis* seedlings in blue light was reduced compared with the dark-grown seedlings. Mutants of B55 α and B55 β of PP2A showed longer hypocotyls elongation than the wild type in both sucrose and without sucrose (Figure: 16 and 17).

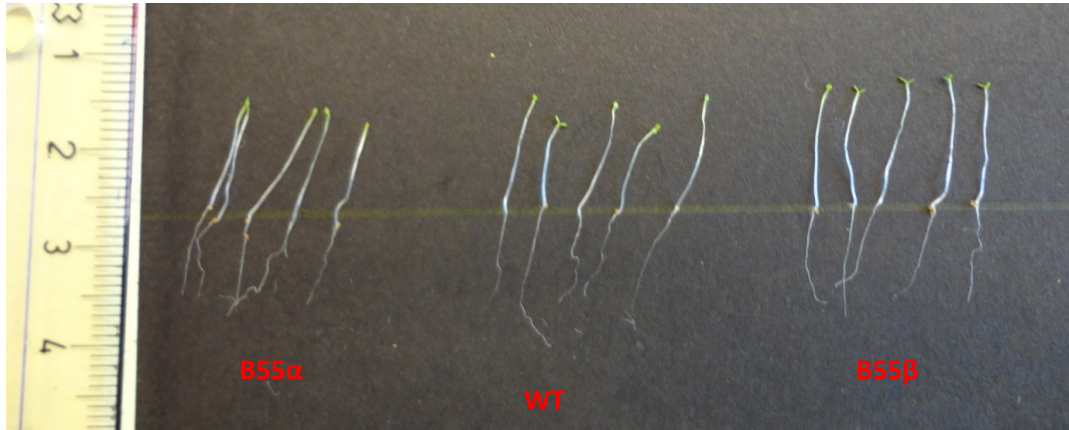


Figure 16: Hypocotyl elongation of *Arabidopsis* seedlings of wild type (Col) grown in blue light for 6 days on 1% sucrose medium are compared with B55 α and B55 β mutants. Seedlings showed short hypocotyls elongation in comparison with those grown in darkness. Cotyledons slightly opened.

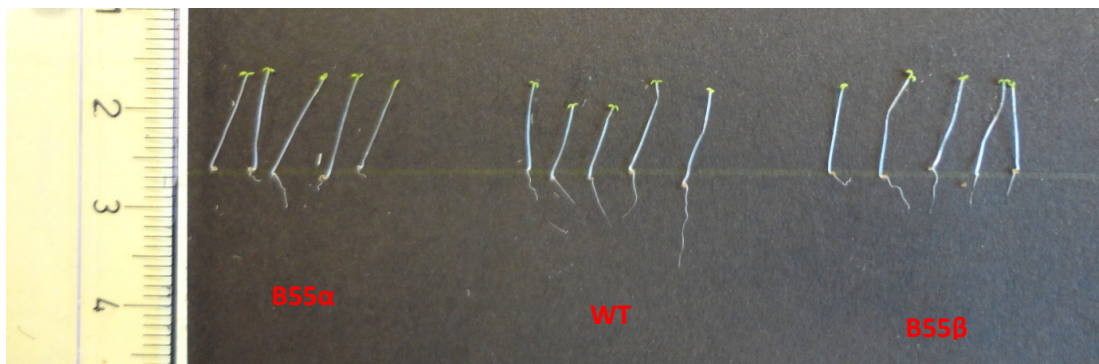


Figure 17: Hypocotyl elongation of *Arabidopsis* seedlings of wild type (Col) grown in blue light for 6 days in the absence of sucrose are compared with B55 α and B55 β mutants. Seedlings exhibited short hypocotyls growth in comparison with those grown with sucrose.

3.1.4 Seedlings grown in red light

Upon treatment with red light, phyB is transported into the nucleus in the form of Pfr, active phytochromes, and phosphorylated the PIFs in the nucleus for subsequent degradation. But, in the recent studies present that COP1 is an E3 ligase for phyB in the nucleus upon continuous red light exposure. PhyB is relatively stable in inactive form in the cytosol than the active form in the nucleus. The photo-conversion of phyB

Results

Pf into Pfr might expose the N-terminal region of phyB and enhances binding of COP1 and subsequently ubiquitinated. Binding of PIFs to both phyB and COP1 is required for promoting photoreceptors ubiquitination (Jang, et al., 2010).

The hypocotyls elongation of *Arabidopsis* seedlings grown in continuous red light are slightly inhibited upon continuous red light exposure. Mutants of B55 α and B55 β of PP2A showed shorter hypocotyls elongation than the wild type (Figure: 18 and 19).

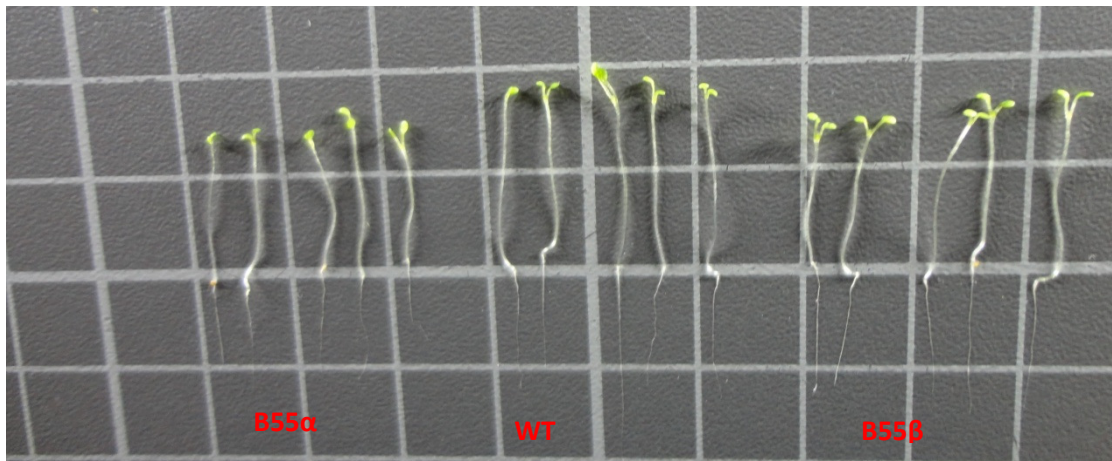
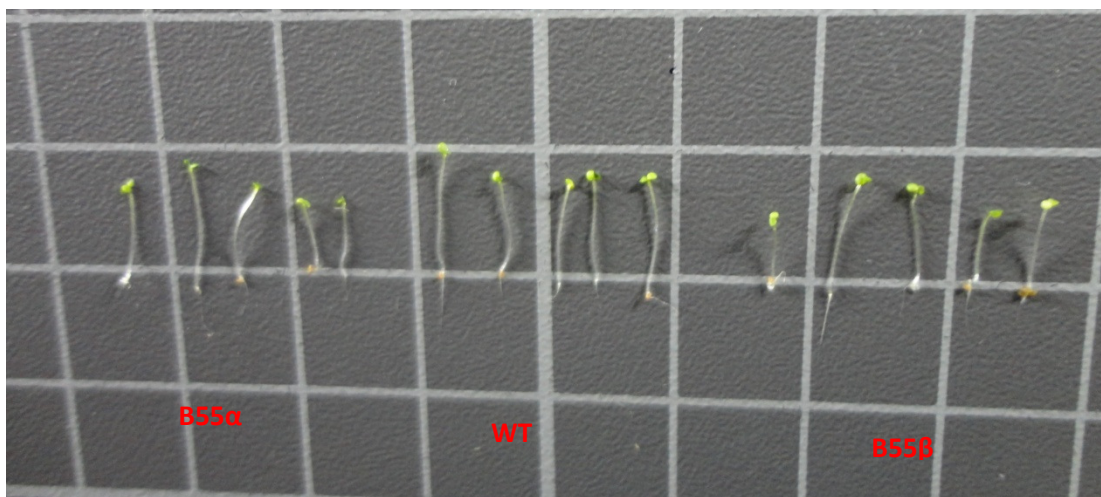


Figure 18: Hypocotyl elongation of B55 α and B55 β mutants of *Arabidopsis* seedlings grown in red light on 1% sucrose for 6 days were compared with the wild type (Col). Seedlings showed longer hypocotyls with opened cotyledons. One square is 10mmx10mm



Figur 19: Hypocotyl elongation of B55 α and B55 β mutants of *Arabidopsis* seedlings grown in red light in the absence of sucrose for 6 days were compared with the wild type (Col). Seedlings showed shorter hypocotyls than those grown on sucrose.

3.1.5 Seedlings grown in far-red light

Upon far-red light exposure, the photo-activated phyA (Pfr) is translocated to the nucleus by **FHY1** and **FHL** proteins and thus triggers the signals to light responses, including the reduction of COP1 in the nucleus and accumulation of HY5. HY5 promotes genes for photomorphogenesis and down-regulates FHY1 and FHL transcript levels (Lin et al., 2007)

Hypocotyls of *Arabidopsis* seedlings in far-red light are inhibited in the presence and absence of sucrose, but those without sucrose is highly inhibited. No phenotypic difference between the wild type and two mutants of B55 α B55 β mutants of PP2A was observed in the same growth condition (Figure: 20 and 21).

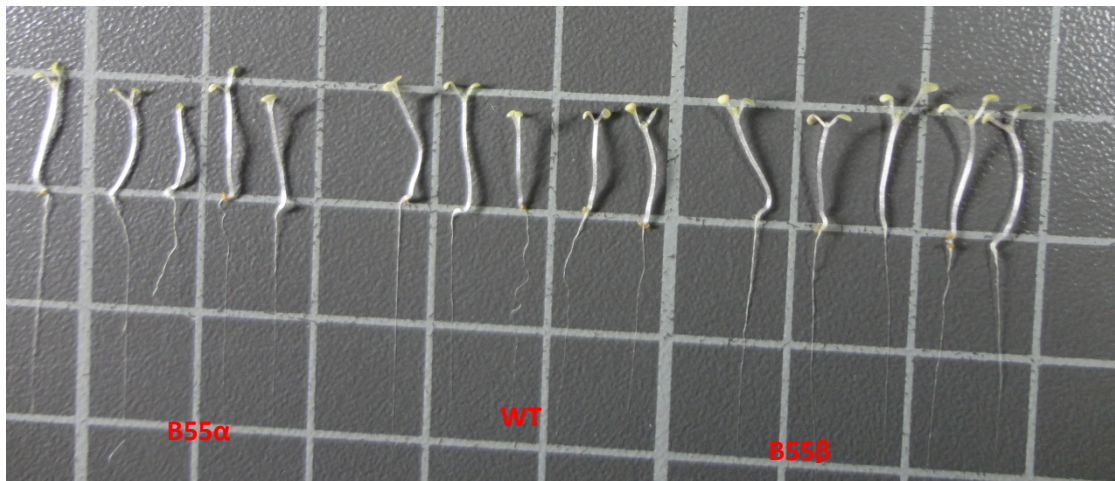


Figure 20: Hypocotyl elongation of B55 α and B55 β mutants of *Arabidopsis* seedlings grown in far-red light on 1% sucrose for 6 days were compared with the wild type (Col). Seedlings exhibited shorter hypocotyls elongation with opened cotyledons.

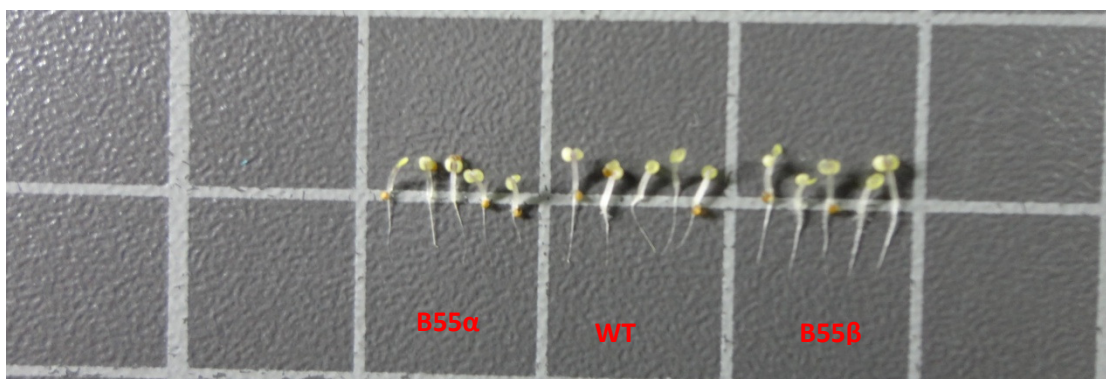


Figure 21: Hypocotyl elongation of B55 α and B55 β mutants of *Arabidopsis* seedlings grown in far-red light in absence of sucrose for 6 days were compared with the wild type (Col). Seedlings showed shorter hypocotyls in comparison with those grown on sucrose.

Results

Table 9: Hypocotyl elongation of 6 days old *Arabidopsis* seedlings of wild type and two mutants of B55 α and B55 β grown in different light conditions on sucrose and in absence of sucrose. Hypocotyl elongation were measured in mm. the mean was obtained from 5 seedlings (n=5).

	Sample	With sucrose					Without sucrose				
		dark	white light	Far-red	blue	red	dark	white light	Far-red	blue	red
	Col	21.1	2	8.8	10.6	16.8	14	1	1.4	8.8	8.8
Mean	B α	21.6	2	8.4	12.6	13.6	14.4	1	1.2	10.2	6.6
	B β	21.6	2	8.8	12.4	13.6	13.2	1	1.4	8.8	6.2
	Col	0.84	0	1.1	1.52	1.25	0.71	0	0.55	0.84	0.99
SD	B α	1.51	0	0.55	0.55	1.68	0.55	0	0.45	0.84	0.74
	B β	0.89	0	0.45	0.55	0.84	0.110	0	0.55	0.84	1.14

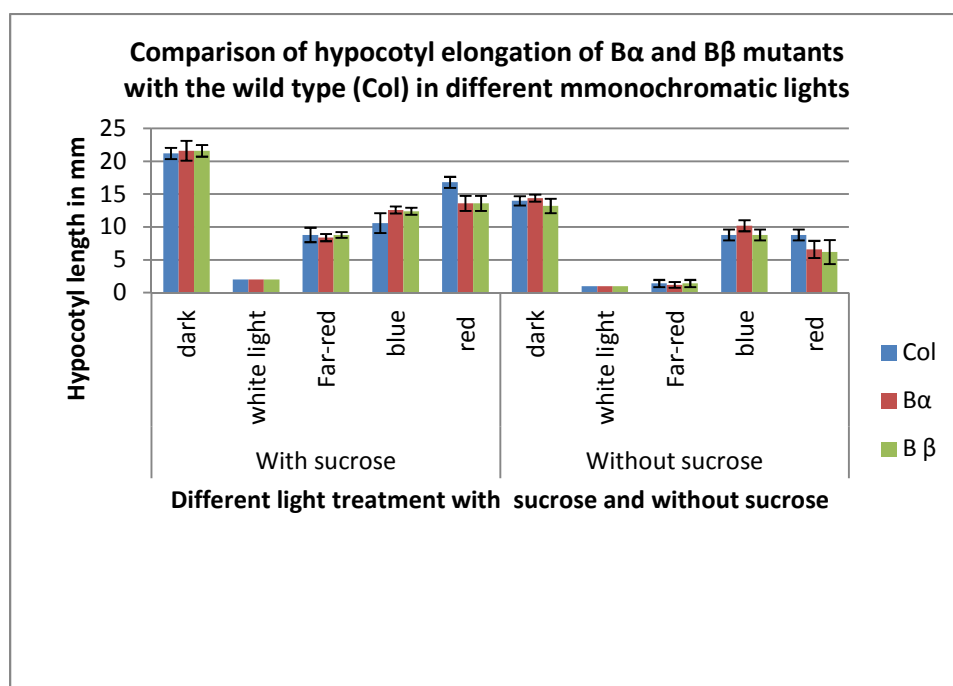


Figure 22: Comparison of hypocotyl length of B55 α and B55 β mutants of PP2A with the wild type (Col) on 1% sucrose and in absence of sucrose under different light conditions. 5 seedlings were measured from each plant. The error bars represent the means \pm SD.

3.2 Cloning of genes of interest

3.2.1 Subcloning of B55 and B β into the expression vector

PCR reactions were carried out using the primers designed for amplification of target genes of B55 α , B55 β and B β in the blue script. The PCR products were checked on agarose gel whether the plasmids contain the right size of the genes. After exposure of UV and then comparing the Hyperladder 1 marker which contains proteins of known sizes, the PCR fragments were found to be the right sizes of 1542bp for B55 α , 1506 for B55 β and 1500 for B β (Figure 23) and this indicates the amplification of the target genes were successful. After confirmation on a gel of 0.8% agarose, the PCR products were excised out of the gel and purified using the GenElute Gel extraction Kit and then digested with the restriction enzymes (Table 1) already incorporated into primers during primer design. The empty PGEX-6p-1 was also isolated, purified and digested with the same restriction enzymes to make sticky ends for ligation. The concentration of plasmid and vector were measured with Nanodrop. A vector to insert ratio of 1:3 were used in the ligation reaction.



Figure 23: The genes from blue script vector were amplified with the designed primers. **Lane 1:** Hyperladder 1 marker. **Lane 2:** B55 α PCR product around 1542bp. **Lane 3:** B55 β PCR product around 1506 bp. **Lane 4:** B β PCR product around 1500bp. The upper bands are plasmids which are around 4000 bp. 4 μ l hyperladder 1 were used.

3.2.2 Agarose gel analysis of expression vector containing the B55 and B`Beta genes

The PGEX-6p-1 with the target gene was introduced into competent cells of bacteria strain, DH5 α , and then cultured LB medium containing ampicillin. Since PGEX-6p-1 vector carries a gene resistance for antibiotic, only those bacteria that acquire these vectors grow in the medium. 10 colonies for each target gene were used as a template in PCR reaction to check the presence of target genes by PCR colony test. Some of the positive colonies in PCR were further confirmed the presence of target genes by using specific primers for genes and vector.

An empty PGEX-6p-1 was run on the gel with positive colonies as negative control to show that the empty PGEX-6p-1 is lacking the target gene and a band of 170 bp was observed on the gel.

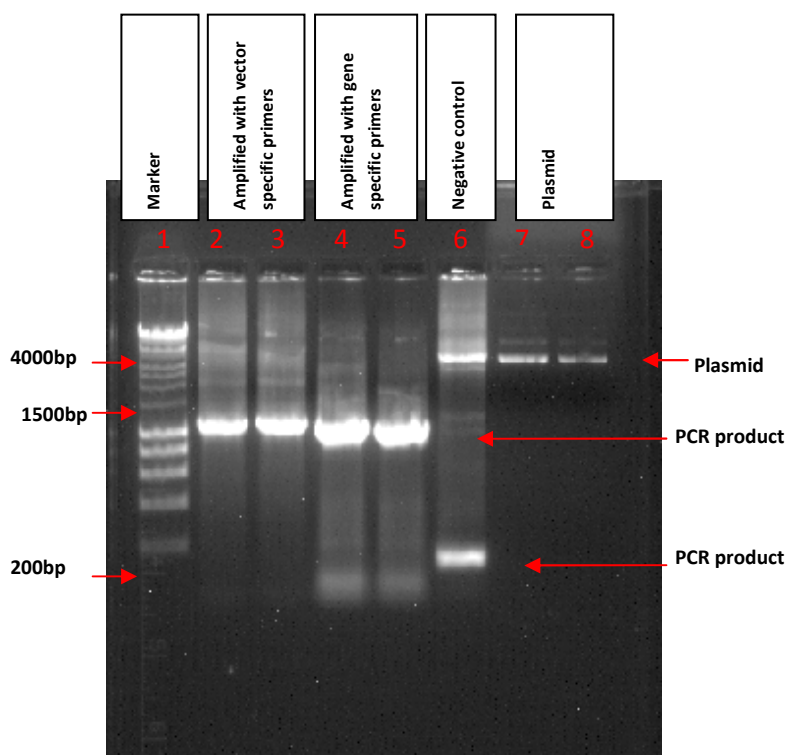


Figure 24: PCR of positive colonies of B55 α was run on 0.8% agarose gel. **Lane 1:** Hyperladder 1 marker. **Lane 2 and 3:** PCR product of positive colonies amplified with vector specific primers. **Lane 4 and 5:** PCR product of positive colonies amplified with insert specific primers. **Lane 6:** Negative control where the plasmid PGEX-6p-1 without insert amplified with vector specific primers. Amplified portion of the empty plasmid showed a band of around 200 bp. **Lane 7 and 8:** Isolated plasmid of the positive colonies.

Results

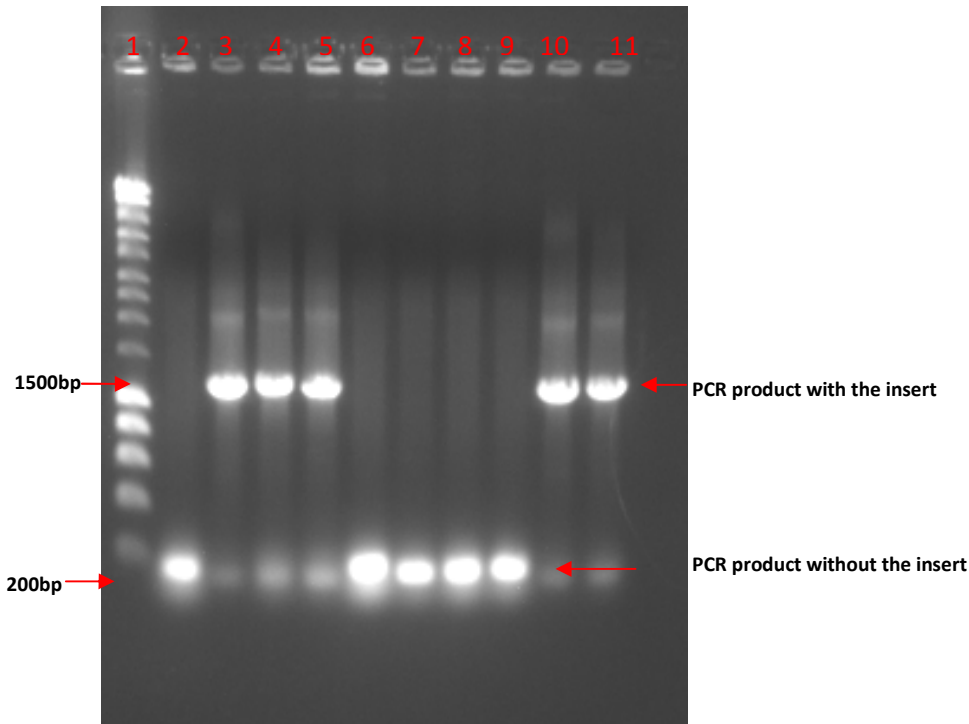


Figure 25: Testing of positive colonies of B55 β after transformation by PCR colony. **Lane 1:** Hypoladder 1. **Lane 2, 6, 7, 8, and 9:** negative colonies. **Lane 3, 4, 5, 10 and 11:** positive colonies. The gel was run on 0.8% agarose.

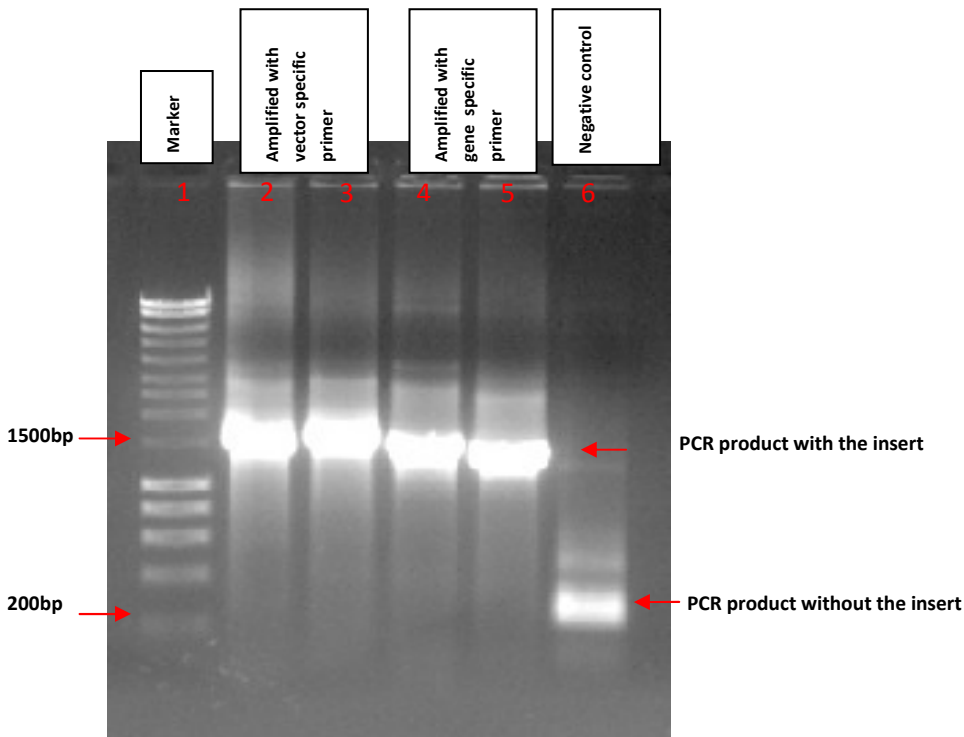


Figure 26: PCR product of positive colonies of B55 β was run on 0.8% agarose. **Lane 1:** hypoladder 1. **Lane 2 and 3:** PCR product of positive colonies amplified with vector specific primers. **Lane 4 and 5:** PCR product of positive colonies amplified with insert specific primers. **Lane 6:** Negative control in which the vector PGEX-6p-1 without insert amplified with vector specific primers.

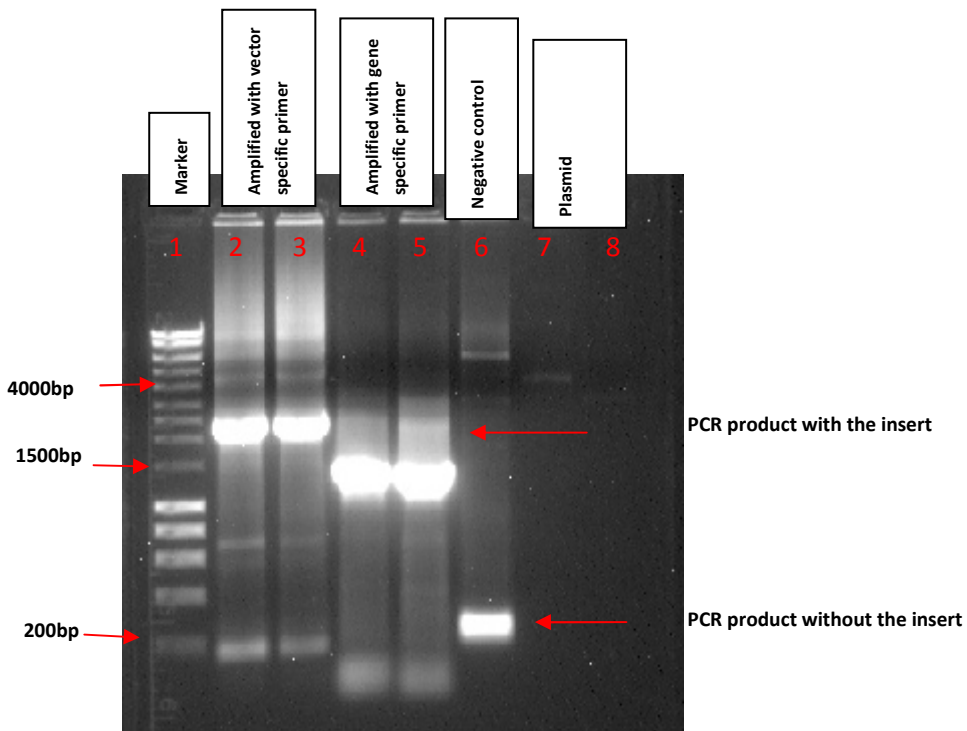


Figure 27: PCR amplification of positive colonies of B β was run on 0.8% agarose gel by using vector primers and insert primers. **Lane 1:** hyper ladder 1 marker. **Lane 2 and 3:** positive colonies of number 4 and 6 amplified with vector specific primers. **Lane 4 and 5:** positive colonies amplified with the insert specific primers. **Lane 6:** Negative control where the vector PGEX-6p-1 without the insert amplified with the vector specific primers. Since there is no insert, only small portion of DNA is amplified. **Lane 7 and 8:** isolated plasmid of the positive colonies (1 μ l is used for running the gel).

The DNA bands amplified with vector specific primers showed more base pairs than those amplified with gene specific primers. After analyzing the PCR products on agarose gel according to size, the expected size of PCR products of B α , B β and B β amplified with the vector specific primers are ~1800 bp, ~1750 bp and ~1750 bp respectively. In case of PCR products of B α , B β and B β amplified with the gene specific primers are ~1542, ~1505 and ~1500 respectively.

HyperLadder™ I

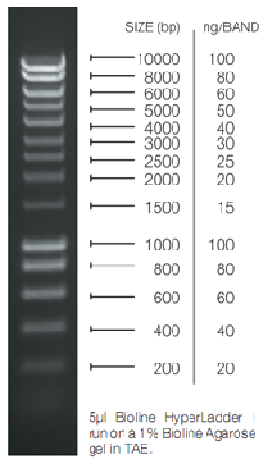


Figure 28: Hyperladder 1 of known band sizes used for comparing with samples.

3.3 SDS-PAGE and western blot analysis

3.3.1 Colony test of transformed expression vector into Rosetta Strain (DE3)

PGEX-6p-1 with the target genes were isolated, purified and transformed into *E. coli*, Rosetta strain, pLysS, which lacks protease that degrades the recombinant protein. Positive colony test were performed to confirm whether our vectors with the target genes were introduced into the Rosetta strain (DE3). All the bands of the three genes of B55 α , B55 β and B β were close to the correct size of 1542, 1506 and 1500 respectively compared with the known size of the marker.

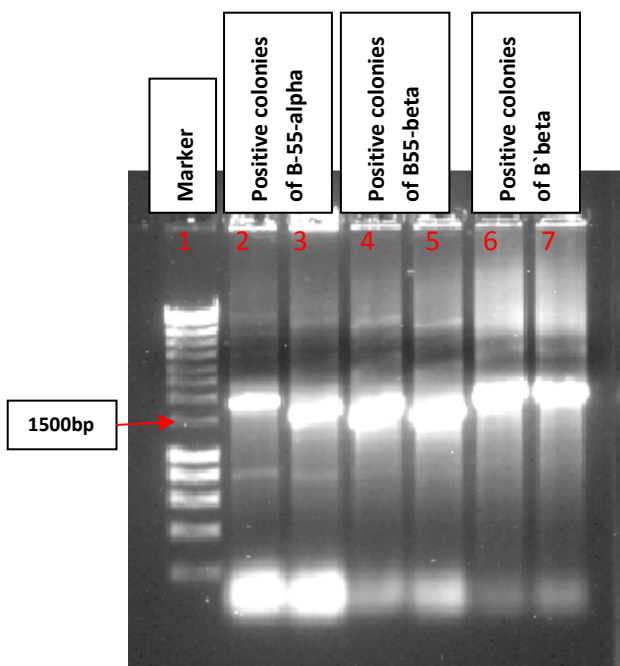


Figure 29: testing of positive colonies after transformation of target genes into Rosetta bacteria (DE3) by PCR. **Lane 1:** Hyperladder 1. **Lane 2 and 3:** B55 α **Lane 4 and 5:** B55 β **Lane 6 and 7:** B β

3.3.2 Fusion protein expression

After confirmation of successful transformation of expression vectors into the expression host of Rosetta, the culture were performed with 2x TY medium until the optical density reaches 0.5-0.7. The expression of GST-fusion construct was induced with 0.1M IPTG. The samples were run on SDS-PAGE gel to visualize and analyze the band sizes of the GST fusion protein by comparing with its migration distance with that of the known band sizes of the marker. Competent cells were also run on the gel as negative control. Visualizing the SDS-PAGE gel by Coomassie stain, there is no visible bands with the expected size of GST fusion protein (81 kDa; GST is 26 kDa

while B55 is 55 kDa) in noninduced samples while there are thick bands with the expected size of the GST-fusion proteins in the induced samples bacterial cultures harbouring PGEX-6p-1 containing the B55 α and B55 β . There are no thick visible bands of the expected size of B β in parallel in the induced state. This indicates that the genes of B55 were expressing the recombinant protein during the IPTG induction. The expression of fusion protein of B55 α and B55 β with GST can be later co-purified or further detected with antibodies.

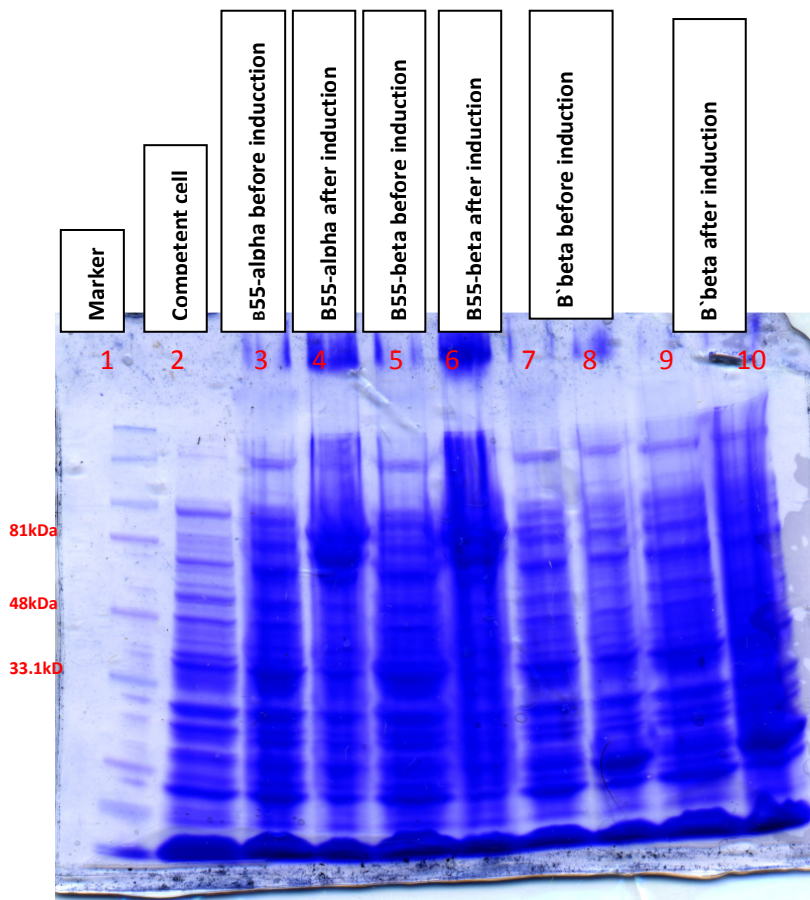


Figure 30: The protein was visualized by incubating the gel with Coomassie Blue stain solution. **Lane 1:** Marker **Lane 2:** Competent cell. **Lane 3:** B55 α before induction. **Lane 4:** B55 α after induction. **Lane 5:** B55 β before induction. **Lane 6:** B55 β after induction. **Lane 7 and 8:** B β before induction. **Lane 9 and 10:** B β after induction. Proteins were separated with 10% mini-protein tax precast gel. Precision Plus protein Western standard was used as marker

3.3.3 Western blot analysis

In order to determine further that our target genes in the PGEX-6p-1 vector were expressed in Rosette strain, the expressed proteins were co-purified with GST and Western blot was carried out by using antibodies as a probe against a short peptide

of the target proteins. The result showed a strong signal of the B55 α recombinant proteins of the expected molecular weight of 81 kDa in different concentration and one B55 β concentration. These are consistent with the expected sizes of target protein (81kDa).

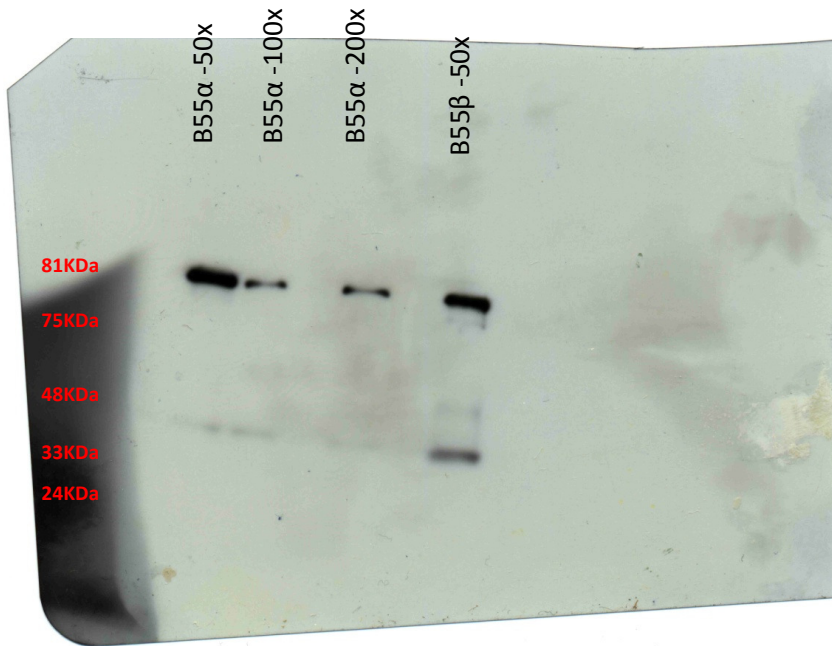


Figure 31: Different dilutions of fusion B55 proteins were used for Western blot. **Lane 1** : (50 X) diluted with GST elution buffer for B55 α , **Lane 2**: (100 X) diluted with GST elution buffer for B55 α and **Lane 3**: (200 X) diluted with GST elution buffer for B55 α . Protein are detected after Western blotting. **Lane 4** (50 x): B-beta are detected. No protein of B'-beta are detected with the antibodies.

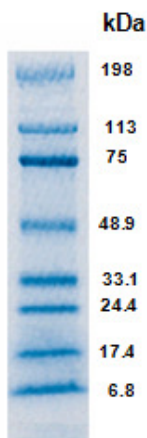


Figure 32: Pre-stained SDS-PAGE standard broad range

The protein extract from leaf Arabidopsis were run on SDS-PAGE alongside with the other samples lysate, eluted protein of B55 expressed in E. coli under induction of

IPTG. There are weak visible bands of extract on the gel at the expected size of the target protein around 81kDa comparing with that of known molecular weight of the marker. There are several bands of the eluted protein on the gel and this could be explained that the protein is degraded. The results show that we have successfully cloned and expressed B55 α and B55 β in *E. coli* as a fusion product to GST, and the antibody did interact with both B55 α and B55 β .

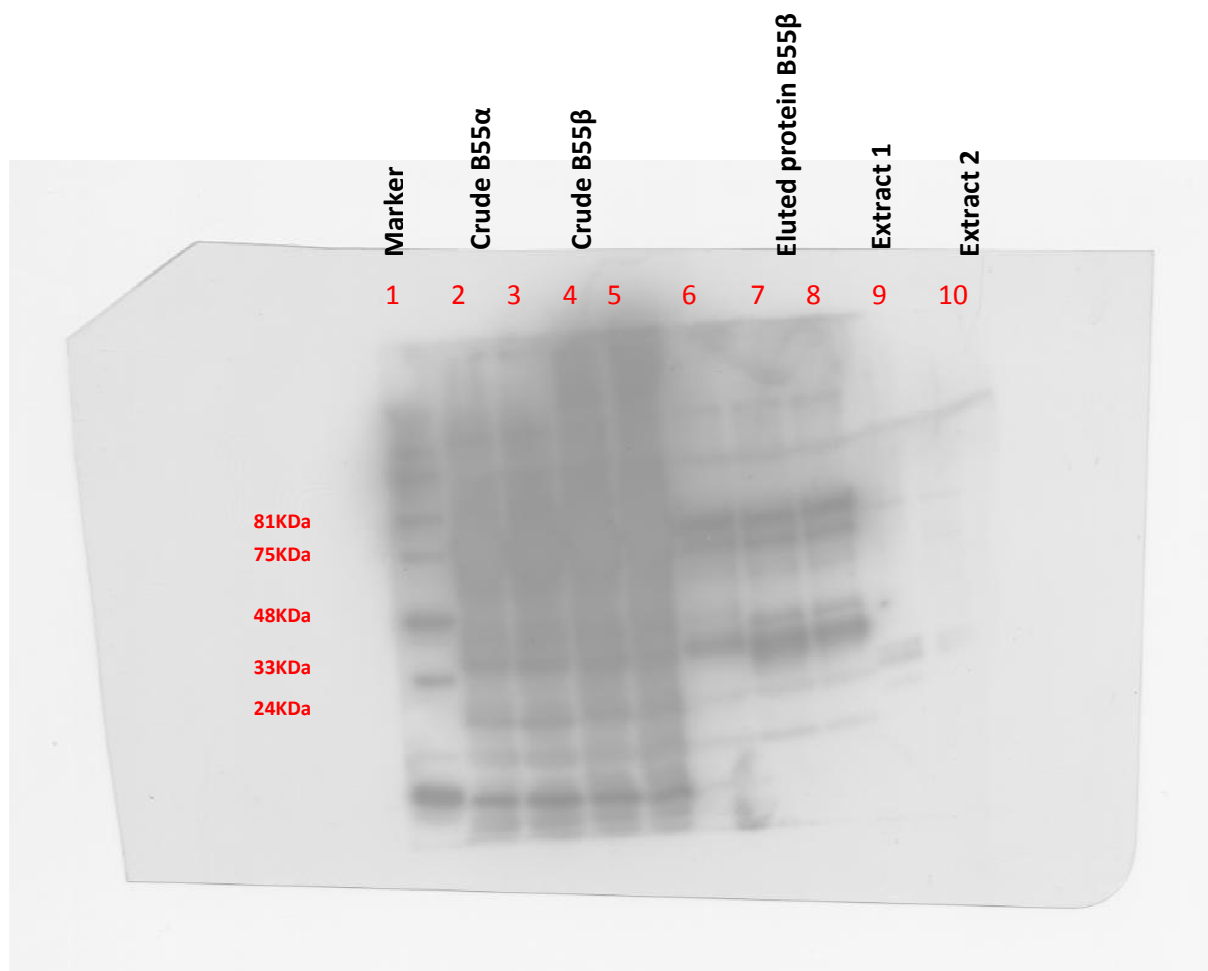


Figure 33: Western blot analyses for B55 protein fused with GST protein. **Lane 1:** Marker, **Lane 2 and Lane 3:** Lysate in B55-alpha, **Lane 4 and 5:** Lysate in B55-beta, **Lane 6:** Eluted protein in B55-alpha, **Lane 7 and 8:** Eluted protein in B55-beta and **Lane 9 and 10:** Tissue extract from wild type (*Col*) of *Arabidopsis*.

4 Discussion

Reversible phosphorylation controls various cellular processes in eukaryotes. Protein phosphatase 2A is a serine/threonine that plays a crucial role in dephosphorylation of many cellular functions in eukaryotes. B subunits of PP2A play an important role in substrate specificity and cellular localization. In this study, we investigate the effect of mutated B55 subunits of Arabidopsis on the phytochromes and cryptochromes in different light conditions. To examine the dephosphorylation site of phytochromes in different light conditions by protein phosphates, we first get a full insight of the light signal transduction and then hypothesize where the dephosphorylation site could be by studying the phenotypic features, particularly the hypocotyls lengths of the single B55 mutated seedlings in PP2A and their comparison with the wild type.

4.1 Dark-grown seedlings

Dark-grown *Arabidopsis* seedlings exhibited no hypocotyls inhibition in comparison with those under light treatment (white, red and far-red). The possible explanation for the result is that, in the darkness, the phytochromes, since they are not photo-activated, are not in the nucleus and there are high PIFs accumulation in the nucleus and high level of GAs. GAs regulates sucrose-induced hypocotyls elongation and plants grown on sucrose medium in the dark accumulate a high level of PIFs in the nucleus resulting in no inhibition of hypocotyls growth (figure: 12 and 13).

Since there is no active phytochromes (Pfr) in the nucleus in the dark, there is no effect of PP2A on phytochromes and thus no inhibition of hypocotyls elongation. *Arabidopsis* mutants of B55 α and B55 β of PP2A showed no phenotypic difference from the wild type in the same experimental condition in darkness.

4.2 White light grown seedlings

Under light, activated phytochromes in their Pfr form phosphorylate PIFs and subsequently degrade them resulting in photomorphogenesis. The combined effects of red, far-red and blue lights are more than the individual effect on hypocotyls inhibition. This is the reason why *Arabidopsis* seedlings under white light treatment exhibited identical hypocotyls inhibition (Figure: 14 and 15). The dephosphorylation of PP2A in the nucleus is minimized due to the combined effect of the phytochromes

and cryptochromes and that is why the mutants of B55 α and B55 β showed no phenotypic difference from the wild type in the white light.

4.3 Blue light grown seedlings

Both cry1 and cry2 also undergo a blue-light dependent phosphorylation and COP1 is inactivated due to its conformational change. HY5 is bZIP transcription factor that is involved in promoting photomorphogenesis under various light conditions (Li et al., 2010). In blue light, HY5 is less dephosphorylated in mutant seedlings of B55 α and B55 β as a result of less specificity of PP2A and genes for de-etiolation are slightly repressed. In case of wild type, HY5 is more dephosphorylated by PP2A due to its high specificity and genes for photomorphogenesis are promoted resulting in hypocotyls inhibition.

4.4 Red light grown seedlings

Red light is less effective than blue light in inhibiting hypocotyls elongation. In the red light, phyB translocated into the nucleus in the form of Pfr is degraded by COP1 and PIFs in the nucleus and that is the reason why *Arabidopsis* seedlings grown in red light has longer hypocotyls in comparison with far-red and blue lights. PIF3 is degraded less under far-red light. PP2A dephosphorylates the phosphorylated phytochromes in the nucleus. The Pfr were more readily dephosphorylated than Pr form by PP2A because Pfr is the physiological active form of phytochromes. Mutant seedlings of B55 α and B55 β of PP2A do not dephosphorylate effectively the Pfr form of phytochromes in the nucleus before binding to the PIFs for degradation and the gene for photomorphogenesis is promoted than the wild type (Figure: 18 and 19).

4.5 Far-red light grown seedlings

In the far-red light, the phyA moves into the nucleus with the help of **FHY1** and **FHL** proteins and this may increase the stability of phyA. COP1 and PIFs have different affinity to phyA and phyB or may regulate light response differently. It is also possible that other factors may involve in signal pathway under far-red light.

Looking at the phenotypic appearance of *Arabidopsis* seedlings of mutants and the wilds type under different light conditions and the described light signaling pathway,

we suggest that photoreceptors are substrate for PP2A in the nucleus in a light dependent manner.

4.5 Subcloning of target genes into the expression vector

In order to fully study the functions of the proteins encoded by these genes of B55 α , B55 β , and B β , they have to be first amplified by PCR before subcloning into the PGEX-6p-1 vector according to the protocol. Examining the comparison of amplified PCR products from PGEX-6p-1 with the known sizes of the DNA ladder, the bands of the amplified PCR products were the correct sizes. The amplified PCR products were subcloned into the expression vector, PGEX-6p-1. The success of subcloning was proved by using gene specific primers and vector specific primers to amplify the genes. It seems that the all the three genes were subcloned into the destination vector, PGEX-6p-1. The PGEX-6p-1 vector encodes N-terminal GST tag which allows the expression of both recombinant proteins in *E. coli* since they have under the control of the promoter. This makes easy the purification of the proteins to study further their roles in the cell.

4.6 Protein production and purification

Once our target genes subcloned into the expression vector, the expression vector had to be introduced into *E. coli*, Rosetta strain, pLysS which is a strain good for protein production due its lack of degrading proteins. Positive colony test was performed to confirm whether the Rosetta strain of *E. coli* acquired the PGEX-6p-1 vector. The production of protein was carried out under the induction of 1M IPTG in the first place and we had some problems of getting enough proteins during purification. We optimized by using 0.1 M of IPTG in inducing the protein production in B55 genes and B β , but we did not get the expression of B β under the induction of IPTG. We have repeated the experiment by optimizing the expression of the gene. Unfortunately, we could not able to get any result. This result could be explained that there could be some mutation of genes and the reading frame of the genes could have been altered.

4.7 Western blot analysis

The recombinant purified target proteins by glutathione agarose beads were detected with antibody. The amount of purified B55 α and B55 β were enough to be detected

with antibody as a probe, but since no protein is expressed in B`β, no western blot was carried out. In case of protein extraction of leaf Arabidopsis, total protein concentration isolated from the leaves were enough to detect with antibody as a probe, but weak signal were visualized after western blotting and we tried to optimize by changing the concentration of antibody and procedure, but no strong signal were visualized.

5 Conclusions

In this study, the hypocotyls elongation of single mutated B55 subunits of PP2A in Arabidopsis plant grown in different light conditions on half MS medium containing 1% sucrose and without sucrose was identified and compared with wild type (Col). The dephosphorylation sites of phytochromes and cryptochromes in the light signal transduction pathway in the nucleus by PP2A was suggested. The phytochromes and cryptochromes possibly have some common components in their signal transduction pathway even though it seems that they act in independent manner. This proposes that HY5, as the gene product, is a common downstream element in red/far-red and blue-light response pathways. The B55 and B`β genes were successfully subcloned into the expression vector, PGEX-6p-1, after amplification of the genes with the designed primers in PCR reaction. The genes of in the PGEX-6p-1 along with GST were expressed under the same promoter in Rosette bacteria, DE3. The purified B55 proteins were detected with antibody as a probe in different concentration. In case of B`β, neither protein expressed in *E. coli* nor detected in western blot. Attempts were made to optimize the protein production by varying the concentration of IPTG and induction times, but none worked for the protein expression.

5.1 Future research

As our goal was to subclone the DNA construct, express them in *E. coli*, purify the proteins and finally detect them in Western blotting. Unfortunately, some problems were encountered about the expression and detection of B`beta in western blotting. To design new primers for the subcloning the target genes and subsequently express in *E. coli* will be possible in the future plan.

6 References

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7 Appendix

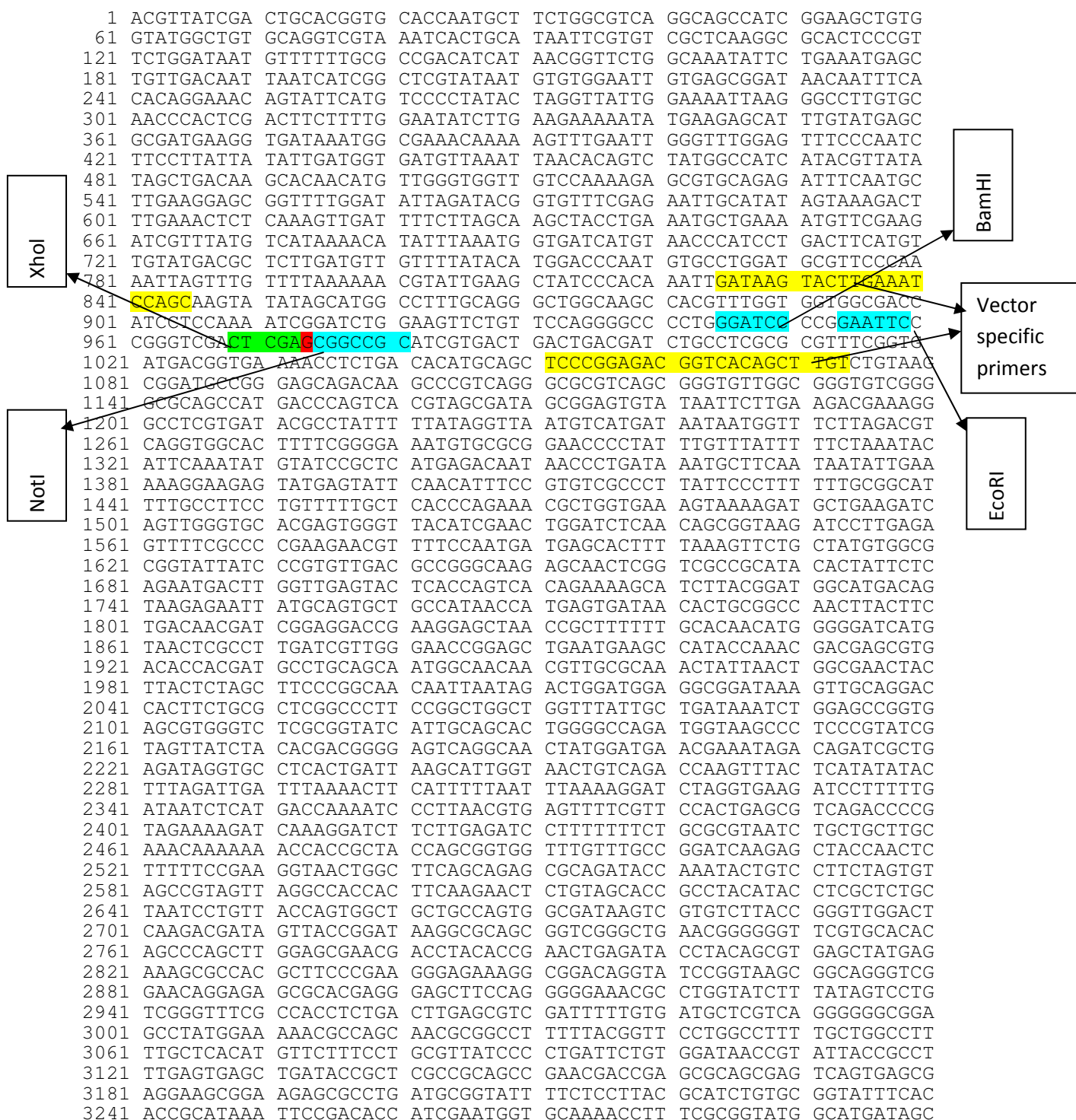
7.1 pGEX-6P-1 sequence

Full length: 4984 bp

Composition: 1228 A; 1207 C; 1297 G; 1252 T; 0 OTHER

Percentage: 25% A; 24% C; 26% G; 25% T; 0%OTHER

Molecular Weight (kDa): ssDNA: 1540.51 dsDNA: 3072.6



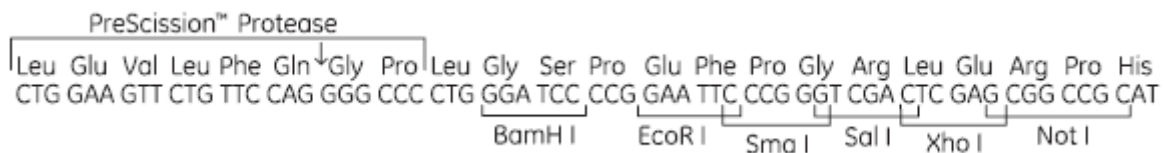
Appendix

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3301 GCCCGGAAGA GAGTCAATTC AGGGTGGTGA ATGTGAAACC AGTAACGTTA TACGATGTCG
3361 CAGAGTATGC CGGTGTCTCT TATCAGACCG TTTCCC CGGT GGTGAACCAG GCCAGCCACG
3421 TTTCTGCGAA AACGCGGGAA AAAGTGGAA GCGGCATGGC GGAGCTGAAT TACATTCCCA
3481 ACCGCGTGGC ACAACAAC TGCGGGCAAAC AGTCGTTGCT GATTGGCGTT GCCACCTCCA
3541 GTCTGGCCCT GCACGCGCCG TCGCAAATTG TCGCGGCGAT TAAATCTCGC GCCGATCAAC
3601 TGGGTGCCAG CGTGGTGGTG TCGATGGTAG AACGAAGCGG CGTCGAAGCC TGTAAAGCGG
3661 CGGTGCACAA TCTTCTCGCG CAACGCGTCA GTGGGCTGAT CATTAACTAT CCGCTGGATG
3721 ACCAGGATGC CATTGCTGTG GAAGCTGCCT GCACTAATGT TCCGGCGTTA TTTCTTGATG
3781 TCTCTGACCA GACACCCATC AACAGTATTA TTTTCTCCA TGAAGACGGT ACGCGACTGG
3841 GCGTGGAGCA TCTGGTCGCA TTGGGTCAAC AGCAAATCGC GCTGTTAGCG GGCCCATTA
3901 GTTCTGTCTC GGCGCGTCTG CGTCTGGCTG GCTGGCATAA ATATCTCACT CGCAATCAAA
3961 TTCAGCCGAT AGCGGAACGG GAAGGCGACT GGAGTGCCAT GTCCGGTTTT CAACAAACCA
4021 TGCAAATGCT GAATGAGGGC ATCGTTCCCA CTGCGATGCT GGTGCGCAAC GATCAGATGG
4081 CGCTGGGCGC AATGCGCGCC ATTACCGAGT CCGGGCTGCG CGTTGGTGCG GATATCTCGG
4141 TAGTGGGATA CGACGATACC GAAGACAGCT CATGTTATAT CCCGCCGTCA ACCACCATCA
4201 AACAGGATTT TCGCCTGCTG GGGCAAACCA GCGTGGACCG CTTGCTGCAA CTCTCTCAGG
4261 GCCAGGCGGT GAAGGGCAAT CAGCTGTTGC CCGTCTCACT GGTGAAAAGA AAAACCACCC
4321 TGGCGCCCAA TACGCAAACC GCCTCTCCCC GCGCGTTGGC CGATTCATTA ATGCAGCTGG
4381 CACGACAGGT TTCCCGACTG GAAAGCGGGC AGTGAGCGCA ACGCAATTAA TGTGAGTTAG
4441 CTCCTCATT AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT GTTGTGTGGA
4501 ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG ACCATGATTA CGGATTCCT
4561 GGCCGTCGTT TTACAACGTC GTGACTGGGA AAACCCTGGC GTTACCCAAC TTAATCGCCT
4621 TGCAGCACAT CCCCCTTTTCG CCAGCTGGCG TAATAGCGAA GAGGCCCGCA CCGATCGCCC
4681 TTCCCAACAG TTGCGCAGCC TGAATGGCGA ATGGCGCTTT GCCTGGTTTC CGGCACCAGA
4741 AGCGGTGCCG GAAAGCTGGC TGGAGTGCGA TCTTCTGAG GCCGATACTG TCGTCGTCCC
4801 CTCAAACCTG CAGATGCACG GTTACGATGC GCCCATCTAC ACCAACGTAA CCTATCCCAT
4861 TACGGTCAAT CCGCCGTTTG TTCCCACGGA GAATCCGACG GGTGTTACT CGCTCACATT
4921 TAATGTTGAT GAAAGCTGGC TACAGGAAGG CCAGACGCGA ATTATTTTTG ATGGCGTTGG
4981 AATT

```

7.1.1 Multiple cloning site of the PGEX-6p-1 expression vector



7.2 >At1g51690, B alpha

Sequence length (bp): 1542

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1 ATGAACGGTG GTGATGAGGT CGTCGCAGCT TCGGCAGACC CATCTCTGCC
51 TTTGGAATGG AGATTCTCTC AGGTCTTCGG TGAACGAAGC GCTGGTGAAG
101 AAGTTCAAGA AGTTGATATA ATCTCAGCGA TTGAATTTGA TAATTCTGGA
151 AACCATCTTG CTACTGGGGA CCGTGGAGGA CGTGTGTTTC TTTTCGAGAG
201 AACCGATACC AATAATAGCA GTGGAACCAG AAGAGAGCTT GAAGAAGCTG
251 ATTATCCACT GAGGCATCCA GAGTTTCGTT ATAAAACAGA GTTTCAGAGT
301 CATGACCCTG AGTTTGATTA CCTCAAGAGT TTGGAGATAG AGGAGAAAAT
351 TAACAAAATT AGATGGTGTC AAACAGCAA TGGAGCTCTT TTTCTCCTCT
401 CGACCAATGA TAAAACCATC AAGTTTTGGA AGGTTCAAGA CAAGAAGATC
451 AAGAAAATCT GTGATATGAA TTCAGATCCT TCAAGAACTG TAGGAAACGG
501 AACCGTTGCA AGCTCGAGCA ATTCAAACAT TACAACTCA TGCCTTGTGA
551 ATGGAGGAGT ATCTGAAGTG AACAACTCCT TATGTAATGA CTTCTCATTG
601 CCAGCAGGAG GCATCTCTTC GCTGCGATTA CCAGTGGTAG TAACTAGCCA
651 TGAGTCGAGC CCTGTGGCTA GATGTCGAAG AGTATATGCT CATGCTCATG
701 ATTATCATAT TAATTCAATC TCAAATAACA GCGACGGCGA AACATTTATT
751 TCTGCTGATG ATTTGCGAAT TAATCTTTGG AATCTGGAAA TTAGCAATCA
801 AAGTTTCAAT ATTGTTGATG TCAAGCCTGC GAAAATGGAA GATCTATCTG
851 AGGTTATCAC ATCAGCAGAA TTTCATCCTA CCCATTGCAA TATGTTAGCC
901 TATAGCAGTT CGAAAGGTTT AATTCGCCTG ATTGATCTGC GCCAGTCAGC
951 TTTATGTGAT TCACATTCAA AATTGTTTGA GGAACCAGAG CAAGCAGGTC
1001 CTAAATCGTT CTTTACTGAG ATAATTGCTT CAGTCTCAGA CATCAAATTT
1051 GCAAAAGAAG GAAGATATCT ACTTAGCCGT GACTATATGA CACTTAAGTT
1101 ATGGGACATC AACATGGACG CAGGTCCAGT AGCAACTTTC CAGGTTTCATG
1151 AATATTTGAA ACCAAAGCTC TGTGATTTAT ATGAAAATGA TTCCATCTTT
1201 GACAAGTTCG AGTGTTGTAT AAGTGGCAAT GGCTTGCGAG CAGCTACAGG
1251 TTCTTACAGC AATCTGTTCC GTGTGTTTGG TGTGCCCCC GGAAGTACTG
1301 AGACGGCAAC CTTAGAAGCA AGCAGAAACC CAATGAGGAG ACATGTCCCA
1351 ATTCCGTCGA GGCCATCCAG AGCACTAAGC AGTATAACGC GTGTCGTAAG
1401 TAGAGGATCA GAGAGTCCGG GAGTCGATGG GAATACTAAT GCTCTTGATT
1451 ACACAACAAA GCTGCTGCAT CTTGCTTGGC ATCCAAACGA GAACTCAATT
1501 GCTTGCGCCG CTGCAAATAG CTTGTACATG TACTATGCTT AA
    
```

7.2.1 Protein data

Length (aa): 513, Molecular weight: 56933.9, Isoelectric point: 5.482

Translation of DNA sequence of the B-beta gene gave the amino sequence shown below using ExpAsy translate tool and the highlighted area is where the antibody toward B-beta made for.

5'3' Frame 1

```

MNGGDEVVAASADPSLPLEWRF SQVFGERSAGEEVQEVDI I SAIEFDNSGNHLATGDRGG
RVVLFERTDTNNSGTRRELEEADYPLRHPEFRYKTEFQSHD PEFDYLKSLEIEEKLNKI
RWCQTANGALFLLSTNDKTIKFWKVQDKKIKKICDMNSDPSRTVGNNGTVASSNSNI TNS
CLVNGGVSEVNNSLNCNDFSLPAGGISSLRPLVVVTSHESSPVARCRRVYAHADYHINSI
SNNSDGETFI SADDLRINLWNLEISNQSFNIVDVKPAKMEDLSEVITSAEFHPTHCNMLA
YSSSKGSIRLIDL RQSALCDSHSHKLFEEPEQAGPKSFFTEIIASVSDIKFAKEGRYLLSR
DYMTLKLWDINMDAGPVATFQVHEY LKPKLCDLYENDSIFDKFECCISGNGLRAATGSYS
NLFRVFGVAPGSTETATLEASRNPMRRHVPIPSRPSRALSSITRVVSRGSESPGVDGNTN
ALDYTTKLLHLAWHPNENSIACAAANSLYMYA-
    
```

Antibody for B55
alpha

7.3 >At1g17720, B beta

Sequence length (bp): 1506

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101  AGGAAGTTGA  CATCATTTCA  GCCATCGAAT  TTGATAAAAAG  TGGAGACCAT
151  CTCGCAACTG  GTGACCGTGG  GGGCCGAGTA  GTTCTTTTTG  AAAGGACAGA
201  TACTAAAGAT  CATGGAGGGT  CCAGGAAGGA  TCTTGAGCAG  ACGGATTACC
251  CAGTTAGGCA  TCCTGAGTTT  CGATATAAAA  CAGAATTTCA  GAGTCATGAA
301  CCAGAGTTTG  ACTATCTCAA  GAGTTTGGAG  ATAGAGGAGA  AAATCAACAA
351  AATCAGATGG  TGTCAGCCAG  CTAATGGTGC  ATTGTTTCTG  CTTTCTACAA
401  ACGATAAAAC  CATCAAATAT  TGGAAGGTAC  AAGAGAAGAA  GATCAAGAAA
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501  ATTAGTTACT  AATGGATTAC  CTGCTGATAA  AGGGCATGAC  TACCTGAGCA
551  AGGACTTTTC  TTTCCCAGCA  GGAGGCATTC  CATCTCTGCG  TTTGCCTGTG
601  GTAGTTACCA  GCCAGGAGAC  TAACCTTGTG  GCTAGATGCC  GAAGAGTGTA
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701  GAGAAACCTT  TATTTCCGGCT  GATGATCTGC  GAGTAAATCT  CTGGAATTTG
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851  GTAATATGCT  CGCATATAGT  AGTTCAAAAG  GCTCCATTCG  TTTGATTGAT
901  ATGCGTCAAT  CAGCTCTGTG  TGATTCTCAT  ACGAAATTAT  TTGAAGAACC
951  GGAAGCACCT  GGTTCAAGAT  CATTTTTCAC  AGAGATAATT  GCCTCAATTT
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1401  GAACGCCTAC  GATTTACAAA  CAAAGTTGCT  GCATATGGCT  TGGCACCCAA
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1501  GCATGA

```

7.3.1 Protein data

Length (aa): 501, Molecular weight: 56274.3, Isoelectric point: 5.612

Translation of DNA sequence of the B-beta gene gave the amino sequence shown below using ExpAsy translate tool and the highlighted area is where the antibody toward B-alpha made for.

5'3' Frame 1

```

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CQPANGALFLLSTNDKTIKYWKVQEKIKIKISEMNIDPSESSNIPPQLVTNGLPADKGH
YLSKDFSFPFGGIPSLRPLVVVTSQETNLVARCRRVYAHADYHINSISNSSDGETFISA
DDLRLVNLWNLEISNQSFNIVDVKPTNMEDLTEVITSAEFHP IHCNMLAYSSSKGSIRLID
MRQSALCDSHTKLFEEPEAPGSRSFTEIIASISDIKFSKDGRIILSRDYMTLKLWDINM
DSGPVASYQVHEHLRPLCDLYENDSIFDKFECLSGDGLRVATGSYSNLFVRFVGFASQGS
TEAATLEASKNPMRRQIQTPARPSRSIGSMTRVVRGSESPGTEANGNAYDFTTKLLHMA
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```

Antibody for B55 -
beta

7.4 >At3g09880, B`beta

Sequence length (bp): 1500

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201  CTCGGTGGAG  CCACTCCCGT  TGTTTAGAGA  TGTGCCGGTT  TCAGAGAGGC
251  AAACCTTTGTT  CTTGAGGAAA  CTTCAGAATT  GCTGTTTCCT  TTTCGATTTT
301  ACTGACACGA  TTAAGAATGC  TAGAGACAAG  GAGATTAAGA  GGCAAACGTT
351  GTTGGAGCTG  GTGGATTTTA  TACAGTCTGG  ATCTAGTAAG  ATCTCAGAGT
401  CGTGTGAGGA  AGAAATGATT  AAGATGATTT  CTGTTAATAT  TTCCGGTCT
451  CTCCCACCAG  CATCACACGA  GAATACGGGT  CAAGAGCCTG  CAGATCCTGA
501  GGAAGAAGAA  CCTTATTTGG  AACCTTCTTG  GCCTCACTTA  CAGCTAGTTT
551  ACGAGTTGCT  CCTGAGATAT  GTTGTTTCGA  CTGACACTGA  TACAAAGGTG
601  GCCAAACGGT  ATATCGACCA  TTCTTTTGTG  TTAAAGTTGC  TCGATTTGTT
651  TGATTCTGAG  GACCCAAGAG  AGAGGGAGTA  TTTGAAAACG  ATTCTTCATA
701  GGATATATGG  GAAGTTTATG  GTCCACAGAC  CCTTTATTAG  GAAAGCAATC
751  AACAACATAT  TCTATAGTTT  TATTTATGAG  ACAGAGAGAC  ACAGTGGGAT
801  TGGGGAAGTC  TTGGAGATTC  TAGGCAGTAT  CATAAACGGG  TTTGCATTGC
851  CTATGAAGGA  GGAACACAAG  CTATTCTTAA  TCAGGGTGTG  GATACCATTG
901  CATAAGCCAA  AACCAATCGT  TGTATATCAT  CAGCAGTTAT  CTTATTGCAT
951  CGTTCAGTTT  GTGGAAAAAG  ATTATAAGCT  TCGCGATACA  GTAATCAGGG
1001  GATTGTTAAA  GTATTGGCCC  GTGACAAACT  GCTCAAAGGA  GAATCTCTTC
1051  CTTGGAGAAC  TTGAAGAAGT  TCTTGAGGCA  ACACAGCCTG  TCGAGTTCCA
1101  GCGTTGCATG  GTTCCATTAT  TCCAACAAAT  TGGTCGCTGT  CTCACTAGCT
1151  CCCACTTTCA  GGTTGCAGAA  CGAGCACTGT  TCTTGTGGAA  CAACGAGCAC
1201  ATTGTGGGTC  TAATCGCGCA  AAACCGAAGC  GTGATCCTTC  CAATCATATA
1251  TCCCACACTG  GAGAAGAACA  TCCAGTCTCA  CTGGAACCAA  GCAGTTCATG
1301  GTCTAACAC  AAATATCAAG  AAAATGTTCA  TGGAAATGGA  TCCTGAACTG
1351  TTTGAAGAAT  GTCAGAGACA  GTATGAAGAG  AAACAAGCGA  AATCCAAAGA
1401  AGTAGAAGAA  CAACGGCAAT  ATACATGGAA  GAGATTAGCA  GAAGCAGCGG
1451  CGGAGCGAGA  TGGAGGAGGA  GGAGAAGAAG  ATCATATGAT  CACTTCCTAG
    
```

7.4.1 Protein data

Length (aa): 499, Molecular weight: 57471.6, Isoelectric point: 6.6161

Translation of DNA sequence of the B-beta gene gave the amino sequence shown below using ExpAsy translate tool and the highlighted area is where the antibody toward B`beta made for.

5`3`Frame 1

Antibody for B`beta

```

MFKKIMKGGHRKPSKSEANEPSSYIGLDPNRSRSGPGSNVVVSHASRGALVNSSPSPVTAT
PPPPPLGSVEPLPLFRDVPVSRQTLFRLKLNCCFLDFDTIKNARDKEIKRQTLLEL
VDFIQSGSSKISESCQEEMIKMISVNIFRSLPPASHENTGQEPADPEEEEEPYLEPSWPHL
QLVYELLLRYVVDTDTKVAKRYIDHSFVLKLLDLFDSEDPREYRKYILHRIYGFV
VHRPFIRKAINNIFYRFIYETERHSGIGELLEILGSIINGFALPMKEEHKLFIRVLIPL
HKPKPIVVYHQQLSYCIVQFVEKDYKLDVTIRGLLKYWPVTNCSKENLFLGELEEVLEA
TQPVEFQRCMVPLFQQIGRCLTSSHFQVAERALFLWNNEHIVGLIAQNRSVILPIIYPTL
EKNIQSHWNQAVHGLTTNIKKMFMEMDPELFEECQROYEEKQAKSKEVEEQROYTWKRLA
EAAAERDGGGGEEDHMITS-
    
```

7.5 Amino acid abbreviations

Table 10: Amino acids with their abbreviation

Single letter code	Abbreviation	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
X	Xxx	Any amino acid
Y	Tyr	Tyrosine

