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IDENTIFICATION AND CHARACTERIZATION OF NOVEL COMPONENTS IN BRASSINOSTEROID SIGNALING AND BIOSYNTHETIC PATHWAYS IN *ARABIDOPSIS THALIANA*

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IDENTIFICATION AND CHARACTERIZATION OF NOVEL COMPONENTS IN BRASSINOSTEROID SIGNALING AND BIOSYNTHETIC PATHWAYS IN *ARABIDOPSIS THALIANA*

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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I dedicate this thesis to my loved twin sons, Bartti and Barggio, and my wife, Sen. Their support, understanding and love make all of these be true. I love you.

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LIST OF ABBREVIATIONS

BAK1: BRI1 ASSOCIATED RECEPTOR KINASE 1

BES1: BRI1-EMS-SUPPRESSOR 1

bHLH: BASIC HELIX-LOOP-HELIX

BKI1: BRI1 KINASE INHIBITOR 1

BKK1: BAK1-LIKE 1

BIM: BES1 INTERCTING MYC-LIKE PROTEIN

BIN2: BRASSINOSTEROID INSENSITIVE 2

BL: BRASSINOLIDE

BR: BRASSINOSTEROID

BR6ox1,2: BR-6-OXIDASE 1, 2

BRI1: BRASSIOSTEROID INSENSITIVE 1

BRL1: BRI1-LIKE 1

BRL3: BRI1-LIKE 3

BRS1: *bri1* SUPPRESSOR

BRZ: BRASSINAZOLE

BSK: BR-SINALING KINASE

BSU1: BRI1 SUPPRESSOR 1

BZR1: BRASSINAZOLE-RESISTANT 1

CPD: CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM

CREST: CHIMERIC REPRESSOR SILENCING TECHNOLOGY

CS: CASTASTERONE

CT: CATHASTERONE

DET2: DE-ETIOLATED 2

DWF4: DWARF 4

GFP: GREEN FLUORESCENT PROTEIN

GSK3: GLYCOGEN SYNTHASE KINASE -3-LIKE PROTEIN

GST: GLUTATHIONE S-TRANSFERASE

GUS: β-glucuronidase

IP: IMMUNOPRECIPITATION

KAPP: KINASE-ASSOCIATED PHOSPHATASE

KD: KINASE DOMAIN

LRR: LEUCINE-RICH-REPEAT

MAPK: MITOGEN-ACTIVATED PROTIEN KINAE

MAPKK: MITOGEN-ACTIVATED PROTIEN KINASE KINASE

MAPKKK: MITOGEN-ACTIVATED PROTIEN KINASE KINASE KINASE

mRNA: MESSENGER RNA

PCR: PLOYMERASE CHAIN REATION

RLK: REEPTOR-LIKE KINASE

TCP: TB1, CYCLIN, PCF1-LIKE

T-DNA: TRANSFER DNA

TE: TEASTERONE

TRIP1: TGF- β RECEPTOR INTERACTING PROTEIN 1

TTL: TRANSTHYRETIN-LIKE

USDA: United State Department of Agriculture

CHAPTER I

INTRODUCTION

1. Brassinosteroid discovery

In 1930-40s, scientists at the USDA noticed that pollen extracts from maize and other plants can promote plant growth. Using a bean-first-internode bioassay, Mitchell and his colleagues screened over 60 kinds of pollen and found that pollen extracts from almost half of them, including *Brassica napus* and *Alnus glutinosa,* were capable of promoting growth of bean first internode (Mitchell and Whitehead, 1941). The novel growth-promoting substance was named as Brassin in 1970 (Mitchell *et al.*, 1970). In the effort to find out what the substance actually is, about 10 mg crystalline brassinolide (BL), the most active BR and the final product of BR biosynthesis in plants, was purified from about 227 kg pollen of *Brassica napus* in a *USDA* laboratory at Maryland in 1979 (Grove *et al.*, 1979). Its structure was determined by spectroscopic analysis and X-ray diffraction with the crystalline BL. It turned to be (22R,23R,24S)-2a,3a,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5a-cholestan-6-one, showing structure similar to animal steroid hormone. The second BR, castasterone (CS), was isolated by a Japanese group in 1982 (Yokota *et al.*, 1982). CS shows the same structure as BL only absent of a Baeyer-Villiger oxidation. So far, over 50 BRs have been isolated from a wide variety of plant species (Figure 1.1) (Ikekawa *et al.*, 1984; Fujioka and Yokota, 2003).

2. Functions of brassinosteroids in plants

It is tempting for scientists to investigate what their functions are in plants after the discovery of the novel plant steroid. After extensive studies, it has been well demonstrated that BRs can regulate many biological processes during normal plant

growth and development. These include: increase yield in crop plants, increase assistance to biotic and abiotic stresses and promote plant development.

2.1. Increasing yield in different crops

It was reported that BL treatment can significantly increase weight of leaves and seeds in rice (Lim *et al.,* 1987; Meudt *et al*.,1983). The yield increase was also observed in corn after BL treatment (Lim and Han, 1988). If treating potato with BL, not only its yield but also its starch content can be increased (Khripach *et al*., 1996). Recently, it was found that application of BL can also increase the yield of soybean (Zullo and Adam, 2002).

2.2 Increasing biotic and abiotic resistance of plants

BL treatment can increase plant resistance to low and high temperature. For example, BL can increase resistance to cold in maize (He *et al.*, 1991; Katsumi *et al.,* 1991); twenty-four epiBL treatment can protect spring rape from freezing (Janeczko *et al*., 2007). In tomato, BRs can significantly increase expression of heat shock proteins, which may account for their resistance to high temperature (Dhaubhadel *et al*., 1999). It was also reported that BRs can increase plant resistance to drought stress. For example, when BL was applied to wheat, plants showed increased growth under drought (Sairam *et al.,* 1994). Cucumber plants also displayed improved drought tolerance with BL application (Pustovoitova *et al*., 2001). In addition, it was also reported that BL treatment can protect barley from salt stress (Kulaeva *et al*., 1991). Besides increasing abiotic resistance, BRs also can increase plant resistance to biotic stresses. BR treatment increased abscisic acid and ethylene levels in potato, which may confer its higher resistance to *Phytophthora infestans* (Krishna *et al.,* 2003). In rice, BL application can

improve its resistance to rice blast and bacterial blight. It was also well documented that BL can induce tobacco resistance against tobacco mosaic virus and the bacterial pathogen *Pseudomonas syringae* (Nakashita *et al*., 2003).

2.3 Promoting plant development

Besides the above physiological roles in the whole plants, BRs' effect at the cellular and sub-cellular level has also been explored. As summarized in Table 1.1, it was well elucidated that BRs can promote cell expansion and elongation by modulating the plasticity and relaxation of cell wall, and BRs can stimulate and enhance cell division in protoplasts (Xu *et al*. 1995; Koka *et al*. 2000). BRs can interact with ABA and GA to regulate seed germination (Steber and McCourt, 2001). BRs also interact with other plant hormones, such as auxin and GAs, to regulate plant growth and development (Takene *et al*. 1982). Furthermore, *Arabidopsis* BR mutants showed delayed senescence of leaf and cotyledon tissues, suggesting their roles in regulating senescence. The male sterility seen in BR mutants also suggests their functions in reproductive biology. BRs also can promote vascular structure development since exogenous BL induces differentiation of tracheary elements (Clouse & Zurek, 1991). In addition, it was widely considered that BRs also regulate photo-morphogenesis, skoto-morphogenesis and embryogenesis in plants (Clouse, 1998). Today, BRs are widely accepted to be essential for regulating normal plant growth and development (Clouse, 1996; Kauschmann *et al.,* 1996; Khripach *et al.,* 1999). The notion was demonstrated by the phenotypes of BR deficient mutant or BR insensitive mutant. As shown in Figure 1.2, both mutants exhibit severe defects including dwarfism, curled leaves, delayed flowering time, male sterility, suggesting its indispensible functions in plant kingdom.

3. BR biosynthesis pathway

Since BRs modulate so many aspects in plant growth and development, it prompts scientists elucidate how the group of plant steroids are synthesized in plants. The BR biosynthesis pathway was first elucidated in a cultured *Catharanthus roseus* cell system by several Japanese groups (Fujioka 1994; Fujioka 2003; Suziki *et al.*, 1995a, 1995b). At the end of the last century, an outline of the BR biosynthesis pathway was successfully established based on their research.

As shown in Figure 1.3, cycloartenol is produced from mevalonate via the mevalonic acid pathway in plants. Cycloartenol can be synthesized to plant sterol precursors including BR precursor, campesterol, through the general sterol biosynthesis pathway. Specific BR biosynthesis pathway then will be started from the BR precursor, campesterol. As shown in Figure 1.4, in the specific BR biosynthesis pathway, campesterol is converted into campestenol first, then BRs will be synthesized through an early C6-oxidation pathway, from campestenol to 6-oxocampestenol to cathasterone to teasterone to 3-dehydroteasterone to typhasterol to castasterone to brassinolide; or through a late C6-oxidation pathway from compestenol to 6-deoxocathasterone to 6-deoxoteasterone to 3-dehydro-6-deoxoteasterone to 6-deoxotyphasterol to 6-dehydocastasterone to castasterone to brassinolide. The early side chain C22-oxidation and C5 hydroxylation was also elucidated from campesterol to (22*S*)-22-hydroxycampesterol to (22*S*,24*R*)-22-hydroxyergost-4-en-3-one to (22*S*,24*R*)-22-hydroxy-5-ergostan-3-one to 6-deoxocathasterone, which leads to the late C6-oxidation pathway (Fujioka *et al.*, 1997, Sakurai *et al.*, 1997, Yokota *et al.*, 1997).

The proposed BR biosynthesis pathway has been established with the identification and characterization of BR biosynthetic mutants. For example, in the early steps of sterol biosynthesis, *smt1, cyp51, fackel* and *hyd* mutants have been identified. These mutants showrd unique defects during embryogenesis that couldn't be rescued by BR application (Jang *et al*., 2000; Schrick *et al*., 2000), which means other sterol pathway may also be disrupted. In the late steps of sterol biosynthesis, *dwf1*, *dwf5* and *dwf7* mutants also showed reduced BR concentration and display developmental defects, however, the phenotypes of these mutants could be rescued by BR application. It was found that the *Arabidopsis dwf1* mutant was defective in the last reaction for synthesizing campesterol (Feldmann *et al*., 1989; Klahre *et al*., 1998); in *dwf5* mutant, 24-methylenecholesterol cannot be synthesized from its substrate 5-dehydroepisterol (Choe *et al*., 2000); and in *Arabidopsis dwf7* mutants, convertion of episterol to 5-dehydroepisterol was blocked (Choe *et al*., 1999; Catterou *et al*., 2001);

In recent years, several BR deficient mutants were identified in the specific BR biosynthesis pathway. For example, *de*-*et*iolated *2* (*det2*) was identified as a de-etiolated mutant from *Arabidopsis* (Chory *et al*., 1991). *DET2* encodes a protein sharing sequence similarity with the mammalian steroid 5α-reductase (Li *et al*., 1997). Biochemical and feeding experiments using intermediates of BR biosynthesis indicated that DET2 is indeed responsible for the reduction step that converts campesterol to campestanol during BR biosynthesis (Fujioka *et al*., 1997). It was found that *det2* mutants were still able to synthesize about 5-10% wild type levels of BRs. Therefore, *det2* null mutant was considered as an intermediate biosynthetic mutant (Fujioka *et al*., 1997; Fujioka and Yokota, 2003). *dw*ar*f 4* (*dwf4*) is another BR deficient mutant isolated from *Arabidopsis*

(Choe *et al*., 1998). The dwarfed stature of *dwf4* can be rescued by brassinolide (BL), the final product of the BR biosynthetic pathway, and the most active form of BRs. *DWF4* encodes a 22-hydroxylase and is responsible for multiple 22-hydroxylation steps during BR biosynthesis. It was proposed that DWF4 catalyses a rate limiting step during BR biosynthesis (Kim *et al*., 2006). Constitutive photomorphogenesis and dwarfism (*cpd*) is another dwarf mutant isolated by T-DNA insertion analysis. It was shown that *CPD* encodes a 23α-hydroxylase and participates in a critical 23α-hydroxylation step in BR biosynthesis (Szekeres *et al*., 1996). Recent feeding and biochemical analyses indicated that two P450 proteins, CYP90C1 and CYP90D1, are the true 23α-hydroxylases (Ohnishi *et al*., 2006). The severe phenotype of the *cpd* mutant indicated that it should be involved in a step prior to the 23α-hydroxylation reaction. Another gene involved in BR biosynthesis is *BR6ox*, which was first identified in tomato by transposon tagging (Bishop *et al*., 1996). BR6ox catalyzed the C-6 oxidation of a number of different 6-deoxoBRs (Bishop *et al*., 1999). BR6ox orthologs from *Arabidopsis* and rice have conserved functions, responsible for linking the early and late C-6 oxidation pathways (Bishop *et al*., 2006).

4. Maintenance of BR homeostasis

Maintaining steroid hormone homeostasis turns out to be extremely critical not only for animals but also for plants. So how is BR metabolism regulated to maintain BR homeostasis to ensure the proper functions of BRs in plants. In the efforts to address this question, some negative mechanisms modulating BR homeostasis have been found recently, however the positive mechanisms are still unknown.

4.1 Inactivation of BRs

As shown in Figure 1.5, it was demonstrated that several BR inactivation mechanisms contribute to reduce BR concentrations in plants, such as epimerization, hydroxylation, conjugation, sulfonation, and oxidation (Asakawa *et al*., 1996). *BNST3*/*AtST1*, a BR sulfotransferase, was isolated from *Brassica napus* and *Arabidopsis thaliana*, respectively. They sulfonated 22-OH of 24-epiBRs to reduce their biological activities (Rouleau *et al*., 1999; Marsolais *et al*., 2007). The *phyB activation-tagged suppressor 1-dominant* (*bas1-D*) was isolated as a suppressor of the *phyB-4* mutant via activation tagging. *bas1-D* exhibited a dwarf phenotype resulted from activation of a cytochrome CYP734A1. BAS1 may convert active BL into an inactive form of 26-hydroxyBL (Neff *et al*., 1999; Turk *et al*., 2003). BAS1 homologs have also been identified to inactivate BL (Takahashi *et al*., Nakamura *et al*., 2005; Turk *et al*., 2005). *UGT73C5*, a UDP-glycosyltransferase enzyme (UGT), catalyzes BL-23-*O*-glucosylation of the BL and CS, resulting inactive CS-23-*O*-glucoside and inactive BL-23-*O*-glucoside, respectively (Poppenberger *et al*., 2005). BEN1 (*bri1-5 enhanced 1- 1 dominant*) was also identified to inactivate BRs through an unknown mechanism (Tong *et al.,* 2007).

4.2 Feedback regulation of BR biosynthesis

Recently it was also revealed that the BR biosynthesis pathway is regulated by a feedback mechanism using BZR1. BZR1 is a transcription factor functioning downstream of the BR signaling pathway to control the expression of several BR response genes. BZR1 directly binds to the promoter of the BR biosynthetic genes (*DWF4, CPD, BR6ox*) and represses their transcription. BL treatment thus will enhance the repression of these

biosynthetic genes, while inhibition of BR biosynthesis can attenuate the repression by disrupting the interaction between BZR1 and its target genes (Wang *et al.,* 1999, He *et al.,* 2002).

5. Brassinosteroid signal transduction pathway

After BRs are produced in plants, a further question is how these essential plant hormones function in plants, such as: how they are perceived in plants, how they eventually transduce BR responses in plants. In the last two decades, especially after the discovery of BR receptor, some important components in BR signaling pathway have been found.

5.1 BRs are perceived by a membrane-bound LRR-RLK BRI1 in plants.

BRs are a group of plant steroid hormones similar to animal steroid hormone. In animals, steroid hormones are perceived by intracellular receptors. Once the ligand binds to its receptor, the ligand-receptor complex is transported to the nucleus where it regulates the expression of hormone-responsive genes (Agarwal, 1992; Beato *et al*., 1995). However, genes encoding for intracellular receptors could not be found in the genome of *Arabidopsis*. Therefore, it is proposed that BRs could be perceived at the plasma membrane to mediate downstream signal transduction (*Arabidopsis* Genome Initiative, 2000; Li and Chory, 1997; McCarty and Chory, 2000; Gerald and Csaba, 2002). In the effort to seek BR receptors, the first BRs receptor mutant *bri*1 was identified in 1996 (Clouse *et al*., 1996). The *bri1* showed extreme dwarfism, with curly leaves and male sterility, similar to the BR deficient mutant. However, unlike BR deficient mutant *bri1* is insensitive to BRs. With more and more allele of *bri1* mutant

were found, BRI1 was successfully cloned in 1997 (Li *et al.*, 1997). As shown in Figure 1.6, BRI1 turned out to be a membrane-bound leucine rich repeat (LRR)-receptor like kinase (LRR-RLK), implying its role in BR perception. BRI1 contains a cytoplasmic kinase domain, a transmembrane domain, and an extracellular domain containing 25 leucine-rich repeats (LRRs) which are interrupted by a 70-amino-acid island between the 21st LRR and the 22st LRR, the island together with nearby LRR forms the ligand binding domain. Further studies demonstrated that BRI1 exists as an inactive homodimer before ligand binding, once BRs associate with receptor BRI1, BRI1 will be auto-phosphorylated and activated, the activated BRI1 will form a heterodimer with its co-receptorm BAK1, to initiate downstream signal transduction (Li *et al.*, 2002; He *et al.*, 2000; Wang *et al.*, 2001; Kinoshita *et al.*, 2005).

5.2 BAK, a co-receptor of BRs

BAK1 was identified independantly by an activation-tagging approach and a yeast two hybrid method at the same time (Li *et al.*, 2002; Nam *et al.*, 2002). BAK1 is an LRR-RLK with structure similar to BRI1, including a cytosolic kinase domain, a transmembrane domain and extracellular LRRs domain (Figure 1.6). But BAK1 only contains five LRRs domains. When BL binds to BRI1, the auto-phosphorylated BRI1 will further phosphorylate BAK1. Phosphorylated BAK1 then transphosphorylates BRI1 to enhance BRI1 activity. The reciprocal phosphorylation between BRI1 and BAK1 will initiate downstream BR signal transduction (Wang *et al.,* 2005).

5.3 BRS1 is an upstream regulator of the BR signal transduction

BRS1 was identified using a weak allele *bri1* mutant by activation-tagging. Over-expression of BRS1 can rescue the weak allele *bri1* mutant, suggesting its positive role in

BR signal transduction. Since BRS1 is a secreted and active serine carboxypeptidase, it probably processes a protein involved in BRs perception in an early event (Li *et al.,* 2001; Zhou *et al.,* 2004)

5.4 BKI1, an inhibitor of BRI1

BKI1 is a transthyretin-like protein (TTL), identified as BRI1 interacting protein in a yeast two hybrid assay. BKI1 specifically interacts with the kinase domain of BRI1 to inhibit BRI1 probably by preventing interaction between BRI1 and BAK1. When BL binds to BRI1, BKI1 is released from BRI1 which allows BRI1 to associate with BAK1 or other substates and initiate BR signal tranduction. It was hypothesized conformation change may underlie the release of BKI1 from BRI1 (Wang *et al.,* 2006).

5.5 BSK1

BSK1 was identified from proteomic studies using two-dimensional difference gel electrophoresis (2-D DIGE). BSK1 becomes phosphorylated upon BL treatment. Phosphylated BSK1 migrat at different rate compared in 2-D DIGE compared to BSK1. BSK1 is a receptor-like cytoplasmic kinase (RLCK), consisting of an N-terminal kinase domain and a C-terminal tetratricopeptide repeat (TPR) domain known to mediate protein-protein interactions. BSK1 is specifically phosphorylated and activated by BRI1. Activated BSK1 disassociates from BRI1 to mediate downstream signal transduction, which is different from BAK1, active BAK1 will interact with BRI1 (Tang *et al.,* 2008).

5.6 BIN2, a GSK3/SHAGGY-Like Kinase, negatively regulates BR signaling

bin2 is identified as a gain-of-function mutant which is insensitive to BL. *bin2* resembles BR insensitive mutants, suggesting its negative role in BR signaling. Later it is found that BIN2 will phosphorylate two important downstream transcription factors,

BZR1/ BES1. Phosphorylated BZR/BES1 are likely degraded through proteasome, which prevents their accumulation in nuclei to induce BR response (Li *et al.,* 2001; Li *et al.,* 2002; He *et al.,* 2002).

5.7 Downsteam components in BR signal transduction pathway

Transcription factors, BZR1 and BES1, are their own two closest homologs in *Arabidopsis thaliana*. A point mutation stabilizes BZR1 and enables BZR1 to constitutively accumulate in the nucleus to regulate the transcription of BR response genes. The resulting gain-of-function mutant, *bzr1-1D*, shows constitutive BR responses, and is insensitive to BR biosynthesis inhibitor in the dark (Wang *et al*., 2002; He *et al.*, 2005). For *bes1-1D*, one point mutation occurs at the same position as *bzr1-1D*. *bes1-1D* not only shows constitutive BR response, but also shows insensitivity to BR biosynthesis inhibitor under both dark and light condition (Yin *et al*., 2002; Yin *et al.*, 2005). When BR is absent, BZR1/BES1 is phosphorylated by BIN2, which stimulates their degradation in the cytoplasm. A 14-3-3 protein can facilitate BZR1/BES1 degradation by binding and retaining these proteins in cytoplasm (Gampala *et al*., 2007). When BRs are present, BSU1, a phophatase, will be activated by BSK1. Active BSU1 de-phosphorylates BIN2 and prevents BIN2 to phosphorylate BZR1/BES1. (Vert and Chory, 2006; Garcia *et al*., 2004; Wang,). De-phosphorylated BZR1/BES1 will accumulate in nuclei to activate BR response genes, such as *SAUR-AC*, *MYB30* (Li L. *et al.,* 2009). They will also repress BR biosynthetic genes, such as *CPD*, *DWF4*, to feedback inhibit BR biosynthesis (Wang *et al*., 2002; He *et al.*, 2005). Other transcription factors, such as BIM, ELF6, REF6 and MYB30, also interact with BZR1/BES1 to control BR responses in plants (Yu *et al.,* 2008; Li L. *et al.,* 2009).

5.8 Proposed model for BR signal transduction pathway

After significant discoveries in the BR signaling pathway, a BR signaling pathway has been proposed, as shown in Figure 1.7. When BR binds to BRI1, inhibitor BKI1 is released from BRI1, and BRI1 undergoes through autophosphorylation to be active. Activated BRI1 will phosphorylate and activate its co-receptor BAK1, and activated BAK1 trans-phophorylates BRI1. Active BRI1 phosphorylates and activates BSK1. BSK1 then activates BSU1, a phosphotase. BSU1 dephosphorylates BIN2 and inhibit its phosphorylation activity to BZR1/BES1. De-phosphorylated BES1/BZR1 accumulates in nuclei to modulate the expression of BR response genes and eventually regulate BR-inducible plant growth and development in *Arabidopsis*. When BR is absent, BIN2 phosphorylates BES1/BZR1, and 14-3-3 proteins contribute to retain phosphorylated BZR1/BES1 in cytoplasm. Phosphorylated BES1/BZR1 will be degraded through an unknown 26S proteasome. Therefore no BR responses can be elicited, which leads to severe defects in plant growth and development (Clouse, 2002; Vert *et al.*, 2006; Belkhadir *et al.*, 2006; Li, *et a.l*, 2007; Kim and Wang, 2010).

6. Perspectives

In last two decades, with the identification and characterization of BR deficiency mutants and BR insensitive mutants, especially after the characterization of BR receptor BRI1, our knowledge about BR biosynthesis pathway and BR signaling pathway has been great advanced, important components functioning in either the BR biosynthesis pathway or the BR signaling pathway have been found. However, many critical questions remain to be answered.

Firstly, BRs are ubiquitously present in the whole plant. However, the levels of endogenous BRs vary among different plant tissues. Pollen, young seeds and tissues usually contain more BRs than shoots and old leaves, suggesting that BR is accumulated in reproductive organs and growing tissues in plants. It will be intriguing to find out how BR biosynthesis is regulated in specific organs and tissues, even at the sub-cellular level (Shimada *et al.,* 2003; Choe *et al.,* 2001). Furthermore, it is known that unlike other plant hormones, BRs do not undergo long distance translocation, which suggests BR homeostasis may be more finely tuned in plants (Symons *et al.,* 2008). So it is critical to understand how BRs homeostasis is spatially and temporally maintained in plants.

Secondly, regulating hormone biosynthesis is a basic wisdom to maintain hormone homeostasis. And regulating the activities of rate-limiting biosynthetic enzymes is fundamental important. In the BR biosynthesis pathway, several key biosynthetic enzymes have been characterized. Among these enzymes, DWF4 is thought to be the rate-limiting enzyme in BR biosynthesis, because DWF4 catalyzes multiple flux-determining steps in BR biosynthetic pathway, in *dwf4* mutant, 95% BR biosynthesis is blocked, resulting in an severe defective phenotype (Choe *et al*., 1998). And *DWF4* expression level is relatively low compared to that of other enzymes, its transcript accumulates in the actively growing tissues, and its expression is correlated to distribution of endogenous BRs in plants (Kim *et al.,* 2006; Ho *et al*., 2006). Other mechanisms are believed to regulate the activity of *DWF4* in addition to the feedback regulation shared with other BR biosynthetic enzymes (Tanak *et al*., 2005). Therefore DWF4 may represent a pivotal point to ensure BR homeostasis in plants. However, the mechanisms underlying DWF4 is largely unknown.

Thirdly, when BRs are over provdued in plants, BR biosynthesis is feedback inhibited and BRs is inactivated using multiple mechanisms. However, positive regulations of BR homeostasis are barely understood. For example, how BR biosynthesis is elicited at the beginning, and how its biosynthesis is accelerated when more BRs are required at particular development stages, are not yet clear.

Fourthly, there are big gaps that need to be filled in the BR signaling cascade. For example, what is the downstream substrate of BRI1/ BAK1 heterodimer? How does BSK1 mediate BR signal transduction? How are BR responses induced after BZR1/BES1 and other transcription factors accumulate in the nucleus? In addition, ligand binding proteins may facilitate the BR perception by BRI1, but their identities are unknown yet.

In order to address these questions and further dissect BR biosynthesis and signaling pathway, more novel components need to be discovered in the field. Since BRs play so important roles in regulating many biological processes in plant growth and development, the gained knowledge in the field will show its great significance not only in plant science but also in agriculture.

Table 1.1 BR functions in plants (According to Clouse *et al*., 1998; Khripach *et al*., 2000)

Cellular and molecular level	**Whole plant level**
Regulate gene expression	promote plant growth
Activate protein and nucleix acid synthesis	Modulate biotic and abiotic stress responses
Control fatty acid composition	Increase the yield of crop and fruits
Interact with different hormones	Promote vascular development
Enhance the photosynthetic capacity and translocation of products	Accelerate senescenc
Promote cell expansion, cell division and cell elongation	Promote fertilization and flowering
Regulate the properties of cellular membranes	Effects on skotomorphogenesis
	Effects on photomorphogenesis

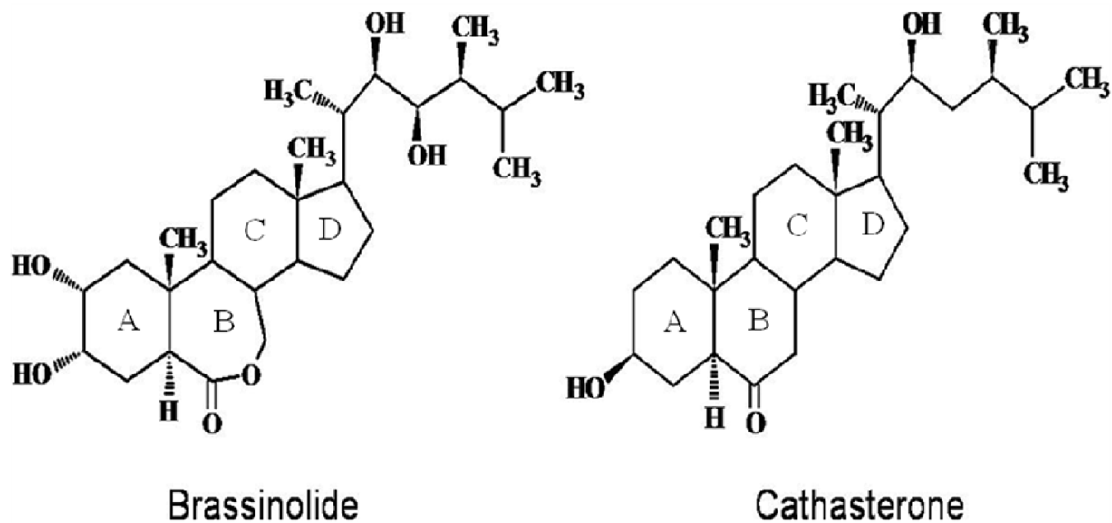

Figure **1.1** BL structure and CS structure. They contain A, B,C and D four rings with side chain.
Their structural variation comes from different modification on the A/B-rings and the side chain.

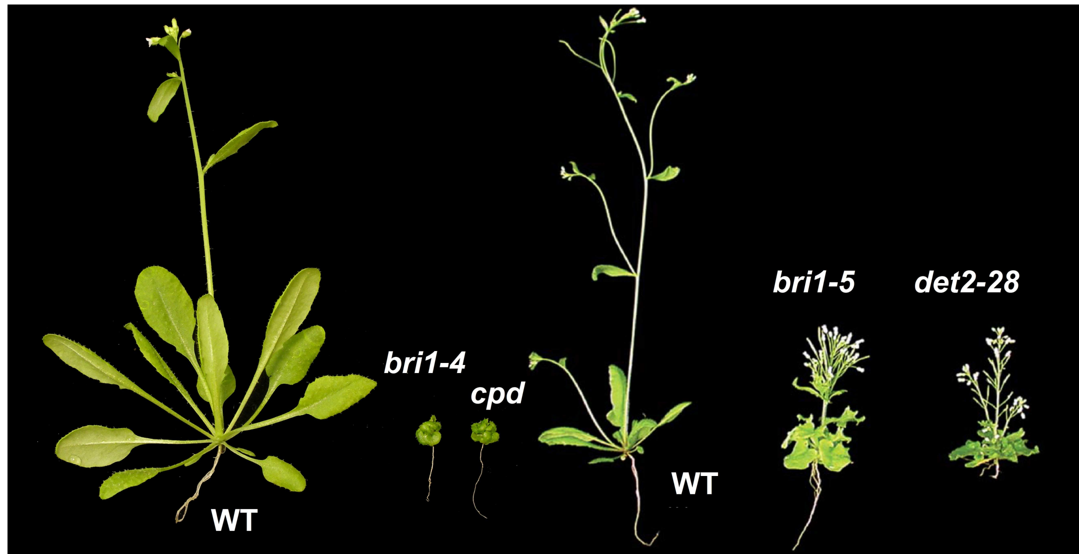

Figure **1.2** BR receptor mutants and BR deficient mutants. Both null allele *bri1* mutant *bri1-4* and BR biosynthesis mutant *cpd* are tiny and sterile with curled leaves. Weak allele *bri1* mutant *bri1-5* and a weak BR biosynthetic mutant *det2* show intermediate phenotypes.

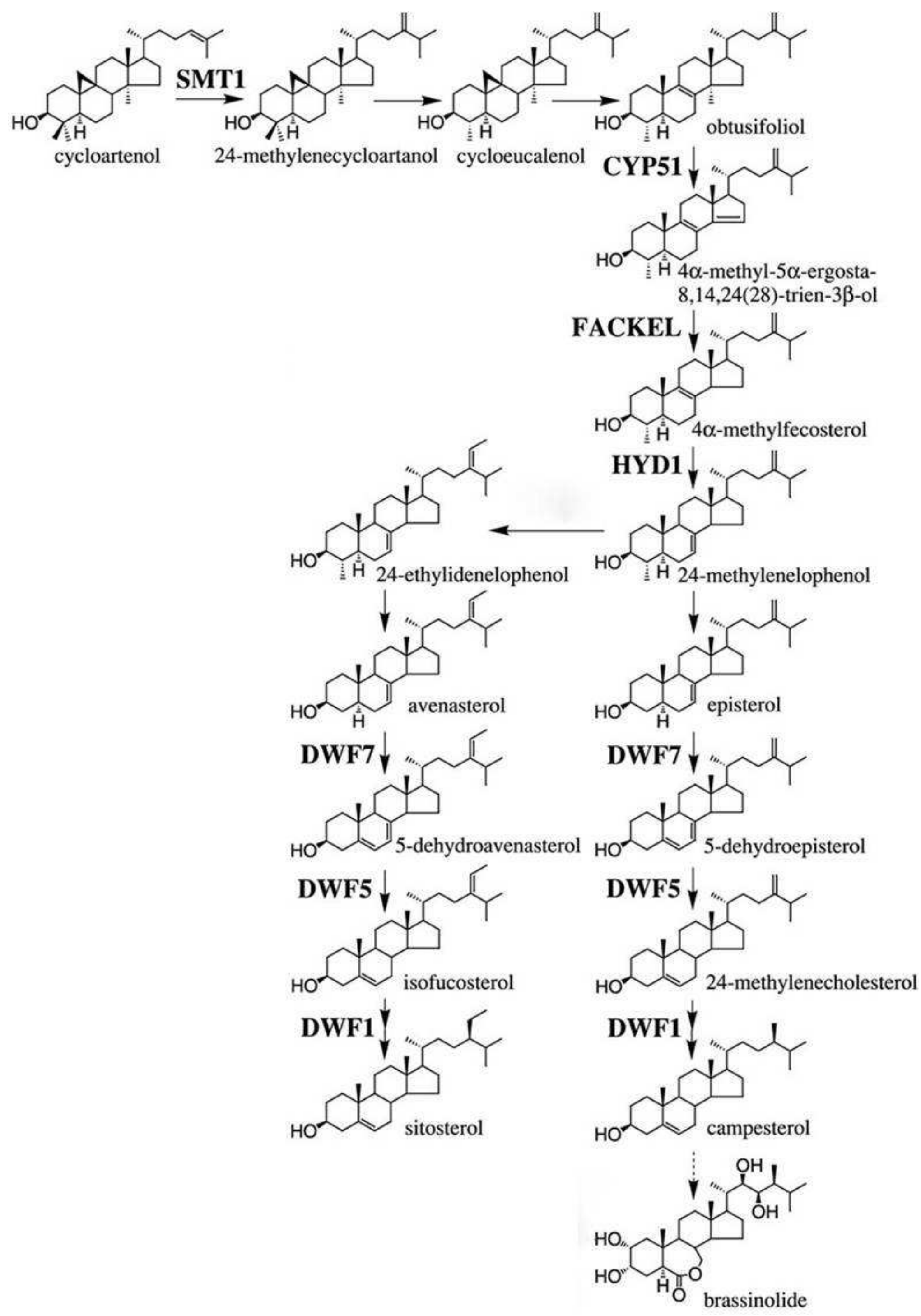

Figure **1.3** The plant sterol biosynthesis pathway (from Fujioka, 2003).

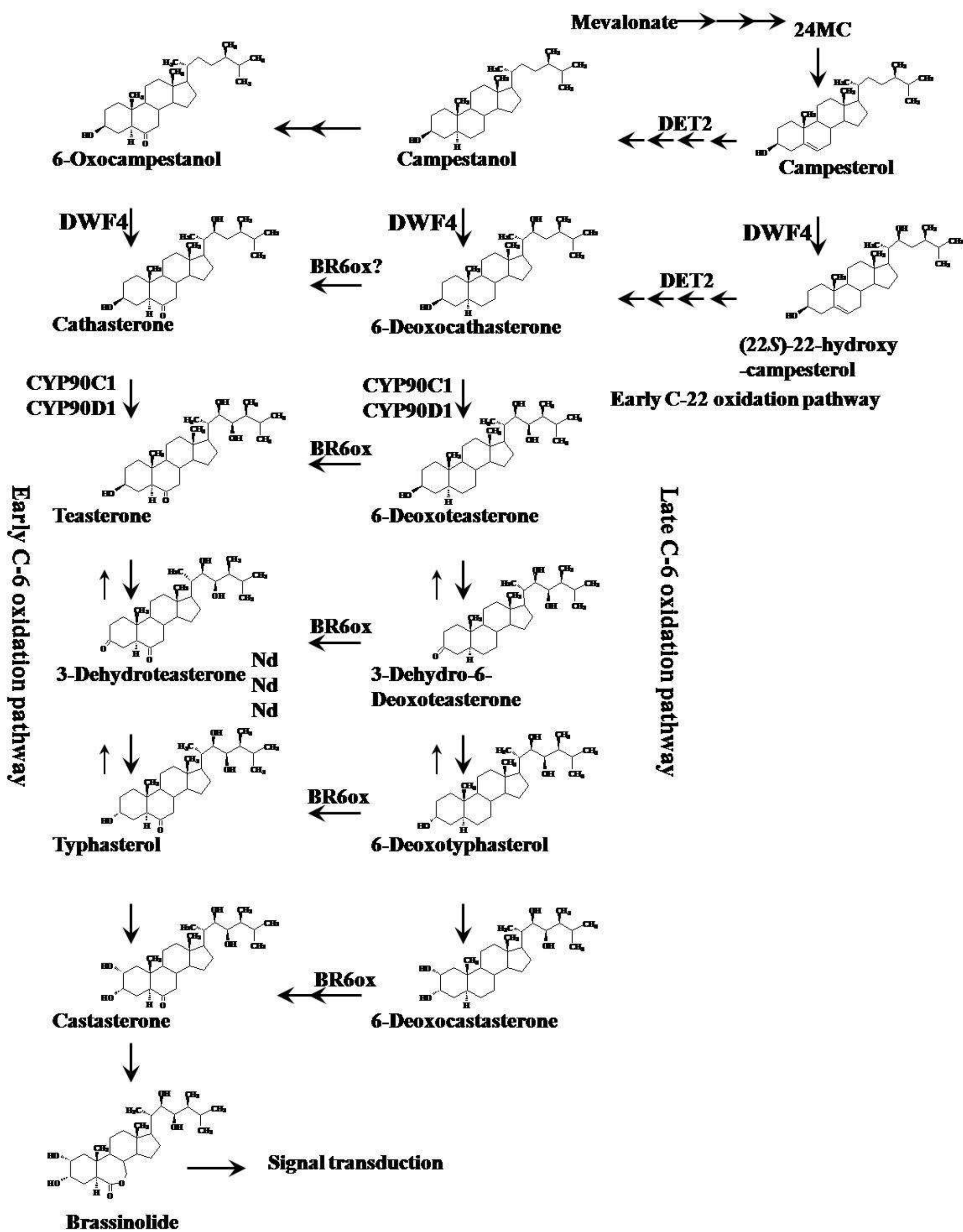

Figure **1.4** BR biosynthesis pathway in *Arabidopsis*. BR precursor, campesterol, enters as a metabolite of the sterol biosynthsis pathway in plants. The final product brassinolide is synthesized through a late C-6 oxidation pathway and an early C-6 oxidation pathway in parallel (Li and Gou , 2007).

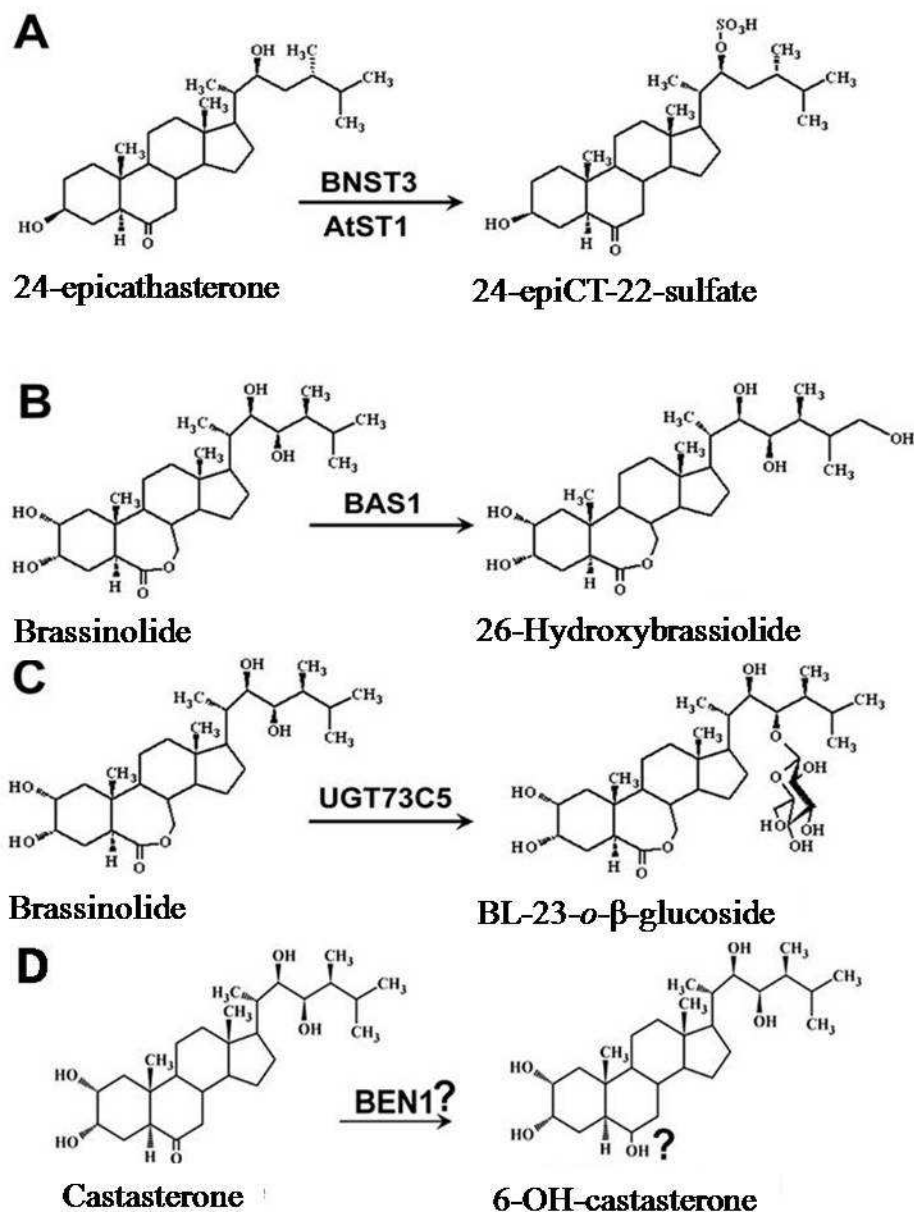

Figure **1.5** BR inactivation mechanisms. **A.** Inactivation through sulfonation by BNST3 and AtST1. **B.** Inactivation through hydroxylation by BAS1. **C.** Inactivation through conjugation with glucose by UGT73C5. **E.** Proposed role of BEN1 in BR inactivation (Modified from Li and Gou, 2007; Yuan *et al*., 2007)

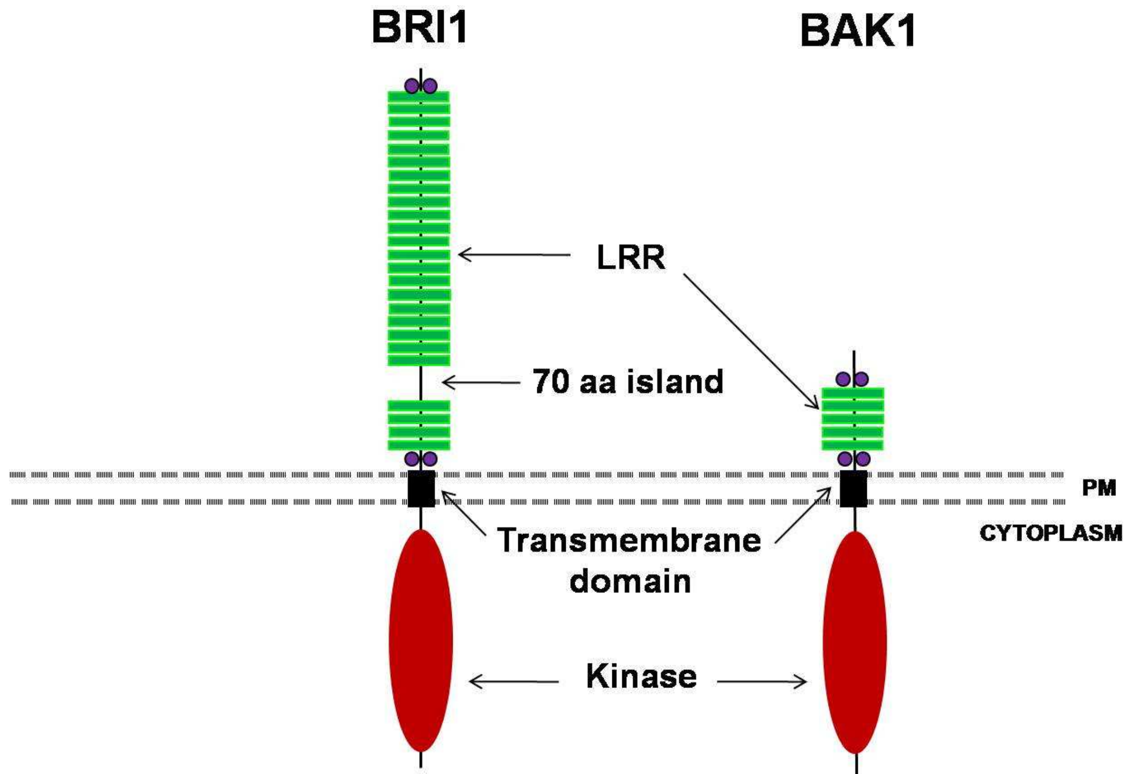

Figure **1.6** BRI1 structure and BAK structure. Both BRI 1and BAK1 contain a cytosolic kinase domain, a transmemebrane domain and extracellular Leucine Rich Repeat (LRR) domains. There are 25 LRRs with a 70 aa island between 21 and 22 LRR in BRI1. The island will bind to BRs. There are only 5 LRRs in BAK1.

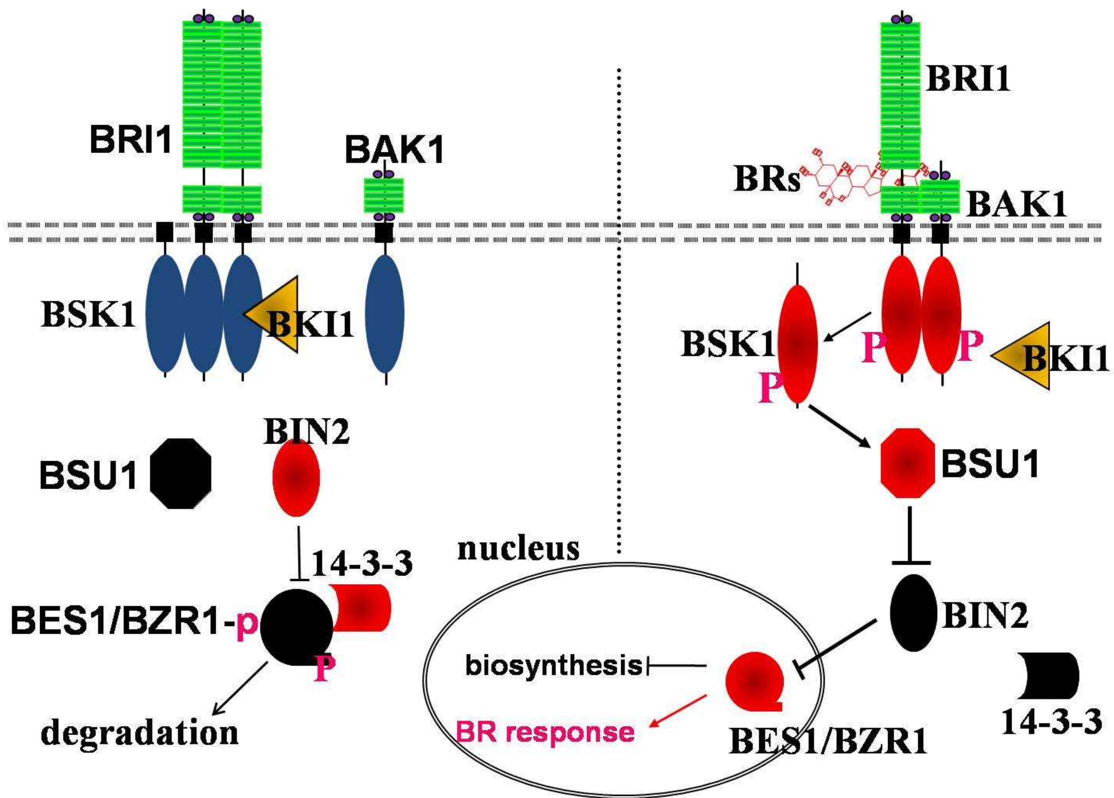

Figure **1.7** BR signal transduction pathway in *Arabidopsis*. When BR binds to BRI1, BRI1 is activated. Inhibitor BKI1 is released from BRI1. BRI1 forms a heterodimer with BAK1. Activated BRI1 phosphorylates and activates its co-receptor BAK1. Activated BAK1 trans-phosphorylates and activates BRI1. Then BRI1 phosphorylates and activates BSK1. BSK1 activates BSU1, a phosphotase. BSU1 dephosphorylates BIN2 to prevent BIN2 to phosphorylate BZR1/BES1. De-phosphorylated BES1/BZR1 accumulate in nuclei promote BR responses. When BRs are absent, BIN2 phosphorylates BES1/BZR1, and 14-3-3 proteins retain phosphorylated BZR1/BES1 in cytoplasm. Phosphorylated BES1/BZR1 is degraded through an unknown 26S proteasome.

References

Asakawa, S., Abe, H., Nishikawa, N., Natsume, M., and Koshioka, M (1996). Purification and identification of new acyl-conjugated teasterones in lily pollen. Bioscience Biotechnol. Biochem. 60, 1416-1420.

Banco, S., Nomura, T., Sato, T., Molnár, G., Bishop, G.J., Koncz C, Yokota. T., Nagy. F., Szekeres. M. (2002). Regulation of transcript levels of the *Arabidopsis* cytochrome p450 genes involved in brassinosteroid biosynthesis. Plant Physiol. 130(1):504-13.

Beato, M., Herrlich, P., and Schutz, G (1995). Steroid hormone receptors—Many actors in search of a plot. Cell 83, 851–857.

Bishop, G.J., Nomura, T., Yokota, T., Harrison, K., Noguchi, T., Fujioka, S., Takatsuto, S., Jones, J.D.G., and Kamiya, Y (1999). The tomato DWARF enzyme catalyses C-6 oxidation in brassinosteroid biosynthesis. Proc. Natl. Acad. Sci. USA. 96, 1761-1766.

Choe S, Fujioka S, Noguchi T, Takatsuto S, Yoshida S, Feldmann KA. (2001). Overexpression of DWARF4 in the brassinosteroid biosynthetic pathway results in increased vegetative growth and seed yield in *Arabidopsis*. Plant J. 26(6):573-82.

Choe, S., Dilkes, B.P., Fujioka, S., Takatsuto, S., Sakurai, A., and Feldmann, K.A (1998). The *DWF4* gene of *Arabidopsis* encodes a cytochrome P450 that mediates multiple 22 α-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell 10, 231-243.

Choe, S., Tanaka, A., Noguchi, T., Fujioka, S., Takatsuto, S., Ross, A.S., Tax, F.E., Yoshida, S., and Feldman, K.A (2000). Lesions in the sterol Δ^7 reductase gene of *Arabidopsis* cause dwarfism due to a block in brassinosteroid biosynthesis. Plant J. 21, 431-443.

Choi, Y.H., Fujioka, S., Harada, A., Yokota, T., Takatsuto, S., and Sakurai, A (1996). A brassinolide biosynthetic pathway via 6-deoxocastasterone. Phytochemistry 43, 593-596.

Choi, Y.H., Fujioka, S., Nomura, T., Harada, A., Yokota, T., Takatsuto, S., and Sakurai, A (1997). An alternative brassinolide biosynthetic pathway via late C-6 oxidation. Phytochemistry 44, 609-613.

Clouse, S.D., Langford, M., and McMorris, T.C (1996). A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol. 111, 671-678.

Clouse, S.D (1996). Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. Plant J.10, 1-8.

Clouse, S.D (1997). Molecular genetic analysis of brassinosteroid action. Physiologia Plantarum 100, 702-709.

Clouse, S.D., and Sasse, J.M (1998). Brassinosteroids: Essential regulators of plant growth and development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 427-451.

Dhaubhadel, S., Chaudhary, S., Dobinson, K.F., and Krishna, P (1999). Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of *Brassica napus* and tomato seedlings. Plant Mol. Biol. 40, 333-342.

Ehsan, H., Ray, W.K., Phinney, B., Wang, X., Huber, S.C., and Clouse, S.D (2005). Interaction of *Arabidopsis* BRASSINOSTEROID-INSENSITIVE 1 receptor kinase with a homolog of mammalian TGF-β; receptor interacting protein. Plant J. 43, 251-261.

Friedrichsen, D.M., Joazeiro, Claudio, A. P., Li, J.M., Hunter, T., and Chory, J. (2000). Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. Plant Physiol. 123, 1247-1256.

Fujioka, S., Inoue, T., Takatsuto, S., Yanagisawa, T., Yokota, T., and Sakurai, A (1995). Identification of a new brassinosteroid, cathasterone, in cultured cells of *Catharantus roseus* as a biosynthetic precursor of teasterone I. Biosci. Biotechnol. Biochem. 59, 1543-1547.

Fujioka, S., and Sakurai, A (1997). Biosynthesis and metabolism of brassinosteroids. Physiologia Plantarum 100, 710-715.

Fujioka, S (1999). Natural occurrence of brassinosteroids in the plant kingdom. Sakurai, A., Yokota, T., Clouse, S.D., Eds., Brassinosteroids. Steroidal Plant Hormones. Tokyo: Springer-Verlag. 253, pp. 21- 45.

Fujioka, S., Noguchi, T., Watanabe, T., Takatsuto, S., and Yoshida, S (2000). Biosynthesis of brassinosteroids in cultured cells of *Catharanthus roseus*. Phytochemistry 53, 549-553.

Fujioka, S., and Yokota, T (2003). Biosynthesis and metabolism of brassinosteroids. Annu. Rev. Plant Biol. 54, 137-164.

Fujioka, S., Takatsuto, S., and Yoshida, S (2002). An early C-22 oxidation branch in the brassinosteroid biosynthetic pathway. Plant Physiol. 130, 930-939.

Fujita, F. (1985). Prospects for brassinolide utilization in agriculture. Chem. Biol. 23, 717-725.

Gampala, S.S., Kim, T.W., He, J.X., Tang. W., Deng, Z., Bai. M.Y., Guan. S., Lalonde, S., Sun, Y.,Gendron, J.M., Chen. H., Shibagaki, N., Ferl, R.J., Ehrhardt, D., Chong, K., Burlingame, A.L.,Wang, ZY (2007) An essential role for 14-3-3 proteins in brassinosteroid signal transduction in *Arabidopsis*. Dev Cell. 13(2):177-89.

Gerard J. Bishop and Csaba, K. (2002). Brassinosteroids and Plant Steroid Hormone Signaling. The plant cell 14, S97-S110.

Grove, M.D., Spencer, G. F., Rohwedder, W. K., Mandava, N., Worley, J. F., Warthen, J. D., Steffens, G. L., Flippenanderson, J. L., and Cook, J. C (1979). Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. Nature 281, 216-217.

He, J.X., Gendron, J.M., Yang, Y.L., Li, J.M., and Wang, Z.Y (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 99, 10185-10190.

He, K., Gou, X., Yuan, T., Lin, H., Asami, T., Yoshida, S., Russell, S.D., and Li, J (2007). BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. Curr. Biol. 17, 1109-1115.

He, Z., Wang, Z-Y., Li, J.M., Zhu, Q., Lamb, C., Ronald, P., and Chory, J (2000). Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. Science 288, 2360-2363.

Heldin, C.-H (1995). Dimerization of cell surface receptors in signal transduction. Cell 80, 213-223.

Ikekawa, T.S., Kitsuwa, T., Saito, H., Morishita, T., and Abe, H (1984). Analysis of natural brassinosteroids by gas chromatography-mass spectrometry. J. Chromatography 290, 289-302.

Janeczko, A., Koscielniak, J., Pilipowicz, M., Szarek-Lukaszewska, G., and Skoczowski, A (2005). Protection of winter rape photosystem 2 by 24-epibrassinolide under cadmium stress. Photosynthetica 43, 293-298.

Janeczko, A., Gullner, G., Skoczowski, A., Dubert, F., and Barna, B (2007). Effects of brassinosteroid infiltration prior to cold treatment on ion leakage and pigment contents in rape leaves. Biologia Plantarum 51, 355-358.

Jiang, J.R., and Clouse, S.D (2001). Expression of a plant gene with sequence similarity to animal TGF-β receptor interacting protein is regulated by brassinosteroids and required for normal plant development. Plant J. 26, 35-45.

Kang, H.-G., and Singh, K.B (2000). Characterization of salicylic acid-responsive, *Arabidopsis* Dof domain proteins: overexpression of OBP3 leads to growth defects. Plant J. 21, 329-339.

Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L., and Altmann, T (1996). Genetic evidence for an essential role of brassinosteroids in plant development. Plant J. 9, 701-713.

Kim TW, Wang ZY., (2010). Brassinosteroid Signal Transduction from Receptor Kinases to Transcription Factors. Annu Rev Plant Biol.61,23.1-23.24

Kim HB, Kwon M, Ryu H, Fujioka S, Takatsuto S, Yoshida S, An CS, Lee I, Hwang I, Choe S (2006) The regulation of DWARF4 expression is likely a critical mechanism in maintaining the homeostasis of bioactive brassinosteroids in *Arabidopsis*. Plant Physiol. 140(2):548-57.

Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Mussig, C., Thomma, B.P.H.J., Albrecht, C., de Vries, S.C., Hirt, H., and Nurnberger, T (2007). The BRI1-Associated Kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. Curr. Biol. 17, 1116-1122.

Khripach, V.A., Zhabinskii, V., and de Groot, A.E. (1999). Brassinosteroids-- a new class of plant hormones (San Diego, USA.).

Kim, T.W., Chang, S.C., Choo, J., Watanabe, T., Takatsuto, S., Takao, Y., Lee, J.S., Kim, S.Y., and Kim, S.K (2000). Brassinolide and [26, 28-H-2(6)]Brassinolide are differently demethylated by loss of c-26 and c-28, respectively, in *Marchantia polymorpha*. Plant Cell Physiol. 41, 1171-1174.

Kinoshita, T., Cano-Delgado, A., Seto, H., Hiranuma, S., Fujioka, S., Yoshida, S., and Chory, J (2005). Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. Nature 433, 167-171.

Kolbe, A., Schneider, B., Porzel, A., Schmidt, J., and Adam, G (1995). Acyl-conjugated metabolites of brassinosteroids in cell-suspension cultures of *Ornithopus-Sativus.* Phytochemistry 38, 633-636.

Kolbe, A., Schneider, B., Porzel, A., and Adam, G (1998). Metabolic inversion of the 3-hydroxy function of brassinosteroids. Phytochemistry 48, 467-470.

Krishna, P (2003). Brassinosteroid-Mediated Stress Responses. J. Plant Growth Regul. 22, 289-297.

Li, J., Lease, K.A., Tax, F.E., and Walker, J.C (2001). BRS1, a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 98, 5916-5921.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell 110, 213-222.

Li, J., and Gou, X (2007). Brassinosteroids. In: Encyclopedia of Life Sciences. John Wiley & Sons, Ltd: Chichester http://www.els.net/ [DOI: 1002/9780470015902. a0020092]

Li, J.M., Nagpal, P., Vitart, V., McMorris, T.C., and Chory, J (1996). A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science 272, 398-401.

Li, J.M., and Chory, J (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90, 929-938.

Li, J.M., Nam, K.H., Vafeados, D., and Chory, J (2001) *BIN2*, a new brassinosteroid-insensitive locus in *Arabidopsis.* Plant Physiol. 127, 14-22.

Li, J.M., and Nam, K.H (2002). Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. Science 295, 1299-1301.

Li, J.M., and Jin, H (2007). Regulation of brassinosteroid signaling. Trends Plant Sci. 12, 37-41.

Li L, Yu X, Thompson A, Guo M, Yoshida S, Asami T, Chory J, Yin Y (2009). *Arabidopsis* MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. Plant J. 58(2):275-86.

Lim, U.K., and Han, S. S (1988). The effect of plant growth regulating brassinosteroid on early state and yield of corn. Seoul. Natl. Univ. Agricul. Sci. 13, 1-14.

Mandava, N.B., Worley, J.F., Mathees D., Warthen Jr., J.D., Jacobson, M., Steffens, G.L., Kenney, H., and Grove, M.D (1978). Isolation of brassins by extraction of rape (*Brassica napus*) pollen. Indust. Engin. Chem. Prod. Res. Develop. 17, 351-354.

Mandava, N.B (1988). Plant growth-promoting brassinosteroids. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 23-52.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M (1995). The nuclear receptor superfamily: The second decade. Cell 83, 835-839.

Marsolais, F., Boyd, J., Paredes, Y., Schinas, A.-M., Garcia, M., Elzein, S., and Varin, L (2007). Molecular and biochemical characterization of two brassinosteroid sulfotransferases from *Arabidopsis*, *AtST4a* (*At2g14920)* and *AtST1* (*At2g03760).* Planta 225, 1233-1244.

Massague, J., Attisano, L., and Wrana, J.L (1994). The TGF-β family and its composite receptors. Trends Cell Biol. 4, 172-178.

McCarty, D.R., and Chory, J (2000). Conservation and innovation in plant signaling pathways. Cell 103, 201–209.

Mitchell, J.W., and Whitehead, M. R (1941). Responses of vegetative parts of plants following application of extract of pollen from *Zea mays*. Botanical Gazette 102, 770-790.

Mitchell, J.W., Worley, J.F., Plimmer, J.R., and Smith, M.V (1970). Brassins --a new family of plant hormones from rape pollen. Nature 225, 1065-1066.

Mitchell, J.W., and Gregory, L.E (1972). Enhancement of overall plant growth, a new response to brassins. Nat. New Biol. 239, 253-254.

Mora-Garcia, S., Vert, G., Yin, Y.H., Cano-Delgado, A., Cheong, H., and Chory, J (2004). Nuclear protein phosphatases with Kelch-repeat domains modulate the response to bras sino steroids in *Arabidopsis*. Genes Dev. 18, 448-460.

Müssig, C (2005). Brassinosteroid-promoted growth. Plant Biol., 110-117.

Nakamura, M., Satoh, T., Tanaka, S., Mochizuki, N., Yokota, T., and Nagatani, A (2005). Activation of the cytochrome P450 gene, *CYP72C1*, reduces the levels of active brassinosteroids *in vivo*. J. Exp. Bot. 56, 833-840.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I., and Yoshida, S (2003). Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. Plant J. 33, 887-898.

Nam, K.H., and Li, J. M (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell 110, 203-212.

Nam, K.H., and Li, J (2004). The *Arabidopsis* transthyretin-like protein is a potential substrate of BRASSINOSTEROID-INSENSITIVE 1. Plant Cell 16, 2406-2417.

Neff, M.M., Nguyen, S.M., Malancharuvil, E.J., Fujioka, S., Noguchi, T., Seto, H., Tsubuki, M., Honda, T., Takatsuto, S., Yoshida, S., and Chory, J (1999) *BAS1*: A

gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 96, 15316-15323.

Nishikawa, N., Abe, H., Natsume, M., Shida, A., and Toyama, S (1995). Epimerization and conjugation of C-14-labeled epibrassinolide in cucumber seedlings. J. Plant Physiol. 147, 294-300.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K.A., and Tax, F.E (1999). Brassinosteroid-insensitive dwarf mutants of *Arabidopsis* accumulate brassinosteroids. Plant Physiol. 121, 743-752.

Nomura, T., Bishop, G.J., Kaneta, T., Reid, J.B., Chory, J., and Yokota, T (2003). The *LKA* gene is a *BRASSINOSTEROID INSENSITIVE 1* homolog of pea. Plant J. 36, 291-300.

Ogweno, J., Song, X., Shi, K., Hu, W., Mao, W., Zhou, Y., Yu, J., and Nogués, S (2007). Brassinosteroids Alleviate Heat-Induced Inhibition of Photosynthesis by Increasing carboxylation efficiency and enhancing antioxidant systems in *Lycopersicon esculentum*. J. Plant Growth Regul (Online, DOI 10.1007/s00344-007-9030-7)

Özdemir, F., Bor, M., Demiral, T., and Türkan, İ (2004). Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidative system of rice (*Oryza sativa L.*) under salinity stress. Plant Growth Regul. 42, 203-211.

Poppenberger, B., Fujioka, S., Soeno, K., George, G.L., Vaistij, F.E., Hiranuma, S., Seto, H., Takatsuto, S., Adam, G., Yoshida, S., and Bowles, D (2005). The UGT73C5

of *Arabidopsis thaliana* glucosylates brassinosteroids. Proc. Natl. Acad. Sci. USA 102, 15253-15258.

Prusakova, L.D., Ezhov, M. N., and Salnikov, A. I (1999). The use of emistim, epibrassinolide and uniconazole to overcome quality difference of buckwheat grains. Agrarian Russia, 41-44.

Pustovoitova, T.N., Zhdanova, N. E., and Zholkevich, V. N (2001). Epibrassinolide increases plant drought resistance. Doklady Biochem. Biophys. 376, 36-38.

Richter, K., Koolman, J (1991). Antiecdysteroid effects of brassinosteroids. In: Cutler H.G., Yokota, T., and Adam, G. Eds., Brassinosteroids - Chemistry, Bioactivity and Applications, pp. 265-278. Am. Chem. Soc., Washington, USA.

Rouleau, M., Marsolais, F., Richard, M., Nicolle, L., Voigt, B., Adam, G., and Varin, L (1999). Inactivation of brassinosteroid biological activity by a salicylate-inducible steroid sulfotransferase from *Brassica napus.* J. Biol. Chem. 274, 20925-20930.

Sairam, R (1994). Effects of homobrassinolide application on plant metabolism and grain yield under irrigated and moisture-stress conditions of two wheat varieties. Plant Growth Regul. 14, 173-181.

Sakurai, A., Yokota, T., Clouse, S.D. (eds), (1999). Brassinosteroids - Steroidal Plant Hormones. Springer Tokyo, Japan. , pp.91-111

Sasse, J (1999). Physiological actions of brassinosteroids. In: Sakurai A, Yokota T, Clouse, S.D. (eds), Brassinosteroids - Steroidal Plant Hormones, Springer Tokyo, Japan. pp.137-161.

Schilling, G., Schiller, C., and Otto, S. (1991). Influence of brassinosteroids on organ relations and enzyme activities of sugar beet plants. In: Cutler, HG, Yokota, T.,

Adam, G. Eds., Brassinosteroids: Chemistry, Bioactivity and Applications. ACS Symp Ser 474, Am. Chem. Soc., Washington, DC, pp 208-219.

Shimada, Y., Goda, H., Nakamura, A., Takatsuto, S., Fujioka, S., Yoshida, S. (2003). Organ-specific expression of brassinosteroid-biosynthetic genes and distribution of endogenous brassinosteroids in *Arabidopsis*. Plant Physiol. 131(1):287-97.

Symons, G.M., Ross, J.J., Jager, C.E., Reid, J.B. (2008). Brassinosteroid transport. J Exp Bot. 59(1):17-24.

Shimofurutani, N., Kisu, Y., Suzuki, M., and Esaka, M (1998). Functional analyses of the Dof domain, a zinc finger DNA-binding domain, in a pumpkin DNA-binding protein AOBP. Febs Lett. 430, 251-256.

Soeno, K., Kyokawa, Y., Natsume, M., and Abe, H (2000). Teasterone-3-*O*-β-D-glucopyranoside, a new conjugated brassinosteroid metabolite from lily cell suspension cultures and its identification in lily anthers. Biosci. Biotechnol. Biochem. 64, 702-709.

Steber C.M. & McCourt P (2001). A role for brassinosteroids in germination in *Arabidopsis*. Plant Physiology 125, 763–769.

Suzuki, H., Fujioka, S, Takatsuto, S, Yokota, T, Murofushi, N, Sakurai, A (1994). Biosynthesis of brassinolide from teasterone via typhasterol and castasterone in cultured cells of *Catharantus roseus*. Plant Growth Regul. 13, 21-26.

Suzuki, H., Fujioka, S., Takatsuto, S., Yokota, T., Murofushi, N., and Sakurai, A (1995). Biosynthesis of brassinosteroids in seedlings of *Catharanthus-roseus*, *Nicotiana-tabacum*, and *Oryza-sativa*. Biosci. Biotechnol. Biochem. 59, 168-172.

Suzuki, H., Inoue, T., Fujioka, S., Saito, T., Takatsuto, S., Yokota, T., Murofushi, N., Yanagisawa, T., and Sakurai, A (1995). Conversion of 24-methylcholesterol to 6-oxo-24-methylcholestanol, a putative intermediate of the biosynthesis of brassinosteroids, in cultured-cells of *Catharanthus-roseus*. Phytochemistry 40, 1391-1397.

Symons, G.M., and Reid, J.B (2004). Brassinosteroids do not undergo long-distance transport in pea. Implications for the regulation of endogenous brassinosteroid levels. Plant Physiol. 135, 2196-2206.

Szekeres, M., Nemeth, K., KonczKalman, Z., Mathur, J., Kauschmann, A., Altmann, T., Redei, G.P., Nagy, F., Schell, J., and Koncz, C (1996). Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell 85, 171-182.

Takahashi, N., Nakazawa, M., Shibata, K., Yokota, T., Ishikawa, A., Suzuki, K., Kawashima, M., Ichikawa, T., Shimada, H., and Matsui, M (2005). *shk1-D*, a dwarf *Arabidopsis* mutant caused by activation of the *CYP72C1* gene, has altered brassinosteroid levels. Plant J. 42, 13-22.

Takeno, K., and Pharis, R. P (1982). Brassinolide-induced bending of the lamina of dwarf rice seedlings: an auxin mediated phenomenon. Plant Cell Physiol. 23, 1275-1281.

Takeuchi, Y (1992). Studies on the physiology and applications of brassinosteroids. Chemical Regul. Plants 27, 1-10.

Tanaka, K., Asami, T., Yoshida, S., Nakamura, Y., Matsuo, T., Okamoto, S. (2005) Brassinosteroid homeostasis in *Arabidopsis* is ensured by feedback expressions of multiple genes involved in its metabolism. Plant Physiol. 138(2):1117-25.

Tang, W., Kim, T.W. (2008). Oses-Prieto JA, Sun Y, Deng Z, Zhu S, Wang R, Burlingame AL,Wang ZY. BSKs mediate signal transduction from the receptor kinase BRI1 in *Arabidopsis*. Science 321(5888): 557-60.

Thompson, M.J., Meudt, W.J., Lusby, W.R., Spaulding, D.W (1981). Synthesis and biological activity of brassinolide and its 22b,23b-isomer: novel plant growth-promoting steroids. Steroids 38, 567-580.

Thompson, M.J., Mandava, N.B., Dutky, S.R., Lusby, W.R., and Spaulding, D.W (1982). Synthesis of brassinosteroids and relationship of structure to plant growth-promoting effect. Steroids 39, 89-105.

Thummel, C.S., and Chory, J (2002). Steroid signaling in plants and insects---common themes, different pathways. Genes Dev. 16, 3113-3129.

Turk, E.M., Fujioka, S., Seto, H., Shimada, Y., Takatsuto, S., Yoshida, S., Denzel, M.A., Torres, Q.I., and Neff, M.M (2003). CYP72B1 inactivates brassinosteroid hormones: an intersection between photomorphogenesis and plant steroid signal transduction. Plant Physiol. 133, 1643-1653.

Upreti, K.K., and Murti, G.S.R (2004). Effects of brassmosteroids on growth, nodulation, phytohormone content and nitrogenase activity in French bean under water stress. Biologia Plantarum 48, 407-411.

Vert, G., Nemhauser, J.L., Geldner, N., Hong, F.X., and Chory, J (2005). Molecular mechanisms of steroid hormone signaling in plants. Annu. Rev. Cell Dev. Biol. 21, 177-201.

Vert, G., and Chory, J (2006). Downstream nuclear events in brassinosteroid signalling. Nature 441, 96-100.

Volynets, A.P., Pschenichanye, L.A., and Khripach, V. A (1997). The nature of protective action of 24-epibrassinolide on barley plants. Plant Growth Regul. Soc. Am. 24, 133-137.

Wachsman, M., Lopez, E.M., Ramirez, J.A., Galagovsky, L.R., and Coto, C.E (2000). Antiviral effect of brassinosteroids against herpes virus and arenaviruses. Antiviral Chem. Chemother 11, 71-77.

Wachsman, M., Ramirez, J.A., Galagovsky, L.R., and Coto, C.E (2002). Antiviral activity of brassinosteroid derivatives against measles virus in cell cultures. Antiviral Chem. Chemother 13, 61-66.

Wada, K., Marumo, S., Ikekawa, N., Morisaki, M., and Mori, M (1981). Brassinolide and homobrassinolide promotion of lamina inclination of rice seedlings. Plant Cell Physiol. 22, 323-326.

Wang, Z.Y., Seto, H., Fujioka, S., Yoshida, S., and Chory, J (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature 410, 380-383.

Wang, Z.Y., Nakano, T., Gendron, J., He, J.X., Chen, M., Vafeados, D., Yang, Y.L., Fujioka, S., Yoshida, S., Asami, T., and Chory, J (2002). Nuclear-localized BZR1

mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev. Cell 2, 505-513.

Wang, X., Goshe, M.B., Soderblom, E.J., Phinney, B.S., Kuchar, J.A., Li, J., Asami, T., Yoshida, S., Huber, S.C., and Clouse, S.D (2005). Identification and functional analysis of *in vivo* phosphorylation sites of the *Arabidopsis* BRASSINOSTEROID-INSENSITIVE1 receptor kinase. Plant Cell 17, 1685-1703.

Wang, X., and Chory, J (2006). Brassinosteroids regulate dissociation of BKI1, a negative regulator of BRI1 signaling, from the plasma membrane. Science 313, 1118-1122.

Watanabe, T., Noguchi, T., Yokota, T., Shibata, K., Koshino, H., Seto, H., Kim, S.K., and Takatsuto, S (2001). Synthesis and biological activity of 26-norbrassinolide, 26-norcastasterone and 26-nor-6-deoxocastasterone. Phytochemistry 58, 343-349.

Watanabe, T., Yokota, T., Shibata, K., Nomura, T., and Seto, H. (2000). Cryptolide, a new brassinolide catabolite with a 23-oxo group from Japanese cedar pollen/anther and its synthesis. J. Chem. Res (S), 18- 19.

Winter, J., Schneider, B., Strack, D., and Adam, G (1997). Role of a cytochrome P450-dependent monooxygenase in the hydroxylation of 24-epi-brassinolide. Phytochemistry 45, 233-237.

Yin, Y.H., Wang, Z.Y., Mora-Garcia, S., Li, J.M., Yoshida, S., Asami, T., and Chory, J (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell 109, 181-191.

Yokota, T (1997). The structure, biosynthesis and function of brassinosteroids. Trends in Plant Sci. 2, 137-143.

Yokota, T, A.M., and Takahashi, N (1982). Castasterone, a new phytosterol with plant hormone activity from chestnut insect gall. Tetrahedron Letters 23, 1275-1278.

Yu, J.Q., Zhou, Y. H., Ye, S.F., and Huang, L. F (2002). 24-epibrassinolide and abscisic acid protect cucumber seedlings from chilling injury. J. Horticult. Sci. Biotechnol. 77, 470-473.

Yu, J.Q., Huang, L.F., Hu, W.H., Zhou, Y.H., Mao, W.H., Ye, S.F., and Nogues, S (2004). A role for brassinosteroids in the regulation of photosynthesis in *Cucumis sativus*. J. Exp. Bot. 55, 1135-1143.

Yuan, T., Fujioka, S., Takatsuto, S., Matsumoto, S., Gou, X.P., He, K., Russell, S.D., and Li, J (2007). *BEN1,* a gene encoding a dihydroflavonol 4-reductase (DFR)-like protein, regulates the levels of brassinosteroids in *Arabidopsis thaliana*. Plant J. 51, 220-233.

Yu, X., Li, L., Li, L., Guo, M., Chory, J., Yin, Y. (2008). Modulation of brassinosteroid-regulated gene expression by Jumonji domain-containing proteins ELF6 and REF6 in *Arabidopsis*. Proc Natl Acad Sci U S A. 105(21):7618-23.

Zhou, A., Wang, H., Walker, J.C., and Li, J (2004). BRL1, a leucine-rich repeat receptor-like protein kinase, is functionally redundant with BRI1 in regulating *Arabidopsis* brassinosteroid signaling. Plant J. 40, 399-409.

Zhou, A., and Li, J (2005). *Arabidopsis* BRS1 is a secreted and active serine carboxypeptidase. J. Biol. Chem. 280, 35554-35561.

Zullo, M.A.T., and Adam, G (2002). Brassinosteroid phytohormones: structure, bioactivity and applications. Plant Physiol. 14, 143-181.

Chapter II

Genetic modifier screen on the weak allele BR receptor mutant via activation tagging

1. Abstract

Identifying and characterizing mutants are efficient methods to dissect a genetic pathway. In the BR pathway, the classic loss of function screening has been extensively utilized to identify related mutants. However, although several genes related to the BR biosynthesis pathway have been successfully identified, only BR receptor BRI1 was repeatedly obtained for the BR signaling pathway, which suggests the functional redundancy may exist for other molecules in BR signaling. To overcome this barrier, a gain of function approach, activation-tagging, has been used to screen for genetic modifiers mutant for the weak allele BR receptor mutants in our lab. In my research, through a large scale activation tagging screening, several promising mutants were successfully isolated for further characterization.

2. Introduction

Screening loss-of-function mutant is routinely used to elucidate genetic mechanisms in plant molecular biology. Although it is an efficient method, it also shows obvious limitations. For example, if one molecule is essential for plant survival, it will be impossible to obtain a loss of function mutant. If one gene has redundantly functional homologs or its functions can be replaced by an alternative pathway, it will be rarely observe a substantial phenotype for its loss of function mutant (Weigel, 2000; Nakazawa, 2003; Tani, 2004). In the genome of *Arabidopsis*, functional redundancy exists for over 70% all of genes, which limits the application of loss of function screening in *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000). To overcome this obstacle, activation tagging screening, a gain of function screening strategy, was developed. In the scheme of activation-tagging, four tandem copies of the cauliflower mosaic virus

(CaMV) 35S enhancer sequence were engineered into T-DNA region in a binary transformation vector. When the vector was transformed into a host plant, the T-DNA containing 35S enhancers will be randomly inserted into the genome of the host plant. Those enhancers will then enhance transcription of neighboring genes on either side of the insertion, which will result in gain-of-function analysis (Hayashi, 1992; Kardailsky, 1999; Weigel, 2000) Activation tagging was first used in identifying a His kinase in tissue culture of *Arabidopsis* (Kakimoto, 1996). From then on, it has been widely applied to study hormone pathways, plant development, metabolism and disease resistance in *Arabidopsis* (Kardailsky, 1999; Neff, 1999; van der Graaff, 2000; 2002; Li J, 2001; Borevitz, 2002; Busov, 2003; Grant, 2003; Hayashi, Niwa, 2006; Perrella, 2006;)

However, since BR mutants are sterile, activation tagging was not used in BR pathway until a weak allele *bri1* mutant was identified. As shown in Figure 1.2 in chapter I, *bri1-5* is an intermediate BR receptor mutant compared to null allele *bri1-4*. Although *bri1-5* still shows typical defects of BR mutants such as dwarfism, curled leaves and small rosette size, it is completely fertile, which makes it an ideal genetic background for activation-tagging screening. BRS1 and BAK1 are the first two regulators of BR signaling identified through activation tagging from *bri1-5* background (Li, 2001; Li, 2002), and both genes have several homologs with high identities in sequence. Especially for BAK1, some of its homologs function redundantly to BAK1 in the BR signaling pathway (Karlova, 2006; He, 2007; Albrecht, 2008). After that, more regulators of the BR pathway were identified using the gain of function screening, such as BEN1 (Tong *et al.*, 2007). Now activation tagging is routinely used for generating genetic modifier

mutants in BR pathway and other genetic pathways in plants (Busov, 2003; Grant, 2003; Niwa, 2006; Perrella, 2006; Zhang, 2006; Kondou, 2008; Aboul-Soud, 2009).

As shown in Figure 2.1, two weak allele BR receptor mutants, *bri1-5* and *bri1-9*, were used to screen genetic modifier mutants via activation tagging. The activation vector was first transformed into *bri1-5* or *bri1-9* through Agro-bacteria mediated transformation. Once the T-DNA containing four copies of 35S enhancers are randomly inserted and integrated into the genome of *bri1-5* or *bri1-9*, the enhancers will activate the transcription of those genes flanking enhancers. If a positive regulator in BR biosynthesis or signaling pathway is activated by enhancers, its over-expression would partially or even completely rescue defects of *bri1-5* or *bri1-9*, which will lead to a suppressor mutant with rescued phenotypes. On the other hand, if the gene plays negative role, its over-expression will negatively impact BR biosynthesis or BR signaling pathway, which will result in an enhancer with more severe defects (Li, 2001). Since the T-DNA is randomly inserted into a genome, all single genes in the genome could be tagged and activated. Theoretically, if a big enough activation tagging transformant pool is generated, all genes functioning in BR biosynthesis or signaling pathway could be identified theoretically.

3. Results

3.1 Suppressors and enhancers identified from activation-tagging screening

Twenty plates of *bri1-5* mutant and 20 plates of *bri1-9* mutants were grown. After 4 weeks, plants bearing flower buds was ready for *Agrobacterium*-mediated transformation. About 8000 transformants were successfully generated from activation-tagging screening. Among these transformants, several suppressors and enhancers were

successfully isolated. As shown in Figure 2.2a, two suppressors and two enhancers were obtained from *bri1-5* background. One suppressor is *serk1-1D*, the other is *tcp1-1D*. Both suppressor have much larger rosette, and are significant taller than the genetic background *bri1-5*. Two enhancers, *bri1-5G19-1D* and *bri1-5 cur-1D*, displayed more severe defects in growth and development compared to *bri1-5*. As shown in Figure 2.2b, from *bri1-9* background, three suppressors, *bri1-9-bri1-1D* and *bri1-9-brl3-1D* and *bri1-9-203-1D*, were obtained. All three suppressors are taller than genetic background and show larger rosette with expanded leaves. Among all suppressors obtained, *bri1-9-bri1-1D* and *bri1-9-brl3-1D* mutants showed best rescued phenotypes, they almost resemble wildtype plants (Figure 2.3).

3.2 Determination of T-DNA insertion site by TAIL-PCR

To find the T-DNA insertion site in the genome, TAIL-PCR was performed. TAIL-PCR is an efficient method to amplify unknown sequences adjacent to known sequences resulting from T-DNA insertion in chromosome. In TAIL-PCR, nested specific primers are designed according to a known sequence. These primers together with arbitrary degenerate primers are then used to conduct PCR with template DNA extracted from transformants (Liu and Whittier, 1995; Terauchi and Kahl, 2000). After sequencing the PCR product, the T-DNA insertion site in the genome can be determined by comparing the sequence with sequence database of TAIR. As shown in Figure 2.4, the T-DNA insertion sites were determined for all above suppressors and enhancers. Among these suppressors or enhancers, SERK1 is known as the homolog of BAK1, the BR co-receptor. SERK1 was originally reported to involve in microsporogenesis, which

is independent of the BR pathway (Hecht, 2001). Recently it was demonstrated that SERK1 also functions redundantly to BAK1 in the BR signaling pathway (Karlova, 2006; Albrecht, 2008). The receptor of BRs, BRI1, and its homolog BRL3 were also identified in our screening. Although BRL3 was thought to be not as important as BRI1, *brl3-1D* activation tagging line almost shows phenotypes similar to wildtype plants (Zhou, 2004; Caño-Delgado, 2004). As to the other two suppressors, TCP1 is a plant specific transcription factor and MYB21 is a member of the MYB transcription factor family in *Arabidopsis*. Transcription factor TFIIB and F-box protein CUR were activation tagged respectively in *bri1-5G19-1D* and *bri1-5cur-1D*. Among these molecules, although BRI1, BRL3 and SERK1 represent known entities in the BR pathway, four new promising molecules were obtained in BR pathway. To understand their potential functions in BR pathway. All four suppressors or enhancers were further characterized. Results for *bri1-5-G19-1D* and *bri1-9-203-1D* are described in this chapter. The detailed analyses of *bri1-5tcp1-1D* and *bri1-5cur-1D* are presented in Chapter III and Chapter IV respectively.

3.3 G19-1D enhancer

Compared to *bri1-5*, *bri1-5G19-1D* showed even more severe defects; it is dwarfer and much smaller with curled leaves. After being crossed with wild-type WS2 to segregate out mutated BRI1, *WS2-G19-1D* also showed dwarf statue with curled leaves, suggesting a function in plant growth and development. After determining T-DNA insertion site, it was found that *35S* enhancers are located nearby *At3g57370*, about 930 bp upstream of the start-codon. *At3g57370* encodes a TFIIB transcription factor, whose function is to recruit RNA polymerase II to the promoter to form a transcription initiation complex

(Greenblatt, 1992; Rowlands, 1994). To find out whether or not the TFIIB transcription factor is truly activated, RT-PCR analysis was performed. The result indicated that the transcription of *At3g57370* was elevated 2-fold in the enhancer mutant compared to background *bri1-5*. To further confirm *At3g57370* is real candidate gene of the *bri1-5G19-1D* enhancer, the *TFIIB* was cloned and over-expressed in either *bri1-5* or WS2, driven by the 35S promoter. Although no transgenic lines were obtained with *bri1-5*, over-expression of *At3g57370* in WS2 generated transgenic lines with phenotypes similar to *WS2-G19-1D* (Figure 2.5), which suggests that the TFIIB transcription factor is responsible for enhanced phenotype in *bri1-5G19-1D*. Since it is a general TFIIB transcription factor, we hypothesized that it may negatively control the expression of BR response genes. However, its target genes need to be identified to clarify its true roles in the BR pathway through further research.

3.4 ***bri1-9-203-1D*** suppressor

bri1-9-203-1D exhibits significantly suppressed phenotypes with higher inflorescence and larger rosettes compared to *bri1-9*. The *35S* enhancer cassette inserted in the promoter of *At3g27810*, *MYB21*, in the *bri1-9~203-1D* suppressor, RT-PCR analysis indicated that the transcription of *MYB21* was elevated about 4 fold in the suppressor compared to *bri1-9*. The recapitulation experiment demonstrated that over-expression of *MYB21* in *bri1-9* resulted in suppressed phenotypes similar to *bri1-9-203-1D* (Figure 2.6). These results suggested MYB21 maybe a positive regulator involved in the BR biosynthesis or signaling pathway in *Arabidopsis*. In recent studies, AtMYB30 null mutants display decreased BR responses and enhance the dwarf phenotype of a weak

allele of the BR receptor mutant *bri1*. AtMYB30 was further identified as a direct target gene of BES1. AtMYB30 could function to regulate the expression of BR response genes (Li L., 2009). These results prompted us to think that its homolog-MYB20 may be also a regulator of BR signaling in *Arabidopsis*.

4. Discussion

Functional redundancy commonly exists in the genome of plants, which greatly limits the application of loss of function analysis in research. The problem also exists in the studies of BR signaling pathway in *Arabidopsis* since loss of function screening only identified BR receptor repeatedly. An alternative gain of function approach, activation tagging, was used to identify those molecules of the BR pathways using weak BR mutants, *bri1-5* and *bri1-9*. When a positive regulator is activation-tagged by enhancers, its activation will rescue the developmental defects of the background mutants, resulting in a suppressor. On the other hand, when a negative regulator is tagged, its activation will produce an enhancer with enhanced developmental defects. Several important regulators of the BR pathway have been identified through the genetic modifier of BR weak allele mutants via activation tagging. In our research, several interesting mutants were obtained in a large scale screening. After cloning the candidate genes, it was found that some well known BR regulators such as BR receptor itself BRI1, its homolog BRL3 (Zhou, 2004; Caño-Delgado, 2004), homolog of BR co-receptor receptor-SERK1, and some novel loci were successfully activation tagged. The result further indicates that genetic modifier screening via activation tagging is an efficient approach for dissecting the BR pathway in *Arabidopsis*.

However, not many suppressors or enhancers were obtained through the large scale activation tagging screening as we originally expected. Even some important loci with known function in the BR signaling were not tagged. For example, as mentioned in Chapter I, BSK1 was identified as the substrate of BRI1 by biochemical analysis, over-expression of BSK1 significantly rescued the weak allele BR mutant, *bri1-5* (Tang, 2008). However, BSK1 was not activation tagged in our research. Probably it is because the transformant pool generated by activation tagging is not big enough. Another reason may be that T-DNA does not insert into specific regions of the genome. It could be true, since the exact mechanism for *Agrobacterium*-mediated transformation is still unclear. In the case, many molecules in the BR pathway will not be activation tagged. In addition, we cannot exclude that over-expression of some genes in the BR pathway may be lethal for plants, which make it impossible to obtain gain of function mutants for the loci via activation tagging.

Another problem in our research is that one genetic modifier mutant may contain multiple T-DNA insertions. In the case, it will be almost impossible to determine the T-DNA insertion sites through TAIL-PCR, since multiple PCR products will be amplified at the same time, which makes the products impossible to be sequenced. In addition, even when all insertion sites can be determined, it will be painstaking to find out which insertion is responsible the mutant, or are several insertions responsible for the mutant together. For this reason, we did not characterize some of mutants. In order to obtain more promising suppressors or enhancers, new transformation strategies need to be developed, and more transformants need to be generated, and alternative efficient cloning techniques need to be applied in the genetic modifier screening via activation tagging.

5. Experimental procedure and methods

5. 1. Plant material and growth condition

Both of *bri1-5* or *bri1-9* seeds are WS2 ecotypes. b*ri1-5* or *bri1-9* seeds were stratified in water under 4°C for two days, and grown at 23-25°C with 18 hours under light and 8 hours under dark until ready for transformation.

5. 2. *Agrobacterium*-mediated transformation through floral dipping method

5.2.1. The activation tagging vector, pBIB-basta-AT2, was first transformed into *Agrobacterium* strain GV3101. The transformants were screened in the Agar LB plate with kanamycin 50ug/ml and gentamycin 30ug/ml to select for transformant carrying the binary plasmid.

5. 2.2. *Agrobacterium tumefaciens* strain GV3101 carrying the activation tagging vector were cultured at 28°C in liquid LB with in liquid LB media with kanamycin 50ug/ml and gentamycin 30ug/ml overnight.

5. 2.3. Spin down Agrobacterium 5000 rpm at room temperature, resuspend in fresh 5% sucrose solution to an OD_{600} = 0.8.

5. 2.4. Silwet L-77 was added to a concentration of 0.05% and mix well.

5. 2.5. Dip plant inflorescence in the Agrobacterium solution for 10-15 seconds, with gentle agitation.

5. 2.6. Cover dipped plants for 16 to 24 hours to maintain high humidity, then water and grow plants normally to produce T1 seeds.

5. 3. Identification of suppressors or enhancers

Plant T1 seeds in soil to screen for transformants with herbicide, and identify suppressor and enhancer according to their phenotypes. Transformants with rescued

phenotypes represent potential suppressors; those displaying more severe phenotypes represent potential enhancers.

5. 4. Determination of T-DNA insertion site by Thermal-Asymmetric-Interlaced PCR (TAIL-PCR).

TAIL-PCR was conducted according to the protocol described previously (Liu and Whittier, 1995; Terauchi and Kahl, 2000).). Briefly DNA is extracted from detected mutants with the Small Prep DNA kit from invitrogen. Three nested primers TR1, TR2, TR3 were designed according to specific sequence of T-DNA. Primary PCR was conducted with TR1 together with random primer AD1 for 10 reduced stringency cycles. Then 100 fold dilution of primary PCR product was used as template to do secondary PCR with TR2 and AD primer for 10 super cycles. Finally 50-fold dilution of secondary PCR products was used to do tertiary PCR with TR3 and AD for 20 normal cycles. Tertiary PCR product was collected back from electrophoresis Gel for sequencing. Sequencing results were compared to a database by BLAST to determine the T-DNA insertion site in the genome.

5. 5. Examination of expression level by RT-PCR

Total RNA was isolated using RNeasy plant mini kits with on-column DNase-treatment (Qiagen; http://www.qiagen.com/). 2 µg total RNA was reversedly transcripted to a first strand of cDNA in a 20 µl volume using the SuperScript III first-strand synthesis system (Invitrogen; http://www.invitrogen.com/). One microliter volume of RT product was used as a template in 20 µl volume PCR. PCR products were separated and visualized by 1% agarose gel with EB by electrophoresis.

RT-PCR primer-pair for *At3g*57370, TFIIB-F: 5'-ATGACGATGAAGTGGGGTCACAG-3' and TFIIB-R: 5'-CTAAGGAGCTCCAAGGTTTTTCAG-3'; RT-PCR primer-pair for MYB21, MYB21-F: 5'-ATGGAGAAAAGAGGAGGAGGAAG-3' and MYB21-R: 5'-TCAATTACCATTCAATAAATGCA-3', RT-PCR primer-pair for *EF1*α, *EF1*α-F: 5'-CAGGCTGATTGTGCTGTCCT-3' and *EF1*α-R: 5'-TCAAGTAGCAAAATCACGGCGCTT-3'.

5. 6. Recapitulation experiment

The CDS of *At3g57370* was amplified from *bri1-5G19-1D* with primer pair TFIIB-attb1: 5'-GTACAAAAAAGCAGGCTATGACGATGAAGTGGGGTCACAG-3' and TFIIB-attb2: 5'-GTACAAGAAAGCTGGGTCTAAGGAGCTCCAAGGTTTTTCAG-3'; The CDS of *Myb21*was amplified from *bri1-9-203-1D* with primer pair MYB21-attb1: 5'-GTACAAAAAAGCAGGCTATGGAGAAAAGAGGAGGAGGAAG-3' and MYB21-attb2: 5'-GTACAAGAAAGCTGGGTTCAATTACCATTCAATAAATGCA-3'. Both CDS were respectively cloned into vector pBIB-BASTA-35S-GW with Gateway strategy as described (Yuan, 2008). MYB21 was over-expressed in *bri1-9* plants. *At3g57370* was over-expressed in *bri1-5* and WS2 respectively. Expression levels of transgenes were further determined by RT-PCR.

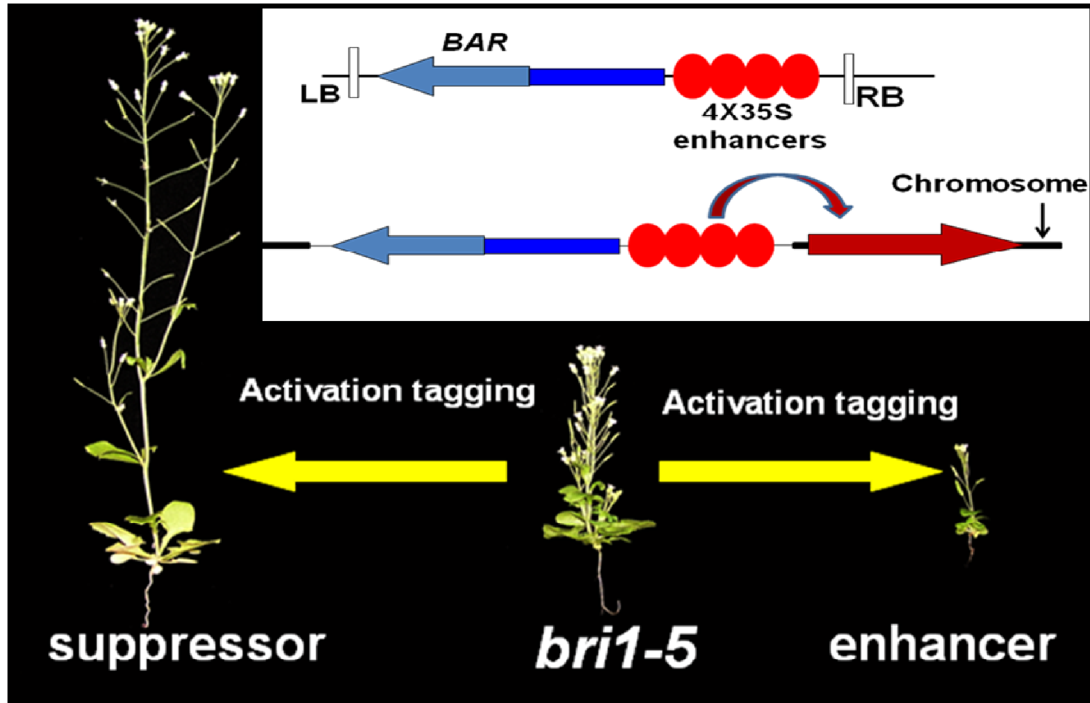

Figure **2.1** Genetic modifier screen though activation tagging. Activation tagging vector is transformed into a weak allele BR mutant *bri1-5*. If a positive regulator is activated, overexpression of the positive regulator will rescue *bri1-5*, resulting in a suppressor mutant. On the other hand, if a negative component is activated, an enhancer will be produced.

Figure **2.2a**, Four-week-old suppressors and enhancers identified from *bri1-5* background.

Figure **2.2b**, four-week-old suppressors identified from *bri1-9* background.

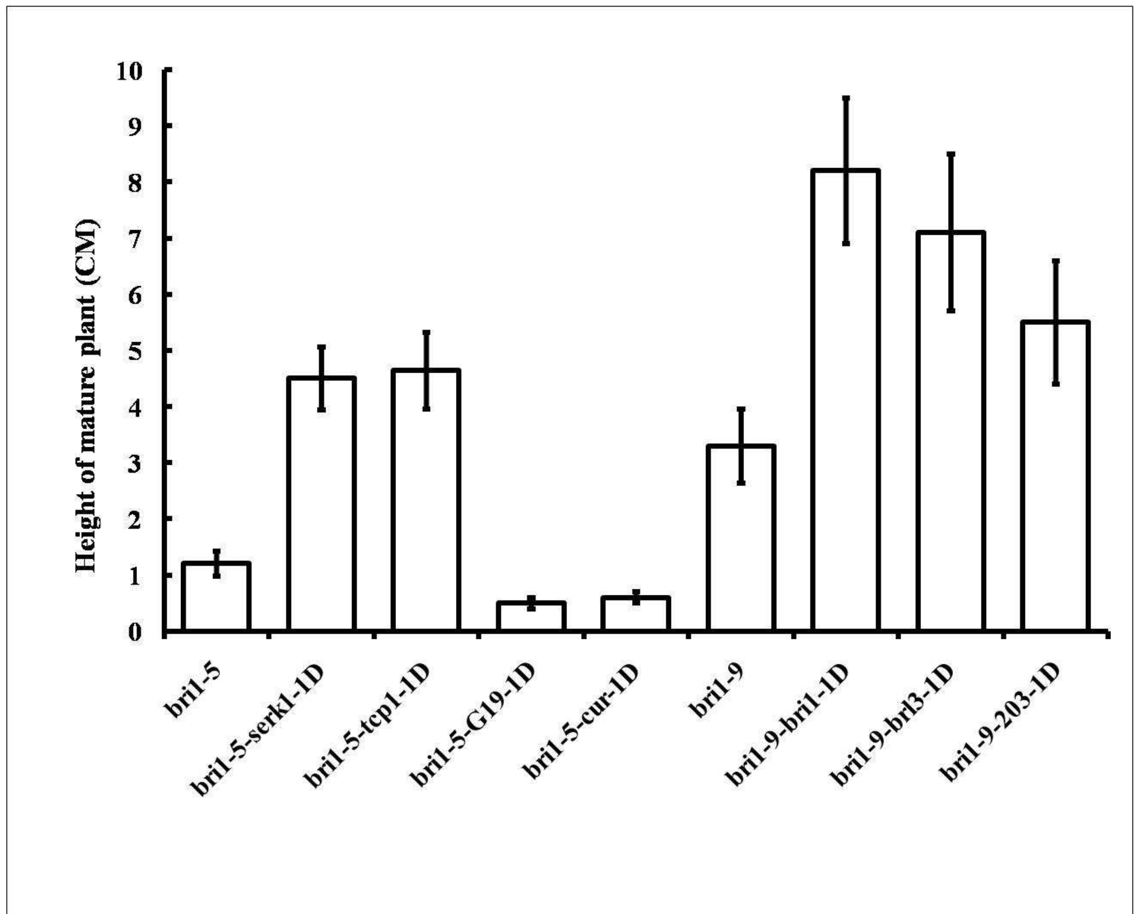

Figure **2.3** Statistical analysis of inflorescence height of suppressors and enhancers. Four-week- old plants were used to measure the inflorescence height. Each bar represents the mean value of 20 plants. All genetic modifiers showed significant difference to their genetic background.

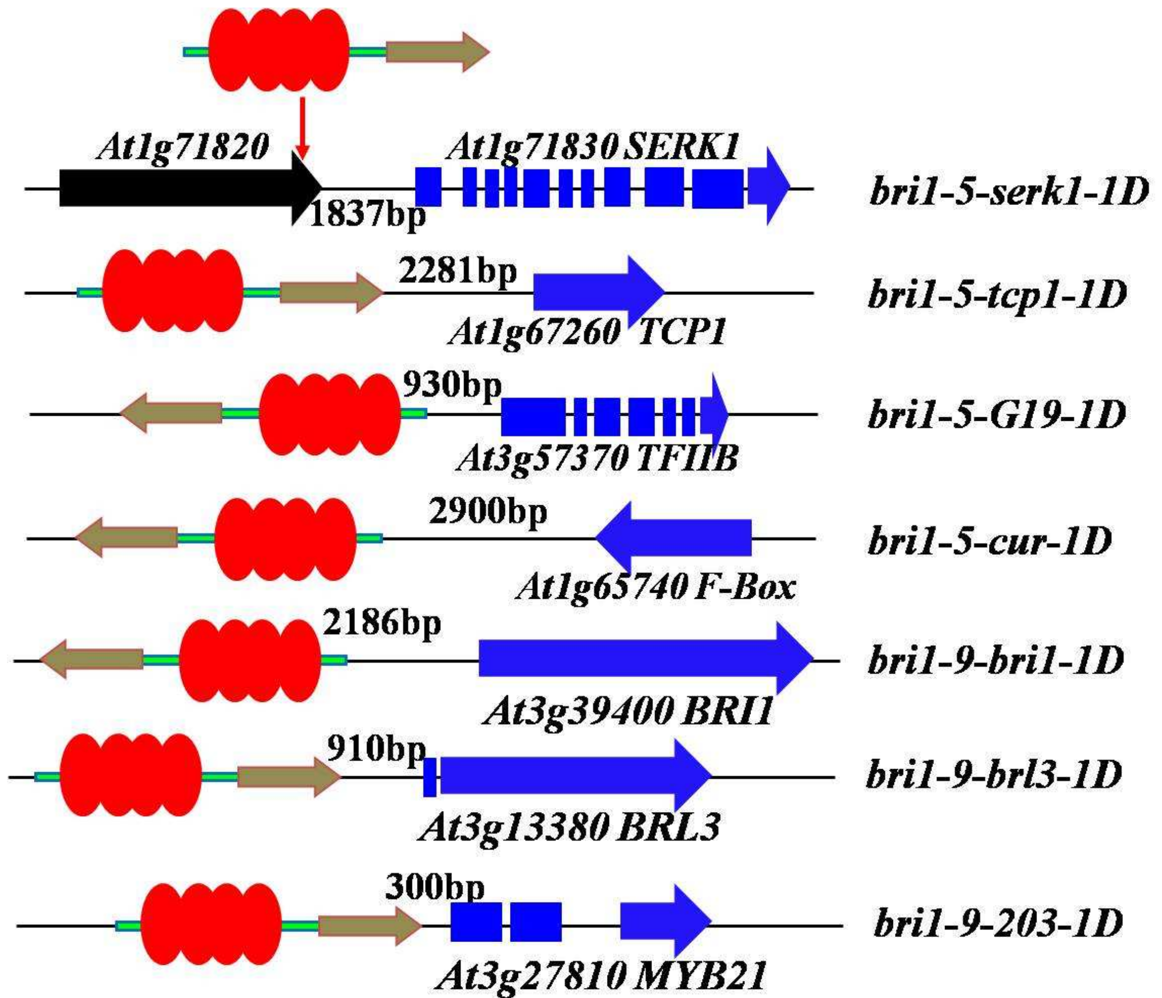

Figure **2.4** T-DNA insertion site in the genome of suppressors and enhancers. Red oval represents enhancers in T-DNA, gray arrow represents *BASTA* marker gene, and blue arrows represent candidate genes tagged in mutants.

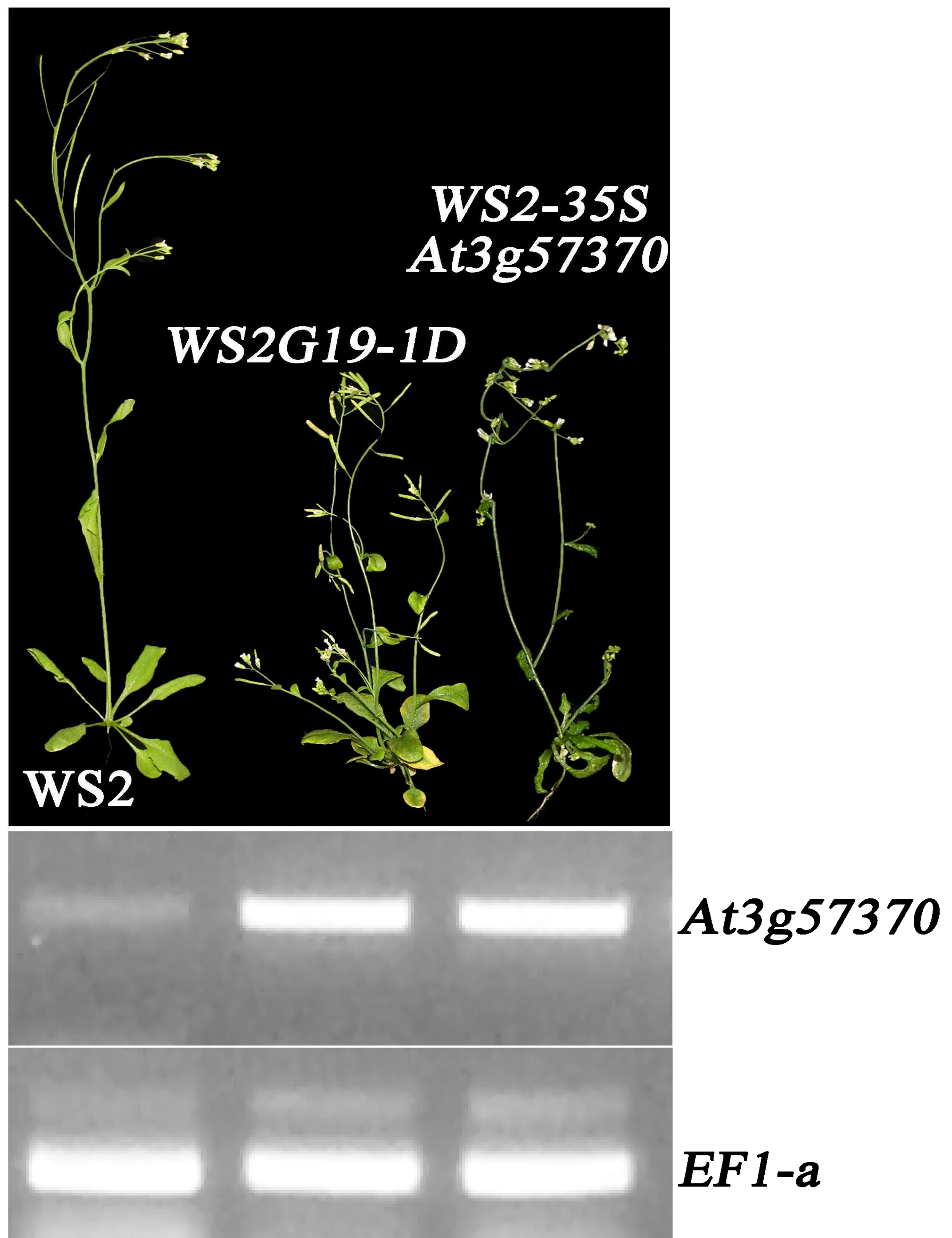

Figure **2.5** Recapitulation results for *WS2G19-1D*. *At3g57370* was overexpressed in WS2 under the control of 35S promoter. RT-PCR was performed to examine its expression level, *EF1-a* was used as control.

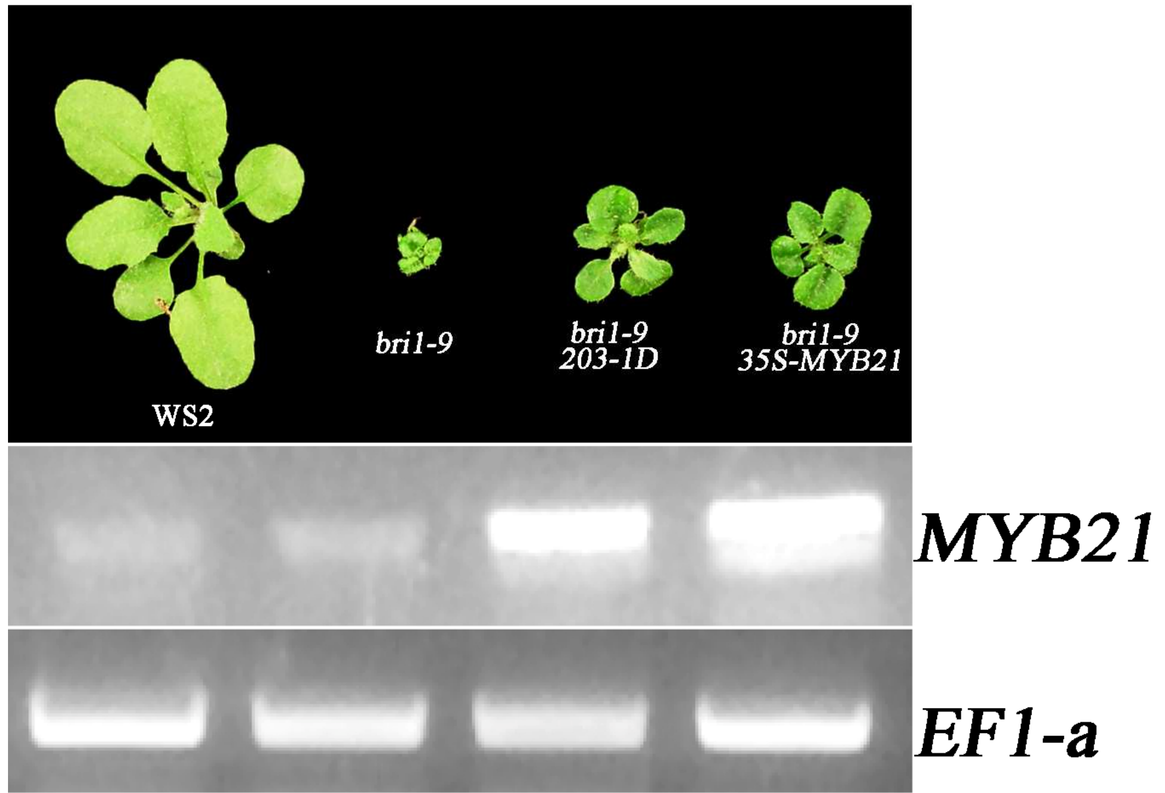

Figure **2.6** Recapitulation results for *bri1-9-203-1D*. *MYB21* was overexpressed in *bri1-9* under the control 35S promoter. RT-PCR was performed to examine *MYB21* expression level, *EF1-a* is used as control.

References

Aboul-Soud MA, Chen X, Kang JG, Yun BW, Raja MU, Malik SI, Loake GJ.(2009) Activation tagging of ADR2 conveys a spreading lesion phenotype and resistance to biotrophic pathogens. New Phytol. 183(4):1163-75.

Albrecht C, Russinova E, Kemmerling B, Kwaaitaal M, de Vries SC.(2008) *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASE proteins serve brassinosteroid-dependent and -independent signaling pathways. Plant Physiol. 148(1):611-9.

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of flowering plant *Arabidopsis thaliana*. *Nature*, 408(6814):796-815

Busov VB, Meilan R, Pearce DW, Ma C, Rood SB, Strauss SH (2003) tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from poplar that regulates tree stature. *Plant Physiol*. 132(3):1283-91.

Caño-Delgado A, Yin Y, Yu C, Vafeados D, Mora-García S, Cheng JC, Nam KH, Li J, Chory J (2004) BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*. *Development*. 131 (21): 5341-51.

Caño-Delgado A, Wang ZY (2009) Binding assays for brassinosteroid receptors. *Methods Mol Biol*. 495:81-8.

Clouse SD, Langford M, McMorris TC (1996) A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. *Plant Physiol*. 111(3):671-8.

Friedrichsen DM, Joazeiro CA, Li J, Hunter T, Chory J (2000) Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. *Plant Physiol*. 123(4):1247-56.

Grant JJ, Chini A, Basu D, Loake GJ (2003) Targeted activation tagging of the *Arabidopsis* NBS-LRR gene, ADR1, conveys resistance to virulent pathogens. *Mol Plant Microbe Interact*. 16(8):669-80.

Greenblatt J.(1992) Transcription. Riding high on the TATA box. *Nature*. 360(6399):16-7. Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt ED, Boutilier K,

Hayashi H, Czaja I, Lubenow H, Schell J, Walden R (1992) Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. *Science* 258: 1350-1353.

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt ED, Boutilier K, Grossniklaus U, de Vries SC (2001) The *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol. 127(3):803-16.

He K, Gou X, Yuan T, Lin H, Asami T, Yoshida S, Russell SD, Li J (2007) BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. Curr Biol. 17(13):1109-15.

He Z, Wang ZY, Li J, Zhu Q, Lamb C, Ronald P, Chory J (2000) Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science*. 288(5475):2360-3.

Kakimoto T (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science*. 274(5289):982-5.

Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer FT. *Science*. 286(5446):1962-5.

Karlova R, Boeren S, Russinova E, Aker J, Vervoort J, de Vries S (2006) The*Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE1. *Plant Cell*. 18(3):626-38

Kondou Y, Nakazawa M, Kawashima M, Ichikawa T, Yoshizumi T, Suzuki K, Ishikawa A, Koshi T, Matsui R, Muto S, Matsui M.(2008) RETARDED GROWTH OF EMBRYO1, a new basic helix-loop-helix protein, expresses in endosperm to control embryo growth. Plant Physiol. 147(4):1924-35.

Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved inbrassinosteroid signal transduction. *Cell*. 90(5):929-38.

Li J, Wen J, Lease KA, Doke JT, Tax FE, Walker JC (2002) BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell*. 110(2):213-22.

Li J, Lease KA, Tax FE, Walker JC. BRS1, (2001) a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A*. 98(10):5916-21.

Li L, Yu X, Thompson A, Guo M, Yoshida S, Asami T, Chory J, Yin Y (2009) *Arabidopsis* MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. *Plant J.* 58(2):275-86.

Liu, Y.G., and Whittier, R.F (1995). Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25, 674-681.

Nakazawa M, Ichikawa T, Ishikawa A, Kobayashi H, Tsuhara Y, Kawashima M, Suzuki K, Muto S, Matsui M (2003) Activation tagging, a novel tool to dissect the functions of a gene family. *Plant J*. 34(5):741-50.

Neff, M.M., Nguyen, S.M., Malancharuvil, E.J., Fujioka, S., Noguchi, T., Seto, H., Tsubuki, M., Honda, T., Takatsuto, S., Yoshida, S., and Chory, J (1999) *BAS1*: A gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 96, 15316-15323.

Niwa Y, Goto S, Nakano T, Sakaiya M, Hirano T, Tsukaya H, Komeda Y, Kobayashi H (2006) *Arabidopsis* mutants by activation tagging in which photosynthesis genes are expressed in dedifferentiated calli. *Plant Cell Physiol*. 47(3):319-31.

Odell JT, Nagy F, Chua N-H (1985) Identification of DNA-sequences required for activity of the cauliflower mosaic virus-35S promoter. *Nature* 313: 810-812.

Pan S, Czarnecka-Verner E, Gurley WB.(2000) Role of the TATA binding protein-transcription factor IIB interaction in supporting basal and activated transcription in plant cells. *Plant Cell*. 12(1):125-36.

Perrella G, Cremona G, Consiglio F, Errico A, Bressan RA, Conicella C (2006) Screening for mutations affecting sexual reproduction after activation tagging in *Arabidopsis thaliana*. *J Appl Genet*. 47(2):109-11.

Rowlands T, Baumann P, Jackson SP (1994) The TATA-binding protein: a general transcription factor in eukaryotes and archaebacteria. *Science.* 264(5163):1326-9.

Shah K, Russinova E, Gadella TW Jr, Willemse J, De Vries SC (2002) The *Arabidopsis* kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1. *Genes Dev*. 16(13):1707-20.

Tang W, Kim TW, (2008) Oses-Prieto JA, Sun Y, Deng Z, Zhu S, Wang R, Burlingame AL,Wang ZY. BSKs mediate signal transduction from the receptor kinase BRI1 in *Arabidopsis*. *Science*. 321(5888):557-60.

Tani H, Chen X, Nurmberg P, Grant JJ, SantaMaria M, Chini A, Gilroy E, Birch PR, Loake GJ (2004) Activation tagging in plants: a tool for gene discovery. *Funct Integr Genomics*. 4(4):258-66.

Terauchi, R., and Kahl, G (2000). Rapid isolation of promoter sequences by TAIL-PCR: the 5'-flanking regions of Pal and Pgi genes from yams (*Dioscorea*). Mol. Gen. Genet 263, 554-560.

Wang ZY, Seto H, Fujioka S, Yoshida S, Chory J (2001) BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature*. 410(6826):380-3.

Weigel D, Ahn JH, Blázquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrándiz C, Kardailsky I, Malancharuvil EJ, Neff MM, Nguyen JT, Sato S, Wang ZY, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation tagging in *Arabidopsis*. *Plant Physiol*. 122(4):1003-13.

Yuan T, Fujioka S, Takatsuto S, Matsumoto S, Gou X, He K, Russell SD, Li J (2007) BEN1, a gene encoding a dihydroflavonol 4-reductase (DFR)-like protein, regulates the levels of brassinosteroids in *Arabidopsis thaliana*. *Plant J*. 51(2):220-33.

Zhang J, Wrage EL, Vankova R, Malbeck J, Neff MM (2006) Over-expression of SOB5 suggests the involvement of a novel plant protein in cytokinin-mediated development. Plant J. 46(5):834-48.

Zhou A, Wang H, Walker JC, Li J (2004) BRL1, a leucine-rich repeat receptor-like protein kinase, is functionally redundant with BRI1 in regulating *Arabidopsis* brassinosteroid signaling. *Plant J*. 40(3):399-409.

Chapter III

TCP1 Modulates brassinosteroid biosynthesis by regulating the expression of the key biosynthetic gene *DWARF4* in *Arabidopsis thaliana*

1. ABSTRACT

Brassinosteroids (BRs) are essential phytohormones regulating normal plant growth and development. Unlike other plant hormones, BRs do not appear to have a long-distance transport system. Elucidating mechanisms regulating bioactive levels of cellular BRs are especially crucial for a better understanding of their roles during entire plant life cycle. *TCP1*, a gene thought to be involved in floral organ symmetric control, was identified as a genetic suppressor of a weak BR receptor mutant, *bri1-5*, in an activation tagging genetic screen. *TCP1* encodes a putative transcription factor possessing a basic helix-loop-helix (bHLH) domain. The dominant allele of *TCP1*, *tcp1-1D*, suppresses the defective phenotypes of *bri1-5*. On the other hand, overexpression of a dominant negative form of *TCP1*, *TCP1-SRDX*, with a 12-amino acid repressor sequence fused to TCP1 at its carboxyl terminus, results in dwarfed plants resembling BR deficient or BR insensitive mutants. Interestingly, the defective phenotypes can be rescued by exogenously applied brassinolide (BL), the final BR biosynthetic product and most active form of BR, but cannot be recovered by other growth-promoting phytohormones such as auxins, GAs, or cytokinins. BR profile assay strongly suggests that TCP1 expression level positively coordinates with the function of DWARF4 (DWF4), a key enzyme in BR biosynthetic pathway. Real-time RT-PCR analysis further demonstrated that TCP1 regulates the transcription levels of *DWF4*. Confocal microscopy analysis indicated that TCP1 is mainly confined to the nucleus. ChIP experiments further showed that TCP1 indeed interacts with the *DWF4* promoter region. The expression of TCP1 appears to be

regulated by BR levels. These studies demonstrate another level of regulation through which BRs mediate plant growth and development.

Key words: TCP1, *bri1-5*, brassinosteroid, transcription factor, SRDX repressor

2. INTRODUCTION

Brassinosteroids (BRs) are a class of polyhydroxyl steroidal hormones naturally found in almost all plant species examined (Clouse and Sasse, 1998). BRs play critical roles in multiple physiological processes during normal plant growth and development, from seed germination to leaf senescence. Mutant plants unable to biosynthesize or perceive BRs exhibit typical defective phenotypes, including extremely dwarfed statures, shortened leaf petioles, rounded and curled leaves, prolonged life spans, reduced male fertility, and de-etiolated open hypocotyls when grown in darkness. Within the last two decades, detailed information regarding BR signal transduction and BR biosynthesis has been uncovered. A number of BR signaling regulators, such as a cell surface BR receptor called brassinosteroid insensitive 1 (BRI1) (Li and Chory, 1997) and its co-receptor named BRI1-associated protein kinase (BAK1) were identified via genetic and biochemical methods (Li *et al*., 2002; Nam and Li, 2002). Other important regulatory proteins, such as a secreted serine carboxypeptidase designated as *bri1* suppressor 1 (BRS1) (Li *et al*., 2001), a BRI1 inhibitory protein BKI1 (Wang and Chory, 2006), several putative BRI1 substrates including an *Arabidopsis* paralog of TGF-beta receptor-interacting protein (TRIP-1) (Jiang and Clouse, 2001; Ehsan *et al*., 2005), a transthyretin-like protein (TTL) (Nam and Li, 2004), and three homologous BR signaling kinases (BSKs) (Tang *et al*., 2008), a negative regulator called brassinosteroid insensitive 2

(BIN2) (Li *et al*., 2001;Li and Nam, 2002), a protein phosphatase (BSU1) (Mora-Garcia *et al*., 2004) , 14-3-3 proteins (Bai *et al*., 2007; Gampala *et al*., 2007; Ryu *et al*., 2007), and two novel transcription factors, BZR1 (Wang *et al*., 2002) and BES1 (Yin *et al*., 2002), have also been identified utilizing various approaches. Although a more detailed mechanistic understanding as to how the aforementioned proteins coordinate various steps in BR signaling is needed, evidence to date strongly indicates that they are key signaling components in BR signal transduction that relay information from the cell surface to nuclear transcription factors. A proposed BR signal transduction starts from ligand (BR) binding to the extracellular domain of BRI1, which triggers a sequential phosphorylation between BRI1 and BAK1 (Wang *et al*., 2008). Activated receptor/co-receptor complex initiates a phosphorylation/dephosphorylation cascade that can transduce the BR signal from the cell surface to cytoplasm, and eventually to nucleus where gene expression patterns are altered through the action of two transcription factors BZR1 and BES1 (Kim *et al*., 2009). As a consequence of these events, the plant is able to fine tune its growth and development.

The entire BR biosynthetic pathway was initially elucidated utilizing cultured *Catharanthus roseus* cells (Fujioka and Yokota, 2003). Several genes encoding key BR biosynthetic enzymes have also been cloned using BR deficient mutants identified from a number of plant species such as *Arabidopsis*, pea, tomato, and rice (Fujioka and Yokota, 2003). For example, *de-etiolated 2* (*det2*) was identified as a de-etiolated mutant from *Arabidopsis* (Chory *et al*., 1991). DET2 encodes a protein sharing sequence similarity with the mammalian steroid 5a-reductase (Li *et al*., 1997). Feeding experiments revealed that DET2 is involved in a 5a-reduction step of multiple related sterols during BR

biosynthesis (Fujioka *et al*., 1997). An ortholog of *Arabidopsis det2*, named *lk*, was also identified from pea as an extremely dwarfed mutant (Nomura *et al*., 2004). *dwarf4* (*dwf4*) is another BR deficient mutant isolated from *Arabidopsis* (Choe *et al*., 1998). The dwarfed stature of *dwf4* can be rescued by brassinolide (BL), the final product of the BR biosynthetic pathway, and the most active form of BRs. *DWF4* encodes a 22-hydroxylase and is responsible for multiple 22-hydroxylation steps during BR biosynthesis. It was proposed that DWF4 catalyses a rate limiting step during BR biosynthesis (Kim *et al*., 2006). *Constitutive photomorphogenesis and dwarfism* (*cpd*) is another dwarf mutant isolated by T-DNA insertion analysis. It was shown that *CPD* encodes a 23a-hydroxylase and participates in a critical 23a-hydroxylation step in BR biosynthesis (Szekeres *et al*., 1996). But recent feeding and biochemical analyses indicated that two P450 proteins, CYP90C1 and CYP90D1, act as true 23a-hydroxylases (Ohnishi *et al*., 2006). The severe phenotype of the *cpd* mutant indicated that it should be involved in a step earlier than the 23a-hydroxylation reaction. Another gene involved in BR biosynthesis is BR6ox, which was first identified in tomato by transposon tagging (Bishop *et al*., 1996). BR6ox catalyzed the C-6 oxidation of a number of different deoxoBRs (Bishop *et al*., 1999). BR6ox orthologs from *Arabidopsis* and rice have conserved functions, which are responsible for linking the early and late C-6 oxidation pathways (Bishop *et al*., 2006).

Unlike other phytohormones, BRs are unable to be transported through a long distance mechanism in a plant (Symons and Reid, 2004). This is uncommon for a typical phytohormone but suggests that homeostasis of bioactive BRs must be precisely controlled at tissue or even at cellular levels to ensure normal growth and development. There are a number of mechanisms for a plant to maintain adequate levels of bioactive

BRs. For instance, excessive amounts of BRs can be inactivated by modifications of BRs (Fujioka and Yokota, 2003). Mechanisms for the inactivation of BRs include sulfonation at a 22-OH group by a steroid sulfotransferase named BNST3, identified in *Brassica napus* (Rouleau *et al*., 1999); 26-hydroxylation by BAS1, found in *Arabidopsis* (Neff *et al*., 1999); conjugation by a UDPglycosyltransferase named UGT73C5 (Poppenberger *et al*., 2005); and a putative reduction step catalyzed by BEN1 (Yuan *et al*., 2007). Plants also use a feedback mechanism to monitor the BR biosynthetic rate, which is tightly linked with the BR signaling pathway (Mathur *et al*., 1998; He *et al*., 2005; Kim *et al*., 2006). If BRs are available, they can trigger a series of cellular processes, resulting in the accumulation of unphosphorylated BZR1 and BES1 in nuclei. Unphosphorylated BZR1 and BES1 have dual roles: repressing biosynthetic gene expression to slow down the biosynthetic rate, and activating BR response genes to promote growth. How BR biosynthesis is positively regulated, however, is still poorly understood. In this paper we describe the identification of a transcription factor, which plays a positive role in regulating BR biosynthesis.

Using a gain-of-function genetic approach, we have identified a number of suppressors for a weak BRI1 mutant allele, *bri1-5* (Noguchi *et al*., 1999; Li *et al*., 2001; Li *et al*., 2002; Zhou *et al*., 2004; Yuan *et al*., 2007). One of these suppressors is *tcp1-1D*. *TCP1* encodes a TCP transcription factor, which contains a basic helix loop-helix domain. Previous studies suggested that TCP1 may play a role in regulating flower organ symmetry (Cubas *et al*., 2001; Busch and Zachgo, 2007).

The activation tagged locus, *tcp1-1D*, can suppress the defective phenotypes of *bri1-5*. Overexpression of a dominant negative mutant *TCP1-SRDX* in wild type plants,

conversely, resulted in dwarfed transgenic plants similar to typical BR deficient mutants such as *det2* (Li *et al*., 1996), or signaling defective mutants such as *bri1-5* (Noguchi *et al*., 1999). Our detailed genetic, biochemical, and molecular analyses demonstrated that TCP1 positively regulates the expression of the key BR biosynthetic gene, *DWF4*, via a direct or indirect interaction with the promoter region of DWF4. Thus, our findings provide a new molecular pathway in the regulation of BR biosynthesis under certain endogenous and external stimuli. The discoveries will significantly advance our knowledge about the functions of BR in regulating normal plant growth, development, and adaptation to various environments.

3. RESULTS

3.1 *tcp1-1D* was identified as a gain-of-function genetic suppressor of *bri1-5*

Activation tagging is a gain-of-function genetic approach that activates gene expression via inserting strong enhancers in the genome. The enhancers are commonly engineered in the T-DNA region of the transformation construct and placed arbitrarily in the genome by floral dipping, an effective *Agrobacterium*-mediated gene transformation method (Clough and Bent, 1998). Usually only the genes in the vicinity of the enhancers can be transcriptionally activated (Weigel *et al*., 2000). This investigation takes advantage of a weak BR receptor mutant named *bri15*, in which a single cysteine has been substituted by a tyrosine at the N-terminus (C69Y) (Noguchi *et al*., 1999). The mutated *bri1-5* protein is largely retained in endoplasmic reticulum and degraded through a proteosome-mediated degradation pathway (Hong *et al*., 2008). *bri1-5* plants show a semi-dwarfed but a fertile phenotype. Activation-tagging-based *bri1-5* genetic modifier

screen has become an effective approach to identify novel components regulating BR signaling, catabolism, and biosynthetic pathways (Li *et al*., 2001; Li *et al*., 2002; Zhou *et al*., 2004; Yuan *et al*., 2007). One of the *bri1-5* suppressors we identified is called *tcp1-1D*. *bri1-5-tcp1-1D* double mutant shows partially suppressed phenotypes compared to the single mutant *bri1-5* (Figure 3. 1A). A *bri1-5* mutant exhibits characteristic BR mutant phenotypes such as rounded leaves, shortened petioles, and delayed flowering time. Although the leaf shapes of the double mutant plants remain unaltered, the petioles of *bri1-5-tcp1-1D* are significantly elongated. The inflorescences of the double mutant plants are twice as tall as those of *bri1-5* plants at maturity. In addition, the delayed flowering time of *bri1-5* was also significantly suppressed. Genetic analysis indicated that the double mutant phenotype was caused by a single dominant locus, because the *bri1-5* suppression phenotype is closely linked with the *basta* resistant gene from the T-DNA of our home-made activation tagging construct, pBASTA-AT2 (Yuan *et al*., 2007).

We cloned the flanking sequences of the T-DNA insertion by tail-PCR (Liu and Whittier, 1995) and found that the T-DNA is inserted at 2,281bp upstream of the initiation codon of *TCP1* (*At1g67260*) (Figure 3. 1B). To determine whether *TCP1* is the gene responsible for the suppression phenotype in the double mutant, we cloned the full length cDNA of *TCP1* and overexpressed it in *bri1-5* driven by a constitutive CaMV35S promoter. Over 50% of the transgenic plants showed elongated petiole phenotypes similar to the originally identified activation tagging line (Figure 3. 1A). Real-time RT-PCR analysis confirmed that the *TCP1* expression levels in both *bri1-5-tcp1-1D* and *bri1-5-35S-TCP1-GFP* were elevated by at least 10 fold compared to those in WS2 and *bri1-5*

(Figure 3. 1C). These results demonstrated that increased expression of TCP1 is the cause of the suppression phenotype seen in the double mutant.

3.2 A functional BRI1 is required for *tcp1-1D* to regulate plant growth and development

To determine whether *tcp1-1D* is a general growth regulator or has a specific role in BR related pathways, we conducted a series of genetic crosses. We first backcrossed the *bri1-5-tcp1-1D* with its background ecotype WS2 and segregated out *bri1-5* after self-pollination. The gain-of-function *tcp1-1D* single mutant showed an elongated leaf phenotype reminiscent of BRI1-overexpressed or DWF4overexpressed transgenic plants (Choe *et al*., 2001; Wang *et al*., 2001) (Figure 3. 2A). A similar suppression outcome was observed when *tcp1-1D* was crossed into *det2* mutant (Figure 3. 2B). *det2* was firstly identified as a de-etiolated mutant when grown in the dark (Chory *et al*., 1991). DET2 encodes a steroid reductase responsible for a reduction step from campesterol to campestanol during BR biosynthesis (Li *et al*.,1996,1997; Fujioka *et al*., 1997). It was found that *det2* mutants were still able to synthesize about 5-10% wild type levels of BRs. Therefore, *det2* null mutant was considered as an intermediate biosynthetic mutant (Fujioka *et al*., 1997; Fujioka and Yokota, 2003). To further test whether BR signaling is necessary for the function of *tcp1-1D* in regulating plant growth, we crossed *tcp1-1D* with *bri1-4*. *bri1-4* has a 10amino-acid-deletion at the N-terminus of BRI1 resulted from an T-DNA insertion event, which causes a premature stop codon after amino acid 153 (Noguchi *et al*., 1999). Therefore, this mutant is regarded as a null mutant of BRI1. *bri1-4 -tcp1-1D* double mutant did not show any leaf suppression phenotypes (Figure 3. 2C).

Thus, our genetic analyses clearly indicated that the role of *tcp1-1D* in regulating leaf growth is dependent on the presence of BRs and the BR signaling pathway.

3.3 Expression of a *TCP1-SRDX* chimeric repressor gene in wild type plants results in a typical BR mutant phenotype

Our genetic data suggested that TCP1 is involved in BR related pathways. To further understand the authentic role of TCP1 in BR-related pathways, we searched for T-DNA null mutants of TCP1 from various resources. Unfortunately, no T-DNA alleles were identified from the available databases. We then tried to generate TCP1 knockdown mutants by using an artificial microRNA strategy (Schwab *et al*., 2006). All the resulting plants did not show any obvious phenotypes, possibly due to gene redundancy. Therefore, we employed a gene silencing system, named chimeric repressor gene-silencing technology (CRES-T), in which TCP1 was fused with a 12amino-acid EAR-motif repressor domain (SRDX) (Figure 3. 3A) (Hiratsu *et al*., 2003). Previous experiments indicated that the chimeric version can be used to effectively repress the expression of the target genes of a number of transcription factors, including TCP genes (Koyama *et al*., 2007). Expression of TCP1-SRDX driven by the constitutive 35S promoter resulted in dwarfed transgenic plants similar to BR deficient or signaling mutants, such as *det2*, *dwf4*, and *bri1-5* (Figure 3. 3B). The dominant negative plants showed phenotypes opposite to those of *tcp1-1D* plants. Whereas *tcp1-1D* plants showed elongated leaves and petioles, *TCP1-SRDX* plants displayed rounded and epinastic leaves, shortened petioles, and reduced statures (Figure 3. 3B, C). When grown under darkness, *TCP1-SRDX* plants exhibited a typical de-etiolated phenotype with opened cotyledons

resembling that of *det2* and *bri1-4* mutants (Figure 3. S1). The dominant negative phenotypes were likely caused by the competitive binding of TCP1-SRDX with native TCP1 and its paralogs to the target gene(s) via the bHLH domain. Overexpression of *TCP1-bHLH* (in which bHLH domain sequence was deleted) or *TCP1-SRDX-bHLH* failed to alter the growth of the transgenic plants (Figure 3. S2), suggesting that the bHLH domain of TCP1 is prerequisite to the functions of TCP1 and TCP1-SRDX in transgenic plants. To ensure that the phenotypes of *TCP1-SRDX* transgenic plants were caused by the overexpression of *TCP1-SRDX*, we crossed the transgenic plants with *tcp1-1D* and isolated homozygous plants for both *TCP1-SRDX* and *tcp1-1D* loci. The fact that *tcp1-1D* can partially complement the dominant negative effect of *TCP1-SRDX* suggests that the phenotypes were indeed caused by *TCP1-SRDX* overexpression (Figure 3. S3). These results suggested that the target gene(s) of TCP1 transcription factor plays a significant role in regulating BR biosynthesis or signal transduction.

3.4 TCP1 regulates BR biosynthesis

The phenotypes of BR deficient and signaling mutants are morphologically similar. It is therefore challenging to determine, by morphology only, whether the dwarfed phenotype of *TCP1-SRDX* plants was caused by the failure of the transgenic plants to respond to endogenous BRs or by disruptions in specific steps of the BR biosynthetic pathway. If the BR signaling pathway is altered, the mutant should show reduced response to exogenously applied BR. Conversely, if the BR biosynthesis pathway is impeded, the dwarf mutant should be rescued by exogenously applied BL. Our results show that the hypocotyl growth of the *TCP1-SRDX* plants can be greatly recovered by the

addition of BL. In ½ MS medium supplemented with 1% sucrose and 1μM BL, the hypocotyl length of the *TCP1-SRDX* seedlings was increased by 4 fold, whereas that of wild type WS2 and *tcp1-1D* seedlings were increased only by 20-40% (Figure 3. 4A, B). Comparison analysis indicated that the growth of the *TCP1-SRDX* transgenic plants can respond to exogenously applied BL in a similar manner as other BR deficient mutants such as *det2* (Figure 3. S4). Application of 1 μM BRZ, a specific BR biosynthetic inhibitor (Asami and Yoshida, 1999; Asami *et al*., 2000), did not further reduce the growth of *TCP1-SRDX* plants. Under the same treatment, the lengths of WS2 and *tcp1-1D* seedlings were reduced by 4 to 8 fold, respectively (Figure 3. 4A, B). Furthermore, none of the other known growth hormones such as GA3, KT, and IAA showed any rescuing effects when they were added into the culture media (Figure 3. S5). These results suggest that expression of *TCP1-SRDX* specifically blocked the BR biosynthetic pathway.

3.5 TCP1 expression levels are positively correlated to the catalytic ability of DWF4-a key enzyme in BR biosynthesis pathway

To investigate how TCP1 is involved in regulating BR biosynthesis, we performed BR profile analyses using four-week old WS2, *tcp1-1D*, and *TCP1-SRDX* plants. It was apparent that *tcp1-1D* enhanced the catalytic capability of DWF4, whereas *TCP1-SRDX* greatly reduced the catalytic ability of DWF4. For example, one of the substrates of DWF4, campestanol, is quantitatively alike in three different genotypes. Although the product of DWF4, 6-deoxocathasterone, is increased by about 2 fold in

tcp1-1D seedlings, it decreased about 10 fold in *TCP1-SRDX* seedlings in comparison with that of wild type seedlings (Figure 3. 5). Similar results were observed for the reaction from campesterol to (22S)-22-hydroxycampesterol, which is also catalyzed by DWF4 (Figure 3. 5). None of the other reactions were affected in such a dramatic way. Because TCP proteins are transcription factors, TCP1 likely regulates DWF4-catalyzed reactions via the control of DWF4 expression.

3.6 The expression level of DWF4 is positively regulated by TCP1

To test our hypothesis that TCP1 can transcriptionally regulate *DWF4* expression, we first generated TCP1p (TCP1 promoter)-ß-glucuronidase (GUS) and DWF4pGUS transgenic plants and examined the expression patterns of TCP1 and DWF4. If TCP1 can directly regulate the expression of DWF4, we would expect to see the overlapped expression patterns of the two genes. We analyzed six independent transgenic lines for each transgenic event. Each transgenic event showed consistent GUS staining results, which clearly indicated that both genes have overlapped expression patterns (Figure 3. S6). Both genes are expressed relatively strong in the roots and at the junctions of roots and hypocotyls. In the leaves, both genes are mainly expressed in vascular tissues. The expression level of DWF4 in the leaves, however, is apparently much less than that of TCP1. Neat DWF4 expression level is determined by both the positive effect of TCP1 and negative action of BZR1/BES1. To examine whether changing expression levels of TCP1 can affect the transcription of *DWF4*, we isolated total RNA from WS2, *tcp1-1D*, and *TCP1-SRDX* entire seedlings and carried out real time RT-PCR analyses. Our results

clearly indicated that the expression level of *DWF4* is elevated in the *tcp1-1D* gain-of-function mutant seedlings, but reduced in the *TCP1-SRDX* dominant negative mutant seedlings (Figure 3. 6A). The expression level of all other known *Arabidopsis* BR biosynthetic genes were not significantly altered in *tcp1-1D* and *TCP1-SRDX* seedlings (data not shown). We also crossed the homozygous lines of *tcp1-1D* and *TCP1-SRDX* with homozygous lines of *DWF41p-GUS* plants respectively and obtained F1 plants. These F1 plants, containing only one copy of each of the transgene, were used to test whether overexpression of TCP1 (*tcp1-1D*) or TCP1-SRDX can influence the expression levels of GUS by an immunoblotting analysis using anti-GUS antibody. If TCP1 controls DWF4 transcription via regulating the function of the DWF4 promoter, we expect to see that overexpression of TCP1 up-regulates the expression level of GUS; whereas overexpression of TCP1-SRDX down-regulates the expression level of GUS. Our immunoblotting results indeed confirmed these predictions (Figure 3. 6B). These results confirmed that TCP1 functions as a positive regulator in controlling DWF4 transcription. In addition, when *tcp1-1D* was crossed into a null DWF4 mutant background, *dwf4-1*, the resulting *dwf4-1- tcp1-1D* double mutant plants did not show any suppression phenotypes (Figure 3. 6C). *dwf4-1* is a T-DNA insertion mutant originally used to clone DWF4 (Choe *et al*., 1998). The null *dwf4-1* mutant is more severe than a null *det2* mutant but much milder than a *cpd* null mutant (Szekeres *et al*., 1996; Fujioka *et al*., 1997; Choe *et al*., 1998). BR profile analysis suggested that *dwf4-1* still makes trace amount of BRs (Choe *et al*., 2001). The inability of *tcp1-1D* to suppress *dwf4-1* is consistent with our notion about TCP1's role in regulating DWF4 expression. Without a functional target gene, overexpression of TCP1 becomes inconsequential as it would not be able to

regulate the expression of the target gene and the subsequent downstream events leading to modified plant growth.

3.7 TCP1 associates with the promoter region of *DWF4*

Our genetic and biochemical analysis data suggested that TCP1 is a transcription factor positively regulating *DWF4* expression. To prove that TCP1 is a transcription factor, which specifically regulates DWF4 expression, we conducted a TCP1 subcellular localization analysis to examine whether TCP1 is localized in nucleus and chromatin immunoprecipitaion (ChIP) assay to determine whether TCP1 associates with the promoter of DWF4. We overexpressed a *TCP1-GFP* fusion gene in WS2 background, selected multiple transgenic lines, and identified homozygous plants for further subcellular localization analysis. Confocal microscopy data of primary roots from 4-day old seedlings expressing TCP1-GFP indicated that fluorescent signals originate exclusively from the nuclei. Representative results are shown in Figure 3. 7A-D. These *TCP1-GFP* homozygous transgenic lines were then used to do ChIP experiments. According to predicted DNA binding sites of TCP family proteins from previous reports in rice, consensus TCP binding sites were found in the promoter regions of both BES1 and BRZ1, but not in the promoter region of DWF4. So we first checked whether the TCP1 protein binds to the promoter regions of *BZR1* or *BES1* through ChIP analysis. As shown in 7E, TCP1 does not seem to associate with either the *BES1* or *BZR1* promoter. ChIP analysis,however, did demonstrate that the TCP1 protein is associated with the DWF4 promoter sequences, either via a novel binding sequence on the DWF4 promoter (Figure 3. 7E), or via its interaction with another transcription factor which directly binds

to the promoter sequence of *DWF4*. Our ChIP analysis results were consistent with our real-time RT-PCR results, because we did not find any altered expression of *BES1* and *BZR1* in *tcp1-1D* or *TCP1-SRDX* plants compared to that in wild type plants (data not shown). We did find, however, that *DWF4* was up-regulated in *tcp1-1D* and down-regulated in *TCP1-SRDX* transgenic plants (Figure 3. 6A-B).

3.8 TCP1 expression can be regulated by BR

To examine whether BR levels can affect the expression of TCP1, seeds from a representative homozygous *TCP1p-GUS* line were germinated in ½ MS medium supplemented with 1% sucrose and 0 μM BL or BRZ, 1 μM BL, and 1 μM BRZ respectively. The plants were grown under either complete darkness or continuous light conditions for 5 days. The seedlings were then stained for GUS activity and were also subjected to immunoblotting analyses using an anti-GUS antibody (Figure 3. 8A-C). Our results indicated that application of 1 μM BL can significantly stimulate TCP1 expression as revealed by GUS staining and immunoblotting results, whereas depletion of endogenous BRs by BRZ treatment can greatly reduce TCP1 expression (Figure 3. 8A-C). We also grew WS2, *det2-28* (in WS2 ecotype), and *bri1-4* (in WS2 background) for 5 days in 1/2 MS medium supplemented with 1% sucrose and 1 μM BL. Real-time RT-PCR analysis indicated that TCP1 expression levels were up-regulated in WS2 and *det2-28* but not in *bri1-4* in the presence of 1 μM BL (Figure 3. S7). The increasing level was quite dramatic in *det2-28*. These data suggest that the expression of TCP1 can be

regulated by exogenous and endogenous levels of BL, and the regulation may relay on a functional BRI1 receptor (Figure 3. 8D).

4. DISCUSSION

BR homeostasis is critical for normal plant growth and development. BR deficiency mutants show a severely retarded growth phenotype mainly due to lack of cell elongation. Excessive amounts of BR, on the other hand, greatly inhibits root growth and triggers leaf senescence (Clouse, 1996; Clouse and Sasse, 1998). Bioactive levels of BR are mainly balanced by the rate of biosynthesis and the speed of inactivation. Within the last a few years, a feedback inhibitory regulation has been elucidated, which involves two novel transcription factors, BZR1 and BES1 (Mathur *et al*., 1998; He *et al*., 2005). Previous studies indicated that the expression levels of five BR-specific biosynthetic genes including *DET2*, *DWF4*, *CPD*, *BR6ox1*, and *ROT3* are up-regulated in plants treated with BRZ (Bancos *et al*., 2002; Tanaka *et al*., 2005). The expression levels of four of the five genes (*DWF4, CPD, BR6ox1*, and *ROT3*) are significantly down-regulated in response to exogenous BL treatment.

Previous studies also revealed a number of biochemical reactions which plants use to inactivate BRs if an excessive amount of BR is present. There have been no reports, however, about how BR biosynthesis is positively regulated. When additional BR is needed at certain developmental stages or under various biotic/abiotic stresses, how plants perceive internal or external signals to trigger the production of more BR is poorly understood. Generally, it is thought that the plants can accumulate additional BR via two different mechanisms including acceleration of the biosynthetic rate and deceleration of

inactivation speed or slowing down the feedback regulation. The most effective way is to speed up BR biosynthesis by elevating the expression of key BR biosynthetic genes.

Among the BR biosynthetic genes isolated, *DWF4* was believed to be one of the key genes in the BR-specific biosynthetic pathway from campesterol to BL (Kim *et al*., 2006). *DWF4* encodes a P450 protein which catalyzes multiple 22ahydroxylation steps in BR biosynthesis (Choe *et al*., 1998). Relative to other BR biosynthetic genes, such as *CPD* and *DET2*, *DWF4* is expressed at an extremely low level (Kim *et al*., 2006). Although BR profile analysis indicated that DWF4 substrates are always plentiful in plants, the products of the DWF4-catalyzed reactions are considerably low or even undetectable. Previous studies also indicated that DWF4 is mainly expressed in actively growing tissues, such as root and shoot apices, where BRs are significantly enriched (Kim *et al*., 2006). In addition, overexpression of DWF4 in wild type *Arabidopsis* or tobacco plants can significantly increase biomass and seed production (Choe *et al*., 2001). These results suggest that DWF4 catalyzes a flux-determining step in the BR biosynthesis pathway (Kim *et al*., 2006). Elucidating how DWF4 expression is regulated will greatly advance our knowledge about the mechanisms controlling BR homeostasis in a plant.

Our detailed analyses clearly demonstrated that TCP1 is an important transcription factor positively regulating the BR biosynthesis by controlling the expression of DWF4. This notion is supported by several key observations. First, wild type plants harboring *tcp1-1D* allele showed a phenotype similar to that of the DWF4-overexpressing plants (Choe *et al*., 2001). The transgenic plants overexpressing a dominant negative chimerical gene, *TCP1-SRDX*, on the other hand, exhibited a typical BR deficiency or signaling mutant phenotype. The dwarfed phenotypes of the transgenic

seedlings can be rescued by supplementing the culture medium with BL, but not with any other growth promoting hormones such as auxins, gibberellins, or cytokinins, suggesting that *TCP1-SRDX* transgenic plants are BR-specific deficient mutants, rather than BR signaling mutants. Secondly, our BR profile analysis results clearly indicated that the expression levels of TCP1 affect the function of DWF4 but not other known BR biosynthetic enzymes. Our real-time RT-PCR results showed that overexpression of *TCP1* up-regulates *DWF4* expression, whereas overexpression of *TCP1-SRDX* down-regulates the expression of *DWF4*. Finally, our ChIP analysis using transgenic plants overexpressing TCP1-GFP demonstrated that TCP1 can interact with the promoter of *DWF4* via a direct or indirect manner but not with the promoters of *BZR1, BES1, EF1a,* and *ACTIN2*.

It is worth noting, however, that although *tcp1-1D* showed an organ elongation phenotype resembling plants overexpressing *DWF4*, BR profile analysis did not reveal any accumulation of CS/BL in *tcp1-1D* plants. As a matter of fact, several intermediates in the later BR biosynthetic pathway, such as CS, were reduced in both *tcp1-1D* and *TCP1-SRDX* plants. However, the phenotypes were opposite. BR profile analysis was also conducted in plants overexpressing *DWF4* by Choe *et al* (2001); and their results also showed there was no CS or BL accumulation in *DWF4-overexpressing* plants. Choe *et al* (2001) proposed that BL, CS or other bioactive BR in *DWF4-overexpressing* plants could be turned over rapidly after triggering the BR signal transduction chain. This idea is supported by the fact that in mutants impaired in BR signaling such as in *bri1*, BL levels became elevated possibly because the signaling pathway has been disrupted in *bri1* mutants (Noguchi *et al*., 1999). The similarity of the BR profiles in *tcp1-1D* and *DWF4-*

overexpressing plants in comparison with wild type plants is another piece of evidence linking TCP1 function to the regulation of *DWF4* expression.

Although significant progress has been made in elucidating both the BR biosynthesis and signal transduction pathways, little is known about how the expressions of BR biosynthetic genes as well as signaling genes are positively regulated. Our identification of TCP1 as a positive regulator for *DWF4* expression provides a new mechanism through which plants are able to modulate BR biosynthesis during normal plant growth and development. TCP proteins are plant specific transcription factors regulating a number of processes during plant growth and development, such as floral symmetry (Luo *et al*., 1999; Broholm *et al*., 2008), embryonic growth (Tatematsu *et al*., 2008), morphology of shoot lateral organs (Koyama *et al*., 2007), and jasmonate biosynthesis and senescence (Schommer *et al*., 2008). The name TCP was used after TEOSINTE BRANCHED 1 (TB1) from maize, CYCLOIDEA (CYC) from *Anthirrinum majus*, and PCF from rice (Cubas *et al*., 1999). These proteins contain a basic helix-loop-helix domain, which is thought to be involved in DNA binding. There is also an "R-domain" whose function is not yet known. In *Arabidopsis thaliana*, there are 24 TCP transcription factors. Based on similarity in the amino acid sequence of the TCP domain, 13 were grouped into class I and 11 were group into class II. It was found that the two classes have distinct but overlapping binding sequences. For example, rice class I TCP proteins prefer to bind GGNCCCAC, whereas class II TCP proteins favor binding to GTGGNCCC (Kosugi and Ohashi, 2002). They share the core sequence GGNCCC. It is not known, however, whether *Arabidopsis* TCP transcription factors bind to the same DNA sequences. Since TCP1 belongs to class I TCP transcription factors, we searched

the promoter region sequence of *DWF4* to see whether it contains GGNCCCAC motif. We failed to identify the consensus sequence for either class I or class II TCPs in the *DWF4* promoter region. This suggested to us that the possible binding sequence for TCP1 in *DWF4* promoter may not be strictly identical to what was reported in rice. Alternatively, even though we have determined that TCP1 and *DWF4* promoter are in the same complex as assayed by ChIP experiments, we cannot rule out the possibility that TCP1 is indirectly involved in activating *DWF4* transcription by its association with another true *DWF4* promoter binding transcriptional factor. Interestingly, in the promoters of both *BZR1* and *BES1*, there are typical TCP binding sequences revealed from rice TCP orthologs. For instance, about 400 bp upstream of the translation initiation codon of *BES1*, there are two putative TCP binding motifs, GGACCCAC and GGCCCCAC. Similar sequences have also been detected in the promoter region of *BZR1*. However, it appears that the expression of *BES1* and *BZR1* are not directly regulated by TCP1, as the expression levels of these two transcription factors are not altered in *tcp1-1D* or *tcp1-SRDX* plants (data not shown). It is possible that these transcription factors are regulated by other members of TCP family. Previous studies suggested that TCP1 is involved in floral symmetric regulation (Cubas *et al*., 2001; Busch and Zachgo, 2007). The detailed molecular mechanism controlling organ mono- or poly-symmetry is poorly understood. Our identification of TCP1 in regulating BR biosynthesis suggests that unequal expression of TCP1 may result in uneven distribution of BRs in floral meristems, which may contribute to the asymmetric regulation of floral organs. Future studies will focus on understanding how the expression of TCP1 is

regulated by internal and external factors. The information about how TCP1 expression is regulated will facilitate our understanding of how BR biosynthesis is controlled.

5. METHODS

5.1 Plant materials and activation tagging for *bri1-5* genetic modifiers

All plants used in these studies including *bri1-5, dwf4-1,* and *bri1-4* were in *Arabidopsis* ecotype WS2 background. Unless specified, plants were grown under 16h light (150–200 µmol /m^2/sec) and 8h dark condition. *bri1-5-tcp1-1D* double mutant was obtained by a large scale activation-tagging screen for *bri1-5* suppressors as described previously (Li *et al*., 2001; 2002; Zhou *et al*., 2004; Yuan *et al*., 2007). Briefly, a homemade activation-tagging construct, pBASTA-AT2, was transformed into bri1-5 to generate transgenic plants with resistance to Basta herbicide. *bri1-5-tcp1-1D* was identified as one of the *bri1-5* suppressors.

5.2 Determination of *bri1-5 tcp1-1D* locus

TAIL-PCR was used to amplify the flanking genomic sequence of the T-DNA of pBASTA-AT2 as described previously (Liu and Whittier, 1995; Terauchi and Kahl, 2000). The T-DNA insertion site was determined by sequencing the flanking genomic DNA. The T-DNA was inserted in the promoter of *TCP1* (*At1g67260*), 2,281bp upstream from its start codon. The activation-tagged gene was determined by RT-PCR and recapitulation experiment was conducted to confirm the result.

5.3 Construction of expression vectors

Full length *TCP1* cDNA was amplified with primers TCP1-attb1, GTACAAAAAAGCAGGCTATGTCGTCTTCCACCAATGACTAC and TCP1-attb2,

GTACAAGAAAGCTGGGTcGTTTACAAAAGAGTCTTGAATCCA, *TCP1-SRDX* was amplified with primers TCP1-attb1, GTACAAAAAAGCAGGCTATGTCGTCTTCCACCAATGACTAC and TCP1-SRDXattb2,GTACAAGAAAGCTGGGTTTATGCAAATCCCAGTCTGAGTTCCAGG TCGAGATCCAGGTTTACAAAAGAGTCTTGAATCCAA. Both sequences were cloned into gateway vector pBIB-BASTA-35S-GWR-GFP as described previously (Yuan *et al*.,2007), and overexpressed in *bri1-5* and WS2 plants. Transgenic lines were analyzed for phenotypes.

5.4 Real-time RT-PCR analyses

Total RNA was extracted using RNeasy plant mini kits with on-column DNase treatment (Qiagen). Total RNA (2 µg) was reversely transcribed to the first strand of the cDNA in a 20µl volume using the SuperScript III first-strand synthesis system (Invitrogen). One to 2 µl RT product was used as PCR template in a 20µl volume reaction. Different PCR cycles were conducted in order to get better result. PCR products were separated by 1% agarose gel electrophoresis and visualized under UV-scanner. Real-time RT-PCR analysis was the same as previously described (Gou *et al*., 2009). *ACT2* was used as a control. Primers for *TCP1* expression analysis: AACTCCTCGATTGGTTCCTTGTAG and CTTCCACCAATGACTACAACGATG, for *DWF4* expression: ATGTTCGAAACAGAGCATCATAC and GAGATCGAGAATTTGCTCCGTC, for *DET2*: CTTCCGATACTGTCTCCTCACTC and GAGTCTTGGGATACTCTTCCTTG, for CPD: GATAGGAGAGACTTTTCAGCTG and CTTCATCGGAAAATCCATCATC, for *BR6OX1*: GTCTTCTCTTGATCATCGTGTCT and

CCTAAGATGGAAGCCTTTTGGTG, for *BR6OX2*: GTACTGCTCTTCTCCGATGGAAC and GGTGCAGAAACTCTTGGAAAGAC, for *EF1a*: CAGGCTGATTTGTGCTGTCCT and TCAAGTAGCAAAATCACGGCGCTT.

5.5 Hypocotyl measurements

All of the seeds used for hypocotyl analysis were surface-sterilized as described previously (Yuan *et al*., 2007) and planted on ½ MS medium supplemented with 1% sucrose, 0.8% agar and 24-epiBL or BRZ. The plates were kept at 4°C for 2 days, and then were grown vertically in white light at room temperature. Hypocotyl length of seedlings was measured after 5 days. All measurements were obtained from three independent experiments and at least 20 seedlings were measured each time.

5.6 Immunoblotting analysis

Immunoblotting analysis was performed as previously described (Li *et al*., 2002; Wang *et al*., 2005). Both anti-GFP (A11120) and anti-ß-glucuronidase (A-5790) antibodies were from Invitrogen at Eugene, Oregon, USA. BR Profile Analysis Aerial parts of 4-week-old WS2, *tcp1-1D* and *tcp1-SRDX* seedlings were harvested and lyophilized. The tissues (15 g of lyophilized tissues per assay) were extracted twice with 250 ml of MeOH. Deuterium-labeled internal standards were added to the extracts. Purification and quantification of BRs and sterols were carried out according to the method described previously (Fujioka *et al*., 2002).

5.7 Confocal microscopy analysis

Seeds of transgenic plants over-expressing *TCP1-GFP* were surface sterilized in 95% ethanol, 20% chlorox and washed with sterile double deionized water prior to planting. Sterilized seeds were germinated in 62 mm X 48 mm glass coverslips coated

with 0.5% agar supplemented with 1/2 MS salts, 0.5 mg/ml pyridoxine-HCL, 0.5 mg/mL nicotinic acid, 1 mg/ml thiamine, 0.10 g/L myo-inositol, 0.5 g/L MES, and 1% sucrose. The pH of the agar-nutrient medium was adjusted to 5.7 with 10 M KOH. After 3-4 days, the primary roots were imaged with a Leica TCS SP2 AOBS laser scanning confocal microscope equipped with a 63X water immersion objective (Leica Microsystems, Exton, PA). Seedlings were counterstained with 5 µM propidium iodide for simultaneous visualization of GFP and the cell wall. Propidium iodide and GFP were excited with the 488 nm line of the argon laser and emission was detected at 510 nm and 620 nm respectively.

5.8 CHIP analysis

EpiQuik Plant ChIP Kit (Epigentek, Brooklyn, NY) was used to conduct ChIP analysis. The experiments were performed based on the specifications from the manufacture. Briefly, seedlings (1-2 g) were used to get chromatin pellets. Re-suspend chromatin pellet in 500 µl CP3F containing protease inhibitor cocktail. Shear DNA to about 500bp fragments by sonicating, then centrifuge to get supernatants. Dilute supernatant and transfer the diluted supernatant to antibody (anti-GFP)-bound strip well and incubate at room temperature for 60-90 min with gentle shaking. TCP1-GFP specifically associated DNA fragments were purified and eluted from the column. The obtained DNA fragments were ready for real-time PCR to test. Primers for amplifying *DWF4p* GATTGGGAATCGGACTTCTACTG and GGAGCATAACGAGGCAACAAAAG; for amplifying for amplifying *BES1p* CTTCTATAATTCCAGCGAAGAAG and TCTGTGTAAGAAAAGGAGCTGA; for amplifying *BZR1p* TGTTCAATGAACTATACAAGTTTTG and

GTGAGTATGTAAAATGAGTAATGAC; for amplifying *EF1ap* AACCACTCTCGTTGCTGATTC and ACAAGATCGATCAGAATGGAAa; for amplifying *ACTIN2p* CGTTTCGCTTTCCTTAGTGTTAGCT and CACAACGCATGCTAAACAGATCTAG.

5.9 Generation of promoter-GUS transgenic plants

A 1.5kb promoter fragment of *TCP1* was amplified from the *Arabidopsis* genomic DNA by PCR with primers GTACAAAAAAGCAGGCTTCCCTTAAACTTACTAGGGTAG and GTACAAGAAAGCTGGGTCGACATCACCGAACTTAAGAAG. A 1.1kb promoter fragment of DWF4 was amplified from genomic DNA by PCR with primers TACAAAAAAGCAGGCTATAGTTGGTGAATTCAAATATCTC and GTACAAGAAAGCTGGGTGGAGCTAGTTTCTCTCTCTCTC. Then they were cloned into gateway vector pBIB-BASTA-GUS-GWR and were transformed into *Arabidopsis* ecotype-WS2. Homozygote transgenic plants were planted on soil or ½ MS medium with 1% sucrose for GUS activity assay. *TCP1p-GUS* plants were planted on 1/2MS medium containing 1% sucrose with or without epi-BL or BRZ for 5 days under light or darkness before determining for GUS activity. Seedlings or tissues were vacuum-infiltrated in X-Gluc solution, followed by overnight incubation at room temperature, then destained with 70% ethanol and visualized for blue color.

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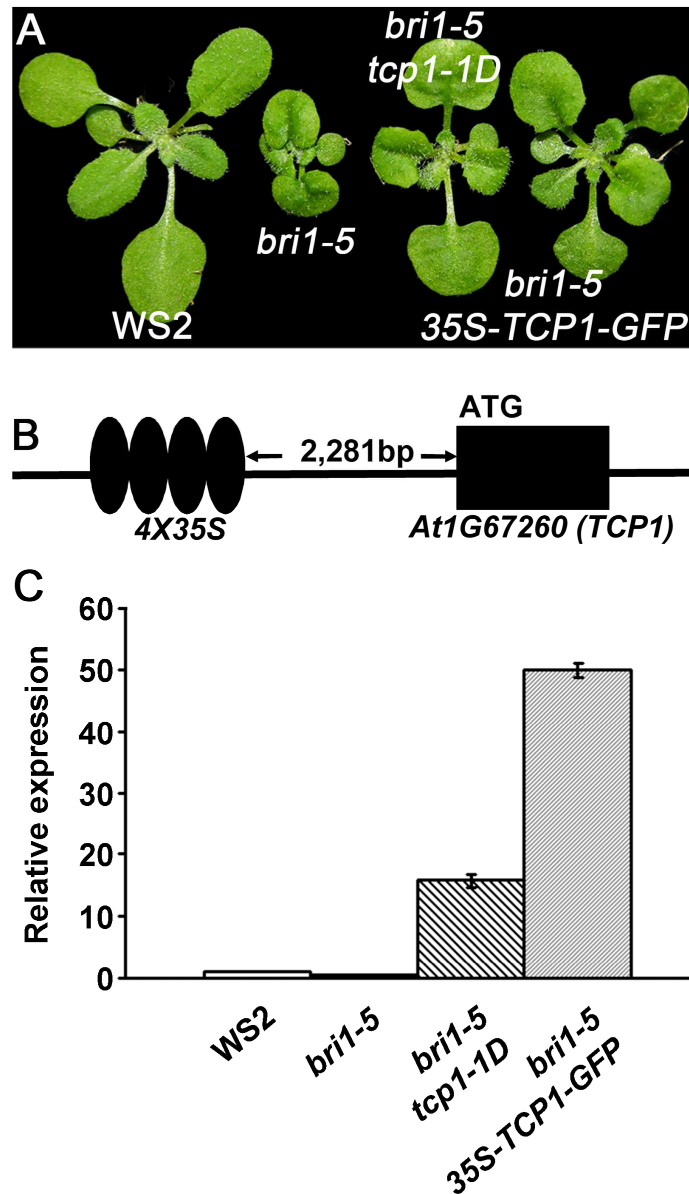


Figure **3. 1.** *tcp1-1D* was identified as a dominant genetic suppressor of *bri1-5* by an activation tagging screen.

(**A**) Phenotypes of WS2, *bri1-5*, *bri1-5 tcp1-1D*, and *bri1-5* 35S-*TCP1-GFP* plants.

(**B**) In *bri1-5 tcp1-1D*, four copies of 35S enhancer were inserted at 2281 bp upstream of the start codon ATG.

(**C**) The expression of *TCP1* was elevated in *bri1-5tcp1-1D* as well as in *bri1-5* 35S-*TCP1-GFP* plants compared to WS2 and *bri1-5* as shown by q-PCR.

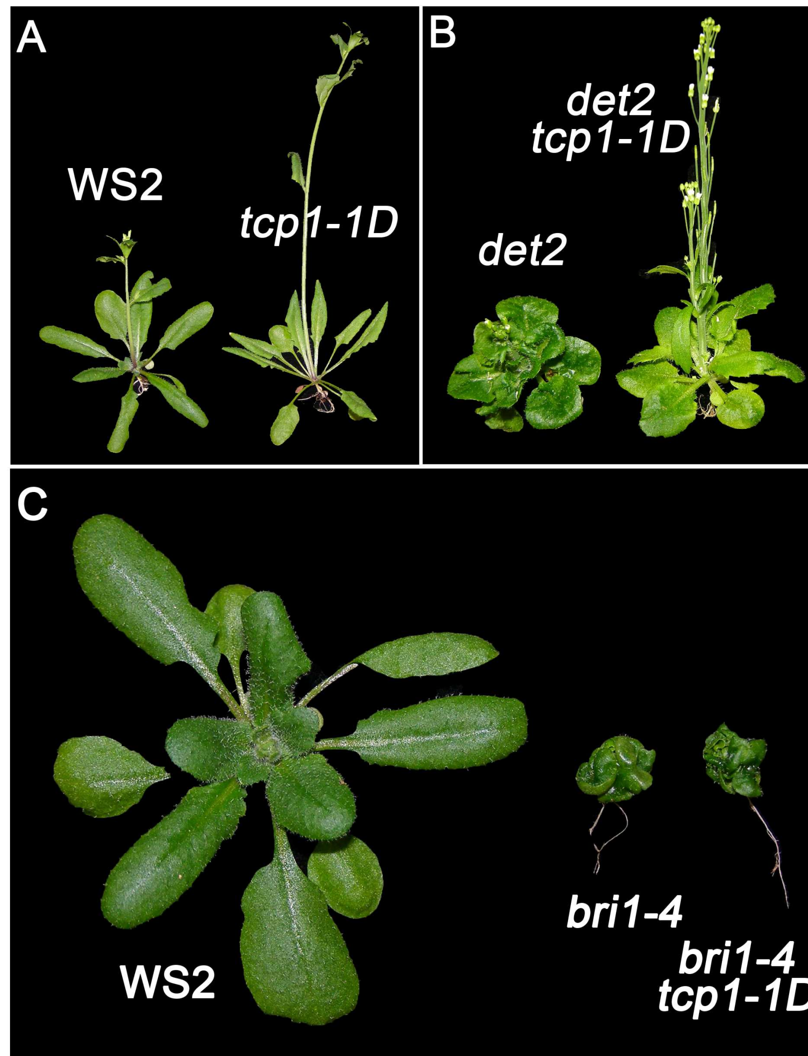


Figure **3. 2.** *tcp1-1D* can suppress weak BR signaling and biosynthetic mutants, but can not suppress a null allele of *BRI1*.

(**A**) WS2 *tcp1-1D* showed a phenotype reminiscent of the phenotypes of *BRI1*- or *DWF4*-overexpressed plants.

(**B**) *tcp1-1D* can partially suppress the defective phenotypes of a BR biosynthetic mutant *det2*.

(**C**) *tcp1-1D* cannot suppress a *BRI1* null allele mutant *bri1-4*.

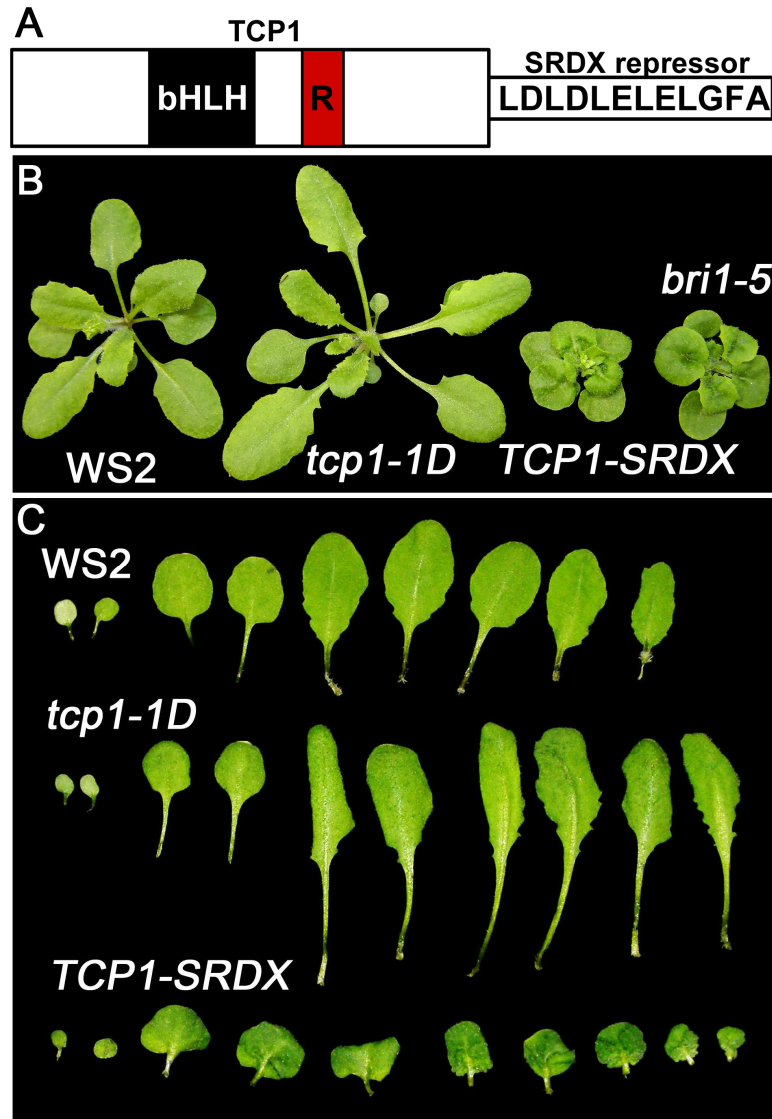
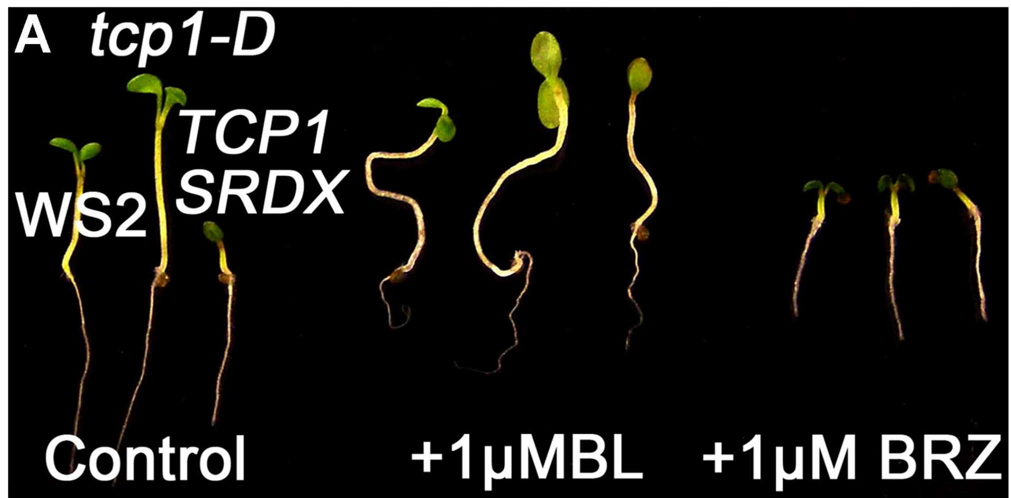


Figure **3. 3.** Transgenic plants overexpressing a *TCP1* dominant negative sequence (*TCP1-SRDX*) show a typical BR mutant phenotype.

(**A**) Protein structures of TCP1. TCP1 contains a typical bHLH domain and an R-domain. For the dominant negative version, a 12-amino acid SRDX repressor sequence was fused at the carboxyl terminus of TCP1.

(**B**) Phenotypes of WS2, *tcp1-1D*, *TCP1-SRDX*, and *bri1-5*. The plants were photographed three weeks after germination.

(**C**) Leaf phenotypes of three-week-old WS2, *tcp1-1D*, and *TCP1-SRDX* plants.



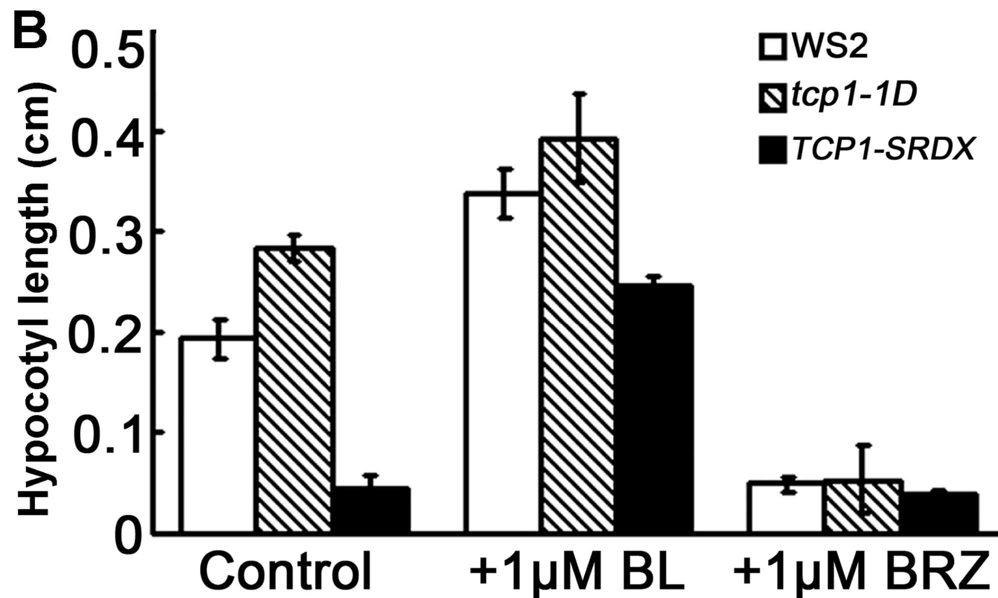


Figure **3. 4.** BL treatment rescues the shortened hypocotyl phenotype of *tcp1-SRDX*.

(**A**) Phenotypes of representative WS2, *tcp1-1D*, and *tcp1-SRDX* seedlings grown on ½ MS medium under light condition with no BL treatment, 1 μM BL treatment, or 1 μM BRZ treatment, respectively. The seedlings were photographed five days after germination.

(**B**) The measurements corresponding to the seedlings shown in (A). Twenty seedlings per genotype were used for the measurements.

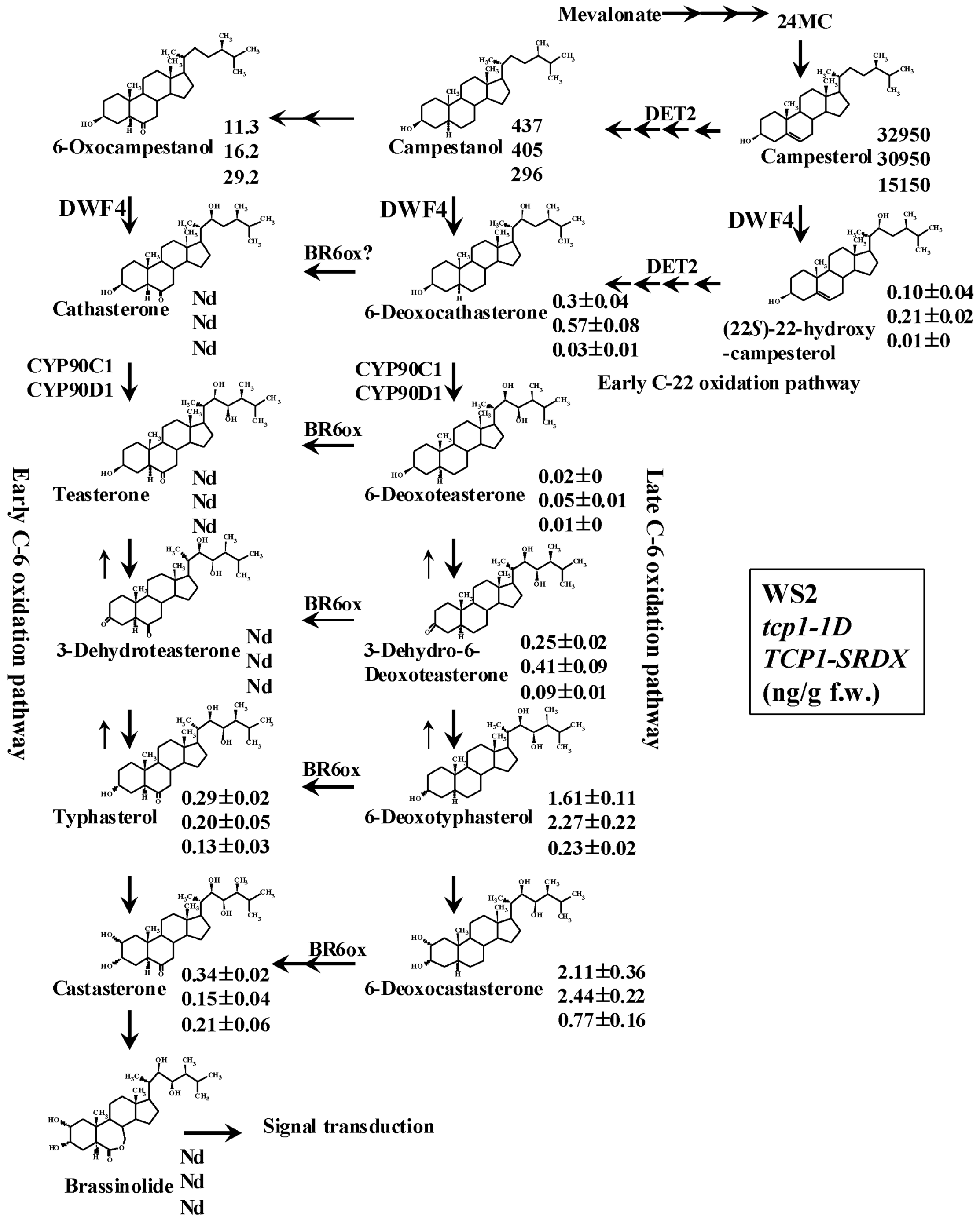


Figure **3. 5.** BR profile analyses of four-week-old soil grown WS2, *tcp1-1D*, and *tcp1-SRDX* Plants.

The numbers shown were the averages from three independent replicas. nd: not detected (below detection limit).

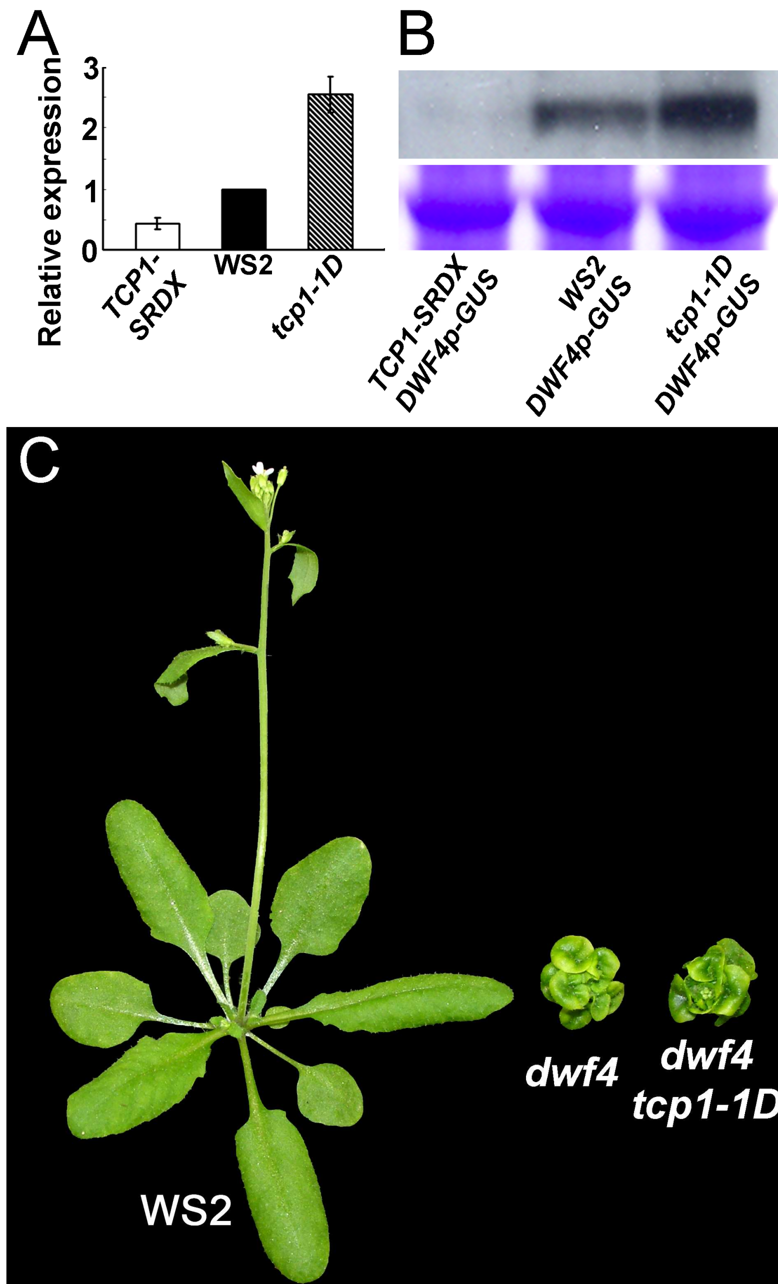


Figure **3. 6.** *TCP1* positively regulates the expression of *DWF4*.

(**A**) q-PCR results indicate that the expression of *DWF4* is drastically reduced in *tcp1-SRDX* but significantly elevated in *tcp1-1D* plants compared to WS2.

(**B**) GUS expression droved by *DWF4* promoter is drastically decreased in *TCP1-SRDX-DWF4p-GUS* plants but significantly increased in*TCP1-1D-DWF4P-GUS* plants compared to *WS2-DWF4p-GUS* plants, as shown by Western-blotting.

(**C**) *tcp1-1D* does not rescue the phenotypes of a null *dwf4* mutant.

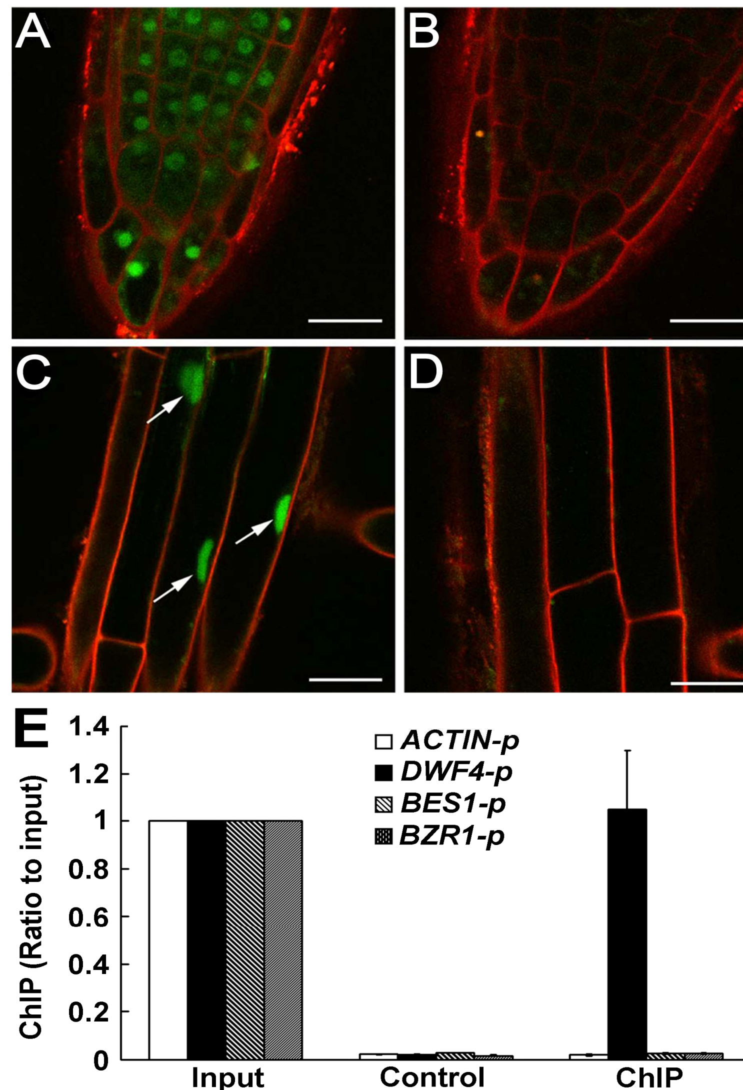


Figure **3. 7.** TCP1 is localized to the nucleus and Chromatin Immunoprecipitation (CHIP) results indicate that TCP1 directly binds to *DWF4* promoter.

(**A**) and (**C**) Confocal microscopy shows that the TCP1-GFP fusion is localized in the nucleus. Transgenic plants harboring 35S-*TCP1-GFP* were used for the analysis.

(**B**) and (**D**) Wild-type plants were used as controls. No fluorescent signals were detected in the analysis. Roots were counterstained with propidium iodide to visualize the outline of the cells. Scale bars = 20 m

(**E**) q-PCR results for CHIP show that anti-GFP antibody can co-immunoprecipitate the *DWF4* promoter region but not the promoter regions of *ACTIN, BES1,* and *BZR1*.

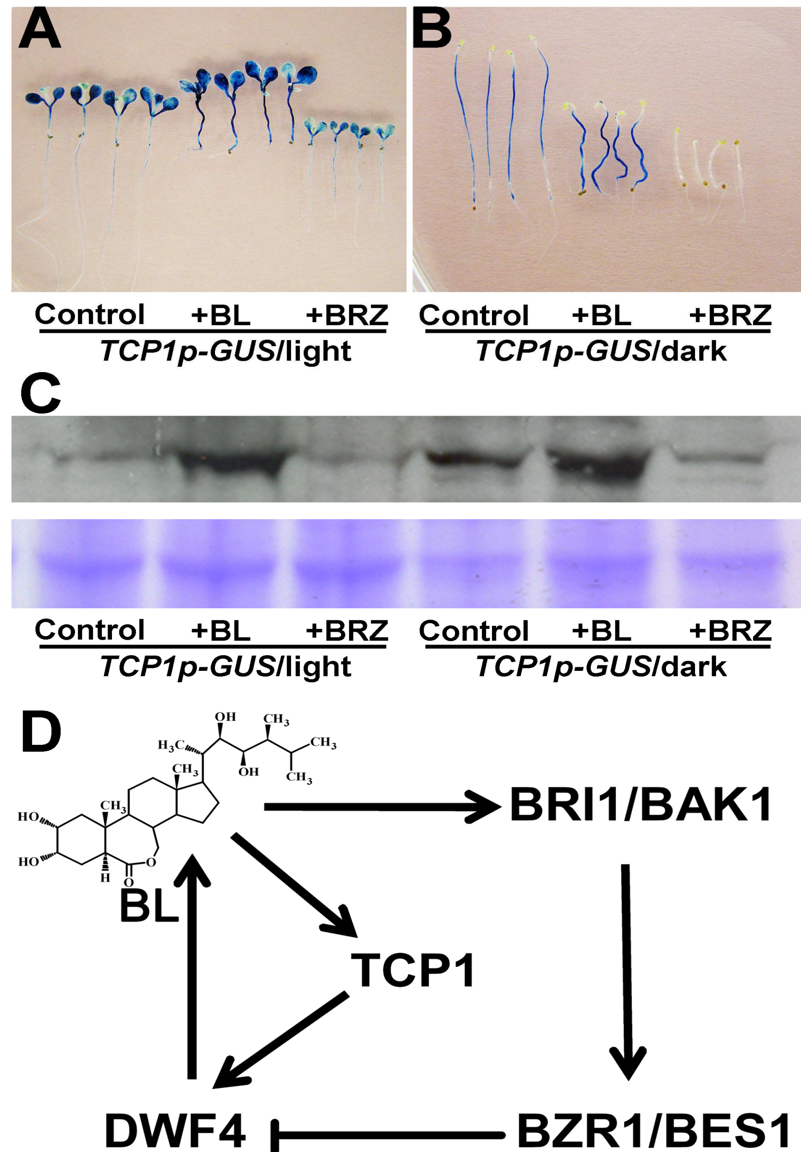


Figure **3. 8.** The expression of *TCP1* is regulated by BL via a positive feed-back mechanism.

(**A**) Light-grown transgenic seedlings harboring *TCP1promoter-GUS* show that the expression levels of *TCP1* can be positively regulated by the BL treatment and negatively regulated by the treatment with BRZ.

(**B**) Dark-grown seedlings are used for the same experiments shown in (**A**).

(**C**) Western-blotting results for GUS expression levels in (**A**) and (**B**).

(**D**) A current model suggesting that the expression of *DWF4* can be negatively regulated by BZR1/BES1 via a feed-back loop, and also can be positively regulated by TCP1 via a Positive Feed-back mechanism.

SUPPLEMENTAL DATA

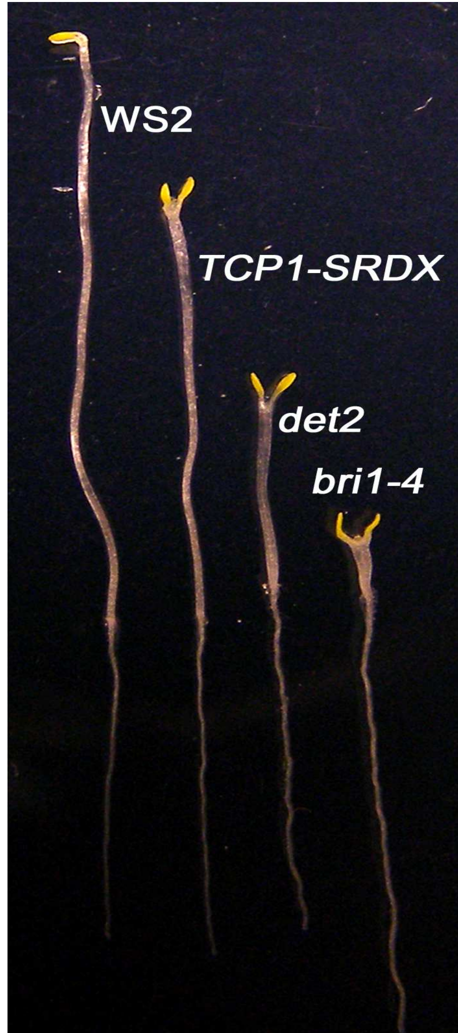
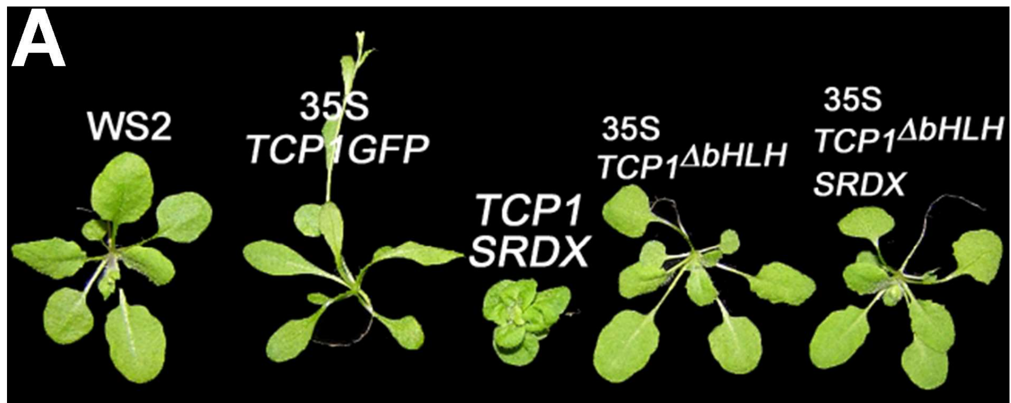


Figure **3. S1.** *TCP1-SRDX* transgenic plants show a typical de-etiolated phenotype when grown in darkness.

The seedlings were grown on ½ MS medium supplemented with 1% sucrose for 5 days before they were photographed. The opened cotyledon phenotype of *TCP1-SRDX* is similar to that of the *det2* and *bri1-4* mutants.



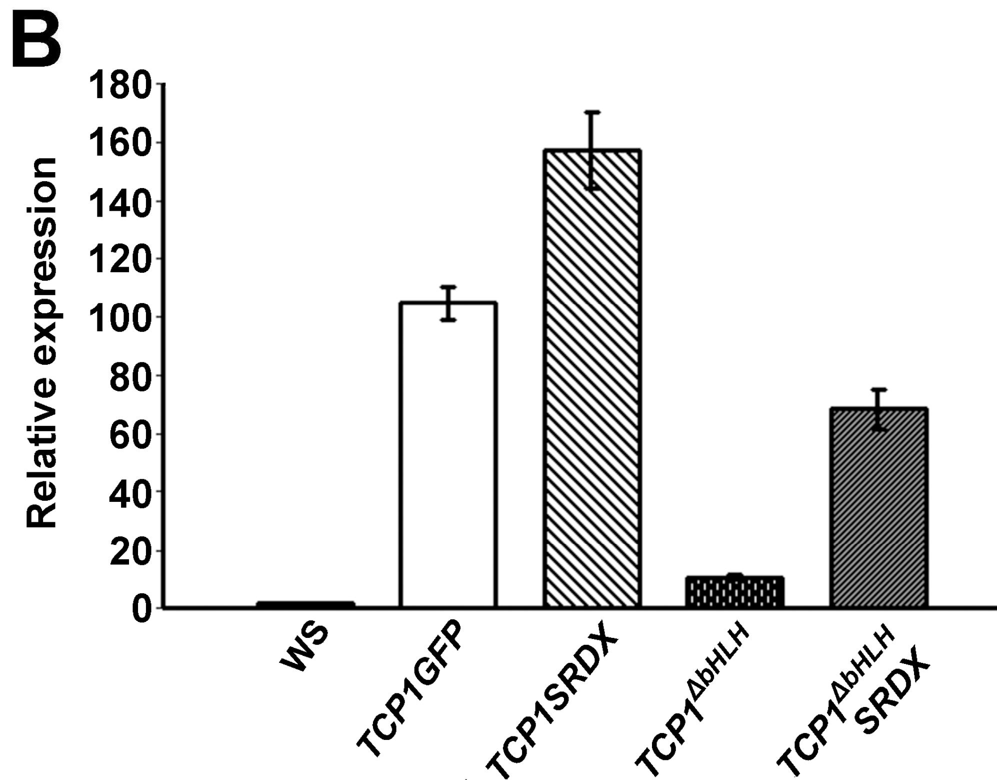


Figure **3. S2.** bHLH domain in TCP1 is critical for the function of TCP1 and TCP1-SRDX in regulating plant growth.

(**A**) The phenotypes of wild type (WS2) and various transgenic plants overexpressing various *TCP1* constructs.

(**B**) q-PCR results for the expression levels of each of the transgenes in (**A**). All plants shown are representative homozygous lines.

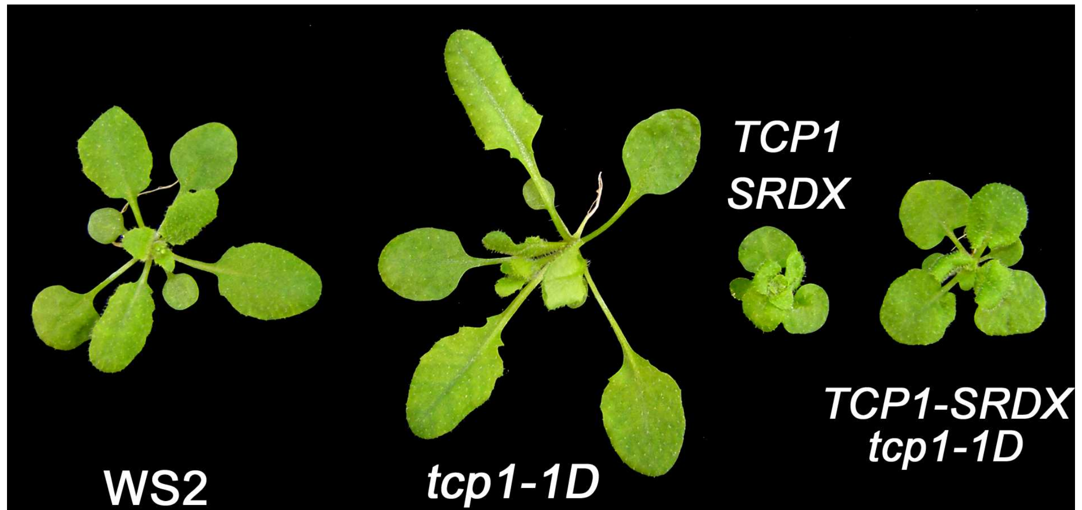
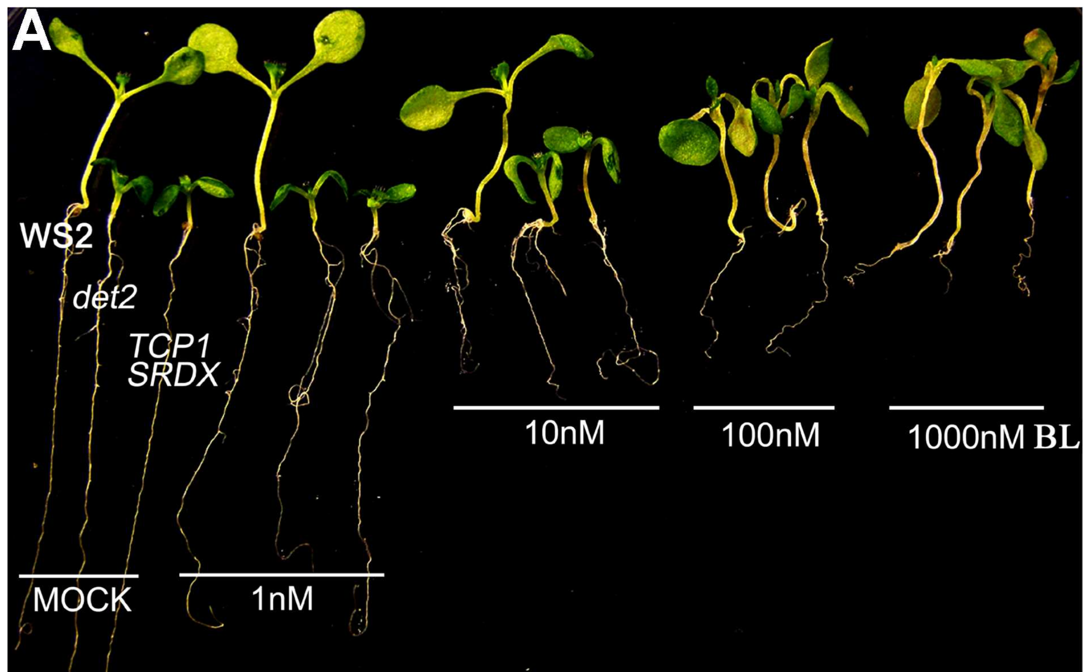


Figure **3. S3.** The dwarfed phenotype of *TCP1-SRDX* plants can be partially rescued by an additional *tcp1-1D* allele.

All the plants shown are homozygous plants.



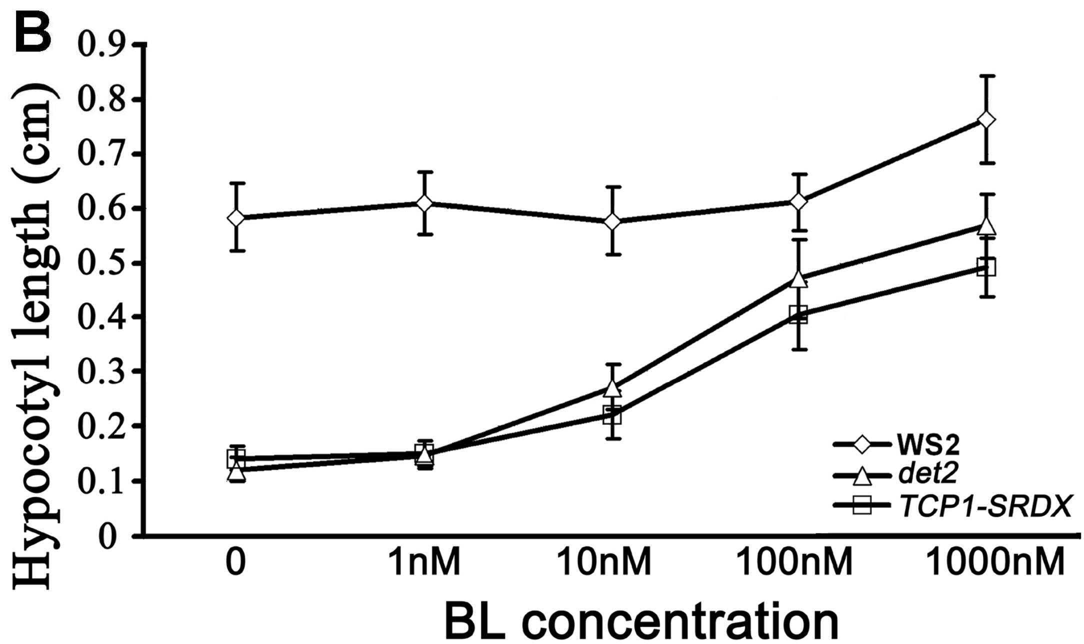


Figure **3. S4.** Growth of *TCP1-SRDX* plants in response to BL treatment is similar to a BR deficiency mutant *det2*.

(**A**) The phenotypes of WS2, *det2*, and *TCP1-SRDX* in response to various concentrations of BL.

(**B**) Hypocotyl length of each genotype in response to different concentrations of BL.

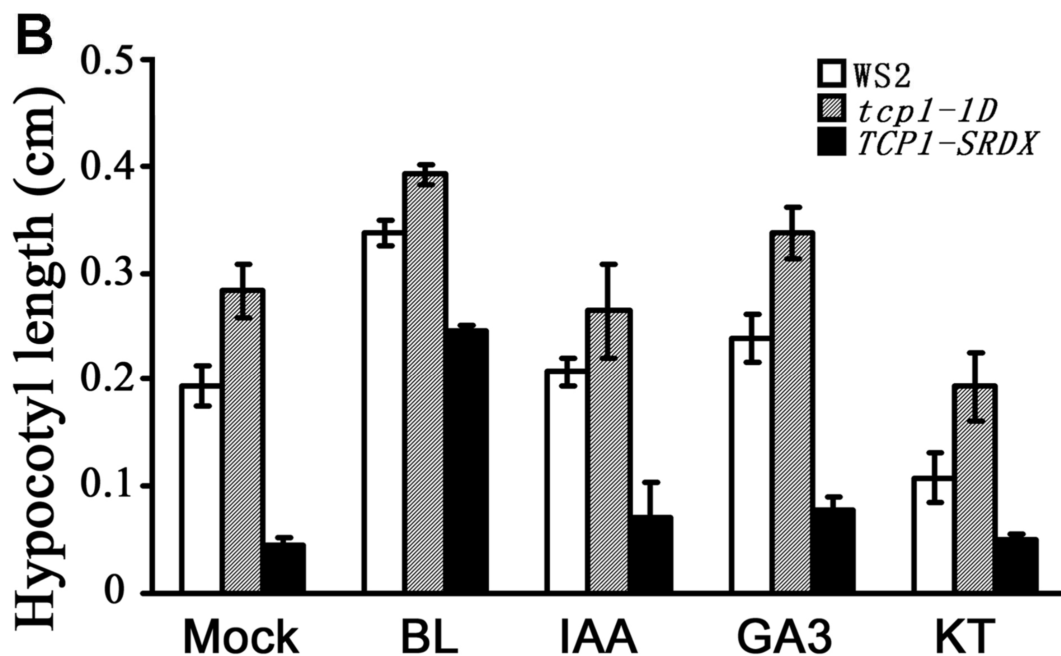


Figure **3. 3.S5**. The dwarfed phenotype of *TCP-SRDX* plants can be rescued by the treatment of BL but not by other growth-promoting plant hormones.

(**A**) Phenotypes of various seedlings grown in ½ MS medium supplemented with different phytohormones.

(**B**) Measurements of the seedlings shown in (**A**).

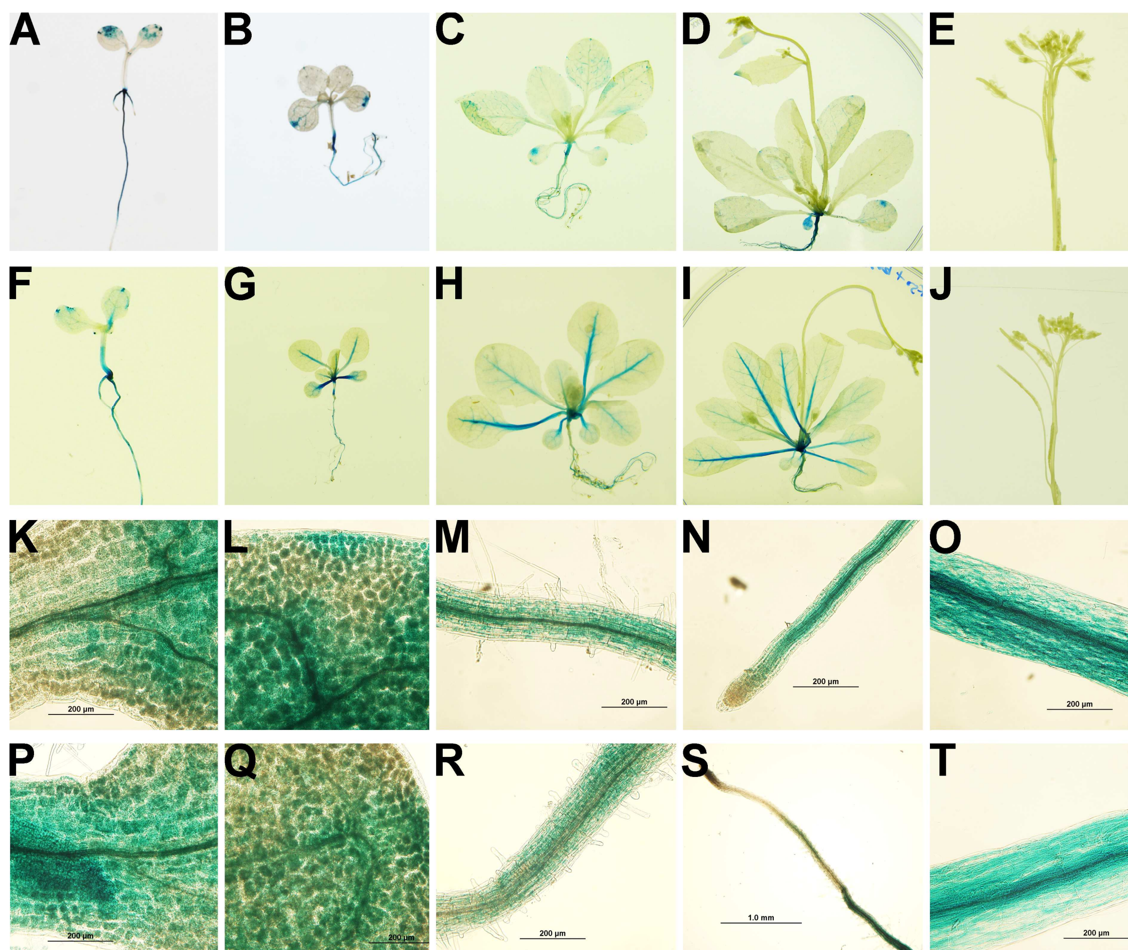


Figure **3. 3. S6.** Comparison of GUS expression pattern between *DWF4p-GUS* transgenic plants and *TCP1p-GUS* transgenic plants.

(**A**)**,** (**B**)**,** (**C**)**,** (**D**) and (**E**) for 5-day-old, 10-day-old, 20-day-old, 30-day-old *DWF4p-GUS* transgenic plants and its flower (**F**) (**G**) (**H**) (**I**) and (**J**) for *TCP1p-GUS* transgenic plants and its flower at the same developmental stages (**K**)**,** (**L**)**,** (**M**), (**N**) and (**O**) for petiole, leaflet, haired region of root, root-tip and hypocotyl of 10-day-old *DWF4p-GUS* transgenic plants photographed under Microscopy (**P**)**,** (**Q**)**,** (**R**), (**S**) and (**T**) for the same tissues of 10-day-old *TCP1p-GUS* transgenic plants photographed under Microscopy.

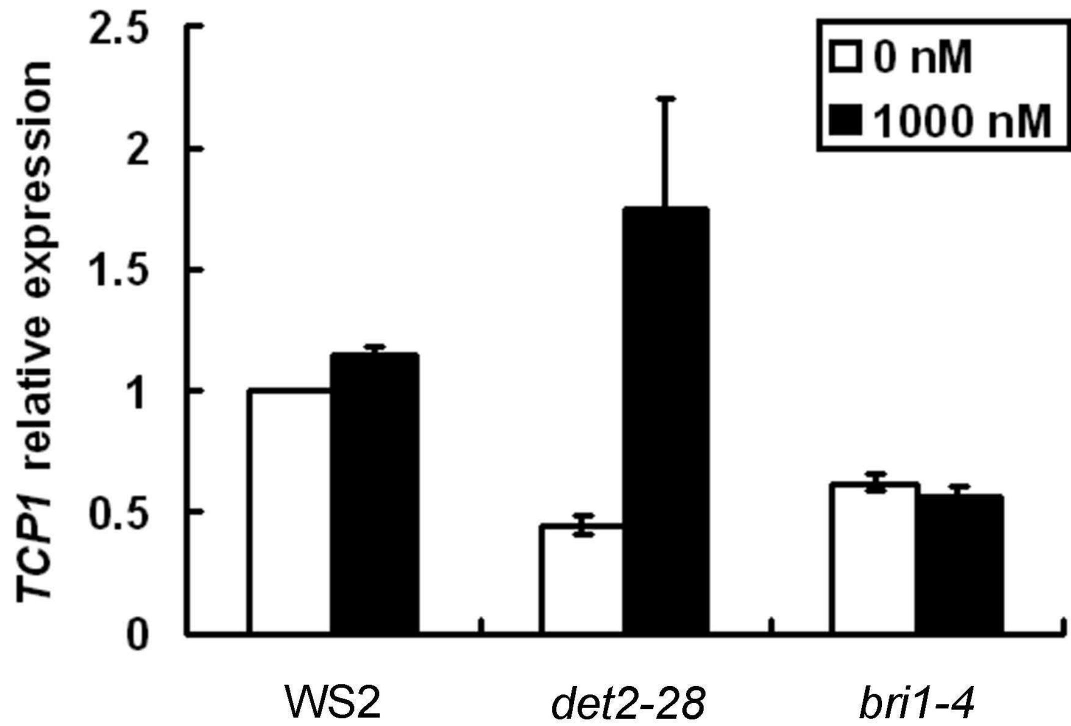


Figure **3. 3.S7.** TCP1 transcription level regulated by BL in different mutant background.

REFERENCES

Asami, T., and Yoshida, S (1999). Brassinosteroid biosynthesis inhibitors. Trends Plant Sci 4, 348-353.

Asami, T., Min, Y.K., Nagata, N., Yamagishi, K., Takatsuto, S., Fujioka, S., Murofushi, N., Yamaguchi, I., and Yoshida, S (2000). Characterization of brassinazole, a triazole-type brassinosteroid biosynthesis inhibitor. Plant Physiol 123, 93-100.

Bai, M.Y., Zhang, L.Y., Gampala, S.S., Zhu, S.W., Song, W.Y., Chong, K., and Wang, Z.Y (2007). Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signaling in rice. Proc Natl Acad Sci U S A 104, 13839-13844.

Bishop, G., Nomura, T., Yokota, T., Montoya, T., Castle, J., Harrison, K., Kushiro, T., Kamiya, Y., Yamaguchi, S., Bancos, S., Szatmari, A.M., and Szekeres, M (2006). Dwarfism and cytochrome P450-mediated C-6 oxidation of plant steroid hormones. Biochem Soc Trans 34, 1199-1201.

Bishop, G.J., Harrison, K., and Jones, J.D (1996). The tomato Dwarf gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. Plant Cell 8, 959-969.

Bishop, G.J., Nomura, T., Yokota, T., Harrison, K., Noguchi, T., Fujioka, S., Takatsuto, S., Jones, J.D., and Kamiya, Y (1999). The tomato DWARF enzyme catalyses C-6 oxidation in brassinosteroid biosynthesis. Proc Natl Acad Sci U S A 96, 1761-1766.

Broholm, S.K., Tahtiharju, S., Laitinen, R.A., Albert, V.A., Teeri, T.H., and Elomaa, P (2008). A TCP domain transcription factor controls flower type specification

along the radial axis of the Gerbera (Asteraceae) inflorescence. Proc Natl Acad Sci U S A 105, 9117-9122.

Choe, S., Dilkes, B.P., Fujioka, S., Takatsuto, S., Sakurai, A., and Feldmann, K.A (1998). The DWF4 gene of *Arabidopsis* encodes a cytochrome P450 that mediates multiple 22alpha-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell 10, 231-243.

Choe, S., Fujioka, S., Noguchi, T., Takatsuto, S., Yoshida, S., and Feldmann, K.A (2001). Overexpression of DWARF4 in the brassinosteroid biosynthetic pathway results in increased vegetative growth and seed yield in *Arabidopsis*. Plant J 26, 573-582.

Chory, J., Nagpal, P., and Peto, C.A (1991). Phenotypic and Genetic Analysis of det2, a New Mutant That Affects Light-Regulated Seedling Development in *Arabidopsis*. Plant Cell 3, 445-459.

Clough, S.J., and Bent, A.F (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16, 735-743.

Clouse, S.D (1996). Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. Plant J 10, 1-8.

Clouse, S.D., and Sasse, J.M (1998). BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development. Annu Rev Plant Physiol Plant Mol Biol 49, 427-451.

Cubas, P., Lauter, N., Doebley, J., and Coen, E (1999). The TCP domain: a motif found in proteins regulating plant growth and development. Plant J 18, 215-222.

Ehsan, H., Ray, W.K., Phinney, B., Wang, X., Huber, S.C., and Clouse, S.D (2005). Interaction of *Arabidopsis* BRASSINOSTEROID-INSENSITIVE 1 receptor kinase with a homolog of mammalian TGF-beta receptor interacting protein. Plant J 43, 251-261.

Fujioka, S., and Yokota, T (2003). Biosynthesis and metabolism of brassinosteroids. Annu Rev Plant Biol 54, 137-164.

Fujioka, S., Takatsuto, S., and Yoshida, S (2002). An early C-22 oxidation branch in the brassinosteroid biosynthetic pathway. Plant Physiol 130, 930-939.

Fujioka, S., Li, J., Choi, Y.H., Seto, H., Takatsuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J., and Sakurai, A (1997). The *Arabidopsis* deetiolated2 mutant is blocked early in brassinosteroid biosynthesis. Plant Cell 9, 1951-1962.

Gampala, S.S., Kim, T.W., He, J.X., Tang, W., Deng, Z., Bai, M.Y., Guan, S., Lalonde, S., Sun, Y., Gendron, J.M., Chen, H., Shibagaki, N., Ferl, R.J., Ehrhardt, D., Chong, K., Burlingame, A.L., and Wang, Z.Y (2007). An essential role for 14-3-3 proteins in brassinosteroid signal transduction in *Arabidopsis*. Dev Cell 13, 177-189.

He, J.X., Gendron, J.M., Sun, Y., Gampala, S.S., Gendron, N., Sun, C.Q., and Wang, Z.Y (2005). BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. Science 307, 1634-1638.

Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. Plant J 34, 733-739.

Jiang, J., and Clouse, S.D (2001). Expression of a plant gene with sequence similarity to animal TGF-beta receptor interacting protein is regulated by brassinosteroids and required for normal plant development. Plant J 26, 35-45.

Kim, H.B., Kwon, M., Ryu, H., Fujioka, S., Takatsuto, S., Yoshida, S., An, C.S., Lee, I., Hwang, I., and Choe, S (2006). The regulation of DWARF4 expression is likely a critical mechanism in maintaining the homeostasis of bioactive brassinosteroids in *Arabidopsis*. Plant Physiol 140, 548-557.

Kosugi, S., and Ohashi, Y (2002). DNA binding and dimerization specificity and potential targets for the TCP protein family. Plant J 30, 337-348.

Koyama, T., Furutani, M., Tasaka, M., and Ohme-Takagi, M (2007). TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in *Arabidopsis*. Plant Cell 19, 473-484.

Li, J., and Chory, J (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90, 929-938.

Li, J., Lease, K.A., Tax, F.E., and Walker, J.C (2001). BRS1, a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 98, 5916-5921.

Li, J., Nagpal, P., Vitart, V., McMorris, T.C., and Chory, J (1996). A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science 272, 398-401.

Li, J., Biswas, M.G., Chao, A., Russell, D.W., and Chory, J (1997). Conservation of function between mammalian and plant steroid 5alpha-reductases. Proc Natl Acad Sci U S A 94, 3554-3559.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell 110, 213-222.

Liu, Y.G., and Whittier, R.F (1995). Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25, 674-681.

Luo, D., Carpenter, R., Copsey, L., Vincent, C., Clark, J., and Coen, E (1999). Control of organ asymmetry in flowers of Antirrhinum. Cell 99, 367-376.

Mora-Garcia, S., Vert, G., Yin, Y., Cano-Delgado, A., Cheong, H., and Chory, J (2004). Nuclear protein phosphatases with Kelch-repeat domains modulate the response to brassinosteroids in *Arabidopsis*. Genes Dev 18, 448-460.

Nam, K.H., and Li, J (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell 110, 203-212.

Nam, K.H., and Li, J (2004). The *Arabidopsis* transthyretin-like protein is a potential substrate of BRASSINOSTEROID-INSENSITIVE 1. Plant Cell 16, 2406-2417.

Neff, M.M., Nguyen, S.M., Malancharuvil, E.J., Fujioka, S., Noguchi, T., Seto, H., Tsubuki, M., Honda, T., Takatsuto, S., Yoshida, S., and Chory, J (1999). BAS1: A gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. Proc Natl Acad Sci U S A 96, 15316-15323.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K.A., and Tax, F.E (1999). Brassinosteroid-insensitive dwarf mutants of *Arabidopsis* accumulate brassinosteroids. Plant Physiol 121, 743-752.

Nomura, T., Jager, C.E., Kitasaka, Y., Takeuchi, K., Fukami, M., Yoneyama, K., Matsushita, Y., Nyunoya, H., Takatsuto, S., Fujioka, S., Smith, J.J., Kerckhoffs, L.H., Reid, J.B., and Yokota, T (2004). Brassinosteroid deficiency due to truncated steroid 5alpha-reductase causes dwarfism in the lk mutant of pea. Plant Physiol 135, 2220-2229.

Ohnishi, T., Szatmari, A.M., Watanabe, B., Fujita, S., Bancos, S., Koncz, C., Lafos, M., Shibata, K., Yokota, T., Sakata, K., Szekeres, M., and Mizutani, M (2006). C-23 hydroxylation by *Arabidopsis* CYP90C1 and CYP90D1 reveals a novel shortcut in brassinosteroid biosynthesis. Plant Cell 18, 3275-3288.

Poppenberger, B., Fujioka, S., Soeno, K., George, G.L., Vaistij, F.E., Hiranuma, S., Seto, H., Takatsuto, S., Adam, G., Yoshida, S., and Bowles, D (2005). The UGT73C5 of *Arabidopsis thaliana* glucosylates brassinosteroids. Proc Natl Acad Sci U S A 102, 15253-15258.

Rouleau, M., Marsolais, F., Richard, M., Nicolle, L., Voigt, B., Adam, G., and Varin, L (1999). Inactivation of brassinosteroid biological activity by a salicylate-inducible steroid sulfotransferase from Brassica napus. J Biol Chem 274, 20925-20930.

Ryu, H., Kim, K., Cho, H., Park, J., Choe, S., and Hwang, I (2007). Nucleocytoplasmic shuttling of BZR1 mediated by phosphorylation is essential in *Arabidopsis* brassinosteroid signaling. Plant Cell 19, 2749-2762.

Schommer, C., Palatnik, J.F., Aggarwal, P., Chetelat, A., Cubas, P., Farmer, E.E., Nath, U., and Weigel, D (2008). Control of jasmonate biosynthesis and senescence by miR319 targets. PLoS Biol 6, e230.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., and Weigel, D (2006). Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell 18, 1121-1133.

Szekeres, M., Nemeth, K., Koncz-Kalman, Z., Mathur, J., Kauschmann, A., Altmann, T., Redei, G.P., Nagy, F., Schell, J., and Koncz, C (1996). Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell 85, 171-182.

Tanaka, K., Asami, T., Yoshida, S., Nakamura, Y., Matsuo, T., and Okamoto, S (2005). Brassinosteroid homeostasis in *Arabidopsis* is ensured by feedback expressions of multiple genes involved in its metabolism. Plant Physiol 138, 1117-1125.

Tang, W., Kim, T.W., Oses-Prieto, J.A., Sun, Y., Deng, Z., Zhu, S., Wang, R., Burlingame, A.L., and Wang, Z.Y (2008). BSKs mediate signal transduction from the receptor kinase BRI1 in *Arabidopsis*. Science 321, 557-560.

Tatematsu, K., Nakabayashi, K., Kamiya, Y., and Nambara, E (2008). Transcription factor AtTCP14 regulates embryonic growth potential during seed germination in *Arabidopsis thaliana*. Plant J 53, 42-52.

Terauchi, R., and Kahl, G (2000). Rapid isolation of promoter sequences by TAIL-PCR: the 5'-flanking regions of Pal and Pgi genes from yams (Dioscorea). Mol Gen Genet 263, 554-560.

Wang, X., and Chory, J (2006). Brassinosteroids regulate dissociation of BKI1, a negative regulator of BRI1 signaling, from the plasma membrane. Science 313, 1118-1122.

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., Huber, S.C., and Clouse, S.D (2008). Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. Dev Cell 15, 220-235.

Wang, Z.Y., Seto, H., Fujioka, S., Yoshida, S., and Chory, J (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature 410, 380-383.

Wang, Z.Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., and Chory, J (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. Dev Cell 2, 505-513.

Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., Nguyen, J.T., Sato, S., Wang, Z.Y., Xia, Y., Dixon, R.A., Harrison, M.J., Lamb, C.J., Yanofsky, M.F., and Chory, J (2000). Activation tagging in *Arabidopsis*. Plant Physiol 122, 1003-1013.

Yin, Y., Vafeados, D., Tao, Y., Yoshida, S., Asami, T., and Chory, J (2005). A new class of transcription factors mediates brassinosteroid-regulated gene expression in *Arabidopsis*. Cell 120, 249-259.

Yin, Y., Wang, Z.Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., and Chory, J (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. Cell 109, 181-191.

Yuan, T., Fujioka, S., Takatsuto, S., Matsumoto, S., Gou, X., He, K., Russell, S.D., and Li, J (2007). BEN1, a gene encoding a dihydroflavonol 4-reductase (DFR)-like

protein, regulates the levels of brassinosteroids in *Arabidopsis thaliana*. Plant J 51, 220-233.

Zhou, A., Wang, H., Walker, J.C., and Li, J (2004). BRL1, a leucine-rich repeat receptor-like protein kinase, is functionally redundant with BRI1 in regulating *Arabidopsis* brassinosteroid signaling. Plant J 40, 399-409.

Note

This chapter was publicated on *The Plant Cell* April 2010. I have obtained permission from *The Plant Cell* press and from all co-authors, Shozo Fujioka, Elison B. Blancaflor, Sen Miao, Xiaoping Gou and Jia Li, to include the full text of the paper in my dissertation.

Chapter IV

CUR, an F-box containing protein, modulates leaf morphogenesis in *Arabidopsis thaliana*

// 1. Abstract

Gain-of-function mutant *cur-1D* was identified as an enhancer of *bri1-5* from a genetic modifier screening using an activation tagging method. The *bri1-5cur-1D* double mutant exhibits enhanced defects in growth and development compared to *bri1-5*. Through TAIL-PCR, it was found that enhancers were inserted in the promoter of CUR, an F-box gene, resulting the elevated expression of *CUR*. Reversion and recapitulation experiments indicate that *CUR* is the authentic gene responsible for the enhancer. Physiological analysis indicated it could be a potential regulator of BR pathway. Further molecular and genetic studies suggested CUR modulates leaf morphogenesis by regulating expression of related homeotic genes in *Arabidopsis*.

2. Introduction

BRs play an essential role in regulating normal plant growth and development. Blocking either BR biosynthesis or BR signaling pathway will cause WT plants to show severe defective phenotypes, such as curly leaves, small rosette, dwarfism and male sterility (Clouse *et al.*, 1996; Szekeres *et al.,* 1996; Li *et al.,* 1997; Clouse, 1998; Choe *et al.,* 1998; Choe *et al.,* 1999). In the efforts to elucidate how BRs function in plants, some important components in BR signaling have been identified and characterized and an outline of BR signaling pathway has been proposed. BRs are perceived by a leucine rich repeat receptor like kinase (LRR-RLK) BRI1 on the cell surface (Li *et al.,* 1997; He *et al.,* 2000; Wang *et al.,* 2001). Upon BR binding BRI1 is activated by a sequential phosphorylation between BRI1 and BAK1 to initiate downstream BR signaling (Wang *et al.,* 2008; Oh *et al.,* 2009; Wang *et al.,* 2005). BIN2, a GSK3- SHAGGY like kinase, was identified as a negative regulator functioning downstream of BRI1-BAK1 in the BR

signal transduction. In the gain-of-function mutant *bin2*, BIN2 acquires constitutive kinase activity, which caused WT plants to show classical BR mutant phenotypes (Li *et al.,* 2001; Li *et al.,* 2002). In the presence of BRs, activated BRI1 phosphorylates and activates BSK1, a BRI1-substrate kinase. BSK1 activates BSU1, which will dephosphorylates and inhibit BIN2, consequently resulting in the accumulation of transcription factors BZR1/BES1 in the nucleus to regulate the expression of BR response genes. If BRs are absent in plants, BIN2 will phosphorylate BZR1/BES1. Phosphorylated BZR1/BES1 will be subsequently degraded through a proteasome (Li *et al.,* 2002; He *et al.,* 2002). Therefore protein degradation machinery may play a critical role in BR signal transduction in plants, although the mechanism underlying the degradation is still unknown.

In our research, *cur-1D* was identified as an enhancer from the *bri1-5* mutant through activation tagging (Weigel *et al.,* 2000). *CUR* encodes a typical F-box protein in *Arabidopsis*. *CUR* is activated in *cur-1D* enhancer, and its over-expression is tied to the enhanced defective phenotypes. *WS2-cur-1D* showed a different response to BL, suggesting a potential role in BR pathway. Furthermore, CUR controls leaf morphogenesis by inducing the ectopic expression of homeotic genes related to leaf development in *Arabidopsis*.

F-box protein is one subunit of SCF complex in ubiquitin-mediated protein degradation machinery. As shown in Figure 4.1, in the ubiquitin-mediated protein degradation pathway, ubiquitin is activated by E1 enzyme. Activated ubiquitin is transferred to conjugating enzyme E2, and ubiquitin then will be transferred to ubiquitin ligase E3. Eventually target substrates becomes polyubiquitinated and is degraded

through proteasome (Hochstrasser *et al.,* 1996; Schwechheimer *et al.,* 2004; Vierstra RD *et al.,* 2009). SCF complex is a major E3 enzyme complex, consisting of four subunits: F-box protein, SKP1, CUL1 and ROC1. The F-box protein is the key subunit for recongnizing and recruiting specific target substrates for degradation (Craig *et al.,* 1999; Willems *et al.,* 2004; Ho *et al.,* 2008; Yee *et al.,* 2009). *Arabidopsis* has about 700 F-box proteins that presumbly mediate the degradation of many specific substrates involved in many biological processes (Lechner *et al.,* 2006). For example, F-box proteins are involved in the signaling pathway of other plant hormones. In auxin pathway, auxin is perceived by TIR1, an F-box protein. After Auxin binding, TIR1 will target AUX/IAA, a negative regulator of auxin pathway, for degradation (Parry *et al.,* 2006; Nemhauser *et al.,* 2005; Mockaitis *et al.,* 2008). The pivotal regulator of jasmonica acid pathway, COI1, is also an F-box protein that may function as receptor of JA and mediate JAZ degradation (Santner *et al.,* 2007; Browse *et al.,* 2009; Chini *et al.,* 2009). In gibberellic acid signaling, two F-box proteins, SLY1 and SNE, target transcription repressor, DELLA, for degradation (Ito *et al.,* 2003; Thomas *et al.,* 2004; Ito *et al.,* 2008). Therefore it is interesting to determine how CUR, an F-box protein potentially involved in the BR signaling pathway, functions in leaf morphogenesis by mediating the degradation of components of the pathway.

3. Results

3.1 *cur-1D* was identified as an enhancer from *bri1-5* background via activation tagging

A large scale genetic modifier screening was conducted as described in Chapter II. *cur-1D* was identified as one of enhancers of the *bri1-5* background. As shown in Figure 4.2, *bri1-5cur-1D* displays dwarfism phenotype with even curled leaves compared to *bri1-5*. After *bri1-5 cur-1D* was crossed with WS2 to segregate out mutated BRI1, *WS2-cur-1D* also shows dwarfism with curled leaves and small flower, suggesting that CUR may be a negative regulator functioning independent of BRI1. However, *WS2-cur-1D* showed earlier flowering phenotype, which is different from the delayed-flowering phenotype of BR biosynthetic mutants or BR signaling mutants.

3. 2 Hypocotyls elongation of *WS2-cur-1D* is hypersensitive to high concentration of BL.

To determine whether or not the mutant is related to the BR pathway, we treated *WS2-cur-1D* with BL using WT as a control. Plants were grown for 5 days in ½ MS media in the light with different concentrations of BL. The hypocotyls length of *WS2-cur-1D* was significantly increased at high concentration of BL compared to WT, which suggests that CUR may be a potential regulator of the BR pathway (Figure 4.3).

3. 3 An F-Box protein is activated in the enhancer

To uncover what gene was activated in *cur-1D* mutant, the flanking sequence of T-DNA insertion was determined using TAIL-PCR. The T-DNA containing 35S enhancers was inserted near *At1g65740* (*CUR*) (Figure 4.4). *CUR* encodes an F-box protein with unknown functions. RT-PCR analysis showed that *CUR* is activated in *cur-1D* compared to *bri1-5* (Figure 4-4).

3. 4 Reversion analysis and recapitulation experiment.

An RNAi vector was developed and transformed into *bri1-5 cur-1D* enhancer to decrease *CUR* expression. The expression level of CUR was reduced in selected transformants, and these transformants showed phenotypes similar to *bri1-5*. The result indicates that reversion of overexpression of *CUR* can restore phenotypes of *bri1-5 cur-1D* to that of *bri1-5* (Figure 4.5). To further confirm this result, a recapitulation experiment was also conducted. *CUR* CDS was cloned into a binary expression vector and was overexpresed in WS2 plants under the control of a 35S promoter. Transgenic WS2 plants resemble the phenotype of *WS2-cur-1D*, like short statue, curly leaves and early flowering. The phenotype was tightly co-related to the expression level of the transgenic gene (Figure 4.6). Therefore, reversion and recapitulation experiments demonstrated that *CUR* is the real gene responsible for the enhanced defective phenotypes of *cur-1D*.

3. 5 Loss of function analysis via microRNA

To further characterize the function of CUR, a loss of functional analysis was also explored. After BLAST in TAIR Database, we found that two tandem duplicated homologs of CUR are present in the genome of *Arabidopsis* (*At1g65760 and At1g65770*). Both share high identities to CUR, suggesting they may function redundantly to *CUR*. Both *At1g65760* and *At1g65770* knockout mutants were obtained from ABRC, but a *CUR* knockout line is still not available. However, *At1g65760* and *At1g65770* knockout mutants do not show substantial phenotypes compared to WT. Moreover it is impossible to generate triple or double mutants by crossing since they are tandem duplicated homologs. To address this problem, microRNA technique was employed to specifically

knockdown *CUR* and *At1g65770* simultaneously. The microRNA was introduced into an *At1g65760* knockout line to diminish the expressions of all three genes at the same time (Rebecca, 2006; Serrano-Cartagena, 2008; Stephan, 2008). As shown in Figure 4.7, Only *CUR* expression was silenced by the microRNA in *At1g65760* knockout. However, the generated loss-of-function plants displayed curly leaves although the symptom of curly leaves is not severe as gain-of–function mutant *cur-1D*. If examined closely, the blades of the leaves are adaxially curled, which is opposite to the abaxially curled leaves of *WS2-cur-1D*. Therefore it seems that CUR together with its homologs may control leaf development in *Arabidopsis*.

3. 6 Ectopic expression of homeotic genes in *WS2-cur-1D*

Leaf morphogenesis starts from leaf primordia emerged from shoot apical meristem. It is well demonstrated that repressing expression of homeobox genes KNAT, STM and AG is required for the normal leaf development in *Arabidopsis* (Goodrich, 1997; Katz A, 2004; Xu, 2008). CURLY LEAF (CLF) together with its partners is responsible for the repression via an epigenetic mechnism (Kim GT, 1998; Köhler, 2002; Schubert, 2006). CLF, the central component of the repression complex, is a polycomb protein (Jiang, 2008; Mayama, 2003). In wild-type plants, the repression on these homeostic genes by CLF is counteracted by trithorax (ATX1) to balance expression of related homeotic genes probably because of the essentiality of these homeotic genes for plant growth and development. In *clf* mutants, disruption of CLF will lead to ectopic expression of these homeotic genes, resulting in abnormal curly leaves (Saleh, 2007; Carles, 2009). In our research, *WS2-cur-1D* phenocopied *clf* mutants, suggesting a ectopic expression of these homeotic genes in *WS2-cur-1D*. As shown in Figure 4.8, AG,

STM and KNAT were ectopically expressed in leaves of *WS2-cur-1D* compared to WT, which suggests a function of CUR in leaf morphogenesis by interacting with CLF or its partners. Coincidently, CLF accumulates in the nucleus to repress related homeotic genes, F-box protein CUR also localizes to nucleus (Figure 4.9), which prompted us to hypothesize that CUR may mediate the ubiquitin-mediated degradation of CLF in the nucleus to regulate leaf morphogenesis in *Arabidopsis*.

4. Discussion

4. 1 CUR is the gene responsible for *bri1-5 cur-1D* enhancer

CUR, an F-box protein, was identified from the enhancer of *bri1-5 cur-1D* mutant obtained by activation tagging screen. Expression of *CUR* was significantly elevated in the enhancer compared to *bri1-5*. Reversion of expression by RNAi restored phenotype of *bri1-5 cur-1D* to *bri1-5*. Furthermore, over-expression of *CUR* in *bri1-5* recapitulated the phenotype of its original enhancer *bri1-5 cur-1D*.

4. 2 CUR is a potential regulator of BR pathway

bri1-5cur-1D enhancer showed more severe defects compared to *bri1-5*. It is extremely dwarf with curly leaves and reduced fertility, which is similar to *bri1* null allele mutant. When crossed with WS2 plants to segregate mutated BRI1, resulted *WS2-cur-1D* showed defective phenotypes similar to weak allele BR mutants. And *WS2-cur-1D* displayed different response to BL application. Hypocotyl length can be significantly increased at high BL concentration compared to wild-type plants, and the response was similar to BR deficient mutant *det2*. The results indicate that CUR, an F-box protein, may

function in the BR pathway by mediating degradation of components involved in BR pathway. Identifying the target proteins could reveal the mechanism underlying.

4. 3 CUR functions in leaf morphogenesis

Leaf is one fundamental structure of plants and is the major organ for photosynthesis. Normal leaf morphogenesis is critical to the success of a whole plant. Leaf morphogenesis begins when the shoot apical meristem (SAM) undergo differentiation to form a leaf primordium. A key step is the differentiation of adaxial and abaxial face of leaves, which will determine the basic shape of a leaf. Once the basic shape of the leaf is established, leaf morphogenesis will give the leaf a final shape (Tsukaya, 2002; Tsukaya, 2003; Tsukaya, 2005). Molecular genetic studies demonstrated that repressing the expression of some homeotic genes such as *AG, KNAT* in leaf is the key to ensure normal leaf morphogenesis in *Arabidopsis* (Scofield *et al*., 2006; Katz, 2004). It is also found that these homeotic genes are epigenetically repressed by polycomb proteins CLF and SWINGER in leaves. Once CLF or SWINGER is disrupted, these homeotic genes are ectopically expressed and produce abnormal leaves (Goodrich, 1997; Katz A, 2004; Xu, 2008). Interestingly, *WS2-cur-1D* shows abnormal leaf morphology. Its phenotypes resemble the CLF knockout mutant. And those epigenetically repressed homeotic genes were also ectopically expressed in *WS2-cur-1D* just like in *clf* mutant. Furthermore, when CUR was silenced using microRNA in a knockout line of *At1g65760*, transgenic plants showed adaxially curled leaves, instead of abaxially curled leaves of *WS2-cur-1D*. These results indicate that F-box protein CUR may modulate leaf morphogenesis by mediating the degradation of the repressors of homeotic genes. Since *WS2-cur-1D* resembles *clf* mutant, and CUR also is a nucleus

protein like CLF, it remains to be seen whether or not CLF is the direct target protein of CUR.

5. Plant materials and experimental methods

5.1 Plant Materials and Activation Tagging for *bri1-5* Genetic Modifiers

All plants used in these studies were in *Arabidopsis* ecotype WS2. Plants were grown under 16h light (150–200 µmol m^{-2} sec^{-1}) and 8h dark conditions. *bri1-5cur-1D* double mutant was obtained by a large scale activation-tagging screen as described previously (Li *et al*., 2001; 2002; Zhou *et al*., 2004; Yuan *et al*., 2007).

5.2 Determination of *bri1-5 cur-1D* locus

TAIL-PCR was used to amplify the flanking genomic sequence of the T-DNA of *pBASTA-AT* as described previously (Liu and Whittier, 1995; Terauchi and Kahl, 2000). The T-DNA insertion site was determined by sequencing the flanking genomic DNA. The T-DNA was inserted in the promoter of *CUR* (*At1g65740*), 2,900bp upstream from the start codon. The expression of the activation-tagged gene was determined by RT-PCR.

5.3 Reversion experiment by RNAi

Antisense fragment of *CUR* CDS was amplified with primer pair CUR-Ri-attb1-GTACAAAAAAGCAGGCTgaaaTCCAAAAGATCAAGGCTCT and CUR-Ri-attb2-GTACAAGAAAGCTGGGTGATCGTTCTCCCTAGTTGAATAC and was cloned into pBIB-BASTA-RNAi-GW vector and was transferred into *bri1-5cur-1D* to induce RNA silencing of *CUR*. *CUR* expression level was further examined by RT-PCR.

5.4 Recapitulation experiment

Full length *CUR* cDNA was amplified with primers CUR1-attb1-GTACAAAAAAGCAGGCTATGGTAGATTGGTCTACCTTACC and CUR1-attb2-CAAGAAAGCTGGGTTCAAAGAAAGCTAGGAAAAAACATT,
And was cloned into gateway vector *pBIB-BASTA-35S-GWR-GFP* as described previously (Yuan *et al*., 2007), and overexpressed in WS2 plants. Transgenic lines were selected by herbicide resistance and then analyzed for phenotypes.

5.5 Generation of knockdown mutant by MicroRNA

MicroRNA specifically mediating degradation of *CUR* (*At1g65740*) and its homolog *At1g65770* was designed using Web MicroRNA Designer 3 (WMD3). Four primers I, II, III, IV were designed to clone the MicroRNA sequence. The microRNA was cloned into vector pBIB-BASTA-35S-GW according to protocol from www.weigelworld.org, and was transformed into knockout Salk_003055 of CUR homolog *At1g65760*, with purpose to silence all three tandem genes (*At1g65740, At1g65760, At1g65770*) at the same time. Transgenic plants were selected by herbicide resistance and then analyzed for phenotypes.
Primer I, gaTAGTATCGGTAGCTATGACAAtctctcttttgtattcc, primer II: gaTTGTCATAGCTACCGATACTAtcaaagagaatcaatga, primer III: gaTTATCATAGCTACGGATACTTtcacaggtcgtgatatg, primer IV: gaAAGTATCCGTAGCTATGATAAtctacatatatattcct. Primer pairs for RT-PCR are listed on Table 4-1.

5.6 RT-PCR Analysis and Primers

Total RNA was extracted using RNeasy plant mini kits with on-column DNase-treatment (Qiagen). Total RNA (2 µg) was reversedly transcribed to the first strand of the

cDNA in a 20 μl volume using the SuperScript III first-strand synthesis system (Invitrogen). A one to two microlitter RT product was used as PCR template in a 20 μl volume reaction. PCR products were separated by 1% agarose gel electrophoresis and visualized under UV-scanner. Primer pairs for RT-PCR was listed on table 4.1.

5.7 Confocal Microscopy Analysis

Seeds of transgenic plants over-expressing *CUR-GFP* were surface sterilized in 95% ethanol, 20% chlorox and washed with sterile double deionized water prior to planting. Sterilized seeds were germinated in 62 mm X 48 mm glass coverslips coated with 0.5% agar supplemented with 1/2 MS salts, 0.5 mg/ml pyridoxine-HCL, 0.5 mg/mL nicotinic acid, 1 mg/ml thiamine, 0.10 g/L myo-inositol, 0.5 g/L MES, and 1% sucrose. The pH of the agar-nutrient medium was adjusted to 5.7 with 10 M KOH. After 3-4 days, the primary roots were imaged with a Leica TCS SP2 AOBS laser scanning confocal microscope equipped with a 63X water immersion objective (Leica Microsystems, Exton, PA). GFP were excited with the 488 nm line of the argon laser and emission was detected at 510 nm and 620 nm respectively.

Sequence Name	Sequence 5'-3'
AG-F	CAA TTG ATG GGT GAG ACG AT
AG-R	CGC GGA TGA GTA ATG GTG AT
AP3-F	GTT CTC TAG CTC CAA CAA GCT TC
AP3-R	CAA GAA CTG AGT CGT AAT CTC CTC
SEP1-F	GCA GCT CCT CAA ACA TGC TC
SEP1-R	CTG AGC TTG ATG ATG CGC G
SEP2-F	CTG CAG CAC CTC CAA CAT GC
SEP2-R	CTC TGA GCA CAC TGG ATG GC
SEP3-F	GTA GTT CGA GCA TGC TTC GG
EP3-R	CAC ACT TGG TCC TGC TCC C
KNAT2-F	GAA GAG ATT CAG CGA GAG AAC C
KNAT2-R	GAA TCG TCC ATC ATA TCA AAC GGC ATG
KNAT6-F	GAT GAT GTC ACC GGA GAG TCT C
KNAT6-R	GAC TCG ACA CCA GTA CAT AGG TTC
STM-F	GTG CTC CTG CCT ATT CTC TAA TG
STM-R	CTC GGA TGA CCC ATT ATT GTT C
EF1a-F	CAG GCT GAT TGT GCT GTT CTT ATC AT
EF1a-R	CTT GTA GAC ATC CTG AAG TGG AAG A
CUR-F	ATGGTAGATTGGTCTACCTTACC
CUR-R	CATTTCGAAGGAACTCTCGGAAG
CLF-F	CAG GAC TGA GTC TGA AAG ACC T
CLF-R	CTA AAG AAG AAG CTT GCA GCT G
At1g65760-F	ATGGTTGATTGCGATTGGTCTAAC
At1g65760-R	CATTTCGAAAGAACTCTTAGAAG
At1g65770-F	ATGGCTGATTGGTCTACCTTAC
At1g65770-R	TCAGAGAAAGCTAGGAACAAAC

Table 4-1, Primer pairs for RT-PCR analyses.

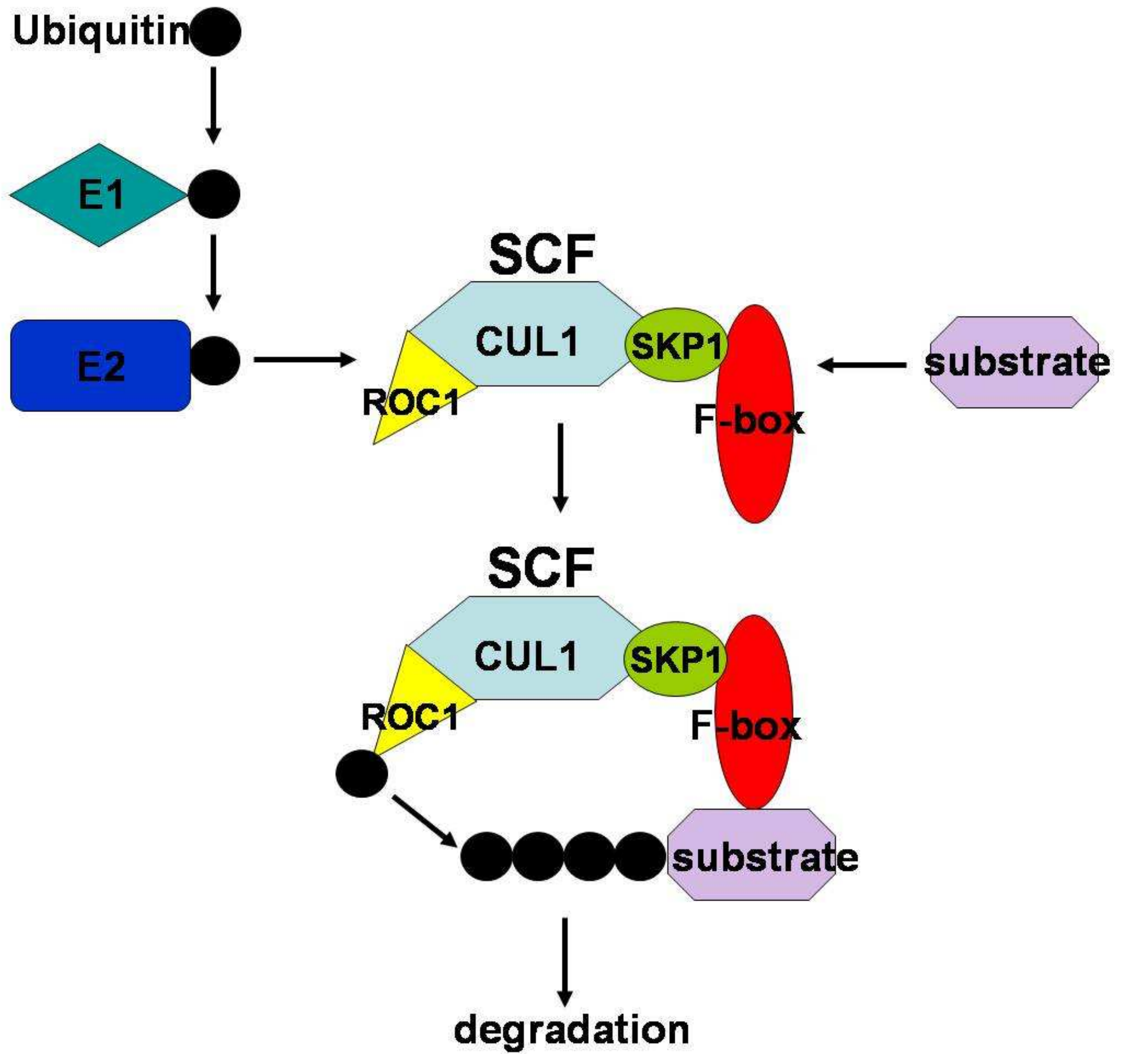


Figure **4.1** Ubiquitin-mediated degradation pathway. Ubiquitin is activated by activating enzyme E1 and is transferred to E2, conjugating enzyme, then it is transferred to E3 complex. Once specific substrate is preyed by F-box, ubiquitin will tag substrate for degradation through proteasome.

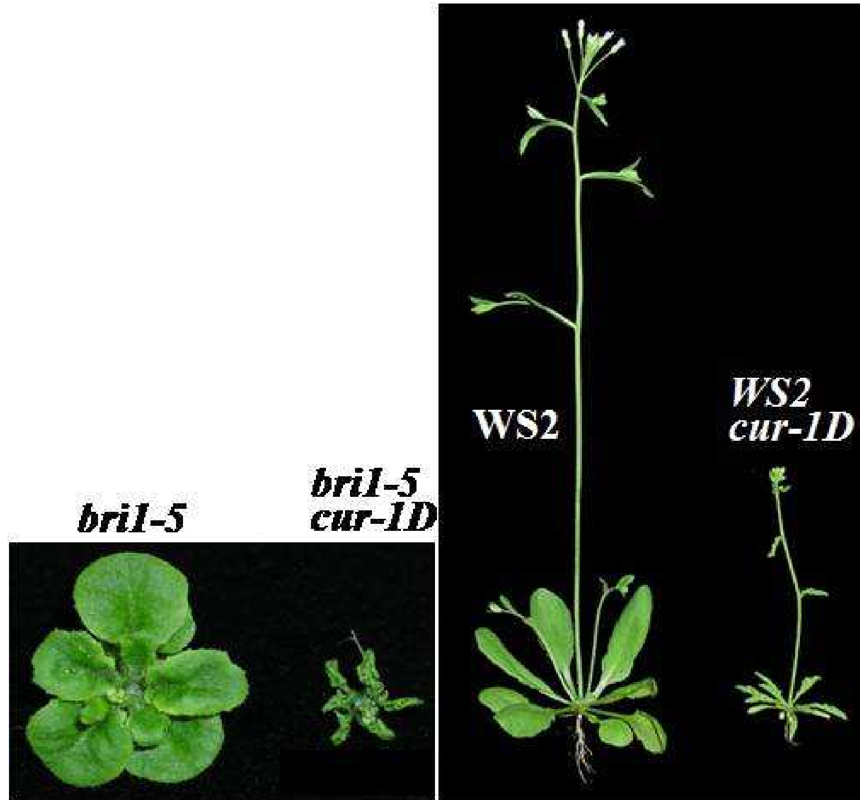


Figure **4.2** Phenotypes of *cur-1D* enhancer mutant. *bri1-5cur-1D* showed enhanced defects compared to *bri1-5* and *WS2-cur-1D* mutants showed small statue with curly leaves, small rosette, and abnormal flowers compared to WS2 plants.

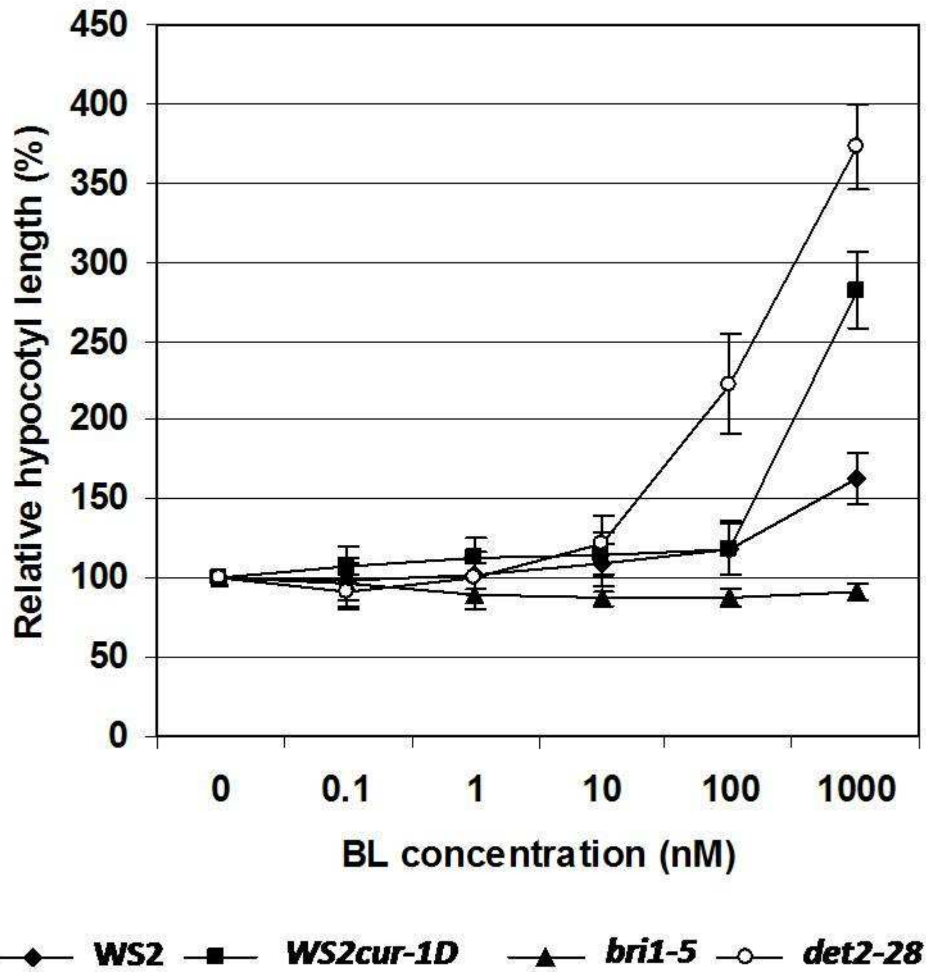


Figure **4.3** Root of *WS2-cur-1D* showed hypersensitive response to BL. Plant seeds were germinated and grown ½ MS media complemented with different concentration of BL. primary root length of were measured after grown for five days under light. For different plants, at least 20 plants were respectively measured for different concentration treatment.

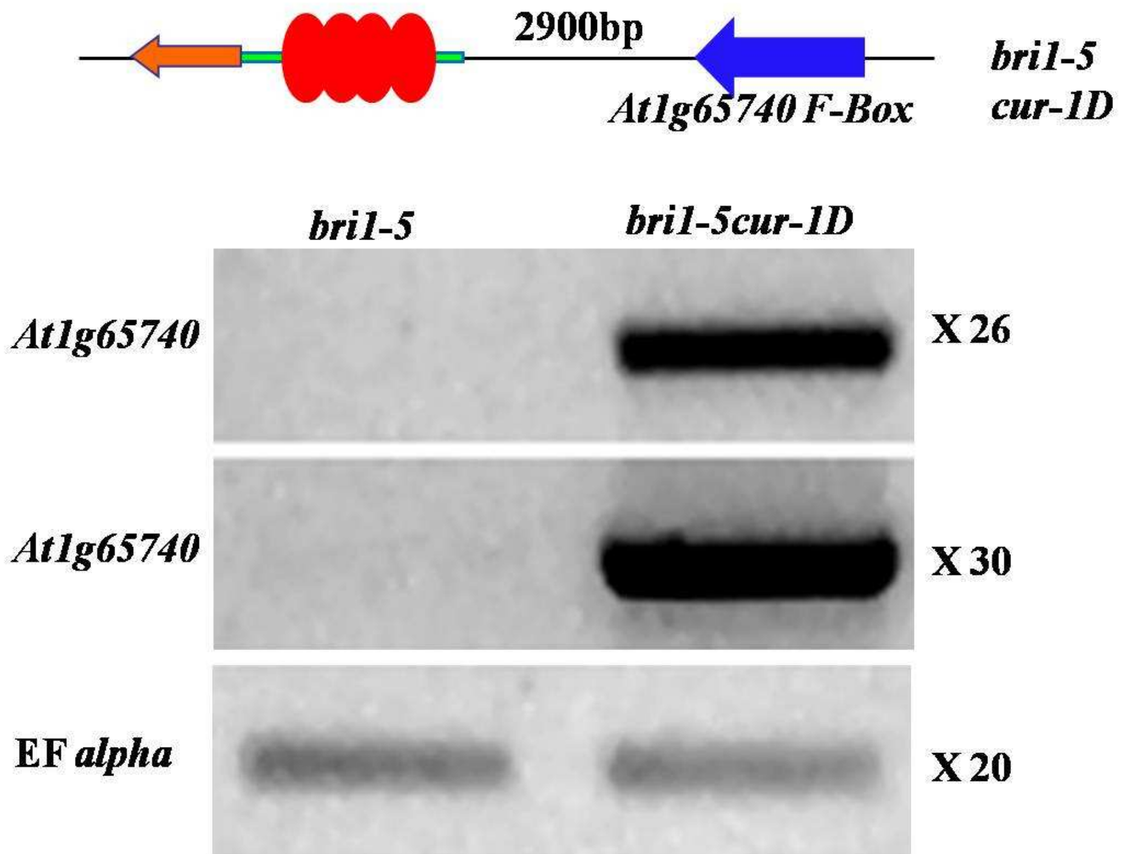


Figure **4-4** Expression level of *At1g65740* is increased in *WS2-cur-1D*. up, Insertion site of T-DNA in *bri1-5cur-1D*. down, RT PCR show *At1g65740* expression is increased in cur-1D, results are showed for two different PCR cycles, EF1 alpha is the control at 20 PCR cycles.

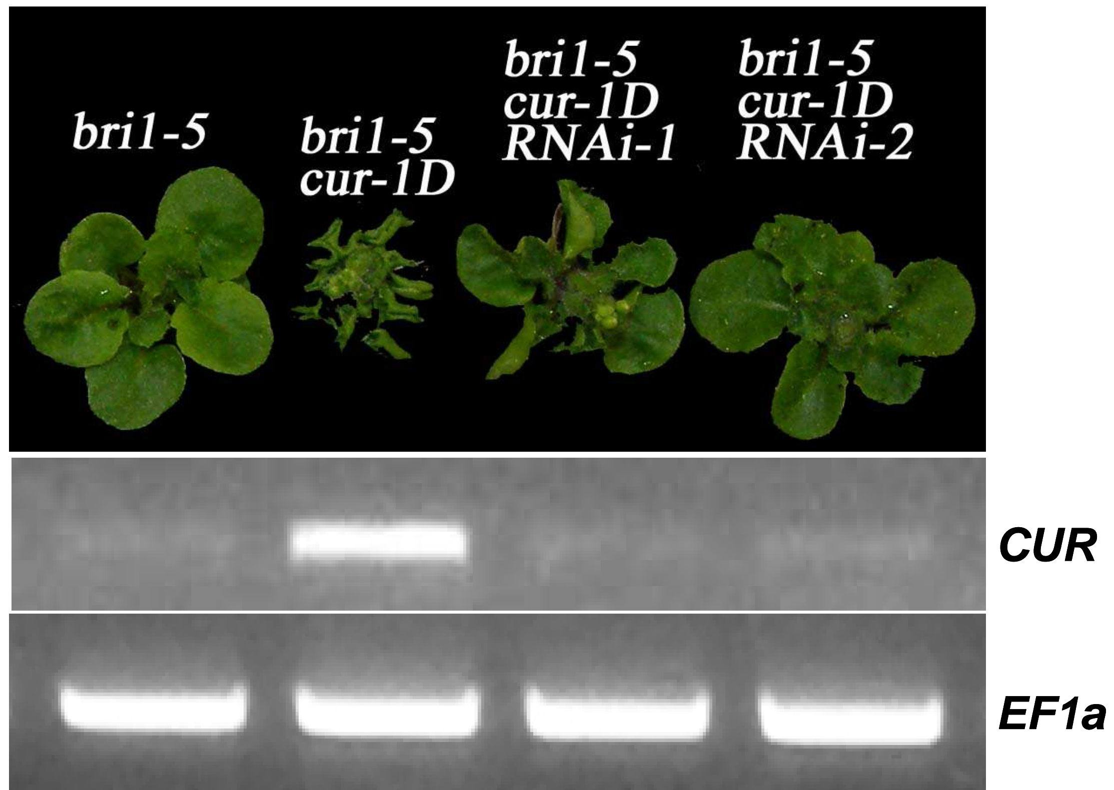


Figure **4.5** Restore CUR expression level in enhancer by RNAi. RNAi vector was introduced into *bri1-5cur-1D*, transgenic plants showed restored phenotypes with reduced expression level of CUR.

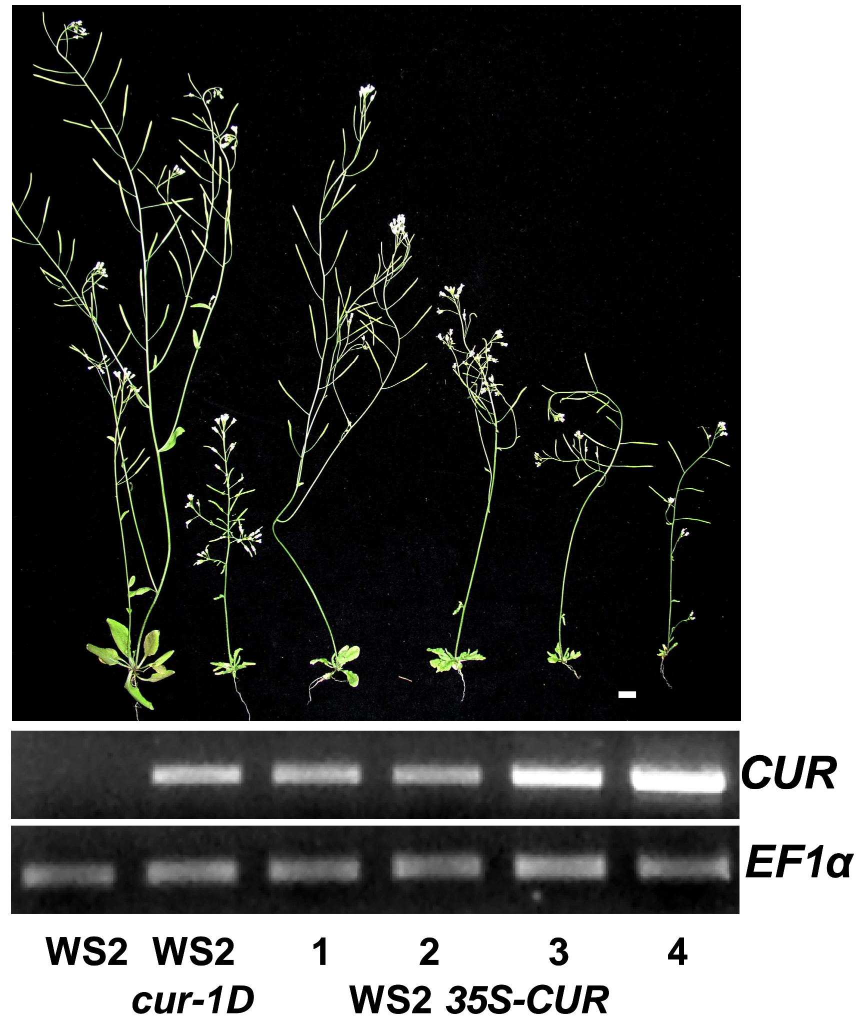


Figure **4.6** Recapitulation results. *CUR* was overexpressed in WS2 under the control of 35S promoter. Transgenic plants show similar phenotypes as *WS2-cur-1D*. RT-PCR results showed over-expression of *CUR* in different transgenic lines, their phenotypes are related to expression level of *CUR*.

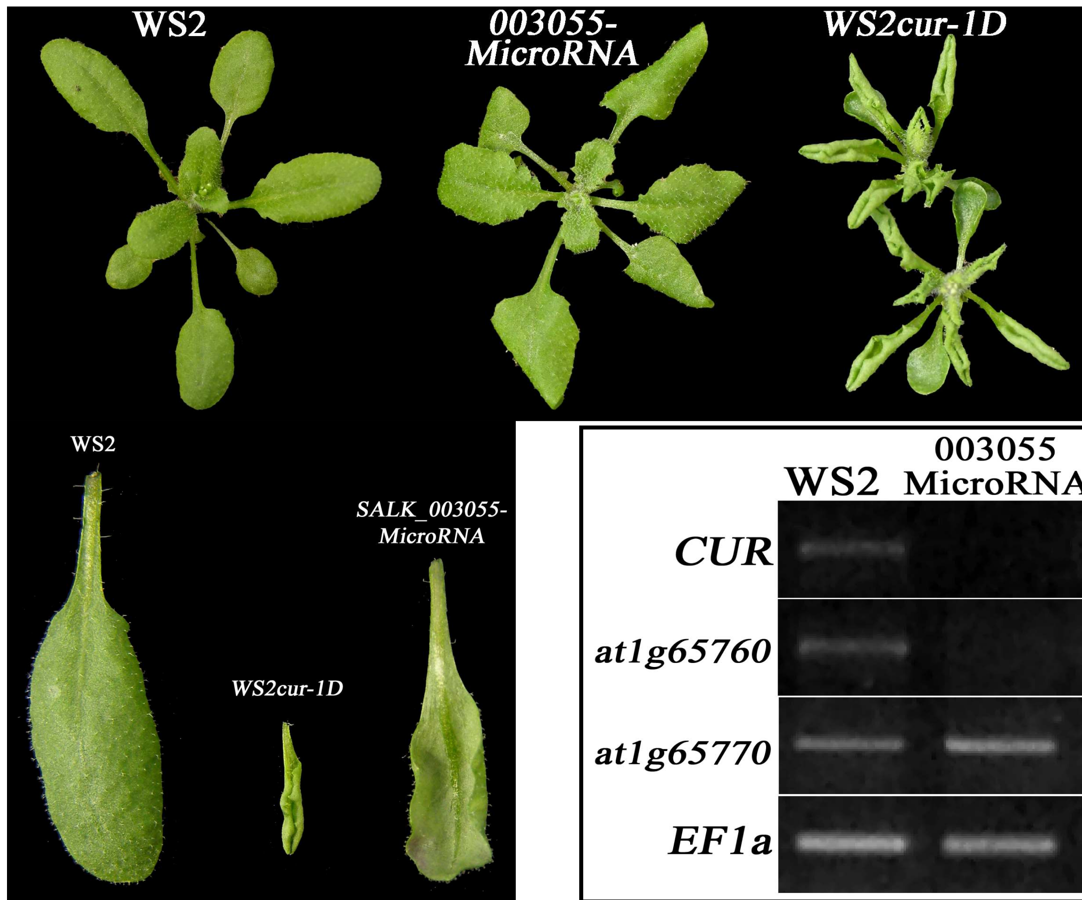


Figure **4.7** Loss of function analysis via MircoRNA. MicroRNA was designed to specifically mediate degradation of CUR and its tandem homolog *At1g*65770 and then was introduced into knockout of *At1g*65760, another tandem homolog for CUR. Transgenic plants display down- curled leaves, which is opposite to *WS2-cur-1D*.

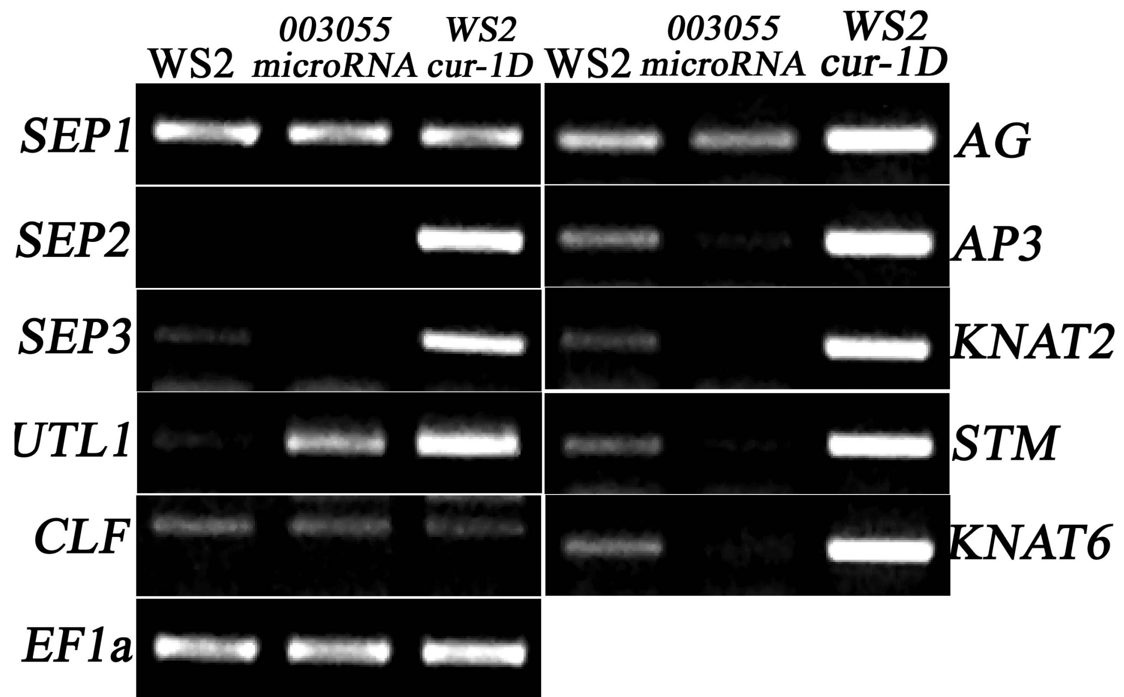


Figure **4.8** RT-PCR results for expression level of homeotic genes. 32 cycles of RT-PCR products were separated by 1% Gel and stained with SYBR green. EF1a was used for control.

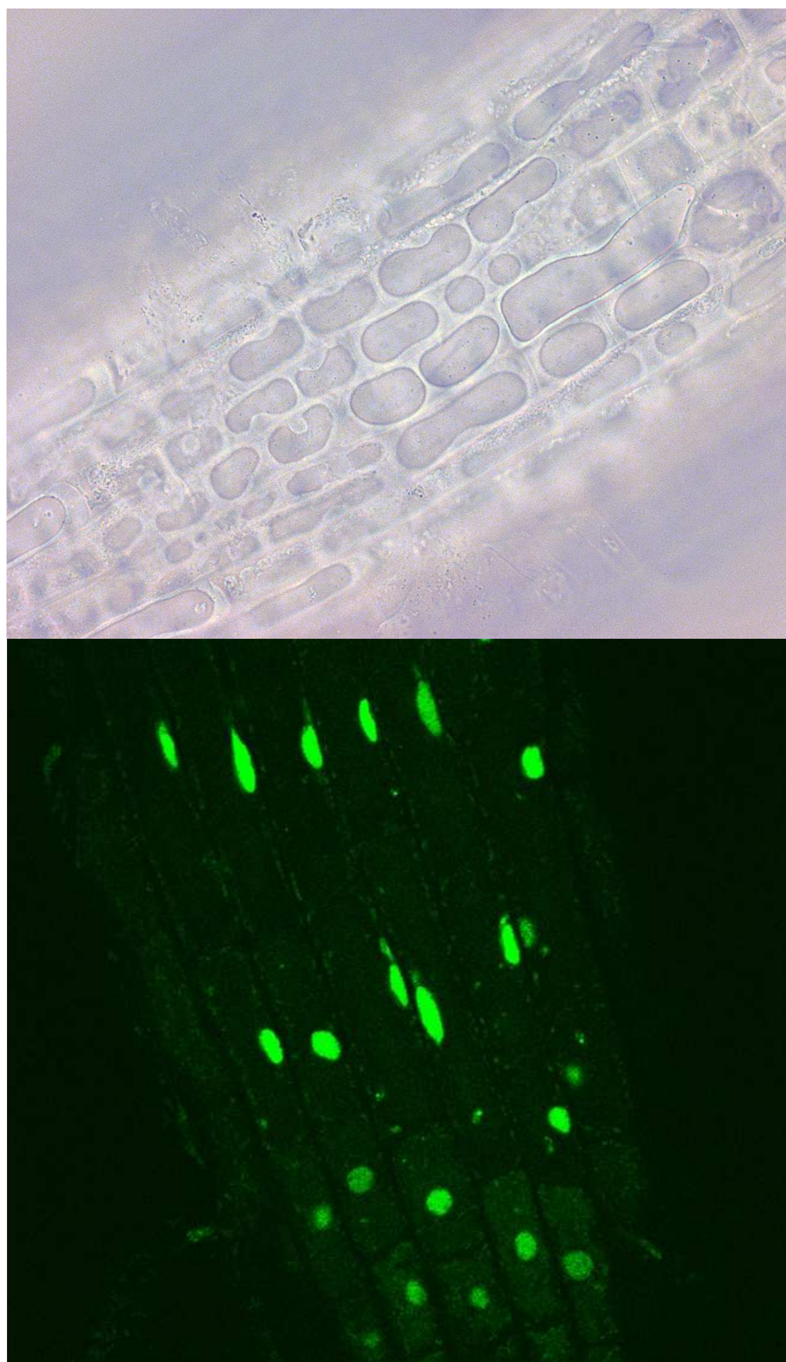


Figure **4.9** Confocal results showed nuclei localization of CUR-GFP in the root tip of *WS2-35S-CUR-GFP* transgenic plant. Up is control.

References

Barkoulas M, Galinha C, Grigg SP, Tsiantis M (2007) From genes to shape: regulatory interactions in leaf development. Curr Opin Plant Biol. 10(6):660-6.

Browse J (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. Annu. Rev. Plant. Biol. 60:183-205.

Carles CC, Fletcher JC (2009) The SAND domain protein ULTRAPETALA1 acts as a trithorax group factor to regulate cell fate in plants. Genes Dev. 23(23):2723-8.

Chini A, Boter M, Solano R (2009) Plant oxylipins: COI1/JAZs/MYC2 as the core jasmonic acid-signalling module. FEBS J. 276(17):4682-92.

Choe, S., Fujioka, S., Noguchi, T., Takatsuto, S., Yoshida, S., and Feldmann, K.A (2001). Overexpression of DWARF4 in the brassinosteroid biosynthetic pathway results in increased vegetative growth and seed yield in *Arabidopsis*. Plant J. 26, 573-582.

Choe, S.W., Dilkes, B.P., Fujioka, S., Takatsuto, S., Sakurai, A., and Feldmann, K.A (1998). The DWF4 gene of *Arabidopsis* encodes a cytochrome P450 that mediates multiple 22 α-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell 10, 231-243.

Choe, S., Schmitz, R.J., Fujioka, S., Takatsuto, S., Lee, M.-O., Yoshida, S., Feldmann, K.A., and Tax, F.E (2002). *Arabidopsis* brassinosteroid-insensitive dwarf12 mutants are semidominant and defective in a glycogen synthase kinase 3-β-like kinase. Plant Physiol. 130, 1506-1515.

Clouse, S.D., and Sasse, J.M (1998). Brassinosteroids: Essential regulators of plant growth and development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 427-451.

Clouse, S.D., Langford, M., and McMorris, T.C (1996). A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol. 111, 671-678.

Craig KL, Tyers M (1999) The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction. Prog Biophys Mol Biol. 72(3):299-328. R

Doyle MR, Amasino RM (2009) A single amino acid change in the enhancer of zeste ortholog CURLY LEAF results in vernalization-independent, rapid flowering in *Arabidopsis*. Plant Physiol. 151(3):1688-97.

Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G. A (1997) Polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. Nature. 386(6620):44-51.

He, J.X., Gendron, J.M., Yang, Y.L., Li, J.M., and Wang, Z.Y (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signaling pathway in *Arabidopsis*. Proc. Natl. Acad. Sci. USA 99, 10185-10190.

Ho MS, Ou C, Chan YR, Chien CT, Pi H (2008) The utility F-box for protein destruction. Cell. Mol. Life Sci. 65(13):1977-2000.

Itoh H, Ueguchi-Tanaka M, Matsuoka M (2008) Molecular biology of gibberellin signaling in higher plants. In.t Rev. Cell. Mol. Biol. 268:191-221.

Itoh H, Matsuoka M, Steber CM (2003) A role for the ubiquitin-26S-proteasome pathway in gibberellin signaling. Trends Plant Sci. 8(10):492-7.

Jiang D, Wang Y, Wang Y, He Y.(2008) Repression of FLOWERING LOCUS C and FLOWERING LOCUS T by the *Arabidopsis* Polycomb repressive complex 2 components. PLoS One. 3(10):e3404.

Katz A, Oliva M, Mosquna A, Hakim O, Ohad N (2004) FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. Plant J. 37(5):707-19.

Kim GT, Tsukaya H, Uchimiya H (1998) The CURLY LEAF gene controls both division and elongation of cells during the expansion of the leaf blade in *Arabidopsis thaliana*. Planta. 206(2):175-83.

Köhler C, Grossniklaus U (2002) Epigenetic inheritance of expression states in plant development: the role of Polycomb group proteins. Curr Opin Cell Biol. 14(6):773-9.

Koo AJ, Howe GA (2009) The wound hormone jasmonate. Phytochemistry. 70(13-14):1571-80.

Lechner E, Achard P, Vansiri A, Potuschak T, Genschik P.2006 F-box proteins everywhere. Curr Opin Plant Biol. 9(6):631-8.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. Cell 110, 213-222.

Li, J.M., and Chory, J (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90, 929-938.

Li, J.M., Nam, K.H., Vafeados, D., and Chory, J (2001b). BIN2, a new brassinosteroid-insensitive locus in *Arabidopsis*. Plant Physiol. 127, 14-22.

Li, J.M., Nagpal, P., Vitart, V., McMorris, T.C., and Chory, J (1996). A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science 272, 398-401.

Makarevich G, Leroy O, Akinci U, Schubert D, Clarenz O, Goodrich J, Grossniklaus U, Köhler C (2006) Different Polycomb group complexes regulate common target genes in *Arabidopsis*. EMBO Rep. 7(9):947-52.

Mayama T, Ohtsubo E, Tsuchimoto S. (2003) Isolation and expression analysis of petunia CURLY LEAF-like genes. Plant Cell Physiol. 44(8):811-9.

Mockaitis K, Estelle M (2008) Auxin receptors and plant development: a new signaling paradigm. Annu. Rev. Cell. Dev. Biol. 24:55-80.

Nemhauser JL, Chory J (2005) A new FronTIR in targeted protein degradation and plant development. Cell. 121(7):970-2.

Noh YS, Amasino RM (2003) PIE1, an ISWI family gene, is required for FLC activation and floral repression in *Arabidopsis*. Plant Cell. 15(7):1671-82.

Oh MH, Wang X, Kota U, Goshe MB, Clouse SD, Huber SC (2009) Tyrosine phosphorylation of the BRI1 receptor kinase emerges as a component of brassinosteroid signaling in *Arabidopsis*. Proc Natl Acad Sci U S A. 106(2):658-

Okamoto H, Qu L, Deng XW (2001) Does EID1 aid the fine-tuning of phytochrome A signal transduction in *Arabidopsis*? Plant Cell. 13(9):1983-6.

Parry G, Estelle M (2006)Auxin receptors: a new role for F-box proteins. Curr Opin Cell Biol. 18(2):152-6.

P, Micol JL (2000) Genetic analysis of incurvata mutants reveals three independent genetic operations at work in *Arabidopsis* leaf morphogenesis. Genetics. 156(3):1363-77.

Rebecca Schwab, Stephan Ossowski, Markus Riester, Norman Warthmann, and Detlef Weigel (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell 18: 1121-1133.

Saleh A, Al-Abdallat A, Ndamukong I, Alvarez-Venegas R, Avramova Z (2007) The *Arabidopsis* homologs of trithorax (ATX1) and enhancer of zeste (CLF) establish 'bivalent chromatin marks' at the silent AGAMOUS locus. Nucleic Acids Res. 35(18):6290-6.

Santner A, Estelle M (2007) The JAZ proteins link jasmonate perception with transcriptional changes. Plant Cell. 19(12):3839-42.

Schubert D, Primavesi L, Bishopp A, Roberts G, Doonan J, Jenuwein T, Goodrich J (2006) Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. EMBO J. 5(19):4638-49.

Schwechheimer C, Schwager K (2004) Regulated proteolysis and plant development. Plant Cell Rep. 2004 23(6):353-64.

Scofield S, Murray JA (2006) KNOX gene function in plant stem cell niches. Plant Mol Biol. 60(6):929-46.

Serrano-Cartagena J, Candela H, Robles P, Ponce MR, Pérez-Pérez JM, Piqueras Stephan Ossowski, Rebecca Schwab, Detlef Weigel (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. Plant Journal 53 (4) , 674-690.

Thomas SG, Sun TP (2004) Update on gibberellin signaling. A tale of the tall and the short. Plant Physiol. 135(2):668-76.

Tsukaya H (2005) Leaf shape: genetic controls and environmental factors. Int J Dev Biol. 49(5-6):547-55.

Tsukaya H (2003) Organ shape and size: a lesson from studies of leaf morphogenesis. Curr Opin Plant Biol. 6(1):57-62.

Tsukaya H.(2002) The leaf index: heteroblasty, natural variation, and the genetic control of polar processes of leaf expansion. Plant Cell Physiol. 43(4):372-8.

Vierstra RD (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. Nat Rev Mol Cell Biol. 10(6):385-97.

Wang X, Kota U, He K, Blackburn K, Li J, Goshe MB, Huber SC, Clouse SD (2008) Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. Dev Cell. 15(2):220-35.

Wang, Z-Y., Li, J.M., Zhu, Q., Lamb, C., Ronald, P., and Chory, J (2000). Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. Science 288, 2360-2363.

Wang, X., Goshe, M.B., Soderblom, E.J., Phinney, B.S., Kuchar, J.A., Li, J., Asami, T., Yoshida, S., Huber, S.C., and Clouse, S.D (2005) Identification and functional analysis of in vivo phosphorylation sites of the *Arabidopsis* BRASSINOSTEROID-INSENSITIVE1 receptor kinase. Plant Cell 17, 1685-1703.

Wang, Z.Y., Seto, H., Fujioka, S., Yoshida, S., and Chory, J (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature 410, 380-383.

Weigel, D., Ji Hoon, A., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., Nguyen, J.T., Sato, S., Wang, Z.Y., Xia, Y., Dixon, R.A., Harrison, M.J., Lamb, C.J., Yanofsky, M.F., and Chory, J (2000) Activation tagging in *Arabidopsis*. Plant Physiol. 122, 1003-1013.

Willems AR, Schwab M, Tyers M (2004) A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. Biochim Biophys Acta. 1695(1-3):133-70.

Xu L, Shen WH (2008) Polycomb silencing of KNOX genes confines shoot stem cell niches in *Arabidopsis*. Curr. Biol. 18(24):1966-71.

Yee D, Goring DR (2009) The diversity of plant U-box E3 ubiquitin ligases: from upstream activators to downstream target substrates. J. Exp. Bot. 60(4):1109-21

Yun HS, Bae YH, Lee YJ, Chang SC, Kim SK, Li J, Nam KH (2009) Analysis of phosphorylation of the BRI1/BAK1 complex in *Arabidopsis* reveals amino acid residues critical for receptor formation and activation of BR signaling. Mol Cells. 27(2):183-90.

Note

The *cur-1D* mutant was identified and cloned by Dr. Jia Li, and Dr. Aifen Zhou did the recapitulation and confocal experiments. I have obtained their permission to include some of post work in this chapter IV of my dissertation.