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The role of Cleavage and Polyadenylation Specificity Factor (*CPSF*) gene in Brassinosteroid signal transduction in *Arabidopsis thaliana*

조선대학교 대학원

생명공학과

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애기장대에서 브라시노스테로이드 신호전달에 관여하는 *CPSF* 유전자의 작용기작

2008 년 8 월 25 일

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A thesis submitted to the graduate school of the Chosun University in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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Abbreviations

24-Cs	.....	24-epicastasterone
ABA	.....	abscisic acid
ALP	.....	alkaline phosphatase
Amp^r	.....	ampicillin resistance
BA	.....	6-Benzyladenine
BAK1	.....	BRI1 associated receptor kinase1
BAP	.....	6-benzyl amino purine
BASTA	.....	glufosinate ammonium
bes1D	.....	bri1-EMS suppressor dominant
BIM2	.....	BES1-interacting Myc-like protein
BIN2	.....	Brassinosteroid insensitive 2
BL	.....	brassinolide
BRI1	.....	brassinosteroid-insensitive
BRs	.....	brassinosteroids
BSA	.....	bovine serum albumin
c-myc	.....	c-myc tag sequence
CaMV35S	.....	cauliflower mosaic virus 35S promoter
cDNA	.....	complementary DNA
Col-0	.....	Ecotype Columbia 0

CPSF	.....	cleavage and polyadenylation specificity factor
Cs	.....	castasterone
DEPC	.....	Diethylpyrocarbonate
det2	.....	de-etiolated 2
DNA	.....	Deoxyribonucleic acid
dNTP	.....	Deoxyribonucleotide triphosphate, A generic term referring to the four deoxyribonucleotides: dATP, dCTP, dGTP and dTTP
DTT	.....	dithiothreitol
Dwf	.....	dwarf
E. coli	.....	*Escherichia coli*
EBL	.....	24-epibrassinolide
ELISA	.....	enzyme-linked immunosorbent assay
EMS	.....	ethyl methanesulfonate
GA	.....	gibberellic acid
GC-MS	.....	gas chromatography-mass spectrometry
gDNA	.....	genomic DNA
GFP	.....	green fluorescent protein
HSPs	.....	heat-shock proteins

IAA	.....	Inole-3-acetic acid
JA	.....	(3R,7R)-jasmonic acid
LB	.....	Lysogeny broth; Luria-Bertani broth
mRNA	.....	Messenger RNA
MS	.....	Murashige-Skoog
NAA	.....	1-Naphtalene acetic acid
ori	.....	origin of replication
PAP	.....	Cytoplasmic Poly(A) polymerase
PCR	.....	polymerase chain reaction
poly A	.....	polyadenylation signal
RLKs	.....	receptor-like protein kinases
RNA	.....	Ribonucleic acid
rpm	.....	revolutions per minute
RT-PCR	.....	reverse transcriptase polymerase chain reaction
SBP	.....	Sterol binding protein
T-DNA	.....	Transfer DNA
TAIL-PCR	.....	Thermal asymmetric interlaced polymerase chain reaction
UTR	.....	untranslated region
WT	.....	wild type
YEP	.....	Yeast Extract-Phosphate Medium

Abstract

The role of Cleavage and Polyadenylation Specificity Factor (CPSF) gene in Brassinosteroid signal transduction in Arabidopsis thaliana

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Brassinosteroids (BRs), a class of plant steroid hormones, are ubiquitously involved in regulating normal growth processes. The hormone controls flowering, leaves shape, color, male fertility, cell elongation and vascular differentiation. Genetic assessments explored a number of genes showing their role in BRs response pathways. We have screened the activation tagged mutants that have severed growth defects and respond to a BRs biosynthetic inhibitor, brassinazole (Brz). Brassinazole is an inhibitor of active BRs that develops characteristic light grown phenotype under dark. The mutant shows taller, dark green, late flowering, short petioles, short and/or curved siliques had fewer seeds with low germination. The mutant had hard texture in maturity. Fruiting was appeared successionally in the mutant having various stages of maturity. TAIL-PCR, genotyping PCR and segregation ratio of T2 plants revealed a single T-DNA insertion at the first exon of Cleavage Polyadenylation Specific Factor (CPSF) gene in chromosome III that encodes a subunit C-terminal domain-containing protein; similar to

DDB1A (UV-damaged DNA-binding protein 1A) in *Arabidopsis thaliana*. To see response of the gene with other BR related mutants, we crossed the mutant between *det2*, a BRs biosynthetic mutant, *bin2* and *bes1D*. We also observed the sense and antisense expression effect of the gene in phenotype. Real Time RT-PCR results showed that there was no alteration in expression level of neighboring genes of the T-DNA insertion. Hypocotyls length of Brz-treated mutant seedlings under dark measured nearly two fold longer than wild type Columbia 0 revealing involvement of *cpsf* in BRs signal transduction. The crossing results reflected that the T-DNA insertion mutation of *CPSF* related to BR perception and it regulate normal growth process through involving in BRs signaling pathways in *Arabidopsis*. Also *cpsf* knockdown or overexpression creates defective siliques and might develop lethality in *Arabidopsis*.

Key word: activation tagging, *Arabidopsis*, BRs signaling, Brassinazole

1 Introduction

1.1 Growth Process and its control

Birth, growth, development, environmental sensation and death are normal phenomenon in living systems that are guided by various extrinsic as well as intrinsic factors. The extrinsic factors such as light, climate, soil, and other external environment involve in such exogenous control in such a way tightly associating with other intrinsic factors. Not only external factors, organisms are guided with internal factors as well. The chemical elements that are variously associating in different proportions govern the normal life processes in several ways. Inorganic chemicals combining together make bio-molecules. The fate and path of synthesis and perception of these bio-molecules designs the fate of the life processes however, only a small portion of this dynamic process is known until now. Therefore seeking living within all non-living chemical bound association in any organisms became a challenge and puzzling the science yet. Understanding chemical state of life and explaining the state of living within all non-living chemical association triggering the science explain the chemical state of life. Macromolecular association in organisms contributed by structural architectural units like proteins, carbohydrates, lipids and other non-architectural units like vitamin, hormones, and so on. Among these several biomolecules, hormones are one of the molecules that control the normal life processes in living system.

1.2 The history of Plant Hormones

Hormones are chemical molecules that are essential for growth and development processes. These chemical compounds synthesized in plant control the overall or part of the development processes acting independently or synergistically. The hormones found in plants are called phytohormones. Various types of phytohormones such as auxin, gibberellins, acetylene, abscisic acids, and brassinosteroids have been found in various plants. Brassinosteroid is one of the phytohormones detected in most recent era in plant physiology. Historically, Charles Darwin and his son Francis Darwin attributed their work of "the power of movement in Plants" 1880 might firstly describe the possibilities of chemical substances that involve in controlling role in plants. The substance later was named as auxin. Dutch scientist discovered cytokinin at middle of 19^{th} century. Similarly, Japanese scientist described gibberellins in 1926. Several types of chemicals responsible for growth and physiological response have been isolated so far until now.

1.3 The history of brassinosteroids

Brassinosteroids (BRs) have been generally accepted as polyhydroxylated steroidal plant hormones only recently. Since the discovery of brassinolide (BL) isolated from rape (*Brassica napus L.*) in 1979 by Grove et al. (Grove *et al.,* 1979) extensive studies have been undertaken worldwide on this notable substance. The characteristic physiological effect of BL on growth and development of plants as well as their potential abilities in agricultural applications have started to be examined and are grouped in new class of plant hormone. Before the isolation of BL, long discussion had been made, whether steroidal hormones exist in plants and was ended in 1979 after isolation of BL

bearing apparent hormonal properties. After the Grove's report, castesterone (Cs) was isolated as the second BRs at the University of Tokyo (Yokota *et al.*, 1982). Since then, a number of related steroidal compounds have been isolated from a variety of plant sources. To date, more then 65 free BRs and 5 BR conjugates have been found and fully characterized by spectrometric methods (Bajguz, 2007). Presumably, there are a number of yet unknown BRs and BR conjugates in plants. BRs and its congeners appear to be ubiquitous and have been isolated from more than 50 plant species, including angiosperms, gymnosperms, a peteridophyte, bryophyte and green algae (Fujioka, 1999; Haubrick and Assamm, 2006) .

Earlier, investigators were focused on describing the biosynthetic pathways of BRs hence major part of the biosynthesis has been elucidated (Fujioka and Sakurai, 1997) putting forward the most conventional hypothetical model. The hypothetical model is based on information from extensive step-wise feeding studies performed with radiolabelled precursors of BL and analysis of their bioconversion products. In the last decade, attention concentrated also to the physiological properties of BRs (Adam *et al.*, 1996; Fujioka and Sakurai, 1997; Sakurai and Fujioka, 1993; Sasse, 1997; Yokota *et al.*, 1997). High interest in the function of BRs has been elicited through the strong responses of intact plants and explants observed upon application of exogenous BRs. They were considered as promising compounds for application in agriculture, because they showed different regulatory activities on growth and development in plants.

Although the BRs are present in plant tissues, their importance for the regulation of plant growth and development has not been accepted as wide as for "classical" plant hormones such as auxins, gibberellins,

cytokinins, jasmonates, ethylene and abscisic acid (Kende and Zeevaart, 1997). This situation changed dramatically with several reports on the identification and characterization of BR biosynthesis and signal transduction mutants (Clouse, 1996; Clouse, 1997; Yokota *et al.*, 1997). The phenotypes of the BR-deficient mutants presumed, that BRs are essential hormones and take part in light-regulated development.

A new attempt to identify BR-insensitive mutants of *Arabidopsis* resulted in the isolation of the dwarf mutant named *bri1* (Clouse *et al.*, 1996; Szekeres *et al.*, 1996). BRI1 was found to have strong homology to leucine-rich receptor kinases. Recent analysis of a range of BR biosynthesis and insensitivity mutants in *Arabidopsis* was decisive in discovering the physiological importance of BRs. The analysis of BR-deficient and BR-insensitive mutants aims to characterize the physiological roles of BRs in growth and development. The cloning of BR-regulated genes is providing insight into the molecular mechanisms of plant steroid hormone action. In future, the research of BRs will become increasingly important in understanding the plant growth regulation. The compounds can be classified as C_{27}, C_{28}, or C_{29} BRs, depending on the pattern of the side. A typical brassinosteroid call brassinoide BL which structure has been illustrated in Figure 1.

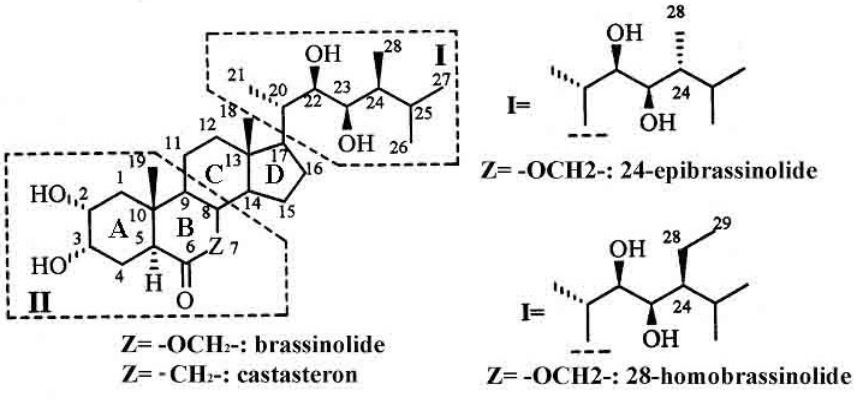


Figure 1: Brassinosteroid structure

BRs are perceived at the plasma membrane by direct binding to the extracellular domain of BRI1 receptor serine-threonine kinase. BR perception initiates a signaling cascade, acting through a GSK3 kinase, BIN2, and the BSU1 phosphatase, which in turn modulates the phosphorylation state and stability of the nuclear transcription factor BES1 and BZR1. Plants deficient either in biosynthesis or perception of these hormones are typically dark green, dwarfs with epinastic leaves, have reduced or no fertility, reduced apical dominance, short petiole, round leaves, de-etiolation in dark, and exhibit delayed development (Bishop and Koncz, 2002; Kyoung and Li, 2002). However, the role of BRs in plant function has demonstrated that the ability to synthesize, perceive and response puzzles sometimes. Brassinazole (Brz) is a triazole compound that specifically blocks BL biosynthesis by inhibiting cytochrome P450 steroid C-22 hydroxylase encoded by DWF4 gene (Asami *et al.*, 2001). BZR causes de-etiolation and dwarf phenotypes similar to those of BR-deficient mutants (Asami *et al.*, 2000).

1.4 Natural occurrence of brassinosteroids in plants

Among plant steroids only BRs are ubiquitously distributed throughout the plant kingdom. They play essential roles in modulating the growth and differentiation of cells at nanomolar to micromolar concentrations (Clouse and Sasse, 1998). The compounds can be classified as C_{27}, C_{28}, or C_{29} BRs, depending on the pattern of the side chains. BRs are widely distributed in all plants parts of the plant in the plant kingdom (Fujioka *et al.*, 1996; Schmidt *et al.*, 1997). Levels of endogenous BRs vary among plant tissues. The highest measured concentration is about 10^{-1} nmol g^{-1} fresh weight (BL in the pollen of *Brassica napus* and *Vicia faba*) and the lowest is about 10^{-7} nmol g^{-1} (homocastasteron in immature

seeds and sheaths of Chinese cabbage, *B. campestris* var. *pekinensis*, (Khripach *et al.*, 2000). Pollen and immature seeds, the original sources of BL, are containing most of the hormones with ranges of 1-100ng x g^{-1} fw, wile shoots an leaves usually have lower amounts of 0.01-0.1ng x g^{-1} fw (Takatsuto, 1994). The average steroid content in mature seeds of *A. thaliana* is illustrated by recent data of (Fujioka *et al.*, 1998): BL, $3.9x10^{-3}$ $nmolg^{-1}$; Cs, $9.5x10^{-4}$ nmol g^{-1}; typhasterol, $3x10^{-3}$ nmol g^{-1}; 6-deoxocastasterone, $3.5x10^{-3}$ nmol g^{-1}; 6-deoxotyphasterol, $2.1x10^{-3}$ nmol g^{-1}; 6-deoxoteasterone, $1.2x10^{-3}$ nmol g^{-1}. Bioassay results suggest that roots also contain BRs, however, possibly due to their low concentration in the tissue, they were not isolated yet (Clouse and Sasse, 1998).

1.5 Biosynthesis

Plant sterols have been extensively studied focusing on biosynthetic and biochemical aspects. Elucidation of BR biosynthesis and metabolism is fundamental to understand how plants regulate the endogenous level of BR for their growth and development which was unclear until recently. BL, the most important BR, has been shown to be synthesized via two pathways from campesterol. Sterols are synthesized via the mevalonate pathway of isoprenoid metabolism. In plant sterols, campesterol and its analogues are assumed to be the biosynthetic precursors of BL, based on the identity of the side-chain skeleton. Early steps of BL biosynthesis were investigated using feeding of cell cultures of *C. roseus* (Fujioka *et al.*, 2000) with ^{2}H-labeled 6-oxocampestanol, 6-deoxocastasterone and 6α-hydroxycastasterone, and the metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS). The cultures produced representatives of C_{28} BRs, such as BL, Cs, typhasterol, testasterone and cathasterone (Choi *et al.*, 1996; Fujioka *et al.*, 1995). The levels of BRs

in cell cultures of *C. roseus* were found to be comparable to those of BRs-rich plant tissues such as pollen and immature seeds.

Campesterol, the major plant sterol was effectively prepared from labeled mevalonic acid. By feeding the cells with radioactive campesterol, campestanol, 6α-hydroxycampesterol, and 6-oxocampestanol were identified as metabolites. Subsequent feeding experiments with labeled campestanol and 6α- hydroxycampesterol revealed the biosynthetic sequence from campesterol to 6-oxocampestanol (Suzuki *et al.*, 1995). This sequence constitutes an early part of the biosynthetic pathway of BL, since the introduction of vicinal hydroxyls in the side chain of 6-oxocampestanol will yield teasterone. Therefore, a biosynthetic pathway for BRs via hydroxylations and epimerisation after the oxidation at C6 of the B-ring was suggested. This pathway leading to BL was proposed as an early C6-oxidation pathway. Transformation of 6-oxocampestanol to teasterone was described using chemically synthesized specimens of possible intermediates as probes. As a result, 22α-hydroxy-6-oxocampestanol was identified and named cathasterone (Fujioka *et al.*, 1995). In the same work cathasterone was identified as is the direct precursor of teasterone. By feeding experiments with deuterium-labeled teasterone, it was shown to be converted to typhasterol. Epimerization would occur via oxidation to the 3-oxo-form. 3-Dehydrotesterone was shown to be reduced to typhasterol as major metabolite and to teasterone as minor one (Suzuki *et al.*, 1995). The conversion of typhasterol to Cs and BL was demonstrated by feeding with deuterium-labeled substrate. Typhasterol was predominantly converted to castasterone, while minor epimerization to teasterone was observed. Conversion of Cs to BL was at first shown by feeding with deuterium-labeled castasterone (Suzuki *et al.*, 1993) , and later

conclusively demonstrated by feeding with tritium-labeled Cs (Yokota *et al.*, 1990). Thus, the biosynthesis of BL via the early C6-oxidation pathway was described.

Among natural BRs, attention was not drawn to the 6-deoxo BRs such as to 6-deoxocastasterone. They were considered to be dead-end in the pathway and not to be converted to active BR. However, reports showing the natural occurrence of 6- deoxo BRs in many plants (Abe *et al.*, 1994; Yokota *et al.*, 1994) have allowed their involvement in the biosynthetic pathway to be reevaluated. Feeding experiments with deuterium-labeled substrate revealed the conversion of 6-deoxoteasterone to 6-deoxotyphasterol. Similarly, 3-dehydro-6-deoxo-teasterone was shown to be converted to 6-deoxotyphasterol. Thus, 6-deoxoteasterone is epimerized to 6-deoxotyphasterol via the 3-oxo form (3-dehydro-6-deoxoteasterone), similar to the conversion of teasterone to typhasterol (Choi *et al.*, 1997). Moreover, 6-deoxocastasterone was found to be converted to Cs and BL (Choi *et al.*, 1996). Therefore, the existence of an alternative BL biosynthetic pathway via late C6-oxidation was demonstrated. Both the early and the late C6-oxidation pathways operate in cell culture of *C. roseus*. The co-occurrence of BRs, belonging to the pathways in the same plant species indicates that these pathways could be ubiquitous in plants (Fujioka and Sakurai, 1997).

1.6 Physiological action of brassinosteroids

In 1970 Mitchell et al. reported about the discovery of new hormones, which they called “brassins”. They were inducing very marked elongation of both second and third internodes of the intact plants of *Brassica napus* (Mitchell *et al.*, 1970). They were found to be a family of plant hormones that appear to have a glyceride structure. Later reports

claimed a wide range of physiological effects of BL in plants: elongation, bending, cell division, reproductive and vascular development, membrane polarization and proton pumping and modulation of stress (Sakurai *et al.*, 1999). In addition to their essential role in development, BRs have the ability to protect from various environmental stresses. Treatment of *B. napus* with 24-epibrassinolide (EBL) leads to a significant increase in their basic thermotolerance, and results in higher accumulation of four major classes of heat-shock proteins (HSPs) as compared to untreated seedlings (Dhaubhadel *et al.*, 2002). Kalinich *et al.* (1986) have shown that BL treatment enhanced the activities of RNA and DNA polymerases as well as the levels of RNA, DNA, and protein and suggested that BL responses are dependent on nucleic acid and protein synthesis. Exogenously applied BL increases graviotropic curvature in maize primary roots (Kim *et al.*, 2000). Morillon *et al.* (2001) hypothesized about the role of BL in the modification of the water-transport properties of cell membranes. Application of BL to the stigmas of castrated flower buds is inducing parthenogenetic haploid seed germination in *A. thaliana* and *B. napus* (Kitani, 1994). *BRs* also interact with environmental signals and can affect insect and fungal development. A study of growth of monocots and dicots showed a promotive effect of a BL isomer on photosynthetic capacity and biomass production in their primary developmental stages (Braun and Wild, 1984). In mustard, fresh weights of the shoots increased, and both elongation and radial growth were stimulated. An increase of 40-50% in CO_2 fixation *in vivo* in wheat chlorophyll levels was only slightly affected. On hydroponically grown *Arabidopsis* plants, with application of BR to the roots, profound effect on petiole elongation and upward bending of the leaf was detected (Arteca and Arteca, 2001). Elongation

was promoted in young stem tissues of many plants after treatment with moderate doses of BR. This could be due to either higher endogenous auxin levels in the tissue or higher sensitivity of young tissue to BR. Evidence for BR-auxin synergism comes from the data of auxin-induced ethylene production in etiolated mung bean hypocotyls segments (Arteca *et al.*, 1985) Several authors proposed that BR-induced effects might be mediated via auxin, that BR treatment might alter the level of endogenous hormones, or that BR might enhance tissue sensitivity to auxin (Mandava, 1988). This synergism, however, occurs only when the tissue is pretreated with BR and then exposed to auxin. When the order is reverse, there is no effect. Stimulatory effects of BRs on elongation are among the most documented physiological effects.

All mutants relating to brassinosteroid in *Arabidopsis thalinana* show male sterility or reduced female fertility, drastic dwarfism and reduced apical dominance. When grown in darkness, these mutants present reduced hypocotyls elongation, unregulated opening of cotyledons and emergence of primary leaves (Pereira-Netto, 2007).

BR stimulates germination in *Arabidopsis*, proposing, that BRs are needed to antagonize seed dormancy and stimulate germination (Steber and McCourt, 2001). It was observed that BL accelerated endosperm rupture of tobacco seeds imbibed in the light (Leubner-Metzger, 2001). His results indicated that BRs and GAs promote tobacco seed germination by distinct signal transduction pathways and distinct mechanisms. GA and light seem to act in a common pathway to release photodormancy, whereas BRs do not release photodormancy. BRs seem to promote seed germination by directly enhancing the growth potential. In addition, a steroid hormone, brassinolide, also plays a role in light

regulated development and gene expression in *Arabidopsis* (Chory *et al.*, 1996; Li *et al.*, 1996; Nagata *et al.*, 2000; Szekeres *et al.*, 1996).

1.7 Molecular genetics of brassinosteroid action

1.7.1 Brassinosteroid signal transduction

Until very recently, most of BR signal transduction research focused to Brassinosteroid Insensitive 1 (BRI1*)*, a single genetic locus in *Arabidopsis* encoding a leucine-rich repeat receptor kinase (Clouse *et al.*, 1996). Eukaryotic organisms use steroids as signaling molecules for physiological and developmental regulation. Two different models of steroid action have been described in animal systems: the gene regulation response mediated by nuclear receptors, and the rapid non-genomic response mediated by proposed membrane-bound receptors (Wang *et al.*, 2001) while plant genomes do not seem to encode members of the nuclear receptor. However, a trans-membrane receptor kinase BRI1, has been implicated in BRs responses (He *et al.*, 2000; Li and Chory, 1997). *Bri1* cannot be rescued by BRs that evidences its essential role in a signaling pathway. The role of BRI1 was demonstrated by binding radiolabeled BL and mitochondrial fraction of wild-type and mutant plants, as well as from transgenic plants expressing BRI1-green fluorescent protein (GFP) fusions (Wang *et al.*, 2001).

A yeast two-hybrid screen for proteins putatively interacting with BRI1 identified a second LRR-K, termed BAK1 (BRI1 associated receptor kinase1), that shares similar structure organization with BRI1 (Nam and Li, 2002). The discovery of BAK1 and the genetic and biochemical evidence showing a role of BAK1 in BR signaling and a physical interaction with BRI is intriguing. Direct physical interaction between

BRI1 and BAK1 was confirmed both in yeast cells and *Arabidopsis* plants by co-immunoprecipitation with target proteins (Li *et al.*, 2002; Nam and Li, 2002). A role of BAK1 in BR signaling was demonstrated by a number of genetic experiments. Knockout mutants of *BAK1* were identified that showed a weak *bri1*-like phenotype and also decreased sensitivity to BR in root inhibition assay. Finally, overexpression of a kinase-deficient mutant form of *BAK1* in *bri* led to a severe dwarf phenotype, suggesting a dominant-negative effect, most likely by the poisoning of a possible heteromeric complex between BRI1 and BAK1 (Li *et al.*, 2002). Nam and Li in 2002 proposed a model for BR signaling in which BRI1 and BAK1 exist as inactive monomers in equilibrium with an active heterodimer. BR binding stabilizes the heterodimer and results in transposphorylation of each cytoplasmic domain by its partner, leading to activate kinases that recognize and phosphorylate currently undefined downstream components. On the other hand, (Li *et al.*, 2002) hypothesize that BRI1/BAK1 interaction is more reminiscent of the TGF-β signaling pathway. They suggest, that BR binds to BRI1, which than activates BAK1 by transphosphorylation. The activated BAK1 would then phosphorylate downstream components. Both of these models are based in part on data from kinase activity of BRI1 and BAK1 expressed in yeast and bacteria. Thus the genetic evidence strongly supports the biochemical experiments that show direct *in vivo* interaction between BRI1 and BAK1 in plant membranes. However, direct evidence of BRI/BAK1 heterodimer as a coreceptor of BR is lacking and will be required before models of BR-dependent heterodimerization and kinase activation can be confirmed. To clarify true nature of BRI1/BAK1 interaction it will be necessary to examine specific phosphorylation sites and mechanisms *in planta* and their dependence on BR.

After plasma membrane perception of BRs the signaling pathway begins to resemble elements of the Wingless/wnt pathway. An unknown number of steps follow BR binding, leading to inactivation of the negative regulator BIN2. Inactivation of BIN2 allows the unphosphorylated form of BES1 and BZR1 to accumulate and translocate to the nucleus. These proteins are obviously involved into the regulation of BR-responsive genes, but the mechanism remains completely unknown. By analogy with β-catenin in the Wingless/wnt pathway, BES1 and BZR1 may complex with transcription factors, and yeast two-hybrid analysis may be effective in identifying such putative binding partners. Once BR response elements have been located in the promoters of BR regulated genes, it will also be possible to conduct yeast one-hybrid analysis to isolate transcription factors associated with BR signaling. The gap between BRI1 and BIN2 represents a major deficiency in our understanding of BR signaling. Substrates for the BRI1 (and BAK1) kinase domain need to be definitively identified and their function characterized. Isolation of receptor kinase substrates will allow a second round of screening for downstream interacting components.

1.7.2 Brassinosteroid regulated gene expression

A number of genes regulated by BR either by transcriptional or posttranscriptional mechanisms have been identified, although a BR response element and interacting protein factors have not as yet been reported (Bishop and Koncz, 2002). Plant cell expansion and division are critical for growth and differentiation in all organs and results from alterations in gene expression and biochemical processes that affect on mechanical properties of cell wall, cell hydraulics, number of cells, and the osmotic potential. Modulating the expression of genes encoding

wall-modifying proteins is one mechanism by which plant hormones, such as BRs, auxins and gibberellins promote cell elongation and many of the known BRs-regulated genes encode such proteins. Another way for plant growth is the cyclin expression regulation. As it was shown by (Hu *et al.*, 2000), *CycD3* is induced by epi-brassinolide. Down-regulation of the genes encoding BR biosynthetic enzymes has been demonstrated, as has for BR-regulated genes encoding proteins associated with environmental adaptation, pathogen attack, assimilate partitioning, biosynthesis of other plant hormones, and translation initiation (Bishop and Koncz, 2002).

Most of the genes were identified by classical methods of studying differential gene expression, like subtractive hybridization. More recently, DNA microarray analysis has been employed to identify several novel genes that appear to be regulated by BR signaling. Examination of BR-regulated gene expression in the *bes1-1D* and *bri1* mutants using Affymetrix GeneChip arrays verified the BR regulation of numerous genes encoding wall-modifying proteins and showed that several genes associated with auxin signal transduction are also BR regulated (Yin *et al.*, 2002). Interestingly, an independent genetic analysis of the *Arabidopsis ucu1* mutant (allelic to *bin2*) suggests that the BIN2/UCU1 kinase may also be involved in auxin signal transduction, indicating the possible crosstalk between BR and auxin, two hormones with pronounced effect on the cell elongation (Perez-Perez *et al.*, 2002). A recent genetic screen for new BR-insensitive mutants, combined with Affymetrix GeneChip analysis, has identified *bin3* and *bin5* as putative subunits of an *Arabidopsis* topoisimerase VI that regulates the expression of numerous genes, including many of those that are also regulated by BRs (Yin *et al.*, 2002). A global

expression analysis using Affymetrix GeneChips and RNA from weak alleles of BR-deficient mutants grown under two conditions has identified a core set of Br regulated genes involved in BR biosynthesis, auxin response, nitrogen transport, and transcriptional activation (Müssig *et al.*, 2000).

While a number of BR-regulated genes have currently been identified in *Arabidopsis,* preliminary microarray experiments suggest that much more of these genes are required to drive BR-promoted developmental processes, that leads to normal plant growth (Clouse, 2002). Global expression studies further defined the downstream events in this pathway, confirming the role of several factors acting in negative feedback regulation on BR levels (Nemhauser and Chory, 2004). The recent availability of full-genome *Arabidopsis* GeneChip from Affymetrix (recognizing more than 24000 distinct genes) provides a unique opportunity to characterize the full spectrum of BR-regulated genes.

1.8 *CPSF* gene and its role

Cleavage polyadenylation and specificity factor (CPSF) is an important multi-subunit component of mRNA 3'-end processing in eukaryotes. Messenger RNA 3'-end processing is a vital step in gene expression. In RNA processing event, a precursor mRNA recognized, cleaved and then polyadenylated at the free 3'-OH generated by processing reaction (Hunt *et al.*, 2008). Polyadenlylation is a ubiquitous feature during processing of all eukaryotic mRNA with exception of cell cycle regulated histone mRNA (Loke *et al.*, 2005; Xu *et al.*, 2004). In animals these factors have been termed as CPSF (cleavage and polyadenylation specificity factor), CstF (Cleavage stimulatory factor), CFI and CFII (Cleavage factors I and II), and PAP [Poly(A) polymerase] (Hunt *et al.*, 2008; Xu *et al.*,

2006). The key functions of poly (A) tails are mRNA stability and translatability and involvement in the export of mature mRNA to cytoplasm. *Arabidopsis* genome contains number of CPSF encoding genes homologues. *AtCPSF160, AtCPSF100, AtCPSF73-I, AtCPSF-II* and *AtCPSF30* have been known in some extent however rest of the other CPSF homologues are yet to be described ((Xu *et al.*, 2004; Xu *et al.*, 2006). Arabidposis genome CPSF160 binds to AAUAA in mRNA processing, CPSF100 function is not known while CPSF73-I and contributes in endonuclease reaction, CPSF30 function for RNA binding and endonuclease activities (Hunt *et al.*, 2008; Proudfoot *et al.*, 2002). It is found that *At3g11060* gene encodes 160 kD protein (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=NP_187802.2) whoever similar sized protein is also encoded by the gene At5g1660 which functioning in binding to AAUAAA during mRNA polyadenlylation process (Hunt *et al.*, 2008). Other role of this gene is yet to be studied.

1.9 Aim of the work and experimental design

The main objective of this study was to assess the gene that involve in BRs signaling cascade in *Arabidopsis* with the use of activation tagging. Knockout or knockdown or knockworst induced in the gene tagged by the activation tagging vector and induction of the expressional modification in it/neighbors to address the gene involvement in the phenotypic variation having firm inter relation with the BRs. Different researches suggested a number of genes involve in BRs signal transduction in *Arabidopsis* and is still under search. In summary, experimental design of the research as been shown in flow chart below (Table 1):

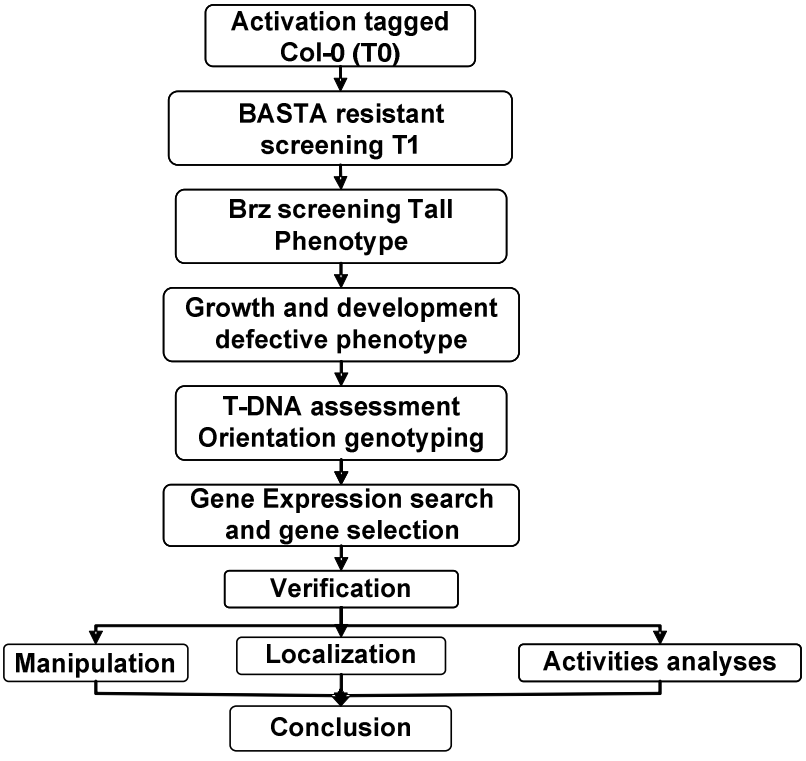


Figure 2: A graphic representation of the experiment design for studying BRs signaling related mutants in *Arabidopsis*

2 Materials and Methods

Wild type *Arabidopsis* Columbia-0 was used for carrying out genetic analysis. Mutant plants unless otherwise specified are derived from the background Columbia-0 (Col-0). Activation tagging vector pSKI015 T-DNA tagged Col-0 seeds was kindly provided by the Kumho Laboratory, Korea.

2.1 Growth media

2.1.1 Media for plant culture

MS medium (Murashige and Skoog, 1962) (MS.3S-1L):

2.22 g of MS (Murashige and Skoog) basal salt mixture (Duchefa, Haarlem, The Netherlands) and 10 g sucrose (Sigma, USA) were dissolved to make 1 L 1/2MS.S medium. pH was adjusted to pH 5.8. To make the solid medium 0.8% agar (w/v) (Duchefa, Haarlem, The Netherlands) was added. Mixture as sterilized by autoclaving and plated in Petri plates for further use.

Infiltration medium (1 L)

1/2 MS (without sucrose) media were prepared Gamborg's vitamin(Duchefa) -1x final concentration, benzylaminopurine (BAP) 10 µL/L of 1 mg/mL stock and Silwet 200 uL/L were added in 1 L medium. pH was adjusted to pH 5.7.

2.1.2 Bacteria culture medium

LB medium (1L):

5g Yeast extract (Difco), 10g Tryptone (Difco), 10g NaCl were dissolved distilled water to final vole 1 L. 8 g agar (Difco) (0.8% - w/v) was added to make solid medium, media was sterilized by autoclaving.

SOC medium (1L):

5g Yeast extract (Difco), 20g Tryptone (Difco), 0.5g NaCl, 0.186 g KCl were dissolved in distilled water to prepare 1 L final volume. pH was adjusted to 7.0. Media was sterilized by autoclaving. Filter sterile glucose solution (20 mM final concentration) was added.

YEP medium (1L):

10 g Yeast extract (Difco), 10 g Tryptone (Difco), 5 g NaCl, were added to prepare 1 L YEP medium. pH of the medium was adjusted to 7.0. 8 g (0.8% -w/v) agar (Difco) was added for solid medium, sterilized by autoclaving and used .

Antibiotics

carbenicillin disodium (Cb) and kanamycin monosulfate (Km) (Duchefa)

2.2 Seed sterilization

Seeds were taken in EP tubes. 1 ml of 70% ethanol and 0.05% tritonX-100 were used for surface sterilization. The seeds containing EP tubes were shaken by inverting them for 20 minutes; Vortexed and centrifuged on table top centrifuge. Solution was replaced with 95% ethanol. The procedure was repeated three times in 95% ethanol. Seeds were washed with sterile 3rd distilled water for three times to make ready to sow.

2.3 Sowing seeds

Murashige and Skoog (MS) medium was used for growing seeds. Ready made MS (Duchefa, Netherlands) was added to prepare 1/2MS plates supplying with 1 % sucrose (w/v). Solid media was prepared adding 0.8% phytoagar (Duchefa, The Netherlands). Media were sterilized and plated. Individual seeds were sown in plates using blunt yellow tips. For the T0 screening, sterilized seeds were spread on to medium. For hormone treatment, desired concentration of hormone is added without using antibiotics. Antibiotic was used only in the case where selection is needed.

2.4 Growth condition

The sown seeds were kept at 4°C for 3 days in the dark for stratification by wrapping them on aluminum foil and transferred to normal growth condition 23°C with a 16-h day/8-h night photocycle. However, seedlings preparation for hypocotyls measurement in different hormone dose response, seeds after 3 days stratification, grown under dark at 23°C for 5-7 days.

2.5 Cell Preparation and Transformation

2.5.1 *E. coli* transformation

Preparation of competent *E. coli* cells 5ml of LB medium was inoculated with a single colony of or DH5a or XL Blue cells and incubated overnight at 37°C. 1ml of the overnight culture was used for the inoculation of 100ml LB medium. The culture was incubated at 37°C by shaking until the optical density at 560 nm reached 0.4. Cells were

centrifuged for 10 min at 4°C (4500rpm), the pellet was resuspended in 20 ml ice cold solution 0.1M $CaCl_2$ with 10 % glycerol and let on ice for 15 min. Cells were centrifuged for 15 min at 4°C (4500 rpm) and the pellet was resuspended in 2 ml ice cold solution 0.1 M $CaCl_2$ with 10 % glycerol. 100µl aliquots of heat shock competent cells were frozen in liquid nitrogen and stored at -80°C

2.5.2 Heat-shock transformation of competent *E. coli* cells

Stored competent cells were refrozen on ice. DNA samples or aliquots of a ligation mixture (10 µl), chilled on ice, were added to competent cells, mixed and incubated for 30 min on ice. The tubes were transferred to 42°C for exactly 90 sec and rapidly transferred to ice for 1-2 min. 900 µl of SOC medium were added to each tube and incubated at 37°C for 45 min to allow bacteria cells to recover and to express the antibiotic marker encoded by the plasmid. Aliquots of transformed cells (up to 200 µl) were transferred onto LB agar containing the selective antibiotic and grown overnight at 37°C.

2.5.3 Electroporation

A pulse (2.5V, 25µF, 200W) was applied from the Bio-Rad Gene-Pulserä on the cuvette containing 50ml of electroporation-competent *E. coli* cells with 1ml of plasmid DNA. Cells were resuspended in 1ml of SOC medium, incubated for 45 min at 37°C and plated on LB medium

2.5.4 Transformation of *Agrobacterium tumefaciens*

Preparation of competent *Agrobacterium tumefaciens* cells

The overnight culture of *A. tumefaciens* in YEP medium was diluted with fresh medium 1:100 (final volume 50 ml) and grown at 28°C till OD600 reached approximately 0.5-0.8. Cells were collected by centrifugation for 20 min at 300 rpm at 4°C and resuspended gently in 10 ml of sterile cold 20 mM $CaCl_2$. The cells were spinned down for 5 min at 300 rpm and resuspended in 1 ml of sterile $CaCl_2$. 100 µl aliquotes of the cell suspension were frozen in liquid N_2 for further storage at –70°C.

2.5.5 Cold shock transformation of competent *Agrobacterium tumefaciens* cells

1-2mg of plasmid DNA was mixed with an aliquote of competent *A. tumefaciens* cells and freeze in liquid nitrogen for 5 min. Then cells were transformed to the 37°C water bath and incubated for 5 min. 1 mL of LB medium were added to the cells. The cells were mixed and incubated for 2-3h at 28°C with shaking. Then 200 µL of the cell suspension were spread onto selective YEP plates and incubated at 28°C for two days to receive visible colonies for further propagation.

2.6 Screening activation-tagged Col-0 and genotyping

pSKI015, an activation tagging vector, tagged Col-0 plants harbors BASTA resistant. The vector characteristically alters the neighboring or the gene of T-DNA insertion making visible phenotypic distinction. BRs related mutant line selection was preceded from BASTA resistant T1 seed lines. Out of 5015 T1 BASTA resistant lines, they were grown in 1/2MS 1S supplied with 3 µL Brz medium for 7 days in dark. Long hypocotyled seedlings in the medium were selected and their hypocotyls were measured. Long hypocotyled BASTA resistant seedlings were

selected and transferred and grown in soil. Their morphology was observed. Mutants having distinct phenotype were selected for determining the site and orientation of T-DNA insertion in its genome. Genomic DNA from the mutant was isolated and Thermal Asymmetric Interlaced Polymerase Chain Reaction (TAIL-PCR) was carried out to find out the site of and orientation of the T-DNA. Single T-DNA insertion harboring, phenotypically distinct plants were selected and further experiments proceeded.

The genotyping was performed using gene specific primers ATNF 5'- and ATNR (Table 1) on either site of the T-DNA insertion and left border primers were used to finding the T-DNA flanking direction in the *Arabidopsis* genome.

2.7 Double mutant genotyping

Double mutants between crossed lines were genotyped and confirmed. Double genotyping was carried out for *det2*cpsf mutant only. Primers used for the *det2cpsf* genotyping were DET2F1 and DET2RA. The PCR condition was used for the amplification was preheating 95º 5'; 35 cycles of denaturation 94 º 30 seconds, annealing 55 º30 seconds, extension 72 º for 45 seconds followed by final extension of 5 minutes holding at 4 º. Ten micro liters of PCR product was restriction digested using Mnl I.

2.8 Total RNA isolation and cDNA synthesis

Total RNA was isolated using Trizol (Life Technologies, USA) method as manufacturer's instruction from two weeks old 1/2MS1S grown plants. Plants were frozen into liquid N_2-replenished mortar and pestle and finely powdered. 100 mg of powdered tissue was taken in 1.5 mL

Epenroff tubes; 1 ml trizol was added and well mixed; and mixture was incubated 10 minutes at room temperature (RT). 200 µL of chloroform was added, shaken vigorously for 30 seconds and incubated for 3 minutes at RT. The mixture was centrifuged for 15 minutes at 4 °C with a speed of 12,000 revolutions per minutes (rpm). Upper clear supernatant was taken and 200 µL of phenol:choloroform:isoamyl alcohol was added and well mixed. The mixture was centrifuged for 10 minutes with a maximum speed at 4 °C. Upper supernatant was taken; equal volume of isopropanol was added; well mixed and incubated for 10 minutes at RT. The mixture was then centrifuged at 12,000 rpm for 15 minutes at 4 °C. Supernatant was removed; pellet was washed with 70% ethanol and dried for 10 minutes. Pellet was dissolved in 30 µL DEPC DW and further reaction was preceded after measuring OD_{260}. cDNA was synthesized by using the superscript II reverse-transcriptase (Invitrogen). 2 µL (about 1-2 µg/µL) of total RNA, 1 µL of the oligo dT (Invitrogen) (500 µg/mL) making 5 µL reaction volume were mixed and heated at 65 °C for 10 minutes. The mixture was immediately cooled on ice. First strand buffer (5x) – 4 µL, DTT (0.1 mM) – 2 µL, dNTP mix (2.5 mM each) – 4 µL and 4 µL DEPC-DW were added and incubated 2 minutes at 42 °C. The enzyme [superscript II reverse-transcriptase (Invitrogen)] (1 µL) was added making total reaction volume 20 µL and incubated at 42 °C for 50 minutes. Then the RT (reverse transcriptase)-PCR (polymerase chain reaction) and Real time –PCR were carried out.

2.9 Full length cDNA construction

Full length cDNA was 4.4 kb sized containing short 5'UTR and and long 3'-UTR regions. Full length cDNA covered the cDNA including its un-

translated regions. PCR amplification was carryout using primers (Fragment-I sized ~2.6 kb: CPSFF2 and CPSFR3; for fragment II sized ~1.7 kb: CPSFF3 and CPSFR2 (Table 1). Double digestion fragments were prepared and ligated them into pUC19 to obtain full-length cDNA clone using stratagene turbo pfu. Conditions for pcr were (For fragment I: 94°, 5'; 56°, 40", 72°, 6' repeated to 35 cycles and final extension of 10 minutes at 72° and fragment II was 94°, 5'; 54°, 40", 72°, 4' repeated to 35 cycles and final extension of 10 minutes). The double restriction sites were XbaI and NcoI in 5' and 3' ends respectively in case of fragment-I and NcoI and XbaI in downstream fragment. Fragments were cloned into cloning vector pUC19 containing ampillicilin selection site. The clone was verified with sequencing. The obtained clone was further sub-cloned into expression vector pBI121-GUS containing 35s promoter from cauliflower mosaic virus (*35SCaMV:cpsf*) to generate sense and anti-sense expression.

The vector pUC19 containing Ampicillin selection has facilities of blue and white selection. The blue colonies were selected and PCR detection was performed. The clone was further verified with restriction digestion and was cloned into expression vector pBI121 containing 35S promoter from cauliflower mosaic virus. Sense and anti-sense constructs were generated to detect the over expression effects.

Agrobacterium mediated transformation was carried out to transform sense or antisense genes into wild type *Arabidopsis* (Col-0) by dipping method. Kanamycin resistant transformants were selected in 1/2MS plates supplied with 1% (w/v) containing Kanamycin (50 mg/L). The plants were transferred into soil and harvested separately. Segregation ratios were obtained and homozygous and heterozygous lines were found out.

2.10 *Agrobacterium*-mediated transformation into plants

Transformation was carried out using *Agrobacterium tumefaciens* (GV3101) by dipping method. Infiltration media was prepared using 1/2 MS supplied with 1xGamborg's vitamin and benzylaminopurine (BAP) 10 µL/L of 1 mg/mL stock maintaining OD_{600} 0.5. Firstly emerged inflorescence was removed few days prior to transformation in order to generate sufficient floral stalks in the plant. The floral buds are then dipped inverting them into the infiltration medium for 15 minutes. After transformation the plants were wrapped for 2 days. Until wilting, plants kept in dry condition without watering approximately for 1 week.

The transformation from each pot were separately harvested and sown in 1/2MS1S supplied with selective antibiotic referring as T0. Thus germinated T1 plants were individually harvested to get T1 seeds. Segregation ratios of transformants were determined and the homozygous lines were separated. While, the cDNA transformant (sense) showed no homozygous line survival all indicating heterozygous whereas antisense were survived to obtain the homozygous transformats.

2.11 Some Primers used

Primers for sequencing and sense and anti-sense analysis and analysis of the cloning confirmation had been done with using following primers (Table -1)

Table 1: List of Primers.

Primer Name	Sequence (5' →3')
Sequencing Primers	
CPIF1	cct tct gaa gac gga ggg aat ggt agc tc
CPIR1	gca cat gct cca aat agt acc aga tat tgc ttg
CPIF2	tgc aga aat ggc aga tgg aac ggt ttt c
CPIF3	gct gcg att cct tct gct atg gag caa gg
CPIF4	gct gtg tag acc cgc tca gtg ggt cag tg
CPIR4	cca gtc tct cca ggt ttg agc ttg tag gatg
CPIF5	gca atc ctg tct tgc aaa gat cat tca ga
Fragment Construction	
CPSFF2	gct cta gag caa gaa gga aaa gca cga cct ttg
CPSFR2	gct cta gag cca act ctt tgc atc cta tac gc
CPSFF3	gcc atg gtt gtt aca aac agc tcg aca aag c
CPSFR3	acc atg gcc tgt cac taa gag cta tga tgt ctg
RT-PCR	
AT2UF	ctt ggc tgc gtt gat caa ttc atc agc
AT2UR	gca gat gtt gac gct gag att cag cag g
AT1UF	gac agt gct tca gct tca tcc gaa gca c
AT1UR	gct gtt aca ttg gag caa acc gcg aga g
AT0TF	gtgatccagcgcagaggttagttgctg
AT0TR	tgatgtgcttggtgatggctgaggtgtc
AT1DF	cgt att cga ctc tct cct cgt cac caa c
AT1DR	ggc gtt cat gct cag ctt ggc tga tat tg
Genotyping primers	
ATNF	gcc gac ttc ctt ctg tta atc atg gc
ATNR	tgt tca cac acc gat tca aca atc c

3 Results

3.1 Activation tagged mutant screening

Activation tagging is breakthrough technology for studying gene function. Activation tagged by vector pSK015 (Figure 3) T0 plants from Columbia-0 background was grown in BASTA for selecting resistant lines. Among more than 5,000 resistant T1 plants were screened in active antagonist to brassinosteroid, brassinazole (3µM). Seven days grown hypocotyls under dark in the medium were measured. The plants showing long hypocotyls (>1.5 times to wild type) in brassinazole were selected and observed for their morphology. Their genomic DNA was isolated. Plants having significant morphological differences and growth impairments were selected and screened by thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) to confirm the T-DNA insertion. It was found that a T-DNA was inserted at the first exon of Cleavage and polyadenylation specificity factor (*CPSF*) gene at At3g11960. After T-DNA insertion confirmation genotyping with T-border and gene specific primers was used to determining orientation. From the screening a plant showing significant morphological and growth impairment with having single T-DNA insertion was selected for the detail study. T-DNA was found inserted in upstream orientation.

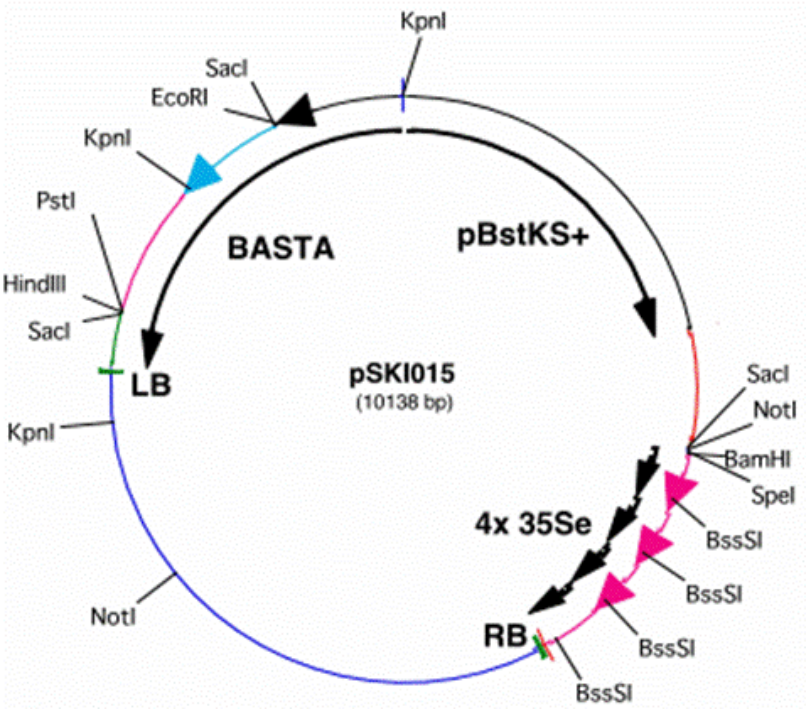


Figure 3: Activation tagging pSK015 vector system. The vector contains 4 enhancers from CaMV35S harboring BASTA resistant gene (Weigel *et al.*, 2000)

3.2 Plant Morphology

cpsf mutant delays about a week to 10 days in flowering than its wild type counterpart. Petioles were shorter while lamina was broader than in mutants. Mutants possess dark green coloration (Figure 4). Number of rosette leaves is higher (1-2 in number) in mutant than wild type. In mutants, stalk was broader and retains hard during maturity turning into violet. Flower appears in succession owing to have different stages of maturity. Hypothalamus was comparatively swollen. Flower interval in the inflorescence was larger. Basal silique matures earlier and dehisces than the apical one while some of the basal silique were empty or and missed seeded. However impairment in silique structure and seedless silique frequently observed at the basal part than the apical region of the inflorescence. Branches are also reduced. Under brassinazole screening, some of the hypocotyls showed nearly double length to wild type however some showed shorter. Under inhibitor treatment on plates, germination was severely affected. Hypocotyls length variation was observed most of the in high frequency. Length was generally irregular. Screening and separation into different categories according to their hypocotyls length, short hypocotyled plants could also show long hypocotyls in next generation. It was noticed that the T-DNA inserted plant showed severe germination defects. Non germinated seeds were observed in control condition (1/2MS.1S) as well as in hormone and inhibitors treatment. In Brz medium all seedlings showed closed cotyledons whether tall or short. Some were also coiled.

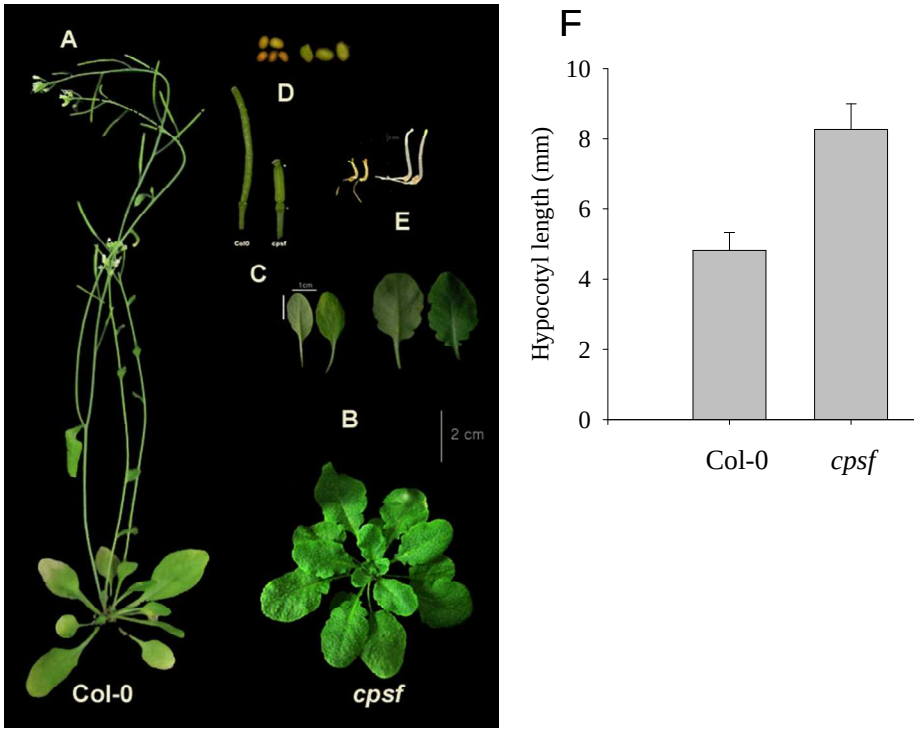


Figure 4: A detail comparative morphology of *cpsf* mutant and wild type Col-0. Leaves, sliliques, seeds and hypocotyls were compared .

Leaves were large, late growing, long hypocotyls Brz medium, irregular silique length and larger seeds were seen in the mutant. Bar graph indicates the hypocotyls length. A. WT, B: *cpsf*; C: Leaves; D: Seeds; E: 7 days grown hypocotyls in dark (3 µM); F: Comparative hypocotyls length of 7 days dark grown seedlings in 3 µM Brz medium.

3.3 T-DNA insertion line selection and its orientation

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) was performed to identify the position of T-DNA insertion in the *arabidopsis* genome. It was found that a T-DNA was inserted at the first exon of At3g11960 encodes cleavage and polyadenylation specificity factor (CPSF) A subunit C-terminal domain-containing protein; similar to DDB1A (UV-damaged DNA-binding protein 1A) [*Arabidopsis thaliana*] (TAIR:AT4G05420); similar to unnamed protein product [*Vitis vinifera*] (GB:CAO23456.1); similar to putative Splicing factor 3B subunit 3 [*Oryza sativa* (japonica cultivar-group)] (GB:BAC79787.1); similar to hypothetical protein [Vitis vinifera] (GB:CAN78747.1); contains InterPro domain Cleavage and polyadenylation specificity factor, A subunit, C-terminal; (InterPro:IPR004871) (www.arabidopsis.org). Orientation of the T-DNA enhancer was confirmed by genotyping assessing its T-borders and the gene specific primers (Figure 5 a and b). Genotyping revealed that the T-DNA right border lie upstream and left border to the downstream facing the enhancers toward upstream direction. Genotyping revealed that enhancers directs towards upstream from the gene of T-DNA insertion. It was found that T-DNA caused knockdown of the gene *cpsf*. Assessment of the neighboring gene indicated that the T-DNA insertion caused no alteration of the neighboring three genes (two upstream and one down stream of the T-DNA insertion gene) expression lying on either side of the T-DNA inserted gene (Figure 6). Therefore, T-DNA insertion, its direction and neighboring genes have been illustrated in figure 6.

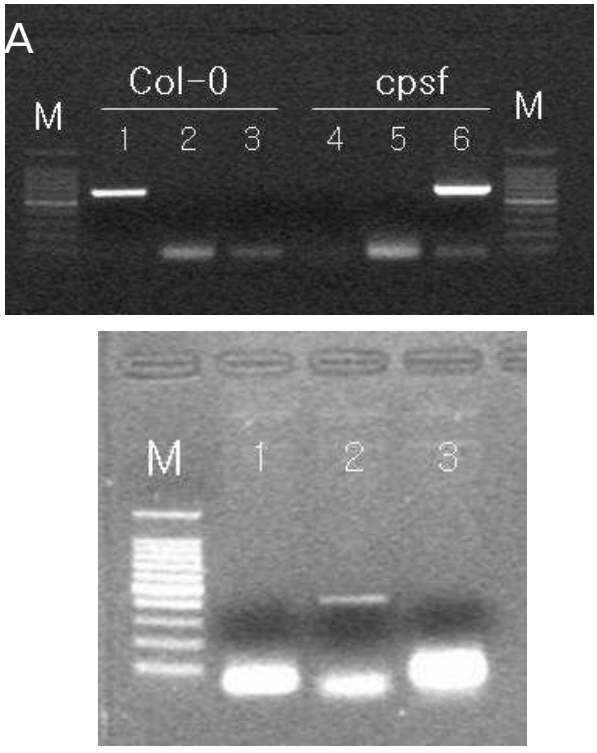


Figure 5: Confirmation of T-DNA insertion borders (right and left borders) and its orientation in activation tagged *Arabidopsis* genome.

A: Left and right T-borders of T-DNA were explored by genotyping. M:100 bp marker; lane 1 and 4 ATNF+ATNR, 2 and 5: ATNF+RB3; 3 and 6: ATNR+LB50. B; Confirmation of right border. 1, ATNF+RB2, 2, RB4 and 3, RB5. Right border was detected by the ATNF+RB4 primers only. M: Marker. Left border primer LB50 with gene specific reverse primer (ANTR) showed the PCR amplification bands in T-DNA insertion mutant while gene specific primers only showed bands in wild type only. ATNF and ATNR (gene specific) and LB50 (left border) and RB (right border) vector primers were used for detection..

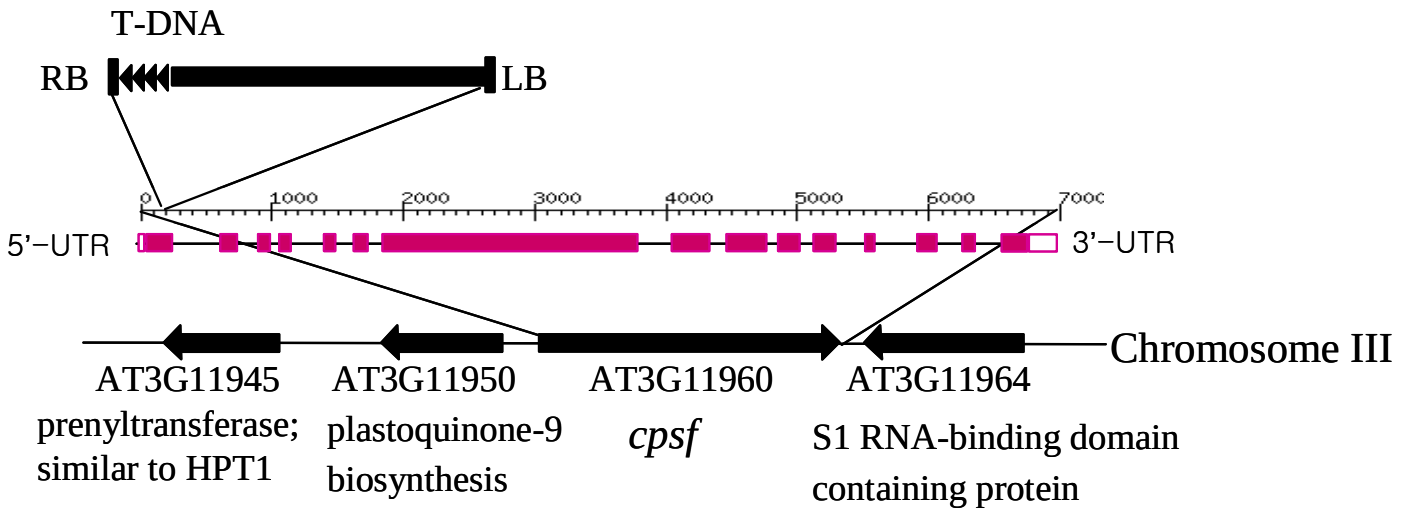


Figure 6: T-DNA insertion and orientation of neighboring genes in chromosome III of *Arabidopsis* genome.

At3g11960 encodes cleavage and polyadenylation specificity factor (CPSF) A subunit C-terminal domain-containing protein; similar to DDB1A (UV-damaged DNA-binding protein 1A) [*Arabidopsis thaliana*] (TAIR:AT4G05420); similar to unnamed protein product [*Vitis vinifera*] (GB:CAO23456.1); similar to putative Splicing factor 3B subunit 3 [*Oryza sativa* (japonica cultivar-group)] (GB:BAC79787.1); similar to hypothetical protein [Vitis vinifera] (GB:CAN78747.1); contains InterPro domain Cleavage and polyadenylation specificity factor, A subunit, C-terminal; (InterPro:IPR004871). T-DNA is inserted at first exon of the gene *At3g11960*.

3.4 Gene Expression analysis

Expression level of the gene of T-DNA insertion and its neighboring genes were carried out. Real Time RT-PCR revealed that neighboring gene expression was not altered due to the insertion of the T-DNA. The expression of gene of T-DNA insertion (*cpsf*) was reduced by 10 folds with compare to wild type (Figure 7) revealing the knockdown of the gene due to T-DNA insertion. In 20 days old seedlings, Real time RT-PCR indicated that *cpsf* gene expression had been reduced by 10 fold in the mutant than wild type. The neighboring gene At3g11950 (first upstream gene from T-DNA insertion) showed nearly 4 fold increase in its expression than wild type while At3g11945, second upstream gene to T-DNA showed no change in its expression. Similarly, At3g11964, first downstream gene to T-DNA insertion showed its expression level same as wild type (Figure 7).

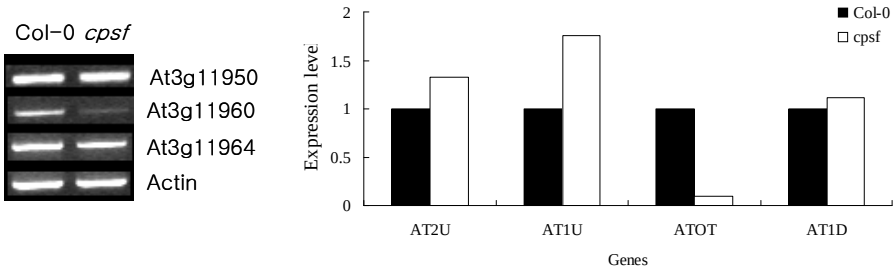


Figure 7: Neighboring genes expression due to insertion of T-DNA.

T-DNA-insertion caused knockdown of the gene without altering expression level of the neighboring genes. AT: Activation Tagged U: Upstream gene and D: Downstream gene from the site of T-DNA insertion. AT2U: *At3g1145*; AT1U: *At3g11950*, AT0T: *At3g11960* (Activation tagged gene) and AT1D: *At3g11964*.

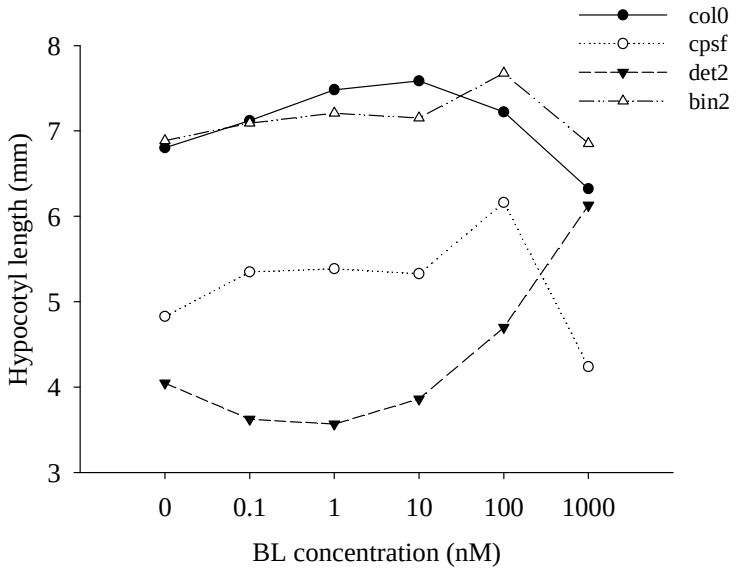
3.5 Hormone dose response and hormonal crosstalk

3.5.1 Hormone dose response

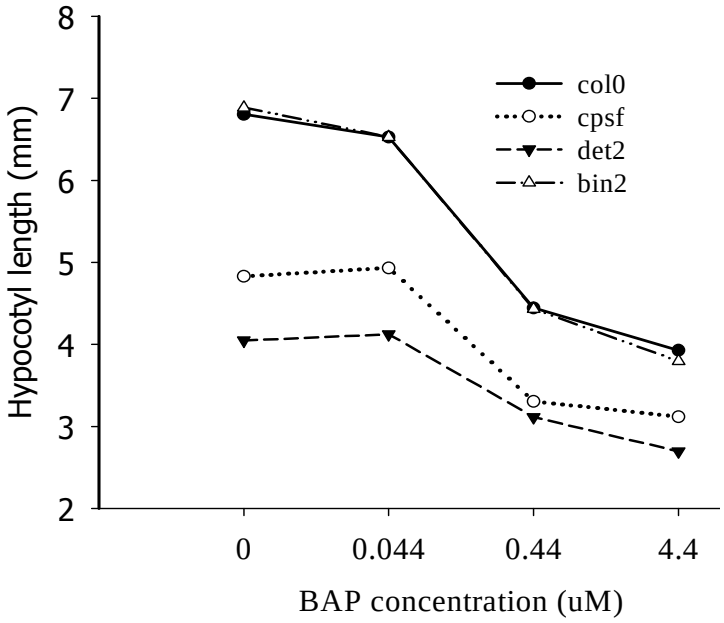
Effects on hypocotyls length during germination under supplements of different phytohormones is one of the best ways in assessing involvement of the gene to the particular hormone biosynthesis or perception pattern. Treatment of the phytohormones like auxin, cytokinin, abscisic acids, brassinosteroid and their co-treatment may also indicate the hormonal cross talk of the particular gene. In this concern, we have tried to explore the cross talk of the gene between BRs with auxin, cytokinin or abscisic acids. Seven days grown seedlings in different hormone concentration under dark were used for analyzing whether the gene involve in particular hormone perception/signaling.

From the active BRs inhibitor, brassinazole treatment it revealed that *cpsf* gene related to BRs perception cascade which is explored for the first time. In this concern we also tried to explore whether the gene functions with other hormone relation. Treatment in auxin, gibberellin, cytokinin, brassinolide indicated no remarkable perception of the gene with the treated hormone. Treatment of BL also couldn't rescue the plant indicating the *cpsf* has defects in BRs perception (Figure 8).

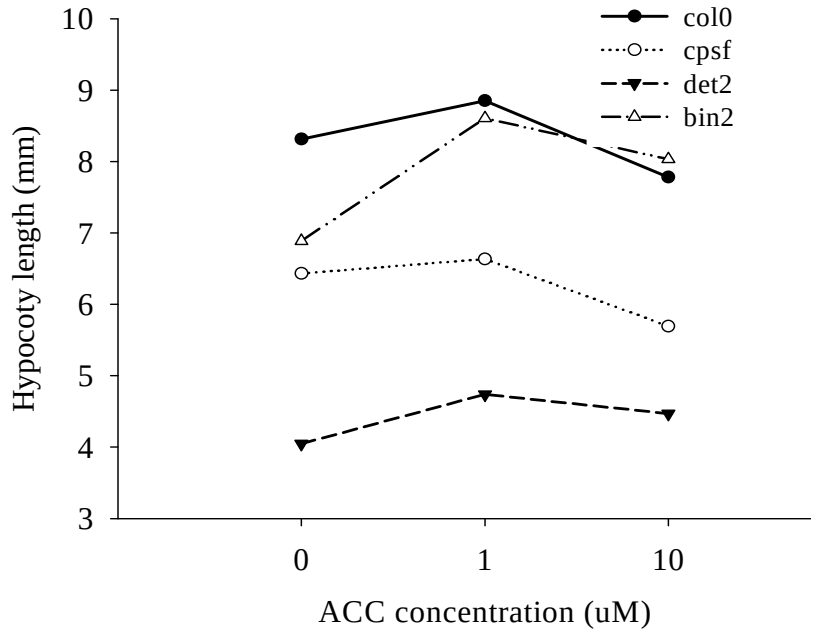
A


col0
cpsf
det2
bin2
Hypocotyl length (mm)
8
7
6
5
4
3
0
0.1
1
10
100
1000
BL concentration (nM)

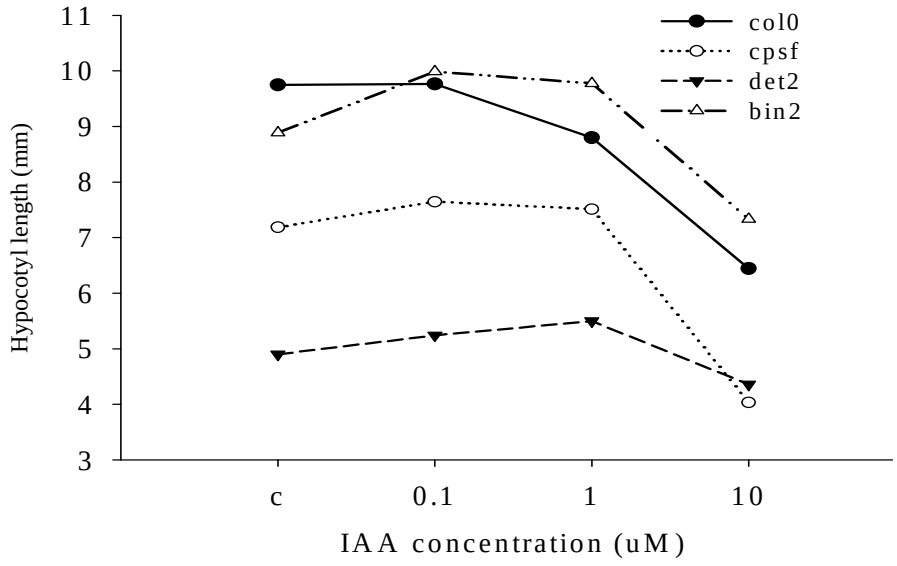
B


col0
cpsf
det2
bin2
Hypocotyl length (mm)
8
7
6
5
4
3
2
0
0.044
0.44
4.4
BAP concentration (uM)

C


col0
cpsf
det2
bin2
Hypocoty length (mm)
10
9
8
7
6
5
4
3
0
1
10
ACC concentration (uM)

D


col0
cpsf
det2
bin2
Hypocotyl length (mm)
11
10
9
8
7
6
5
4
3
c
0.1
1
10
IAA concentration (uM)

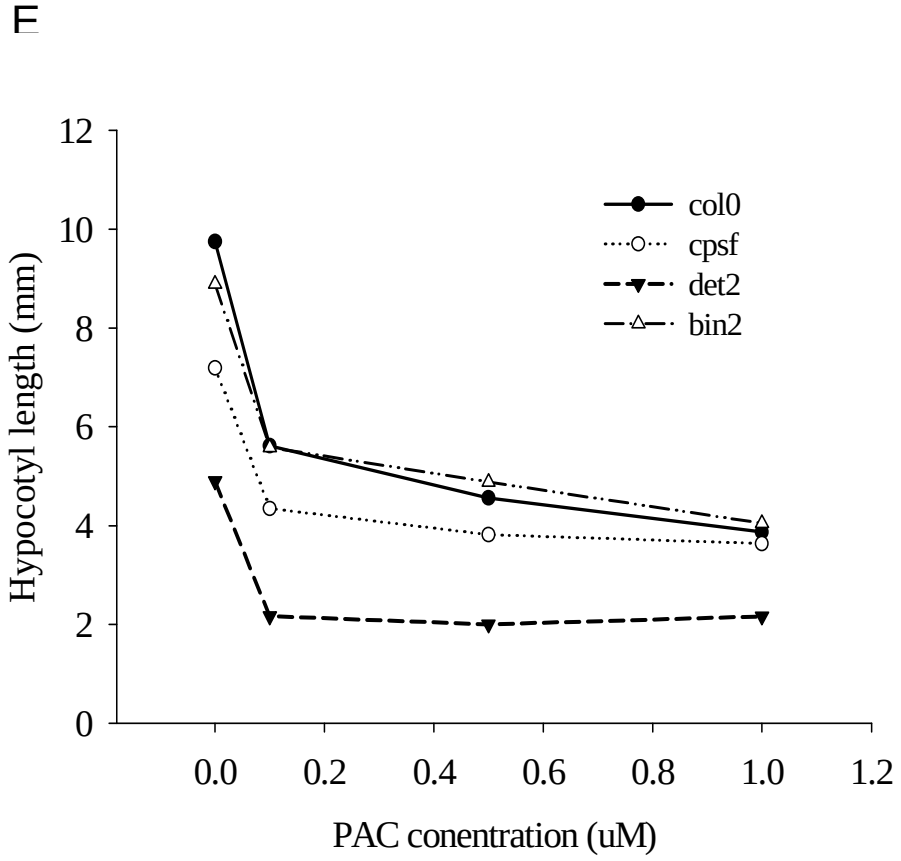


Figure 8: Dose responses of different phytohormones on hypocotyls length of the mutant *cpsf* and other BRs-related mutants in dark.

A. BL: Brassinolide; B: BAP; C: ACC; D: ICC and E: PAC treatment in dose dependent manner. It was found that BL treatment could rescue hypocotyls in *det2* mutants while *cpsf* mutant has shown similar pattern of response with wild type

3.5.2 Hormone cross talk

Mutant and its crossed lines showed no major relationship with the other plant hormones except brassinosteroid. Relationships of the gene with other phytohormones were tested. It has no such a close affinities with other phytohormones could be seen (Figure 8).

3.6 Crossing with brassinosteroid related mutants

Different BRs defective mutants (Table 2) had been used for crossing experiments between *cpsf* mutant to see its response with other BRs related genes.

Table 2: Other brassinosteroid mutants and their properties

Plant Line	Phenotye	Properties
Col-0	wild type	Normal growth
BIN2	Brassinosteroid insensitive mutant	Unresponsive to exogenous BL / negative regulator
det2	De-etiolated2	Rescued by exogenous BL
bes1D	bri1-EMS suppressor Dominant	Suppressor of bri1

BIN2 (Brassinosteroid Insensitive 2) is a semi-dwarf mutant that is unresponsive to the exogenous application of BL. It is negative regulator of the BR signaling. It evidences the crossed lines with the different hormone dose treatment respond to up or downstream signaling component in the BRs signaling cascade. While *det2* a BR related biosynthetic dwarf mutant is rescued by exogenous BL. *bes1D* is a *bri1*- ethyl methanesulfonate suppressor dominant rescues bri1 type mutation.

Wild type Col-0 plant showed average height of 35 cm flowering begins around 35 ±3 days while *cpsf* mutant had shown sprouting of the first flower in 45±3 days. *bes1D* mutant possesses highly epinastic curling leaves and short but broad silique (Figure 8a and b). Similarly, *bin2* heterozygous mutant showed semi dwarf structure possessing siliques similar to wild type Col-0. *det2* showed rounded leaves with dwarf phenotype with short siliques while the mutant *cpsf* possessed rounded leaves with short, curved as well as long siliques (Figure 4).

The results obtained from the crossed between the *cpsf* mutant with other brassinosteroid related mutants indicated that *cpsf* rescued the dwarf or semi-dwarf stature of the plants. However, the mutation caused the fruiting impairments in all crossed lines (Figure 9 and 11). Double mutant between *det2* and *cpsf* showed tall phenotype identical with *cpsf* mutant. The double mutant showed leaves, height and inflorescence similar to the *cpsf*. It was found that flowering period also showed similar with *cpsf*.

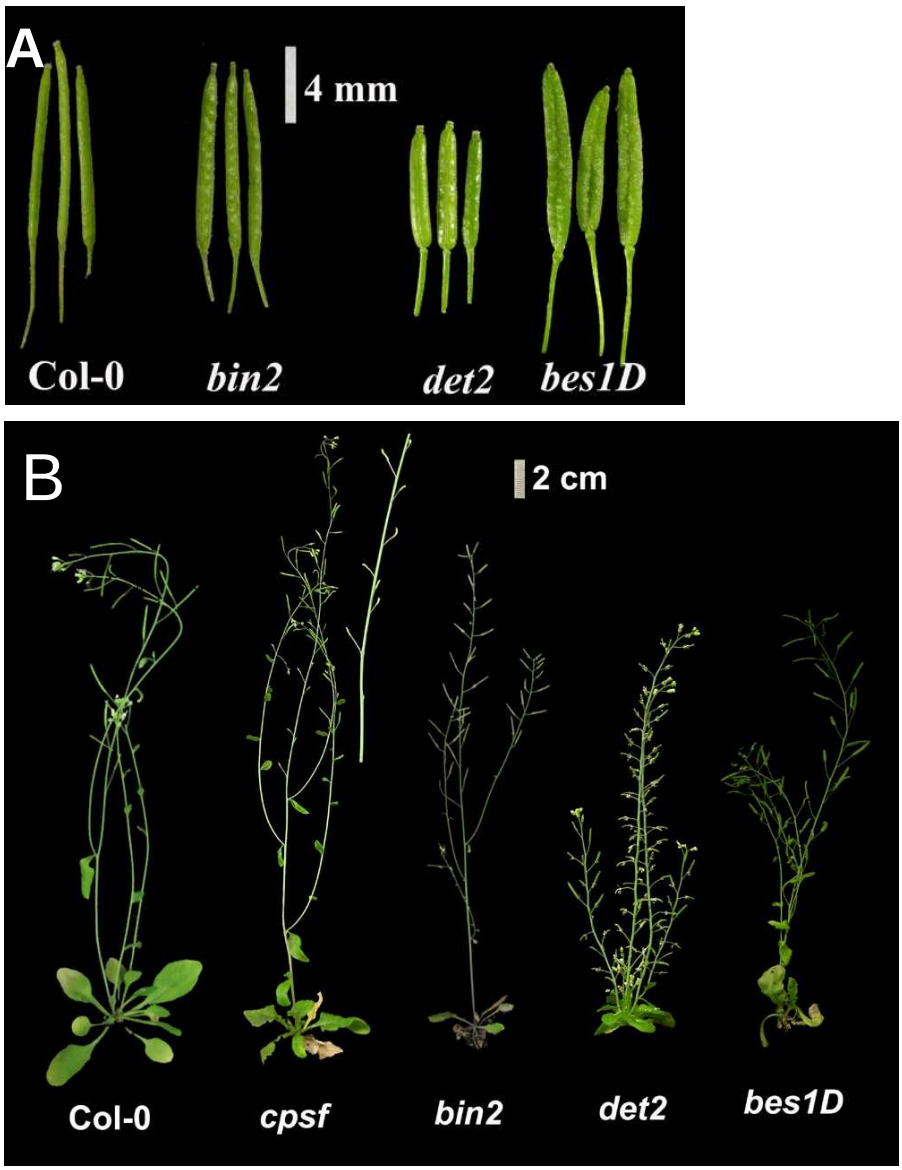


Figure 9: Morphology of wild type and BR related mutants with their silique morphology. A. silique morphology of wild type and other BRs related mutants. B. Phenotype of wild type and BR related mutants

3.6.1 Morphology of crossed lines

F1 crossed plants between all BRs defective mutants and wild type showed many common characters including dark green color, large leaves, late flowering, and elongated internodal region. Siliques are short; some are curved, missing fruits (seeds), and bulged hypothalamus. Silique contained few but larger seeds. F1 plants between *det2cpsf* showed phenotypic identical structures with mutant *cpsf*. *bes-1*D lost its curvedness and *det2* lost its dwarfness. *bin2* (heterozygous in original line) also showed phenotypic identical structures to all F1 plants in crossed lines having functional flowers and fruits. In aggregate, all crossed lines displayed tall phenotypes with large leaves losing their original phenotype. They all lost their original phenotype revealing *cpsf* develops dominant mutation in *Arabidopsis*.

For first few weeks until the cauline leaves development, crossed plant possessed wild type phenotype. Morphology was gradually changed after emergence of inflorescence. *det2cpsf* double mutant rescued the dwarf phenotype of *det2* mutant in height, petiole stature and silique sizes. The double mutant line showed phenotypic similarities with *cpsf* in structure, silique and inflorescence characters (Figure 11).

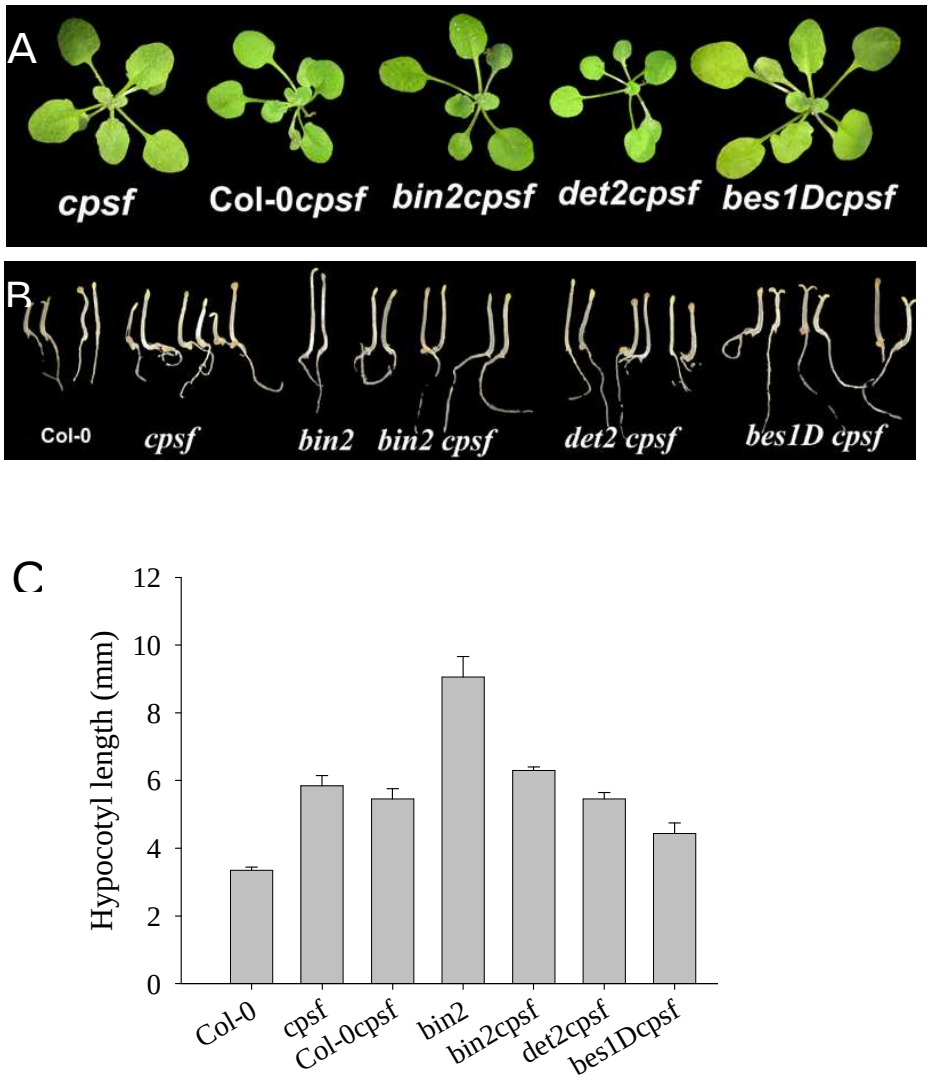


Figure 10: Hypocotyls length and morphology of crossed plant lines

A. 19 days old seedlings on soil. B. Brz grown seeds, C. Comparison of 7 days dark grown seedlings of different lines Brz medium.

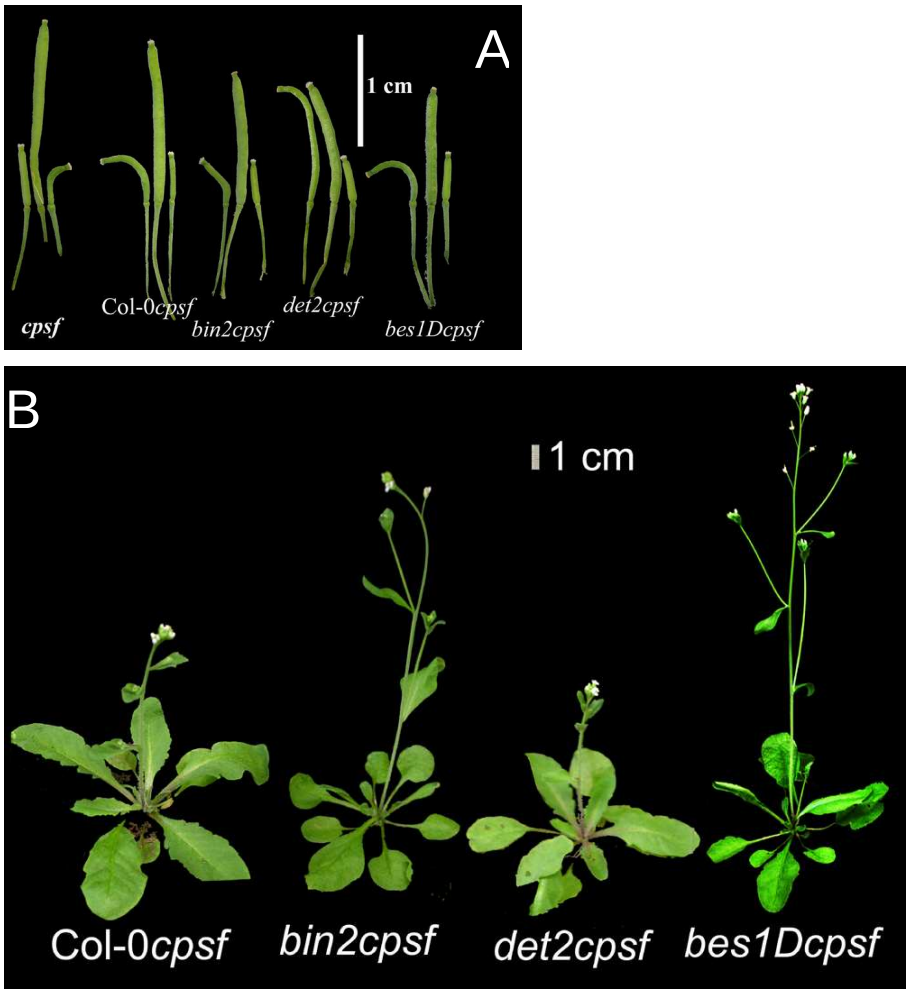


Figure 11: Morphology of crossed lines with their siliques structures. A. Siliques B: Morphology of crossed plant lines. Siliques *cpsf,* Col-0*cpsf, bin2cpsf, det2cpsf* and *bes1Dcpsf* (from left to right order)

3.7 Germination impairments in mutant and its crossed lines

The mutant possesses fewer seeds growing in successive maturities. The seeds shows germination impairments when they are grown in any medium. While BAP (0.44 µM) promoted the germination of the seeds. Hypocotyls grown in 1/2MS with 1% (w/v) sugar containing Brz (3 µM) also showed variation in length ranging from shorter to almost 2 fold taller to wild type. However, Brz also inhibited germination of the *cpsf* seeds in the ½ MS 1S medium.

In basta, all crossed F1 seeds had shown 100% germination. Later in F2, it showed variable ratio of survival showing critical germination and resistance irregularities. Germination impairment was a severe impairment observed in this experiment (Table 3).

Table 3: Segregation test results between crossed lines with *cpsf* mutant

Crossed line	BASTA resistant	Dead	NG	Ratio of resistant with		
				Dead	NG	Dead: NG
Col-0						
F1	35	0	0	-	-	-
F2	60	140	117	1:2.3	1:1.95	1:4.3
bin2						
F1	25	0	0	-		-
F2	137	240	76	1:1.75	2.08:1	1:2.3
det2						
F1	28	0	0	-	-	-
F2	100	167	16	1:1.67	6.25:1	1:1.83
bes1D						
F1	15	0	0	-	-	-
F2	90	173	30	1:1.92	3:1	1:2.25

NG: Not germinated

3.8 *cpsf* sense and antisense expression Construct

Full length genomic sequence of the gene constitutes 6.99 kb having cDNA flanking length of 4.4 kb attributing into 15 exons and 14 introns with short 5-prime untranslated region (5'-UTR) and longer 3-prime untranslated regions (3'UTR). Detail of the cDNA and its amino acid translation has been shown in figure 12. cDNA construct had included untranslated regions along with ORF flanking regions. Both of the termini of the construct having XbaI restriction sites was ligated randomly in either direction in the vector. Sense and antisense orientation of the designed construct is given in Figure 13.

```
  1 AAAGAAGGAA AAGCACGACC TTTGCCGACT TCCTTCTGTT AATCATGGCG
                                                M  A     Frame 3
 51 GCTCCGGAGG ATGAATCTTC CGCTCAGTCT CAATCATCAC CTGCCACGGC
    A  P  E  D   E  S  S   A  Q  S   Q  S  S  P   A  T  A   Frame 3
101 GGCTCCTACG CCTCCTCCTT CCTCTTCCCC TTCCTCTGCT GGGGATCACT
     A  P  T   P  P  P  S   S  S  P   S  S  A   G  D  H  Y   Frame 3
151 ACCTAGCTAA GTGCATCCTC CGCCCCAGCG TCGTTCTCCA GGTTGCTTAT
      L  A  K   C  I  L   R  P  S  V   V  L  Q   V  A  Y    Frame 3
201 GGCTACTTCC GCTCCCCTTC CTCCCGCGAC ATAGTTTTCG GCAAGGAGAC
    G  Y  F  R   S  P  S   S  R  D   I  V  F  G   K  E  T   Frame 3
251 CTGCATAGAA TTGGTGGTTA TTGGTGAAGA CGGGATTGTT GAATCGGTGT
     C  I  E   L  V  V  I   G  E  D   G  I  V   E  S  V  C   Frame 3
301 GTGAACAGTA TGTATTTGGA ACAATTAAGG ATTTGGCTGT CATACCTCAG
      E  Q  Y   V  F  G   T  I  K  D   L  A  V   I  P  Q    Frame 3
351 AGTAGCAAGC TTTATTCGAA TAGTTTGCAG ATGGGAAAAG ACCTTCTGGC
    S  S  K  L   Y  S  N   S  L  Q   M  G  K  D   L  L  A   Frame 3
401 TGTTCTTTCT GATTCTGGAA AGCTCTCATT TCTCTCATTT AGCAATGAAA
     V  L  S   D  S  G  K   L  S  F   L  S  F   S  N  E  M   Frame 3
451 TGCACAGGTT CTCGCCAATA CAACATGTTC AACTTTCTAC TCCAGGTAAC
      H  R  F   S  P  I   Q  H  V  Q   L  S  T   P  G  N    Frame 3
501 TCAAGGATTC AACTCGGAAG AATGCTCACA ATAGATTCCA GTGGTCTCTT
    S  R  I  Q   L  G  R   M  L  T   I  D  S  S   G  L  F   Frame 3
551 TCTTGCTGTC AGTGCGTATC ATGACCGTTT TGCTCTGTTT TCCCTCTCAA
     L  A  V   S  A  Y  H   D  R  F   A  L  F   S  L  S  T   Frame 3
601 CATCGTCTAT GGGTGATATT ATTCATCAGA GGATTTCTTA TCCTTCTGAA
      S  S  M   G  D  I   I  H  Q  R   I  S  Y   P  S  E    Frame 3
651 GACGGAGGGA ATGGTAGCTC TATTCAAGCA ATATCTGGTA CTATTTGGAG
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     M  C  F   I  S  K  D   F  N  E   S  K  E   Y  A  P  I   Frame 3

751 TTCTTGCCAT TGTTATAAAT AGGAAAGGGT CCCTCATGAA TGAGCTGGCT

      L  A  I   V  I  N   R  K  G  S   L  M  N   E  L  A     Frame 3

801 TTGTTTAGAT GGAATGTCAA AGAGGAATCC ATATGTTTAA TATCAGAGTA

    L  F  R  W   N  V  K   E  E  S   I  C  L  I   S  E  Y    Frame 3

851 TGTTGAAACT GGGGCTCTGG CACATAGTAT TGTCGAAGTT CCTCACTCCT

     V  E  T   G  A  L  A   H  S  I   V  E  V   P  H  S  S   Frame 3

901 CTGGATTTGC CTTTCTGTTT AGGATTGGTG ATGTTCTCTT AATGGATCTA

      G  F  A   F  L  F   R  I  G  D   V  L  L   M  D  L     Frame 3

951 AGAGATCCTC AAAACCCTTG CTGTCTGTTT AGGACATCCT TAGATTTTGT

    R  D  P  Q   N  P  C   C  L  F   R  T  S  L   D  F  V    Frame 3

1001 TCCTGCTTCA TTAATGGAAG AGCATTTTGT TGAAGAGTCG TGTAGAGTAC

     P  A  S   L  M  E  E   H  F  V   E  E  S   C  R  V  Q   Frame 3

1051 AAGATGGAGA CGATGAGGGC TGTAATGTTG TTGTATGTGC TTTGTTAGAG

      D  G  D   D  E  G   C  N  V  V   V  C  A   L  L  E     Frame 3

1101 CTCAGGGATC ATGAGGTCAG GGATCATGAT CCCATGTTCA TAGATACTGA

    L  R  D  H   E  V  R   D  H  D   P  M  F  I   D  T  E    Frame 3

1151 AAGTGACATT GGAAAGTTGA GTTCTAAGAA CGTCTCTTCA TGGACTTGGG

     S  D  I   G  K  L  S   S  K  N   V  S  S   W  T  W  E   Frame 3

1201 AGCCGGAAAA TAATCATAAT CCTCGGATGA TTATTTGCTT AGATAATGGT

      P  E  N   N  H  N   P  R  M  I   I  C  L   D  N  G     Frame 3

1251 GATTTTTTCA TGTTTGAACT CATATATGAA GATGATGGGG TTAAAGTAAA

    D  F  F  M   F  E  L   I  Y  E   D  D  G  V   K  V  N    Frame 3

1301 TCTATCAGAG TGTTTATACA AAGGTTTACC ATGCAAGGAT ATTTTATGGA

     L  S  E   C  L  Y  K   G  L  P   C  K  D   I  L  W  I   Frame 3

1351 TCGAAGGTGG ATTCCTTGCT ACATTTGCAG AAATGGCAGA TGGAACGGTT

      E  G  G   F  L  A   T  F  A  E   M  A  D   G  T  V     Frame 3
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       Q  I  F   A  C  C   G  V  T  P   E  G  S   L  R  I    Frame 3
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     I  R  S  G   I  N  V   E  K  L   L  K  T  A   P  V  Y   Frame 3
1601 TCAAGGAATA ACTGGTACTT GGACTGTTAA AATGAAGCTT ACTGATGTAT
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     V  V  G  K   K  D  N   L  P  V   N  L  L  L   I  A  T   Frame 3
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      R  R  I   G  I  T  P   V  F  L   V  P  F   S  D  S  L  Frame 3
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2851 CGTCTGATAT ATGCTGTGTA GACCCGCTCA GTGGGTCAGT GTTATCATCC

       S  D  I   C  C  V   D  P  L  S   G  S  V   L  S  S    Frame 3

2901 TACAAGCTCA AACCTGGAGA GACTGGAAAG TCAATGGAGC TTGTACGTGT

     Y  K  L  K   P  G  E   T  G  K   S  M  E  L   V  R  V   Frame 3

2951 GGGAAATGAA CATGTCCTTG TGGTTGGGAC GAGTTTGTCT TCTGGTCCTG

      G  N  E   H  V  L  V   V  G  T   S  L  S   S  G  P  A   Frame 3

3001 CTATACTACC CAGTGGTGAA GCTGAAAGTA CAAAAGGACG AGTAATCATC

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3101 TTCAAAGGCT TGTTCGTCTT CCCAACGTAC ATCACCTTTC CATGATGTTG

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3251 GTTAAGATTG GCCTCTTCAA CCACTTGGCC GGGTATGGTA CTTGCAATCT

      L  R  L   A  S  S  T   T  W  P   G  M  V   L  A  I  C   Frame 3

3301 GTCCATACCT TGATCATTAC TTCCTGGCAT CTGCCGGCAA TGCTTTCTAT

       P  Y  L   D  H  Y   F  L  A  S   A  G  N   A  F  Y    Frame 3

3351 GTCTGTGGTT TTCCGAACGA TAGTCCTGAG AGAATGAAGA GGTTTGCGGT

     V  C  G  F   P  N  D   S  P  E   R  M  K  R   F  A  V   Frame 3

3401 AGGAAGGACC CGTTTCATGA TAACATCTCT GCGCACATAC TTCACTAGAA

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3451 TTGTTGTTGG GGATTGCCGT GATGGTGTTC TATTTTATTC TTATCACGAG
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      V  V  G   D  C  R   D  G  V  L   F  Y  S   Y  H  E     Frame 3

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      A  D  C   F  L  M  D   A  N  S   V  A  V   S  D  R  K   Frame 3

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      G  S  I   A  I  L   S  C  K  D   H  S  D   F  G  M     Frame 3

3651 AAGCACCTTG AATATTCAAG TCCGGAGTCC AACCTGAATC TAAACTGTGC

     K  H  L  E   Y  S  S   P  E  S   N  L  N  L   N  C  A   Frame 3

3701 TTATTACATG GGAGAGATTG CCATGTCTAT AAAAAAGGGT TGCAACATCT

      Y  Y  M   G  E  I  A   M  S  I   K  K  G   C  N  I  Y   Frame 3

3751 ACAAACTTCC AGCTGATGAT GTACTGCGAA GTTATGGTCT TAGTAAAAGT

      K  L  P   A  D  D   V  L  R  S   Y  G  L   S  K  S     Frame 3

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     I  D  T  A   D  D  T   I  I  A   G  T  L  L   G  S  I   Frame 3

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      Q  A  K   L  G  I   H  P  L  T   A  P  V   L  G  N     Frame 3

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      L  D  G   D  M  L  A   Q  F  L   E  L  T   N  R  Q  Q   Frame 3

4051 AGGAGTCAGT TTTATCGACA CCTCAGCCAT CACCAAGCAC ATCAAAAGCA

      E  S  V   L  S  T   P  Q  P  S   P  S  T   S  K  A     Frame 3

4101 AGTTCAAAGC AACGTTCTTT CCCGCCTCTC ATGCTACACC AAGTTGTACA

     S  S  K  Q   R  S  F   P  P  L   M  L  H  Q   V  V  Q   Frame 3

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     L  L  E   R  V  H  Y   A  L  H   STOP                  Frame 3

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4251 AACGAATTCA CTAATTTGAT CTGGTGCGTA ACTGAGTCCA AAGTAGTTTT

4301 AGTTGGAAAA TGTATATGGA ATTTATTAAA TAACTGGTAG TGAATATTGG

4351 ATGTTTACAA GCTTATTGAC ACTATTGCGT ATAGGATGCA AAGAGTTGG
```

Figure 12: Full-length cDNA and its amino acids translations of *At3g11960*.

(Note: Start ATG; stop CTA codons and untranslated regions are highlighted with changed font color.)

Segregation in T2 generation of cDNA transformants indicated that the over expression lines showed 2.5:1:1.7 (survived:dead:not germinated). Nearly half of seed of over expression line to the survived seedling were not germinated in the selection medium. Not germinated seeds proportion remained nearly double with the number of dead seedlings. The results indicated that the proportion of resistant seedlings to the sum of dead and not germinated nearly remain equal (1:1.1). In antisense transformants, all seeds were germinated. The proportion of the resistant seeds to susceptible was obtained as 3:1 (survived:dead). It indicated that sense expression lines were heterozygous.

T3 generation of the sense expression line also showed similar ratio to the T2 generation. Proportion of resistant and dead and dead and not germinated were found as T2. While in T3 antisense, all resistant seedlings could be obtained indicating homozygocity. Homozygous line could be achieved in antisense transformants whereas, all sense transformants were heterozygous. Sense expression lines of the gene were all obtained as heterozygous. Homozygous line could be obtained in antisense transformants only. The characteristic germination impairment was also observed in the sense lines.

CaMV35S:*cpsf* cDNA over expression construct revealed most of the phenotypic characters similar to wild type Col-0. While it showed characteristic differences in siliqua structure with wild type and showed it similarities with the mutant *cpsf* (Figure 14). Flowering time had been slightly delayed in sense transformants while antisense flower time was similar to wild type.

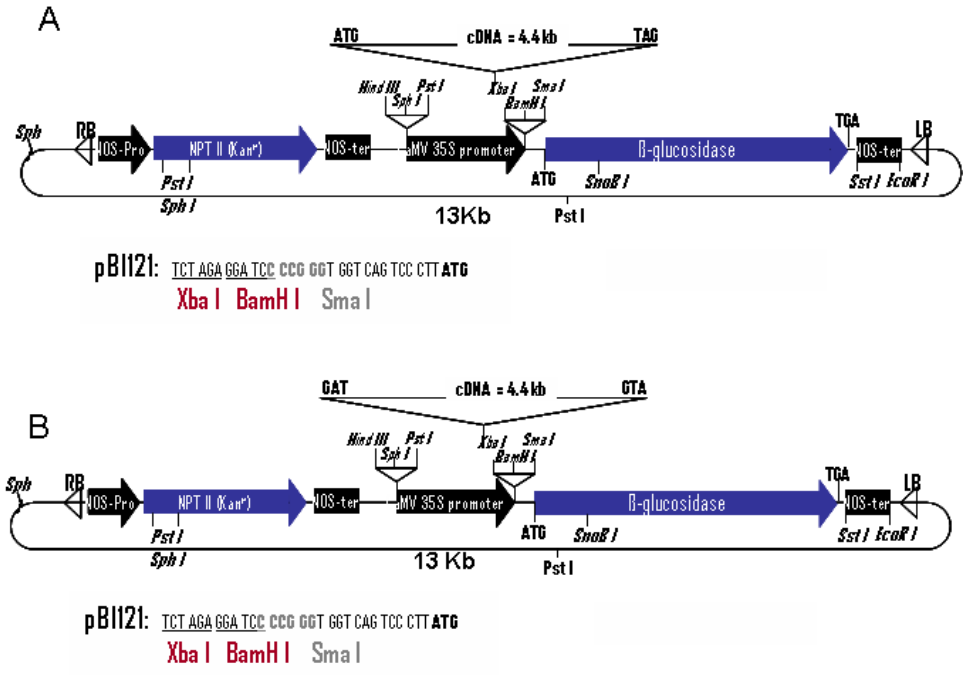

Figure 13: pBI121 vector construction for *CPSF* cDNA expression.

A. Sense (Over expression) construction and B. Antisense construction scheme of the full length cDNA. cDNA of 4.4kb including 5'and 3' untranslated regions were cloned into expression vector pBI121. Both termini containing XbaI restriction site were inserted into the multiple cloning site of the vector pBI121 to generate the cDNA expression construct.

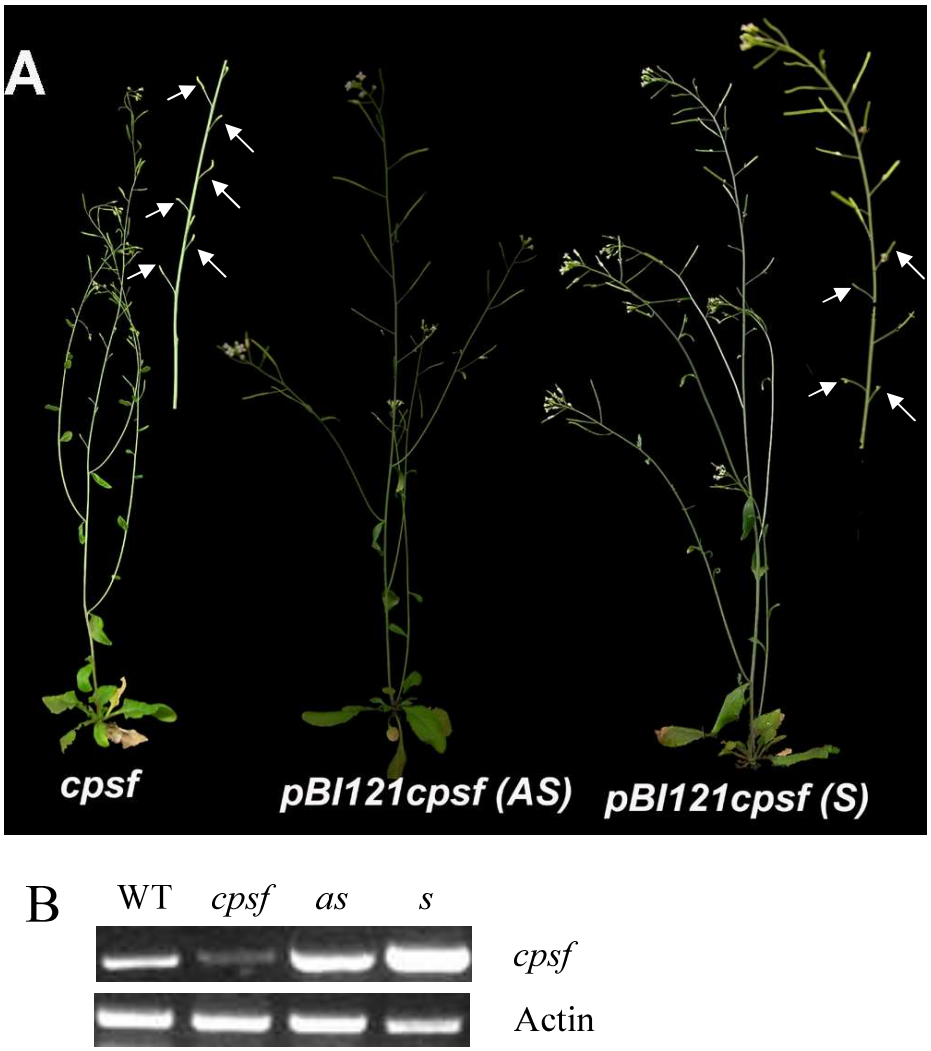

Figure 14: Morphology of *cpsf* (35S:cDNA) transformants harboring 35S promoter from CaMV in *Arabidopsis thaliana*.

A. Wild type and *35S:cpsf* [antisense (as) and sense (S)]. B. *cpsf* gene expression in different lines. Arrow indicates the similar silique in *cpsf* mutant and 35S:cDNA transformants

4 Discussions

Eukaryotic organisms utilize steroids as signaling molecule for physiological and developmental processes. Present study was conduced to shed more light on brassinosteroids mediated gene function in growth and developmental regulation. The genes are integrated in the complex signaling network and numerous BRs effects appear to be mediated via a modulation of levels and sensitivities of the other phytohormones. Correspondingly, BR-deficient and insensitive mutants among another phenotypical alteration display delayed flowering, reduced vascular development, reduced fertility and male sterility, dark green coloration and rounded leaves. There is a great affinity of the hormone in relation to germination control as well. In application of triazole type of active BR antagonist brassinazole, develops de-etiolation and opening cotyledon, reduced hypocotyls length in dark avoiding shading effect in normal plants. Mutation impaired in BR biosynthesis or perception revealed key role for BRs in normal growth and development (Friedrichsena *et al.*, 2002).

In this concern, almost all BR signal transduction research focused to Brassinosteroid Insensitive 1 (BRI1*)*, a single genetic locus in *Arabidopsis* encoding a leucine-rich repeat receptor kinase (Clouse *et al.*, 1996). Two different models of steroid action have been described in animal systems: the gene regulation response mediated by nuclear receptors, and the rapid non-genomic response mediated by proposed membrane-bound receptors (Wang *et al.*, 2001) while plants do not

seem encode members of nuclear receptor superfamily. However, a trans-membrane receptor kinase BRI1, has been implicated in brassinosteroid responses in plants (He *et al.*, 2000). *Bri1* cannot be rescued by BR treatment, this is the evidence of its essential role in genes that involve in BR signaling pathway.

A putative interactive to BRI1 screen evidenced BAK1 (BRI1 associated receptor kinase1), that shares similar structure organization with BRI1 (Nam and Li, 2002). The discovery of BAK1 and the genetic and biochemical evidence showing a role of BAK1 in BR signaling and a physical interaction with BRI is intriguing. Direct physical interaction between BRI1 and BAK1 was confirmed in *Arabidopsis* by co-immunoprecipitation experiments with target proteins (Li *et al.*, 2002; Nam and Li, 2002). Knockout mutants of *BAK1* were identified that showed a week *bri1*-like phenotype and also decreased sensitivity to BR. Overexpression of a kinase-deficient mutant form of *BAK1* in *bri* led to a severe dwarf phenotype, suggesting a dominant-negative effect, most likely by the poisoning of a possible heteromeric complex between BRI1 and BAK1 (Li *et al.*, 2002). BRI1 and BAK1 exist as inactive monomers in equilibrium with an active heterodimer (Nam and Li, 2002). BR binding stabilizes the heterodimer and results in transposphorylation of each cytoplasmic domain each other, leading to activate kinases that recognize and phosphorylate currently undefined downstream components. The genetic evidence strongly supports a direct *in vivo* interaction between BRI1 and BAK1 in plant membranes. However, direct demonstration of a BRI/BAK1 heterodimer as a coreceptor of BR is lacking and will

be required before models of BR-dependent heterodimerization and kinase activation can be confirmed.

After plasma membrane perception of BRs signals inters to cytoplasm after the transphosphorylation of the inner domains of the BRI1 and BAK1. An unknown number of steps follow BR binding, leading to inactivation of the negative regulator BIN2. Inactivation of BIN2 allows the unphosphorylated form of BES1 and BZR1 to accumulate and translocate to the nucleus in associating with BIM1 leading to activate BR-responsive gene while the mechanism remain completely unknown. While phosphoryated form of BES/BZR1 interrupts translocating BR signals downstream leading to cause proteosomic degradation. However, there is a possibility of active role of BSU1 in the cytosol to dephosphoryle BES1 and BZR1 avoiding degradation. There are several nuclear components that participate transcription of BR responsive genes that are yet to determind. It is evidenced that BZR1 a nuclear component that inactivate the BR feedback-regulated biosynthetic gene transcription.

A number of genes regulated by BR either by transcriptional or posttranscriptional mechanisms have been identified, although a BR response element and interacting protein factors are not as yet reported (Bishop and Koncz, 2002). Plant cell expansion and division are critical for growth and differentiation in all organs and results from alterations in gene expression and biochemical processes that affect on mechanical properties of cell wall, cell hydraulics, number of cells, and the osmotic potential. Modulating the expression of genes encoding wall-modifying proteins is one mechanism by which plant hormones, such as BRs,

auxins and gibberellins promote cell elongation and many of the known BRs-regulated genes encode such proteins. Another way for plant growth is the cyclin expression regulation as it was shown by (Hu *et al.*, 2000), *CycD3* is induced by epi-brassinolide.

Classical differential gene expression and DNA microarray analyses have been employed in recent days in identifying novel genes that appear to be regulated by BR signaling. Examination of BR-regulated gene expression in the *bes1-1D* and *bri1* mutants verified the BR regulation of numerous genes associated with auxin signal transduction (Yin *et al.*, 2002). An independent genetic analysis of the *Arabidopsis ucu1* mutant (allelic to *bin2*) suggests that the BIN2/UCU1 kinase may also be involved in auxin signal transduction, indicating the possible crosstalk between BR and auxin, two hormones with pronounced effect on the cell elongation (Perez-Perez *et al.*, 2002). A recent genetic screen for new BR-insensitive mutants, combined with Affymetrix GeneChip analysis, has identified *bin3* and *bin5* as putative subunits of an *Arabidopsis* topoisimerase VI that regulates the expression of numerous genes, including many of those that are also regulated by BRs (Yin *et al.*, 2002).

Not only the positive role of in the pathways, expression studies also defined the downstream events, confirming the role of several factors acting in negative feedback regulation on BR levels (Nemhauser and Chory, 2004; Pereira-Netto, 2007). The full-genome *Arabidopsis* GeneChip from Affymetrix recognized more than 24000 distinct genes that provide a unique opportunity to characterize the full spectrum of Br regulated genes. Full functional analyses of these newly discovered

genes would increase our understanding of the range of physiological events influenced by BRs.

Additionally, genes like *dwf* (dwarf) group, *cpd, cpy, det2* (*deetiolated2*), *bas1-d, sax, brd1, bls1* have been characterized in their possible involvement in brassinosteroid biosynthetic pathways. The biosynthetic pathway consist sterol biosynthesis and BR-specific pathways where the BR homeostasis is maintained through negative feedback regulation of numerous genes (Tanaka *et al.,* 2005). In the connection, the impairments in BRs biosynthetic genes are expectedly rescued by use of exogenous BRs while the perception mutants are unable to rescue with the supply of exogenous BRs. Similarly, the genes like *bri1, bzr1 and bes1, back1-1d, brs1, bin2-1, bin2-2, bsu1, dwf12, bee1, bee2, bee3, bru1, tch4, cycd3, bru2* and *exo* have been identified as their involvement in BR signal transduction pathways (Pereira-Netto, 2007).

Eukaryotic gene expression is a complex process, which generally includes transcription, pre-mRNA processing and translation of mRNA into proteins. The key functions of poly (A) tails are mRNA stability and translatability and involvement in the export of mature mRNA to cytoplasm. Thus defects in mRNA 3'-end formation can profoundly alter cell viability, growth and development. The process also affects pre-mRNA splicing and transcription termination. As CPSF play a central role in the cleavage and polyadenylation process it also obviously involves in releasing of mature mRNA to cytoplasm (Xu *et al.,* 2004). While information about the gene function is extremely low.

It is indicated that the gene localizes at the nucleus and involves in transcription factor activation during mRNAs transcription (Adamson *et al.*, 2005).

cpsf mutants show remarkable relation with BR perception that has been determined through brassinazole screening. As the mutant possesses germination defects, it might be the indication of the gene which overexpression or knockout homologue showed early embryonic lethality. Xu et al, 2004 also found that homozygous *cpsf* 73 mutants, a *cpsf* homologue in plant, possessed lethal while heterozygous retained vegetative growth displaying empty seed spaces as well as aborted seeds with embryos aborted at the globular stage (Xu *et al.*, 2004). It was also found that CPSF 73 kD of cleavage and polyadenylation specificity factor complex affects reproductive development in *Arabidopsis* (Xu *et al.*, 2006). Also over expression lines of the gene were all obtained as heterozygous. Homozygous line could be obtained in antisense transformants only. The characteristic germination impairment was also observed in the sense lines. Xu et al, 2006 also obtained that the over expression line of CPSF homologue CPSF73-II were lethal. Xu et al., 2006 found that overexpression line created in *cpsf73* fusing with 35S promoter showed no lethality and phenotypic alteration however, fusion with its native promoter showed phenotypic lethality in over expression line of the *cpsf73* transformants also knockdown of the gene also develop the lethal phenotype. T-DNA insertion line segregation also develop some seedling-lethal mutants (Budziszewski *et al.*, 2001).

Fujioka et al., 1997 revealed that *det2* mutant is blocked early in BRs biosynthesis in *Arabidopsis* in missing reduction of 24-methylcholest-4-en-3-one which is further modified into campestanol (Fujioka *et al.*, 1997). Exogenous application of BL thus can rescue the mutant *det2*. It can be noted that rescue of the *det2* phenotype in *det2cpsf* double mutant reveal *cpsf* either involves in downstream to the component guided by the *det2* if it involves in BRs biosynthetic process or it might possess alternate signaling pathway or is relation only in BRs perception. In this connection application of exogenous BL the mutant did not rescue the *cpsf* phenotype indicating the *cpsf* links with the BR perception but not to biosynthetic process hence it could rescue the *det2* phenotype in double mutant between *det2* and *cpsf* (Figure 12).

In present study, germination ambiguities appeared in T-DNA insertion mutants and overexpression line of cDNA transformants and its alteration in some of the silique structures indicated that the 35S promoter also functions in some extent. However lacks in gDNA construct including is native promoter in the present study, it can not be establishes the effects in expression pattern altered by 35S promoter and native *CPSF* promoter. In study of tomato genes *DUMPY* and *CURL*-1, it was also found out that mutations in other genes involved in BRs signal transduction produce lethality (Koka *et al.*, 2000; Pereira-Netto, 2007).

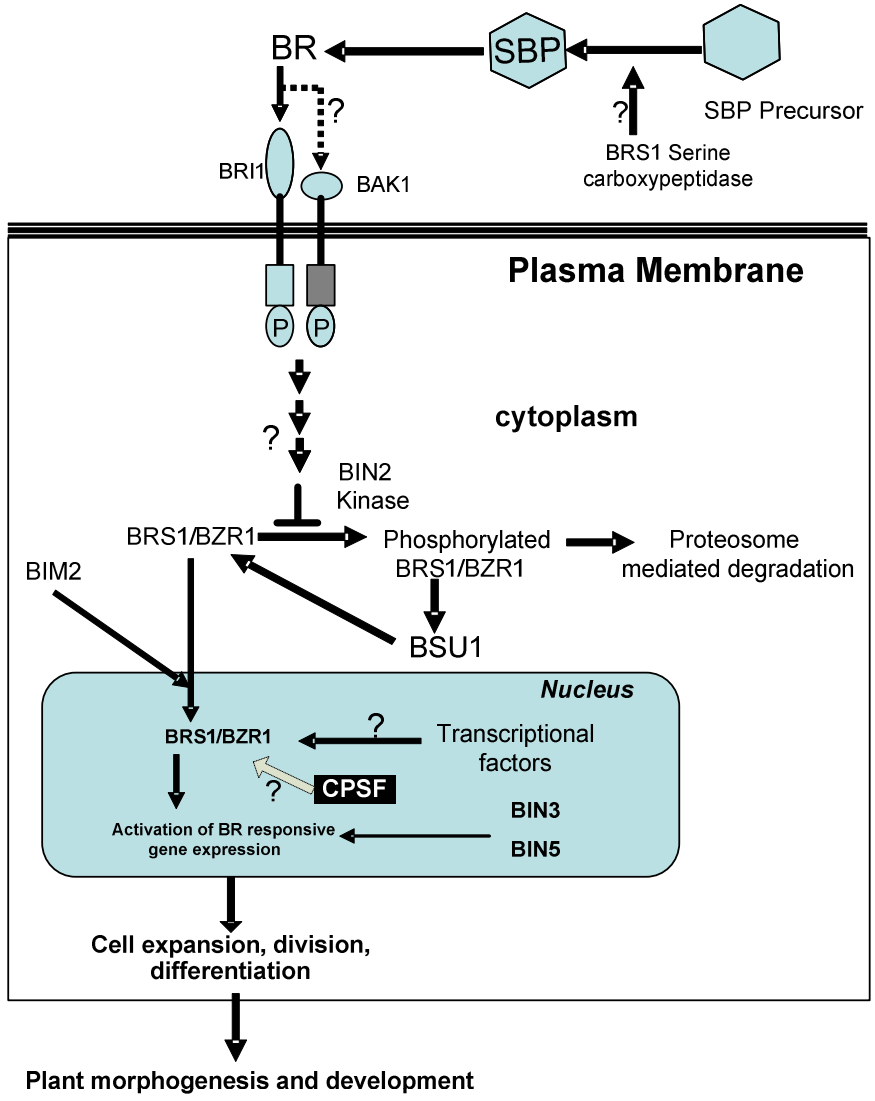

Figure 15: Suggested position of CPSF in BRs signal transduction.

The pathway is modification of pathway suggested by Clouse (2002a).

CPSF probably involves in activating BRs responsive gene expression at nuclear region because various studies suggested nuclear localization of CPSF however, how its involvement and are associations are yet to be confirmed. Mark ? denotes the proposed but not characterized step.

The study reveals that not only T-DNA insertion knockdown but also over expression of the gene *cpsf* develops germination impairment. The gene mutation links with the missing seeds in siliques. Double mutant analysis revealed that the *cpsf* appears in BRs perception mutation in BRs signaling pathway in *Arabidopsis*.

From the study it can be concluded that *CPSF* carries two major tasks in *Arabidopsis*. First, it relates to mRNA processing in plants and second, it controls normal growth process through BRs signal transduction in *Arabidopsis*.

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