Transcriptional mechanisms of brassinosteroid regulated plant growth and stress responses

by

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Abstract

Plant Steroid hormones, Brassinosteroids (BRs), play important roles in plant growth, development and responses to various stresses. BR signal through receptor BRI1 and BAK1 and a series signaling intermediates to control the activities of BES1/BZR1 family transcription factors that control the expression of thousands of genes, half of which are induced and the other half repressed by BR. While BES1 is known to activate BR-induced genes by itself or cooperating with co-activators, such as transcription factors, histone modification enzymes and transcription elongation factors, how BES1 mediates the BR-repressed gene expression is not known. In chapter Ⅱ, MYBL2, a small MYB family transcription repressor, was found to interact with BES1 to down-regulate BR-repressed gene expression. The loss-of-function *mybl2* mutant enhances the phenotype of a weak allele of *bri1* and suppresses the constitutive BR-response phenotype of *bes1-D,* suggesting that suppression of BR-repressed gene expression is required for optimal BR response. Moreover, MYBL2 is a substrate of GSK3-like kinase BIN2, a negative regulator functioning in inhibiting the activities of BES1/BZR1 through its phosphorylation in BR pathway. Unlike BIN2 phosphorylation of BES1/BZR1 leading to protein degradation, BIN2 phosphorylation stabilizes MYBL2, which demonstrated a dual role of BIN2 phosphorylation in BR pathway, similar to the function of GSK3 in WNT signaling pathway. Our results thus establish the mechanisms for BR-repressed gene expression and the integration of BR signaling and BR transcriptional network.

In addition to promote the growth, BRs are known to be involved in drought response, but the mechanism of interactions between these two pathways remains to be established. In chapter Ⅲ, the NAC family transcription factor RD26 and its close homologs mediate crosstalk between drought and BR signaling pathway. RD26 is a direct target of BES1 and functions to inhibit BR-regulated growth as overexpression of RD26 leads to decreased plant growth and knockout of RD26 and its close homologs results in increased BR response. Global gene expression analysis revealed that RD26 modulates BR-regulated gene expression in a complex way. RD26 represses many BR-induced genes including BR-activated cell elongation genes and activates many BR-repressed genes, thereby inhibiting BR functions. On the other hand, BR signaling also inhibits drought responses through repressing the expression of RD26, its homologs and RD26-mediated drought-induced genes. The reciprocal inhibitory effects of BES1 and RD26 are mediated by their interactions on different promoter elements. This mechanism ensures that BR-induced plant growth is inhibited under drought condition that induced RD26

expression, while this mechanism also prevents unnecessary activation of drought response when plants undergo BR-induced growth, during which BES1 accumulates. Our results thus revealed a previously unknown mechanism coordinating plant growth and drought tolerance.

Organization of the thesis and author contributions:

Introduction (Chapter I): A comprehensive review of the BR field, largely based on a published invited review, is firstly presented as introduction (Chapter I). Myself, a former graduate student in the lab, Lei Li, and my supervisor Dr. Yanhai Yin all contributed to the writing of the review.

The characterization of MYBL2 (Chapter II) is accepted for publication in PNAS. Myself, and my supervisor Dr. Yanhai Yin designed research; I performed most of the research; Lei Li contributed new reagents/analytic tools; Myself, Lei Li and Hongqing analyzed data; and myself., Hongqing Guo and my supervisor wrote the paper.

The characterization of RD26 (Chapter III) will be submitted to Nature Genetics for consideration of publication soon.

Myself, and my supervisor Dr. Yanhai Yin designed research; I performed most of the molecular and genetic studies; Dr. Sanzhen Liu in Dr. Patrick Schnable's lab analyzed the RNA-seq data; Dr. Maneesha and Dr. Srinivas Aluru constructed the gene regulatory network; myself, and my supervisor wrote the paper.

CHAPTER I. GENERAL INTRODUCTION

Recent Advances in the Regulation of Brassinosteroid Signaling and Biosynthesis Pathways

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The chapter has small changes to reflect the current status of the field and to present the hypotheses of the thesis.

Abstract

Brassinosteroids (BRs) play important roles in plant growth, development and responses to environmental cues. BRs signal through plasma membrane receptor BRI1 and co-receptor BAK1, and several positive (BSK1, BSU1, PP2A) and negative (BKI1, BIN2 and 14-3-3) regulators to control the activities of BES1 and BZR1 family transcription factors, which regulate the expression of hundreds to thousands of genes for various BR responses. Recent studies identified novel signaling components in the BR pathways and started to establish the detailed mechanisms on the regulation of BR signaling. In addition, the molecular mechanism and transcriptional network through which BES1 and BZR1 control gene expression and various BR responses are beginning to be revealed. BES1 recruits histone demethylases ELF6 and REF6 as well as a transcription elongation factor IWS1 to regulate target gene expression. Identification of BES1 and BZR1 target genes established a transcriptional network for BR response and crosstalk with other signaling pathways. Recent studies also revealed regulatory mechanisms of BRs in many developmental processes and regulation of BR biosynthesis. Here we provide an overview and discuss some of the most recent progress in the regulation of BR signaling and biosynthesis pathways.

Introduction

A group of plant steroid hormones, named Brassinosteroids (BRs), regulate many processes in plant growth and development, including cell elongation, cell division, senescence, vascular differentiation, reproduction, photomorphogenesis， responses to various stresses (Mandava 1988; Clouse and Sasse 1998; Li and Chory 1999; Divi and Krishna 2009). Loss-of-function mutants, deficient in BR biosynthesis or perception, display dwarf phenotypes with reduced cell elongation, dark-green and epinastic leaves, reduced apical dominance, delayed flowering and senescence, altered vascular patterning and male sterility (Clouse et al. 1996; Li et al. 1996; Szekeres et al. 1996). Genetic and molecular studies in *Arabidopsis* have greatly advanced our understanding of the BR signaling pathway (Fig 1) (Kim and Wang 2010; Li 2010b; Clouse 2011; Yang et al. 2011). BRs are perceived by a membrane-bound receptor called BRI1 (BRASSINOSTEROID INSENSITIVE 1), a leucine rich repeat (LRR) receptor like kinase (Li and Chory 1997; He et al. 2000; Wang et al. 2001; Kinoshita et al. 2005). Many alleles of BRI1 have been identified through various genetic screens(Clouse et al. 1996; Li and Chory 1997; Noguchi et al. 1999; Friedrichsen et al. 2000; Zhao et al. 2009). In the absence of BRs, BRI1's activity is inhibited by the negative regulator, BKI1 (BRI1

KINASE INHIBITOR 1), which exerts its effect by binding the C-terminal tail of BRI1 (Wang and Chory 2006). However, binding of BRs to BRI1 relieves this repression and results in BRI1 association with the co-receptor BAK1 (BRI1-Associated Receptor Kinase 1) and a series of phosphorylation events (Li et al. 2002; Nam and Li 2002; Wang et al. 2005a; Wang et al. 2005b; Wang et al. 2008; Oh et al. 2009b). BRI1 signals through BSK1 (BR SIGNALING KINASE 1) and CDG1 kinases as well as BSU1 (BRI1-SUPPRESSOR 1) to regulate a negative regulator BIN2 (BRASSINOSTEROID INSENSITIVE 2), a GSK3/Shaggy-like kinase (Choe et al. 2002; Li and Nam 2002; Pérez-Pérez et al. 2002; Mora-Garcia et al. 2004; Tang et al. 2008b; Kim et al. 2009; Kim et al., 2011). Other BRI1 substrates such as Arabidopsis TRIP-1 (TGF RECEPTOR-INTERACTING PROTEIN-1) and TTL (TRANSTHYRETIN-LIKE PROTEIN) may function to regulate BR signaling as well (Nam and Li 2004; Ehsan et al. 2005). The dephosphoryaltion of BIN2 by BSU1 inhibits BIN2 function, which allows the nuclear accumulation of two transcription factors BES1 (*BRI1*-EMS SUPPRESSOR 1) and BZR1 (BRASSINAZOLE RESISTANT 1), two bHLH (BASIC HELIX-LOOP-HELIX)-like transcription factors that play major roles in regulating BR target gene expression (Wang et al. 2002; Yin et al. 2002b; Zhao et al. 2002; He et al. 2005; Yin et al. 2005).

Significant progress has been made in the past few years. After the biochemical confirmation that BRI1 is indeed BR receptor, proteomics and functional studies revealed a sequential phosphorylation model for the activation and signaling mechanisms of BRI1 and its coreceptor BAK1 (Wang et al. 2005a; Wang et al. 2008). In the process, BRI1 and BAK1 have been discovered to have both serine/threonine (S/T) and tyrosine (Y) phophorylation activities, which are important for kinase activation, substrate modification and specific BR responses (Oh et al. 2009a; Oh et al. 2009b; Oh et al. 2010; Jaillais et al. 2011; Oh et al. 2011). Several important signaling components that function between BRI1 and BIN2 have been identified by proteomics and genetic approaches; and the mechanisms of signal transduction have been revealed (Kim and Wang 2010; Tang et al. 2010). The regulatory mechanisms of BES1 and BZR1 by BIN2 phosphorylation appear to be much more complex than originally thought and PP2A phosphatase that dephophorylates BZR1 has been identified (Li and Jin 2007; Ryu et al. 2010a; Ryu et al. 2010b; Tang et al. 2011). In addition, the mechanisms and transcriptional network through which BES1 and BZR1 control BR responses have been revealed by identification and characterization of BES1 and BZR1 target genes by ChIP-chip and BES1 partners by protein-protein interaction and genetic screens (Yu et al. 2008; Li et al. 2009; Li et al. 2010b; Luo et al. 2010; Sun et al. 2010; Yu et al. 2011). Additional transcriptional factors involved in BR signaling have been identified in both Arabidopsis and rice (Li 2010b). BR functions

in various developmental processes are covered in details in two recent reviews (Clouse 2011; Yang et al. 2011). The molecular mechanisms of BR function in pollen and root have been revealed (Ye et al. 2010; Gonzalez-Garcia et al. 2011; Hacham et al. 2011). The studies confirm BR function in cell division (Hu et al. 2000) and BR action from epidermis to control root meristem cell division and expansion, similar to that found in shoots (Savaldi-Goldstein et al. 2007). Finally, several regulators for BR biosynthesis have been identified (Guo et al. 2010; Je and Han 2010; Je et al. 2010; Chung et al. 2011; Poppenberger et al. 2011). In this review, we will discuss the progress and provide some perspectives on the future directions in this fast-evolving field.

BR receptor BRI1 and its regulation

BRI1 is a membrane-localized receptor kinase that contains 25 LRRs (leucine-rich repeat) with an island domain between 21st and 22nd repeat in the extracellular region, a single transmembrane domain, a juxtamembrane region, a kinase domain and a C-terminal regulatory region (Li and Chory 1997). BRI1 has been established as the BR receptor by several biochemical and molecular experiments, including domain-swapping analysis with Xa21 receptor kinase, binding of immunoprecipitated BRI1 with radio-labeled brassinolide (BL, the most active BR), and binding assays with recombinant BRI1 protein (He et al. 2000; Wang et al. 2001; Kinoshita et al. 2005). In addition to BRI1, two of three BRI1 homologs were also shown the ability to bind BRs, and genetic studies showed that they played a major role in vascular development and are partially redundant with *BRI1* (Caño-Delgado et al. 2004; Zhou et al. 2004).The identification and functional characterizations of BRI1 phosphorylation sites, its cellular localizations as well as identification and characterization of BRI1 partners have greatly increased our understanding of BRI1 kinase activation and signaling outputs (Kim and Wang 2010). Homo-dimerization of BRI1 was detected in plasma membrane by fluorescence resonance energy transfer analysis, and was confirmed by co-immunoprecipitation experiment with BRI1-GFP and BRI1-FLAG transgenic plants (Russinova et al. 2004; Wang et al. 2005b; Hink et al. 2008). It was further found that this homo-dimerization of BRI1 was promoted or stabilized by BRs (Wang et al. 2005b). In the absence of BRs, two mechanisms operate to inhibit the basal BRI1's activation. The C-terminal domain of BRI1 can function to inhibit BRI1 kinase activity. In addition, BKI1 can bind BRI1 at the plasma membrane and inhibit BRI1 function. BR binding leads to the release of the auto-inhibition by the C-terminal and releasing of BKI1 into cytosol, allowing BRI1 association with its coreceptor BAK1 (Wang and Chory 2006). BAK1, a small LRR-RLK with 5 LRRs, was identified as BRI1' coreceptor by activation tagging screen of *bri1-5* suppressor and yeast two hybrid screen (Li et al. 2002; Nam and Li 2002). Based on careful mapping

of BRI1 and BAK1 phosphorlation sites both in vitro and in vivo as well as their functional studies, a sequential transphorylation model was proposed for the activation of BR signaling (Wang et al. 2005a; Wang et al. 2008). In this model, BR binding to BRI1 leads to autophosphorylation on many of the phosphorylation sites to activate its kinase activity, which then phosporylates and activates BAK1 kinase activity. Activated BAK1 can in return phosphorylate BRI1 at the juxtamembrane and C-terminus to fully activate BRI1 function. The model suggests that BAK1 is involved in the fully activation of BRI1 kinase activity, but not required for ligand binding. Recent studies demonstrated that in addition to functioning in BR signaling, BAK1 and its homologs also have roles in cell death and plant defense responses by acting as a coreceptor for flagellin receptor FLS2 (Chinchilla et al. 2007; He et al. 2007; Heese et al. 2007; Kemmerling et al. 2007; Albrecht et al. 2008; He et al. 2008; Kemmerling and Nurnberger 2008; Shan et al. 2008; Chinchilla et al. 2009; Gao et al. 2009; Jeong et al. 2010; Li 2010a). The phosphorylation site mapping and functional studies also revealed that BRI1 and BAK1 are kinases with dual specificity. Interestingly, while both S/T and Y phosphorylations are important for kinase functions in both BRI1 and BAK1, Y phosphorylation appears to be important for specific BR responses and for the regulation of BKI1 activity.

Unlike several tyrosine residues that are required for BRI1 kinase activity, Y-831 phosphorylation in the juxtamembrane appears to account for some specific BR responses (Oh et al. 2009a; Oh et al. 2009b). Expression of BRI1Y831F rescued *bri1* mutant phenotype, but displayed a larger leaf and early flowering phenotypes compared with the expression of wild-type (WT) BRI1. The results imply that Y831 control some specific BR responses, likely by altering BR signaling specificity by interacting with specific proteins.

Similarly, a recent study showed that BAK1 Y610 at the C-terminal was a major site for BAK1 tyrosine autophosphorylation (Oh et al. 2010; Oh et al. 2011). Interestingly, functional studies demonstrated that Y610 is required for BAK1 function in BR signaling as the BAK1Y610F transgenic plants in *bak1 bkk1* mutant background displayed BR insensitive phenotype including reduced growth, accumulation of unphosphorylated BES1 and reduction of BR-regulated genes. Similarly, Flagellin signaling in defense response is also impaired. However, other BAK1 responses, such as Flagellin-mediated inhibition of plant growth and BAK1-mediated cell death are not affected. It will be interesting to determine how specific tyrosine phosphorylation events differentially affect various responses mediated by BRI1 and BAK1.

Tyrosine phosphorylation also plays an important role regulating the cellular localization of the BRI1 inhibitor BKI1 (Jaillais et al. 2011). The N-terminal lysine-arginine-rich (KR) motif targets BKI1 to the plasma membrane and a C-terminal 20-residue conserved domain mediates the

interaction between BKI1 and BRI1. Deletion analysis showed that the KR repeats around motif-3 (amino acids 200-221) was sufficient for BKI1 localization to plasma membrane. Phosphorylation of a conserved tyrosine (Y211) in motif-3 of BKI1 was essential for the dissociation of BAK1 from plasma membrane. Mutation in this tyrosine (Y211F) constitutively targeted BKI1 protein to plasma membrane and overexpression of BKI1Y211F resulting in the plants with dwarf phenotype. In contrast, a phosphorylation-mimicking mutant of BKI1 (Y211D) leads to constitutive cytoplasmic localization of the mutant protein and lost ability in inhibiting BR signaling. Taken together, BR-activated BRI1 phosphorylates BKI1 at Y211, which lads to its disassociation from BRI at the plasma membrane and allows BRI1 association with BAK1.

BRI1 also phosphorylates other positive-acting substrates to transduce BR signal to downstream targets. By proteomic analysis of two-dimensional difference gel electrophoresis, several early BR-response proteins, BSKs, were identified (Tang et al. 2008b). These BSKs are the members of cytoplasmic receptor-like kinases (RLCKs) that are likely associated with plasma membrane through N-myrisylation. BSKs are positive regulators of BR response as overexpression of several members in the family suppressed *bri1* phenotype and knockout of one of the members, BSK3, shown a weak BR insensitive phenotype likely due to functional redundancy among BSKs. BSK1 was phosphorylated on Serine residue 230 (S230) by BRI1 in vitro and co-immunoprecipitaion experiments indicated that BSK1 interacted with BRI1 in vivo. BRI1 and BSK1 interaction is reduced upon BR treatment, suggesting that BSKs are disassociated from BRI1 after being phosphorylated. As we'll discuss in the next section, BSKs play an important role in transducing BR signal to downstream components.

BRI1 likely has additional substrates in the regulation of various BR responses. Arabidopsis TRIP-1, an essential subunit of the eIF3 eukaryotic translation initiation factor, is phosphorylated by BRI1 at several amino acids in vitro and interacts with BRI1 in vivo (Ehsan et al. 2005). As expected for an essential component in translation initiation, RNAi knock-down of *TRIP-1* lead to pleiotropic phenotype with some resemblance to BR loss-of-function mutants (Jiang and Clouse 2001). It remains to be determined if TRIP-1 functions in both translation and transcription, as its mammalian homolog does, and if so, how BRI1 phosphorylation affects its activities. Arabidopsis TTL interacts with BRI1 kinase domain in yeast two-hybrid assays and is phosphorylated by BRI1 in vitro (Nam and Li 2004). Genetic studies indicated that TTL function as a negative regulator for BR-regulated plant growth and the mechanisms by which TTL modulate BR regulated plant growth remain to be determined. It was recently found that BRI-GFP is associated with a plasma-membrane Proton ATPase (P-ATPase) in a BR and BRI1 kinase dependant manner, which accompanies BR-induced cell

expansion (Caesar et al. 2011). Since phosphorylation of P-ATPase is known to activate the enzyme activity, it would be interesting to determine if BRI1 can directly phosphorylate P-ATPase (Fig 1).

Regulation of negative-acting kinase BIN2

In addition to the large number of BRI1 loss-of-function alleles, screens for BR-insensitive mutants identified a negative regulator, BIN2 (Choe et al. 2002; Li and Nam 2002; Pérez-Pérez et al. 2002). While gain-of-function in BIN2 displayed *bri1*-like dwarf phenotype, loss-of-function of BIN2 and its homologs displayed a constitutive BR response phenotype (Yan et al. 2009). BIN2 is homologous to GSK3/Shaggy kinase that plays an essential role in WNT signaling pathway that is essential for animal development and is affected in many cancers (Cadigan and Nusse 1997; Polakis 2000). Recent studies suggest that BIN2 is regulated by targeted protein degradation in response to BR signaling as well as by BSK kinase and CDG1 kinase through protein phosphatase BSU1. The initial evidence that BIN2 is regulated at protein degradation comes from the observation that several gain-of-function mutations of BIN2, localized in a "TREE" domain, stabilizes BIN2 protein (Peng et al. 2008). Further studies clearly indicated that BIN2 protein accumulation is decreased by BR treatment. The decrease of BIN2 is apparently mediated by proteasome-mediated degradation pathway, as treatment with 26S proteasome inhibitor (MG132) reversed the BR-mediated decrease of BIN2 protein.

Although how BR signaling promotes BIN2 degradation remain to be established, a Kelch-repeat containing protein phosphatase, BSU1, has been shown to directly dephosphorylates and regulate BIN2 function (Kim et al. 2009). BSU1 phosphatase, identified by activation tagging with *bri1-5*, plays a positive role in BR signaling pathway and was found to act downstream of BIN2 (Mora-Garcia et al. 2004). However, recent genetic experiments suggest that BSU1 acts downstream of BRI1 but upstream of BIN2 (Kim et al. 2009). BIN2 can autophosphorylate at Y200, which is required for BIN2 function. BSU1 binds and dephosphorylates BIN2 at Y200, thereby inhibiting BIN2 function. Co-immunoprecipitation and BiFC assay suggested that BSK1 and BSU1 interact with each other in vivo and the phosphorylation of BSK1 at serine 230 (S230) by BRI1 promotes this interaction. In addition to BSK1, a receptor-like cytoplasmic kinase, CDG1, may also play an important role in BR signal transduction (Kim et al. 2011). Transgenic studies indicated that CDG1 was a positive regulator, which functioned upstream of BIN2 and downstream of BRI1, in the BR pathway. BiFC and co-immunoprecipitated experiments indicated that CDG1 interacted with BRI1 in vivo and CDG1 was phosphorylated by BRI1 in vitro. Ser234 of CDG1 phosphorylated by BRI1 is a major mechanism to activate the function of CDG1. Yeast two hybrid experiment, BiFC and co-

immunoprecipitated experiments indicated that CDG1 also interacted with BSU1. In vitro kinase assay and substitution experiments showed that CDG1 phosphorylated Ser764 of BSU1 to enhance the interaction between BSU1 and BIN2 to promote BSU1 dephosphorylation of BIN2. These studies therefore connected all the components between BRI1, its substrate BSK1, CDG1, BSU1 and BIN2 in a linear pathway. It remains to be determined if BIN2 is also regulated by additional mechanisms and how different mechanisms coordinate to control this important regulator in the BR pathway.

Regulation of BES1 and BZR1

BES1 and BZR1 are two major transcription factors that are regulated by BIN2 and mediate BR-regulated gene expression (Wang et al. 2002; Yin et al. 2002a; Zhao et al. 2002; He et al. 2005; Yin et al. 2005). BES1 and BZR1 are 88% identical and are composed of DNA binding domain (DBD), BIN2 phsophorylation domain with more than 20 putative BIN2 phosphorylation sites (S/TxxxS/T), and a C-terminal domain (CTD) (Fig. 2). BES1 and BZR1 DBDs are predicated to form a bHLH structure although they are not classified as typical bHLH transcription factors. The CTD is required for BES1 function as deletion of this domain leads to accumulation of inactive BES1 that acts as a dominant-negative form (Yin et al. 2005). The C-terminal domain mostly likely acts as a transcription activation domain as it activates reporter gene expression in yeast when fused with GAL4 activation domain. In addition, the C-terminal domain also contains a 12 amino acid docking motif (DM) that binds BIN2, allowing BIN2 to phosphorylate BZR1 (Peng et al. 2010). Since the same domain is conserved in BES1, it's likely that BIN2 interacts with DM to phosphorylate BES1 as well. BIN2 phosphosphorylates BES1 and BZR1 at their central phosphorylation domain and inhibits their function likely through several different but non-exclusive mechanisms, including targeted protein degradation, nuclear export and cytoplasmic retention by 14-3-3s and decreased DNA binding of the BIN2-phosphorylated protein (He et al. 2002; Yin et al. 2002b; Vert and Chory 2006; Bai et al. 2007; Gampala et al. 2007; Ryu et al. 2007; Ryu et al. 2008; Ryu et al. 2010a).

BIN2 phosphorylates BES1 and BZR1 at many of putative phosphorylation sites (Fig 2). Phosphorylation of different sites could affect different aspects of BES1 and BZR1 functions. For example, phosphorylation at T177 of BZR1 and T175 of BES1 is required to interact with 14-3-3 for cytoplasmic retention (de Vries 2007; Ryu et al. 2010a). Recent studies also suggest that BSU1 and BIN2 preferably act at different locations to regulate BES1 functions. While the cytoplasmic location is more important for BSU1, BIN2 likely phosphorylate BES1 and BZR1 in the nucleus to trigger its nuclear export (Ryu et al. 2010b).

A phosphatase that dephosphorylates BZR1 has been identified by looking for BZR1-interacting

proteins through tandem affinity purification (Tang et al. 2011). Protein phosphates 2A (PP2A) is a heterotrimeric serine/threonine phosphatase, which contains as scaffolding subunit A, catalytic subunit C, and a regulatory B subunit that interacts with substrates (Janssens et al. 2008). In vitro and in vivo experiments showed that BZR1 was indeed able to interact with several PP2A B′ isoforms, such as B′α, β and η through the PEST domain of BZR1. While the loss-of-function PP2A mutant accumulated more phosphorylated BZR1, overexpression of B′ components increased both phosphortylated and unphosphorylated BZR1, suggesting that PP2A affects both BZR1 dephosphorylation and protein degradation. Pharmacological studies with GSK3 kinase inhibitor bikinin and PP2A inhibitor okadaic acid also support a role of PP2A in BZR1 dephosphorylation. Immunoprecipitated PP2A can dephosphorylate BZR1 in vitro, which likely affects the inhibitory effects of phosphorylation on BZR1 including the binding of 14-3-3 protein. Finally, deletion of PEST motif in BZR1 leads to the accumulation of phosphorylated protein and a dwarf phenotype, consistent with a negative role of PEST in protein degradation and positive function in recruiting PP2A. Yeast two-hybrid experiments showed that PP2A B′α and β also interacted with BES1, implying that PP2A regulated the function of BES1 as well.

Network and mechanism for BES1 and BZR1 regulated gene expression

BRs affect many growth and developmental processes and much of them are likely due to BR-mediated changes in gene expression. Several genome-wide microarray experiments in *Arabidopsis* have demonstrated that BRs regulate hundreds to thousands of genes (Goda et al. 2004; Nemhauser et al. 2004; Nemhauser et al. 2006; Guo et al. 2009). Proteomics study also identified many proteins in response to BRs in both Arabidopsis and rice (Tang et al. 2008a; Wang et al. 2010). Understanding how BES1 and BZR1 coordinate with other proteins to control the expression of the large number of genes in a transcriptional network is important to understand how BRs regulate various biological processes at different stages of growth and development under various environmental conditions. Identification and characterization of BES1 and BZR1 partners and target genes can help address this question.

BES1 and BZR1 direct target genes have been recently identified by Chromatin immunoprecipitation followed by genomic tiling array (ChIP-chip) (Sun et al. 2010; Yu et al. 2011). In total, 1609 BES1 targets were identified with 2-week-old *bes1-D* seedlings and an ant-BES1 antibody, at least 250 of them are regulated by BRs (Yu et al. 2011). On the other hand, 3410 BZR1 target genes were identified with 4-week-old transgenic BZR1-CFP plants treated with BL with an anti-CFP antibody, 953 of them are regulated by BRs (Sun et al. 2010). The different plant ages used

(seedlings vs. adult plants) probably account for some of the differences in numbers of target genes identified for BES1 and BZR1. Nevertheless, about half of BES1 target genes are also BZR1 targets, which is consistent with the fact that these two factors function redundantly with distinctive functions (Wang et al. 2002; Yin et al. 2002b). Several important conclusions can be drawn from the characterization of BES1 and BZR1 target genes.

The analysis of enriched promoter elements in BR-regulated BES1 and BZR1 target genes confirmed previously identified BES1 and BZR1 DNA binding sites and changed previous perception about the activation and repression of these two transcription factors. It was previously shown that BES1 binds to E-box sequences to activate a BR-induced gene expression and BZR1 binds to BRRE on *CPD* (*CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM*) promoter to repress its expression (He et al. 2005; Yin et al. 2005). However, promoter elements analysis indicated that both BES1 and BZR1 can bind both BRRE and E-boxes (particularly CATGTG and CACGTG that is also termed G-box) in vivo with BRRE mostly enriched in BR-repressed genes and E-boxes are mostly enriched in BR-induced genes (Sun et al. 2010; Yu et al. 2011). Since E-box and BRRE are also enriched in both BR-induced and BR-repressed genes, additional promoter sequence elements and/or BES1 and BZR1 interacting proteins likely determine if these transcription factors either activate or repress gene expression.

BES1 and BZR1 can bind and induce its own expression, probably in a positive feedback loop (Yu et al. 2011). At the same time, BES1 and BZR1 inhibit many genes involved in BR biosynthesis and signaling, likely as a feedback inhibition mechanism (Sun et al. 2010). These observations also point out that at least several regulators in the BR pathway, such as BES1, BZR1, BRI1 and BIN2, can be regulated by BRs at both transcription and post-transcriptional levels. Both BES1 and BZR1 target gens suggest interactions between BR and other signaling pathways (Clouse 2011). In particular, the genes involved in light and other hormones responses including auxin, gibberellin (GA), abscisic acid (ABA), ethylene, jasmonic acid (JA) and cytokinin are enriched in both BES1 and BZR1 targets. The crosstalk between auxin ABA and BRs have been determined at molecular levels (Hardtke 2007; Zhang et al. 2009b).

BR actions are closely regulated by light, which is directly demonstrated by the constitutive photomorphogenesis/deetiolation phenotype of BR mutants (Chory et al. 1991; Li et al. 1996; Szekeres et al. 1996). Microarray studies with *det2* mutant indicated that BRs negatively regulate genes involved in photomorphogenesis (Song et al. 2009). The interaction between BZR1 and light signaling was further confirmed by the finding that BZR1 represses light signaling components (Sun et al. 2010). The comparison between targets between BZR1 and regulated transcription factors

indicated that about 1/3 of target genes are in common between BZR1 and HY5, a transcription factor mediating light regulated gene expression (Oyama et al. 1997; Lee et al. 2007). The crosstalk is further confirmed with one of BZR1 targets (Luo et al. 2010). Genetic studies indicated that GATA2 is a negative regulator in the BR pathway. The protein level of GATA2 was regulated by light through COP1-dependent proteasome degradation. GATA2 is therefore inhibited by BRs at transcription level and promoted by light at protein level, providing a link between BR and light signaling pathways. Finally, BES1 and BZR1 repress the expression of two related transcription factors, GLK1 and GLK2, which function redundantly to promote chloroplast development (Waters et al. 2008; Waters et al. 2009; Sun et al. 2010; Yu et al. 2011). It's well known that BR loss-of-function mutants have premature chloroplast development but the mechanisms are not known (Chory et al. 1991). It's conceivable that BRs function through BES1 and BZR1 to repress GLK1 and GLK2 expression and thus chloroplast development in the dark. Consistent with the hypothesis, *bes1-D* mutant, in which BES1 accumulated to high level, have reduced expression of GLK1 and GLK2, reduced expression of GLK target genes and altered chloroplast function (Waters et al. 2008; Waters et al. 2009; Sun et al. 2010; Yu et al. 2011). The connection between BR signaling and chloroplast development is further supported by characterization of BPG2 (BRZ-INSENSITIVE-PALE GREEN 2), a chloroplast protein involved in BR response (Komatsu et al. 2009). Taken together, the identification and characterization of BES1 and BZR1 target genes provided more evidence that light and BR pathways have extensive crosstalk in the regulation of plant growth, photomorphogenesis and chloroplast development.

Some of the BES1 and BZR1 target genes were further confirmed by functional studies to mediate BR response or BR-regulated plant growth. In the case of BES1, knockout mutants for 15 BES1-targeted Transcription Factors (BTFs) were tested and 12 of them shown BR response defects in hypocotyl elongation assays (Yu et al. 2011). Interestingly all of these 12 BTFs are also BZR1 targets. With a few exceptions, BES1 and BZR1 in general promote the expression of BTFs that are positive regulators of BR response and repress BTFs that are negative regulators of BR response. In the case of BZR1 targets, overexpression of DREPP, one of BR-regulated BZR1 targets, increased the cell length of *det2* mutant (Sun et al. 2010). On the other hand, the transgenic plants overexpressing *BZS1*, another BZR1-repressed transcription factor gene, were hypersensitive to BRZ, while co-suppressed lines of *BZS1* showed longer hypocotyls grown on BRZ medium compared with wild type plants. Several BES1 and BZR1-targeted receptor-kinases mediate vegetative plant growth in adult plants (Guo et al. 2009; Sun et al. 2010). Apparently, BES1 and BZR1 target genes depend on developmental stages and tissue specificity. BRs are known to regulate male fertility, but the

mechanisms were not well defined. A recent study indicated that BR mutants are defective in several aspects of anther and pollen development, including reduced filament length, fewer numbers of pollen grains, and defects in tapetal development, pollen wall formation and pollen release (Ye et al. 2010). Consistent with the mutant phenotype, several key genes involved in the process, including SPL/NZZ required for microspore mother cell development, TDF1, AMS and AyMYB103 involved in microspore development, MS1/MS2 required for tapetal development and pollen wall formation are reduced in BR mutants. ChIP experiment with chromatin isolated from flower tissues indicated that most of these genes are direct BES1 targets. Since most of these genes are not detected as BES1 targets with 2-week-old seedlings, these results clearly indicated that BES1 targets different genes in different tissues and developmental stages.

Besides BES1 and BZR1 targets, BES1 interacting proteins provide additional dimension to modulate BR-regulated gene expression in response to developmental and environmental cues. The first identified partner of BES1 in BR pathway was BIM1, a basic helix-loop-helix protein, which was found from yeast two hybrid screen by using BES1-C domain as bait (Yin et al, 2005). Further protein interaction analysis indicated that there were two different interaction domains: BES1 N-terminal domain and bHLH domain of BIM1 as well as BES1 C-terminal domain and BIM1. Genetic evidences indicated that *bim1 bim2 bim3* triple mutant showed more resistance to BRZ, while BIM1OX plant displayed reduced resistance to BRZ. Combining with the fact that overexpression of BIM1 in *bri1* background partially suppressed the dwarf phenotype of bri1, the genetic results demonstrated that BIM1 was a positive regulator in BR pathway. EMSA experiment, transient experiments and ChIP assay indicated that BES1 and BIM1 synergistically bound to E-box to promote BR-induced genes expression.

Then BES1 was found to interact with its direct target to amplify the BR signal transduction. From microarray data and chromatin immunoprecipitation experiment results, MYB30, a BR-induced transcription factor belonging to MYB family, was identified as a direct target of BES1 (Li et al, 2009). Loss-of-function mutant *myb30* displayed insensitive to BL and enhanced the phenotype of bri1-5, suggesting that MYB30 was a positive regulator in BR pathway. GST pull-down experiment and BiFC experiment indicated that MYB30 and BES1 interacted with each other in vitro and in vivo. DNA binding and gene expression analysis showed that MYB30 and BES1 bound to MYB binding site and E-box, respectively, to cooperatively amplify and promote the expression of a subset of BR targets.

BES1 recruits two related histone demethylases, ELF6 and REF6, to modulate BR-regulated gene expression and BR responses (Yu et al. 2008). ELF6 and REF6 were originally identified as two

genes that regulate flowering time (Noh et al. 2004). It was found that ELF6 and REL6 interact with BES1 both in vitro and in vivo through the basic region in the bHLH DNA binding domain (Fig 2). ELF6 and REF6 belong to JHDM3 subfamily of Jumonji family histone demethylases that function by removing methyl groups from various histone residues (Klose et al. 2006). ChIP assay indeed suggest that histone methylation is elevated at the promoter of BR-induced gene *TCH4* in *elf6* and *ref6* mutants. The study demonstrated that BES1 recruited ELF6 and REF6 to change chromatin structure and regulate genes expression. BRs are known to modulate flowering time as loss-of-function BR mutants have delayed flowering, which is accompanied by increased expression of FLC (Domagalska et al. 2007; Li et al. 2010a). ELF6 and REF6 therefore provide a molecular link between plant growth and reproduction (Clouse 2008).

In addition to histone modifying enzymes, BES1 also recruits IWS1 (INTERACTING-WITH-SPT6-1), a conserved protein implicated in transcription elongation, to regulate BR target genes. IWS1 was identified in a genetic screen for genes required for BES1 function by looking for suppressors for constitutive BR response mutant *bes1-D* (Li et al. 2010b). The *iws1* mutants suppress *bes1-D* phenotypes, displayed a semidwarf phenotype and reduced BR response in hypocotyl elongation assays. Gene expression studies indicated that about 1/3 of BR-induced genes are compromised in the *iws1* mutants. IWS1 interacts with BES1 in vitro and in vivo through the central domain of BES1 (aa 140-271, Fig. 2). AtIWS1 is a homolog of IWS1 in the yeast/human and interacts with histone chaperone and transcription elongation factor Spt6. Yeast IWS1, also termed SPN1, is implicated in inducible gene expression in yeast and it's function involves histone remodeling complex SWI/SNF (Fischbeck et al. 2002; Zhang et al. 2008). In human cells, IWS1 is required for splicing of HIV gene and global RNA export (Yoh et al. 2007; Yoh et al. 2008). Recent structure studies revealed that IWS1 has structure feature similar to transcription elongation factor TFIIS (Pujari et al. 2010). Genomic studies suggest that the expression of up to 30% genes can be regulated at steps after transcription initiation (Kim et al. 2005; Guenther et al. 2007); but the mechanisms for such regulation are not well defined. The study established IWS1 as a target for BR signaling, providing a potential new mechanism for the regulation of gene expression.

Other transcription factors involved in BR signaling

Other family transcription factors have been found to be involved in BR signaling in Arabidopsis and rice (Li 2010b; Clouse 2011). Several small and atypical HLH (helix-loop-helix) proteins, ATBS1 (ACTIVATION TAGGED BRI1 SUPPRESSOR 1), its Arabidopsis homologs including KIDARI and PRE1 (PACLOBUTRAZOL RESISTANT 1), and rice orthologs, ILI1 (INCREASED LAMINA

INCLINATION 1) and BU1 (BRASSINOSTEROID UPREGULATED 1), were identified as positive regulators for BR response as overexpression of these genes display increased BR responses (Tanaka et al. 2009; Wang et al. 2009a; Zhang et al. 2009a). ATBS1/PRE/ILI cannot bind DNA and therefore likely function by blocking the DNA binding activity of AIF (ATBS1-INTERACTING FACTOR)/IBH1 (ILI1–BINDING bHLH) bHLH proteins that function as negative regulators of the BR pathway. Overexpression of AIF1/IBH1 resulted in the plants with BR-like dwarf phenotype. Interestingly, AIF1/IBH1 and PRE1 are BZR1 targets that are repressed and induced by BZR1, respectively. These results suggest AtBS1/PRE/ILI1/BU family proteins are positive factors for BR pathway by sequestering AIFs/IBH1, the negative regulators of the BR pathway. How AIFs/IBH1 inhibits BR response remain to be established. AtBS1/PRE/ILI1/BU family proteins appear to be involved in other signaling processes as well (Clouse 2011).

In rice, BES1 and BZR1 homolog, OsBZR1, functions as a positive regulator of BR response (Bai et al. 2007). Interestingly, rice DLT (DWARF AND LOW TILLERING), a member of unique GRAS family transcription factors, also acts as a positive regulators of BR response as loss-of-function mutants display BR-like dwarf phenotype and have increased expression of BR biosynthesis genes (Tong et al. 2009). Several MADS box proteins, OsMDP1, OsMADS22 and OsMADS55 are negatively regulated by BRs and function as negative regulators in the BR pathway (Duan et al. 2006; Lee et al. 2008).

Regulation of BR biosynthesis

BR biosynthesis pathway is well established (Fujioka and Yokota 2003; Asami et al. 2005). It's well known that BR signaling inhibits BR biosynthesis through BES1 and BZR1 inhibition of the expression of *DWF4*, *CPD* and other biosynthesis genes (Noguchi et al. 1999; Choe et al. 2002; Mora-Garcia et al. 2004; Sun et al. 2010). Recent studies expanded our understanding how BR biosynthesis is positively regulated (Fig 3). BRX (BREVIS RADIX) was identified as a gene required for optimal root growth by promoting the expression of *CPD* gene expression; and was later found to promote shoot growth as well (Mouchel et al. 2004; Mouchel et al. 2006; Beuchat et al. 2010a; Beuchat et al. 2010b). Since *BRX* expression is induced by auxin and feedback inhibited by BR signaling, BRX apparently regulates BR level by coordinating auxin signaling and BR feedback pathway in the regulation of root growth (Mouchel et al. 2006). Interestingly, BRX protein also translocates from membrane to nucleus in the presence of auxin, suggesting that auxin regulate BRX activity through multiple mechanisms (Scacchi et al. 2009). Auxin signaling was recently found to induce the expression of *DWF4*, possibly through an Auxin Responsive Element (AuxRE) and its

interacting protein, which may reduce BZR1/BZR1 binding to *DWF4* promoter (Chung et al. 2011).

T-DNA activation tagging identified two bHLH transcription factors, TCP1 and CTSTA (CES), both of which positively regulate BR biosynthetic gene expression. The T-activation tagged allele of *TCP1*, *tcp1-D*, suppresses the dwarf phenotype of weak allele of BRI1, *bri1-5*, but not *bri1-4*, a null BRI1 allele, suggesting that TCP1 functions upstream of BRI (Guo et al. 2010). Loss-of-function of *TCP1* created by TCP1 fusing with EAR transcription repressor domain (SRDX) resulted in a dwarf phenotype in adult plant and short hypocotyls that can be recovered by exogenous BL. BR measurement in the TCP1 mutants suggest that DWF4 was the target of TCP1. Gene expression and ChIP assay confirmed that that DWF4, but not other biosynthetic genes, was directly regulated by TCP1. TCP1 is a member of a unique family of bHLH proteins that have an additional conserved region (R-domain) (Cubas et al. 1999). The characterization of TCP1 binding sites will be crucial to understand how exactly it regulates *DWF4* expression.

Similarly, overexpression of *CESTA* (*CES*) leads to increased plant growth that resembling BR gain-of-function mutants (Poppenberger et al. 2011). In contrast, loss-of-function mutant created by CES-SRDX caused a BR-related dwarf phenotype. BR levels are increased, and BR biosynthesis genes, *DWF4* and *CPD*, are increased in *ces-D*. In addition, CES protein was shown to bind *CPD* promoter in vitro and in vivo likely through G-box (CACGTG). Interestingly, CES appears to interact with BEE1, BEE2 and BEE3, which were previously shown to be induced BR and play positive role in BR-regulated growth (Friedrichsen et al. 2002). Although *CES* gene is not regulated by BRs, CES-YFP protein accumulates as distinct nuclear bodies in response to R treatment. These results raise a possibility that CES protein is regulated by BR signaling. It would be interesting to determine how BES1 and BZR1, TCP1, and CES/BEEs function together in the regulation of BR biosynthesis.

In rice, B3-domain containing transcription factor, RAVL1 (RELATED TO ABI3/VP1, ABA INSENSITIVE 3/VIVAPARIOUS 1), was identified as a positive regulator of BR response in rice (Je and Han 2010; Je et al. 2010). RAVL1 regulates the expression of BR receptor *OsBRI1* and several BR biosynthesis genes, including *D2*, *D11* and *BRD1* through E-box sequences (Yamamuro et al. 2000; Hong et al. 2002; Hong et al. 2003; Tanabe et al. 2005).

In addition to the regulation of BR biosynthesis genes at transcription level, regulatory proteins can directly regulate a couple of BR-biosynthesis enzymes. A small G-protein from Pea, Pra2, is expressed in the dark and interacts with BR biosynthesis enzyme DDAWF1 to promote BR level in the dark (Kang et al. 2001). GSR1 (GA STIMULATED TRANSCRIPT IN RICE 1, a small cystein-rich protein) binds to DIM1/DWF1 to regulate its enzyme activity and BR level in rice, providing another mechanism for the regulation of BR biosynthesis (Wang et al. 2009b). OsGSR1 is regulated

by GA and thus provide a link between BR and GA pathways.

In addition to the regulation of biosynthesis, BRs such as castasterone and BL can be inactivated by conserved P450 moonooxygenases, CYP734A1/BAS1 and CYP72C1/SOB7/CHI2/SHK1, through C26 or C22 oxidations (Neff et al. 1999; Nakamura et al. 2005; Takahashi et al. 2005; Turk et al. 2005; Ohnishi et al. 2006; Thornton et al. 2010; Sakamoto et al. 2011).

Hypotheses

Although much is known about how BES1 activates gene expression, how BRs repress gene expression is largely unknown. MYBL2 is a small MYB protein containing only one R3-MYB (SANT) domain and a transcription repression domain. Previous data showed that MYBL2 was involved in the repression of anthocyanin biosynthesis genes expression by interacting with TT8, a bHLH protein (Dubos et al. 2008; Matsui et al. 2008). The finding that MYBL2 is a BES1 target gene raises the possibility that MYBL2 plays a role in BR-repressed gene expression. I used genetic, genomic, and biochemical approaches to test the hypothesis.

Another BR-repressed transcription factor, RD26, was also studied in this thesis. RD26, belonging to NAC (petunia NAM and Arabidopsis ATAF1, ATAF2 and CUC2) transcription factor family, is involved in drought responses (Fujita et al. 2004; Tran et al. 2004; Tran et al. 2010). We hypothesize that RD26 plays an important role in mediating crosstalk between BR and drought pathways. Multidisciplinary approaches are used to test the hypothesis.

Conclusion

Many new regulatory components have been identified in the BR signaling or biosynthesis pathways. Identification of these components is just the beginning for the understanding how

BRs function to regulate a large number of biological processes at different stage, tissues and environmental conditions. Identifications of interacting proteins for many of the BR signaling components have provided important insight into the functions and regulation in BR signaling and will continue to do so in the future. As we lean from some of the components, regulation for each protein can be very complex involving multiple mechanisms. Identifications of both in vitro and in vivo phosphorylation sites and mapping the functional domains in combination with genetic studies will reveal new mechanisms in the regulation of BR pathways. Although a large number of BES1 and BZR1 target genes have been identified, it's still a daunting task to figure out how all these genes are regulated and more importantly how they function together to control specific processes. BES1 and BZR1 activate and repress about equal number of genes, how they interact with different partners to

perform the opposite functions in transcription remain to be defined. An initial focus on BES1 and BZR1 targeted transcription factors and their transcriptional partners seem to be reasonable to move forward. Eventually, computational modeling, in combination with genetic and genomic studies, are needed to put hundreds to thousands of BR target genes in order to fully understand how a simple hormone can lead to fundamental changes in plant growth and development, not only by itself, but also with many other hormonal and environmental pathways.

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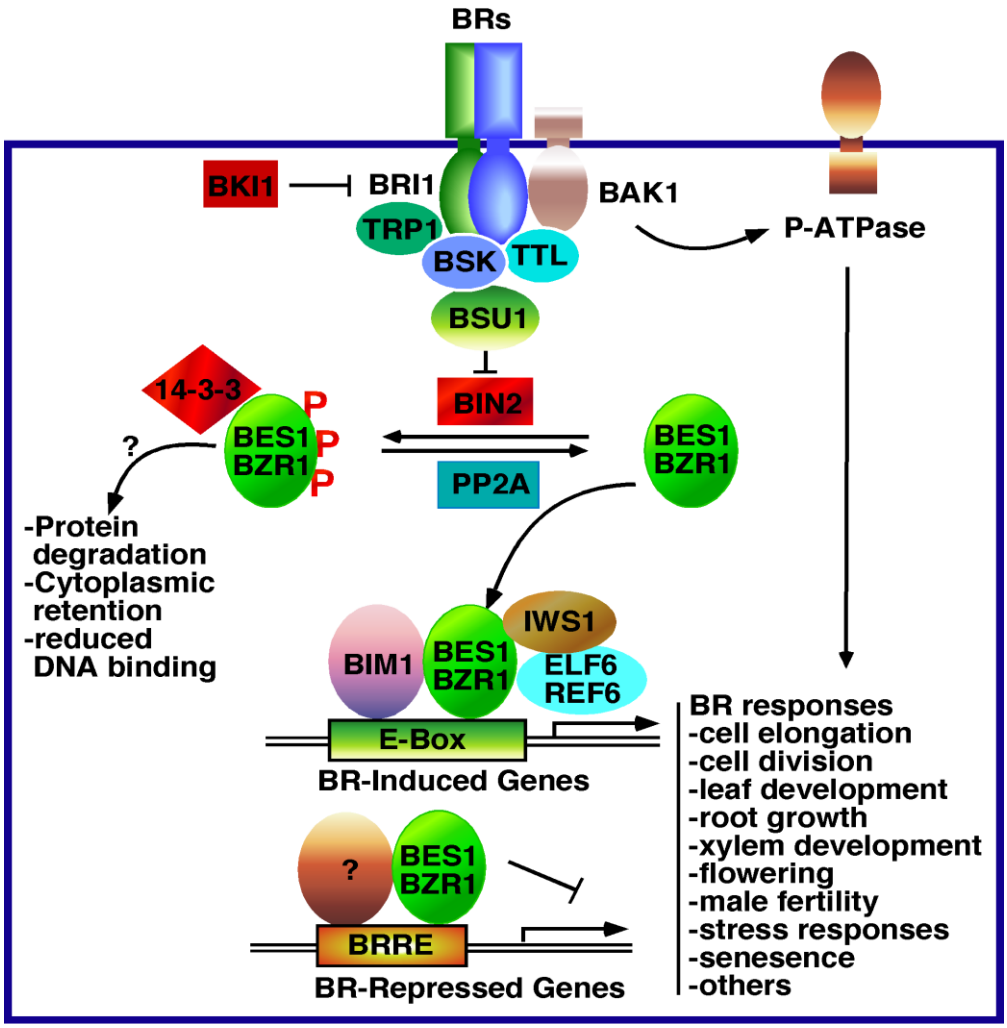

Fig.1: A model for Brassinosteroid (BR) signaling. BRs are perceived by membrane localized BRI1 receptor kinase. In the absence of BRs, BKI1 binds and inhibits BRI1 function; at the same time, GSK3-like kinase BIN2 phosphorylates BES1 and BZR1 family transcription factors and inhibits their functions by several mechanisms including protein degradation, reduced DNA binding, and/or cytoplasmic retention by 14-3-3 proteins. In the presence of BRs, activated BRI1 phosphorylates BKI1, which leads to dissociation of BKI1 from the plasma membrane and association of BRI1 with its coreceptor BAK1. BRI1 phosphorylates and activates BAK1, which in return phosphorylates and further activates BRI1. Activated BRI1 phosphorylates several substrates such as BSKs, TRIP-1, TTL and potentially a proton-ATPase (P-ATPase) to transduce the BR signal to downstream targets. While the targets for TRIP-1 and TTL remain to be identified, BRI1 phosphorylation of BSK1 leads to its association with BSU1 phosphatase, which can function to inhibit BIN2 kinase. At the same time, PP2A phosphatase can dephophorylate BZR1 and likely BES1 as well. These signaling events result in accumulation of unphophorylated BES1 AND BZR1 in the nucleus, where they can interact with other transcription regulators such as BIM1, histone demethylases ELF6/REF6 and transcription elongation factor IWS1 to regulate genes for various BR responses. BES1 and BZR1 may interact with additional factors to repress gene expression.

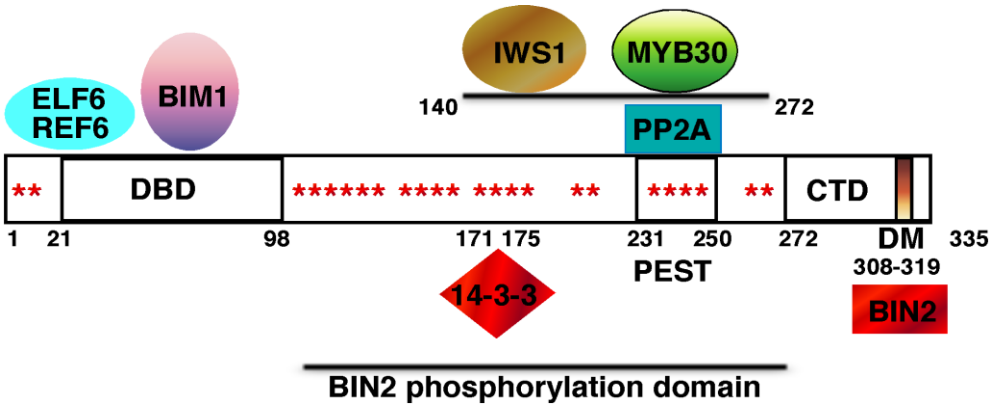

Fig.2: BES1 AND BZR1 protein structure and their interacting proteins. BES1 and BZR1, highly related to each other, have bHLH DNA binding domain (DBD), a BIN2 phosphorylation domain that contains 22 BIN2 phosphorylation sites (indicated by *), a PEST motif that is implicated in protein degradation, and a 14-3-3 binding motif that interacts with 14-3-3 when BES1 AND BZR1 are phophorylated. The C-terminal domain (CTD) includes a docking motif (DM) for BIN2 binding and a transcription activation function. Histone demethylases ELF6/REF6 and bHLH protein BIM1 interact with BES1 through their basic region and HLH dimerization domain of DBD, respectively. Phosphorylation of T175 for BES1 and T177 for BZR1 are important for interaction with 14-3-3s. While PEST domain is also required for interaction with PP2A, IWS1 and MYB30 interacts with BES1 through a region that contains part of the phosphorylation sites and PEST motif. The amino acid numbers are based on BES1.

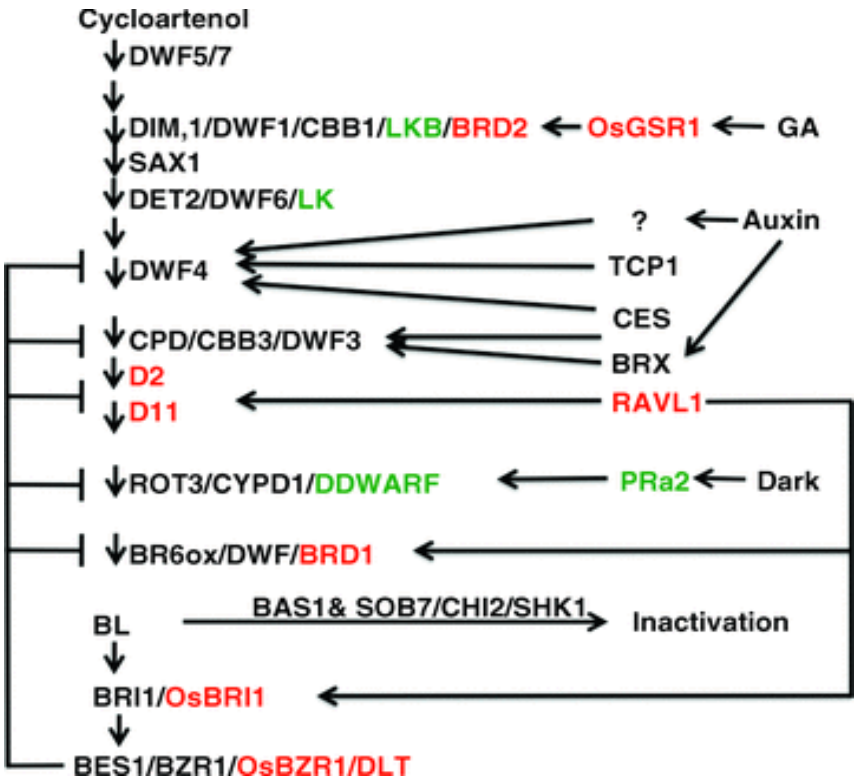

Fig. 3: Regulation of BR biosynthesis. BR biosynthesis pathway is shown from cycloartenol to brassinolide (BL). The enzymes catalyze most of the steps are shown (see text for references). Different color indicates enzymes/proteins from different species (black: Arabidopsis; red: rice and green: pea). BES1 AND BZR1, OsBZR1, and DLT function to inhibit many of the BR biosynthetic genes. In Arabidopsis, *DWF4* is induced by TCP1; CES and auxin (likely through an unidentified ARF protein) and *CPD* expression is promoted by BRX and CES. In rice, RAVL1 promotes the expression of *D2*, *D11* and *BRD1* as well as BR receptor gene *OsBRI1*. BL and its precursor, castasterone, can be inactivated by cytochrome P450 enzymes BAS1 and its homolog SOB7/CHI2 /SHK1.

CHAPTER II.
MYBL2 IS A SUBSTRATE OF GSK3-LIKE KINASE BIN2 AND ACTS AS A COREPRESSOR OF BES1 IN BRASSINOSTEROID SIGNALING PATHWAY IN ARABIDOPSIS[1]

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Abstract

Plant Steroid hormones, Brassinosteroids (BRs), play important roles in plant growth, development and responses to various stresses. BRs regulate the expression of several thousand genes, half of which are induced and the other half repressed by the hormone. BRs signal through plasma membrane-localized receptor kinase BRI1, BAK1 and several intermediates to regulate the protein levels, cellular localizations and/or DNA binding of BES1/BZR1 family transcription factors. While BES1 is known to interact with other transcription factors, histone modifying enzymes and transcription elongation factors to activate BR-induced genes, how BES1 mediates the BR-repressed gene expression is not known. Here we show that BES1 interacts with MYBL2, a small MYB family transcription repressor, to down-regulate BR-repressed gene expression. The loss-of-function *mybl2* mutant enhances the phenotype of a weak allele of *bri1* and suppresses the constitutive BR-response phenotype of *bes1-D*. The results suggest that suppression of BR-repressed gene expression is required for optimal BR response. Moreover, MYBL2 is a substrate of GSK3-like kinase BIN2, which has been well established as a negative regulator in the BR pathway by phosphorylating and inhibiting the functions of BES1/BZR1. Unlike BIN2 phosphorylation of BES1/BZR1 leading to protein degradation, BIN2 phosphorylation stabilizes MYBL2. Such dual role of phosphorylation has also been reported in WNT signaling pathway in which GSK3 phosphorylation destabilizes β-catenin and stabilizes Axin, a scaffolding protein facilitating the phosphorylation of β-catenin by GSK3. Our results thus establish the mechanisms for BR-repressed gene expression and the integration of BR signaling and BR transcriptional network.

Introduction

A group of plant steroid hormones, named Brassinosteroids (BRs), regulate many processes in plant growth, development and responses to biotic and abiotic stresses. Loss-of-function mutants display dwarf phenotypes with reduced cell elongation, dark-green and epinastic leaves, reduced apical dominance, altered vascular patterning, delayed senescence, male sterility, and late flowering (1-3). By contrast, gain-of-function mutants such as *bes1-D* have long hypocotyls, leaf petioles, curly leaves and early leaf senescence (4).

Genetic and molecular studies in *Arabidopsis* have greatly advanced our understanding of the BR signaling pathway (5-8). BRs are perceived by a plasma membrane-bound receptor BRI1 (9-11). BR signaling leads to the dephosphorylation of a family of plant-specific transcription factors, defined by their founding members BES1 and BZR1 with an atypical basic helix-loop-helix (bHLH) DNA

binding domain (12, 13). In the absence of BR, the negative regulator BKI1 binds to BRI1 and inhibits its function (14, 15). BR binding to BRI1 leads to the release of BKI1, which in turn sequesters 14-3-3 proteins that inhibit BES1/BZR1 function (16). At the same time, BR also promotes the association of BRI1 with co-receptor BAK1 and a series of phosphorylation events (17-20). Activated BRI1 likely signals through BSK1 and CDG1 kinases as well as BSU1 phosphatase to inhibit BIN2 kinase (21-24). The inhibition of BIN2 and dephosphorylation by PP2A phosphatase allow accumulation of BES1/BZR1 in the nucleus (25). LCMT, a Leucine C-terminal Methyltransferase, activates PP2A, which dephosphorylates BRI1 and appears to turn off the BR signaling (26). BRI1 autophosphorylation can also lead to self deactivation (27).

In the absence of BRs, BIN2 phosphorylates BES1/BZR1 and their homologs to inhibit their function (28, 29). In the presence of BRs, BIN2's kinase activity is inhibited, leading to the accumulation of dephosphorylated BES1 and BZR1 in the nucleus and subsequent regulation of gene expression.

Several genome-wide microarray experiments in *Arabidopsis* have demonstrated that BRs regulate thousands of target genes, activating and repressing about equal numbers of them (4, 30-35). BES1 and BZR1 target genes have been identified using ChIP-chip (Chromatin Immunoprecipitation followed by genomic tiling arrays) methods (36, 37). The genome-wide analysis suggests that both BR Responsive Element (BRRE) and E-box sequences are enriched in BES1 and BZR1 targets with BRRE site preferred in BR-repressed genes and E-boxes more predominant in BR-induced genes. Both BES1 and BZR1 target many transcription factors, some of which have been functionally characterized (38-43).

BES1 interacts with transcription regulators to activate BR target gene expression (12, 34, 43, 44). However, how BES1/BZR1 act to repress gene expression remains largely unknown. In this paper, we demonstrate that MYBL2, previously shown to be involved in anthocyanin biosynthesis gene expression (45, 46), is a substrate of BIN2 and is stabilized by BIN2 phosphorylation. We show that MYBL2 interacts with BES1 to repress BR-repressed genes. Our study thus identified a previously unknown substrate of BIN2 in the BR pathway and established a mechanism by which BES1 represses target genes expression.

Results

MYBL2 is a direct target of BES1 and its expression is repressed by BR and BES1

Recent ChIP-chip and gene expression studies indicated that MYBL2 was a direct target of BES1 and BZR1 (36, 37). We performed an independent ChIP PCR experiment to confirm the results (Fig 1A).

Anti-BES1 antibody and a control antibody were used to perform the ChIP assay. There are two putative BES1 binding sites in MYBL2 promoter: an BRRE at -9 bp relative to transcription start site and an E-box at-500 bp. ChIP-qPCR results indicated that BES1 was enriched significantly at BRRE site, but not at the E-box or at 3'-untranslated region (3'UTR). The preference of BES1 binding to BRRE on *MYBL2* promoter suggests that BES1 represses *MYBL2* expression. To test the hypothesis, *MYBL2* expression was examined in wild type (WT) and *bes1-D* seedling plants with or without BL (Brassinolide, the most active BR) treatment (Fig 1B). The expression of *MYBL2* is reduced by BL to about 60% in WT and to less than 20% in *bes1-D* mutant. These results confirm that MYBL2 is a direct target of BES1 and is repressed by BR through BES1.

MYBL2 is a positive regulator in the BR pathway

To determine the function of MYBL2 in BR responses, we obtained a T-DNA insertion line (SALK_126807) at 3'UTR of *MYBL2* (Fig.S1). RT-PCR analysis indicated that there is no detectable transcript in the mutant (Fig. S1A), as reported previously (45). We created double mutants of *mybl2* with *bri1-5*, a weak loss-of-function allele of BR receptor BRI1 (47) and *bes1-D*, a gain-of-function mutant in the BR pathway (4). Although the single mutant *mybl2* did not display obvious growth phenotype in either seedling or adult stage, double mutant analysis indicated that *mybl2* enhanced *bri1-5* phenotype and suppressed *bes1-D* phenotype (Fig. 1C-D, Fig. S1B). The *bri1-5 mybl2* double mutant showed more severe reduced growth or dwarf phenotype than *bri1-5* either in vegetative or inflorescent stage (Fig. 1C, D). The *bes1-D mybl2* double mutant had shorter petioles in adult plants and shorter hypocotyls at seedling stage compared with *bes1-D* (Fig. 1E and Fig. S1C). These genetic studies demonstrate that MYBL2 plays a positive role in the BR pathway.

MYBL2 interacts with BES1 in vitro and in vivo

We have previously showed that BES1-induced MYB30 interacts with BES1 to activate BR-induced genes and thus amplify the BR signaling (43). We hypothesize that BES1-repressed MYBL2 may interact with BES1 in the down-regulation of BR-repressed genes. Yeast two-hybrid experiments indicated an interaction between full-length BES1 and MYBL2 (Fig. S2A). GST pull-down experiment confirmed this interaction (Fig. 2A). GST-MYBL2, but not GST alone, pulled down significant amount of MBP-BES1 protein, demonstrating a direct interaction between MYBL2 and BES1. Several truncated GST-BES1 were used to map the domain in BES1 required for the interaction with MYBL2. While BES1 with deletion to amino acid (aa) 89 still interacts with MYBL2, BES1 with deletion to aa 140 lost the interaction, suggesting that aa 89-140 in BES1 are important for

the interaction (Fig. S2B). Indeed, yeast two-hybrid experiment confirms that aa 89-140 in BES1 was sufficient for the interaction with MYBL2 (Fig. 2B). To map the specific domain in MYBL2 for the interaction, a series of truncated-MYBL2 constructs were generated for yeast two-hybrid experiments. Fig. 2C showed that the SANT domain of MYBL2, aa 34-82, is both necessary and sufficient to interact with BES1. These results indicate that part of the BIN2-phosphorylation domain of BES1 (aa 89-140) and the SANT domain (aa 34-82) of MYBL2 are required for the interaction between these two proteins (Fig. 2D).

We further tested in vivo interaction between MYBL2 and BES1 by Bimolecular Fluorescence Complementation (BiFC) experiment with MYBL2 fused to N-terminal YFP (MYBL2-nYFP) and BES1 fused to C-terminal YFP (BES1-cYFP). When MYBL2-nYFP and BES1-cYFP were co-transformed into *Arabidopsis* protoplasts, strong fluorescence signal was observed in the nucleus (Fig. 2E-F). In contrast, there was no fluorescence observed when MYBL2-nYFP and cYFP were co-transfected (Fig. 2G-H). Taken together, these results demonstrate that MYBL2 and BES1 interact with each other in vitro and in vivo.

MYBL2 facilitates BES1 in down-regulating BR-repressed gene expression

It has been shown previously that MYBL2 represses gene expression by interacting with bHLH transcription factor TT8 (45). Based on the interaction between BES1 and MYBL2, we hypothesize that MYBL2 is recruited by BES1 to down-regulate BR-repressed genes. The expression of seven BR-repressed genes, which are direct targets of BES1, were examined in *bri1-5 mybl2* and *bri1-5* mutants. The expression of five of the seven tested genes increased significantly in *bri1-5 mybl2* compared to *bri1-5* (Fig. 3A), suggesting that MYBL2 is responsible for the repression of a portion of the BR-repressed genes. To confirm that MYBL2 indeed are targeted to these genes, we performed ChIP PCR assays with MYBL2-GFP transgenic plants using anti-GFP, anti-BES1 antibodies and normal IgG as control. Both MYBL2 and BES1 binding were enriched on all 5 target gene promoters (Fig. 3B, Fig. S3). To further confirm that BES1 and MYBL2 cooperate in the down-regulation of the BR-repressed genes, we constructed promoter-luciferase (LUC) reporter constructs with At2g45210 and *DWF4* genes and assayed their regulation by BES1 and MYBL2 in tobacco leaves by a transient expression experiment (Fig. 3C). While BES1 led to reduced gene expression on both reporter genes, MYBL2 alone showed no effect. However, when MYBL2 and BES1 were co-expressed, the expression of the reporter genes was further reduced compared to BES1. We also tested the repression effect of BES1 with truncated MYBL2 protein that lacked interaction domain (SANT domain), there was no synergistic repression effect on these two reporter constructs (Fig. S4). All the results

demonstrated that MYBL2 forms a complex with BES1 to inhibit BR-repressed genes expression.

MYBL2 is a novel substrate of BIN2 and is stabilized by BIN2 phosphorylation

BIN2 is a negative regulator in the BR pathway, which phosphorylates and inhibits BES1 and BZR1 (4, 48-50). BIN2 belongs to glycogen synthase kinase 3 (GSK3) family, whose substrates contain repeats of a short consensus sequence S/TxxxS/T (x corresponds to any amino acid residues). There are 19 potential phosphorylation sites for BIN2 in the predicted MYBL2 protein (Fig. 4A), which promoted us to test if MYBL2 was a substrate of BIN2. We first tested the interaction between BIN2 and MYBL2 by GST pull-down experiment. Fig. 4B showed that there was a direct interaction between MYBL2 and BIN2 in vitro. We then performed an in vitro kinase assay with MBP-BIN2 and MBP-MYBL2. BIN2 indeed phosphorylated MYBL2, but not MBP (Fig. 4C). Bikinin, a small molecule, and lithium chloride (LiCl) have been found to inhibit the kinase activities of BIN2 and its close homologs (48, 51). Both Bikinin and Li^+ can inhibit BIN2 phosphorylation of MYBL2 as well as BIN2 autophosphorylation (Fig. 4D-E).

To test if MYBL2 exists as phosphorylated form in plants, MYBL2-GFP protein was immunoprecipitated from MYBL2-GFP transgenic plants and subjected to phosphatase (CIP) treatment (Fig. 4F). CIP treatment leads to several fast-migrating bands on a SDS-PAGE gel containing Phos-tag reagent that binds to phosphorylation groups and reduce protein mobility (see Materials and Methods), indicating that the MYBL2-GFP exists as phosphorylated form in plants.

To further investigate the function of the BIN2 phosphorylation on MYBL2, MYBL2-GFP protein level in transgenic plants was examined by Western blotting. Fig. 5A showed that in the absence of BL, MYBL2-GFP protein level remains mostly constant. However, in the presence of BL, MYBL2-GFP protein decreased more than 10 folds after 4 hours of treatment while its mRNA level only decreased by about 40%. As previously demonstrated, BL treatment leads to the accumulation of unphosphorylated BES1 (Fig. 5A, middle panels). Since BL treatment is well established to reduce BIN2 activity, we conclude that BIN2 phosphorylated MYBL2 is stable in the absence of BL and is unstable as unphosphorylated form in the presence of BL.

To test the hypothesis, BIN2 inhibitors Bikinin was also applied to plants to test the effect of BIN2 phosphorylation on MYBL2. MYBL2 protein decreased significantly after 2h with Bikinin treatment (Fig. 5B top panels), while the dephosphorylated form BES1 accumulated at the same time (Fig. 5B middle panels). We further examined MYBL2 accumulation in *bri1-5*, in which BR signaling is blocked and BIN2 is thus constitutively active. The protein level of MYBL2 significantly accumulated compared to WT (3.9X, Fig. S5), which is more than the *MYBL2-GFP* transcript

accumulation (2.3X) in the *bri1-5*.

Proteasome inhibitor MG132 was used to test whether BL-induced decrease of MYBL2 protein was due to proteasome-mediated degradation. When the plant samples were treated by BL together with MG132, the MYBL2 protein is significantly accumulated (Fig. S6). This result indicated that MYBL2 degradation by BL is dependent on the proteasome-mediated pathway. Taken together, all the results demonstrate that MYBL2 is regulated by BIN2.

Discussion

Recent studies indicated that BRs activate and repress about equal number of genes and BES1/BZR1 transcription factors play an essential role in mediating BR-regulated gene expression (36, 37). BES1/BZR1 interacts with other transcription factors (BIM1, MYB30 and PIF4), chromatin-modifying enzymes (REF6/ELF6 histone demethylase) and transcription elongation factor (IWS1) to regulate BR-induced gene expression. However, how BES1 and its homologs repress gene expression is not well established. In this study, we found that BES1 interacts with one of its targeted transcription factors, MYBL2, to repress the BR-repressed gene expression. This MYBL2-mediated repression is required for BR-regulated plant growth, as *mybl2* knockout mutant enhances the weak loss-of-function BR mutant (*bri1-5*) and suppresses gain-of-function BR mutant (*bes1-D*). We further found that MYBL2 is a substrate of BIN2 kinase, and BIN2 phosphorylation of MYBL2 stabilizes the protein. The regulation of MYBL2 by BIN2 is opposite of BIN2 phosphorylation of BES1, which destabilizes BES1. The study thus provides new insights into the regulation of BR transcriptional network by BR signaling.

MYBL2 is a small MYB protein that has only one R3-MYB (SANT) domain. MYBL2 interacts with TT8, a bHLH protein, to repress anthocyanin biosynthesis gene expression (45, 46). MYBL2 has a novel repression motif (TLLLFR) at its carboxyl terminus (45), which might interact with TOPLESS family co-repressors (52). Several lines of evidence support the role of MYBL2 as a transcription corepressor for BES1. First, BES1 interacts with MYBL2 both in vitro and in vivo, through the SANT domain of MYBL2 and a specific region of BES1 (aa 89-140). It was proposed that the SANT domain of MYBL2 does not interact with DNA directly due to the replacement of several critical residues required for DNA binding (45). Our findings that the SANT domain of MYBL2 doesn’t bind to DNA and is involved in interaction with BES1 corroborate this conclusion. Second, MYBL2 and BES1 act cooperatively to repress BR-repressed gene expression in a transient expression assay. Lastly, several BR-repressed genes are up-regulated in *mybl2* mutant in *bri1-5* background.

In addition to the regulation by BES1 at transcription level, MYBL2 is regulated by BR signaling through BIN2 kinase. BIN2 phosphorylates and inhibits BES1 and its homolog, BZR1, through several mechanisms, including targeted protein degradation, cytoplasmic retension by 14-3-3 proteins and reduced DNA binding (6, 29, 53). Recent studies suggest that BIN2 can also phosphorylate and regulate other factors involved in BR-regulated processes. For example, it was recently reported that BIN2 phosphorylates and inhibits MAP kinase kinase YODA and transcription factor SPCH to regulate stomatal development (54, 55). BIN2 was also reported to phosphorylate and inhibit ARF2 DNA binding (56). AIF, a BES1/BZR1 target bHLH protein involved in BR-responses and CESTA implicated in BR biosynthesis are both reported to be phosphorylated by BIN2 in vitro (41, 57). Rice transcription factor DLT is a BIN2 kinase substrate and mediate BR signaling in rice (58). BIN2 phosphorylation of MYBL2, like that of DLT, is not as effective as BIN2 phosphorylation of BES1/BZR1, which may explain the higher concentrations of bikinin and LiCl required to observe their effects on MYBL2 phosphorylation and protein accumulation.

In contrast to BES1/BZR1 and SPCH that are destabilized by BIN2 phosphorylation, BIN2 phosphorylation of MYBL2 stabilizes the protein. Our results thus suggest that BIN2 phosphorylation can have different functional consequences. The dual role of GSK-like kinase phosphorylation has been observed in WNT pathway. In the WNT signaling pathway, GSK3 kinase phosphorylates positive regulator β-catenin in a protein complex including scaffolding protein Axin, leading to β-catenin degradation (59). Interestingly, Axin is also phosphorylated by GSK3 kinase and such phosphorylation stabilizes Axin (60), much like the effect of BIN2 phosphorylation on MYBL2 (Fig. S7).

Although MYBL2 acts as a transcriptional repressor, it functions as a positive regulator in the BR pathway, as *mybl2* mutant enhances and suppresses the phenotype of *bri1-5* and *bes1-D*, respectively. Why the positive regulator MYBL2 is repressed by BRs and in *bes1-D*? One possibility is that this regulation represents a feedback regulation mechanism in which excessive BRs or BR signaling down-regulates the pathway through MYBL2. We therefore propose that MYBL2 function as a “buffer” to fine-tune BR responses (Fig. S8). Global gene expression studies with *mybl2* mutants and functional studies of BIN2 regulation of MYBL2 phosphorylation are needed to further test the model.

In conclusion, our studies showed that MYBL2 formed a complex with BES1 to down-regulate BR-repressed genes and BIN2 phosphorylates and stabilizes MYBL2 protein. Like BES1-mediated gene activation, BES1-MYBL2 complex likely interacts with additional cofactors in the regulation of the BR-repressed genes. Identification of these factors and elucidation of the transcriptional network

through which BES1 and its corepressors act to repress gene expression are important steps to understand how BRs regulate plant growth and various responses.

Materials and Methods

Plant materials and growth condition

T-DNA insertion mutant, *mybl2*, was obtained from ABRC (Arabidopsis Biological Resource Center), corresponding to line SALK_126807 (61). All the plants were grown on 1/2MS plates and/or in soil under long day conditions (16h light/ 8h dark) at 22 ℃.

Plasmid constructs

For GFP-tagged transgenic plants, MYBL2 genomic sequence including its native promoter was cloned from wild type and fused with GFP tag into pZP211 vector (62). For recombinant protein purification and GST pull-down assay, MYBL2 coding region was cloned into pETMALc-H vector, while BIN2, BES1 and truncated BES1 fragments were incorporated into pET42a(+) (Novagen) respectively. For yeast two-hybrid assays, BES1, the DNA binding domain of BES1 and phosphorylation domain of BES1 were cloned into pGBKT7, while MYBL2 as well as its deletion mutation were clone into pGADT7 (Clontech). For BiFC assay, the constructs of N or C-terminus of EYFP used were previously reported (44). The coding region of MYBL2 and BES1 were inserted into YFP-N construct and YFP-C construct, respectively.

Transgenic plants

The construct of MYBL2-GFP driven by its native promoter was transformed into *Agrobacterium tumefaciens* (stain GV3101) which were used to transform plants by the floral dip method (63). Transgenic lines were selected on 1/2 MS medium plus 50ug/ml kanamycin. Transgene expression was analyzed by western blotting.

Gene expression analysis

Total RNA was extracted and purified from 4-week-old plants of different genotypes using RNeasy Mini Kit (Qiagen). Mx4000 multiplex quantitative PCR system (Stratagene) and SYBR GREEN PCR Master Mix (Applied Biosystems) were used in quantitative real-time PCR analysis. At2g45210 promoter (1021bp including 5'UTR) and *DWF4* promoter (972bp including 5'UTR) were cloned and used to drive luciferase reporter gene expression. MYBL2, MYBL2ΔSANT and BES1 coding region driven by CaMV 35S promoter were cloned into pZP211 vector respectively. Tobacco

leaf transient assay (64) was used to examine the repression effect of MYBL2 on reporter gene expression in the presence or absence of BES1 and/or MYBL2. Equal amount of Agrobacterium cells (measured by O.D., adjusted to same with vector-containing strain) were injected to tobacco leaves. The luciferase activities were measured from protein extracts from triplicate samples and measured using Berthold Centro LB960 luminometer with luciferase assay system (Promega). The luciferase levels were normalized by the total protein from each sample.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as previously described (36). GFP antibody was used to precipitate chromatin from MYBL2-GFP overexpression plants, while antibody against BES1 and normal IgG (Sigma) were used as control.

In vitro Kinase assay and detection of in vivo MYBL2 phosphorylation

The in vitro kinase assay was performed as described (4). MYBL2 protein was immunoprecipitated from transgenic plants and treated with or without calf alkaline phosphatase (CIP) as described (4). The in vivo phosphorylated-MYBL2 was examined by Phos-tag reagent (NARD Institute) with or without CIP treatment as described (65).

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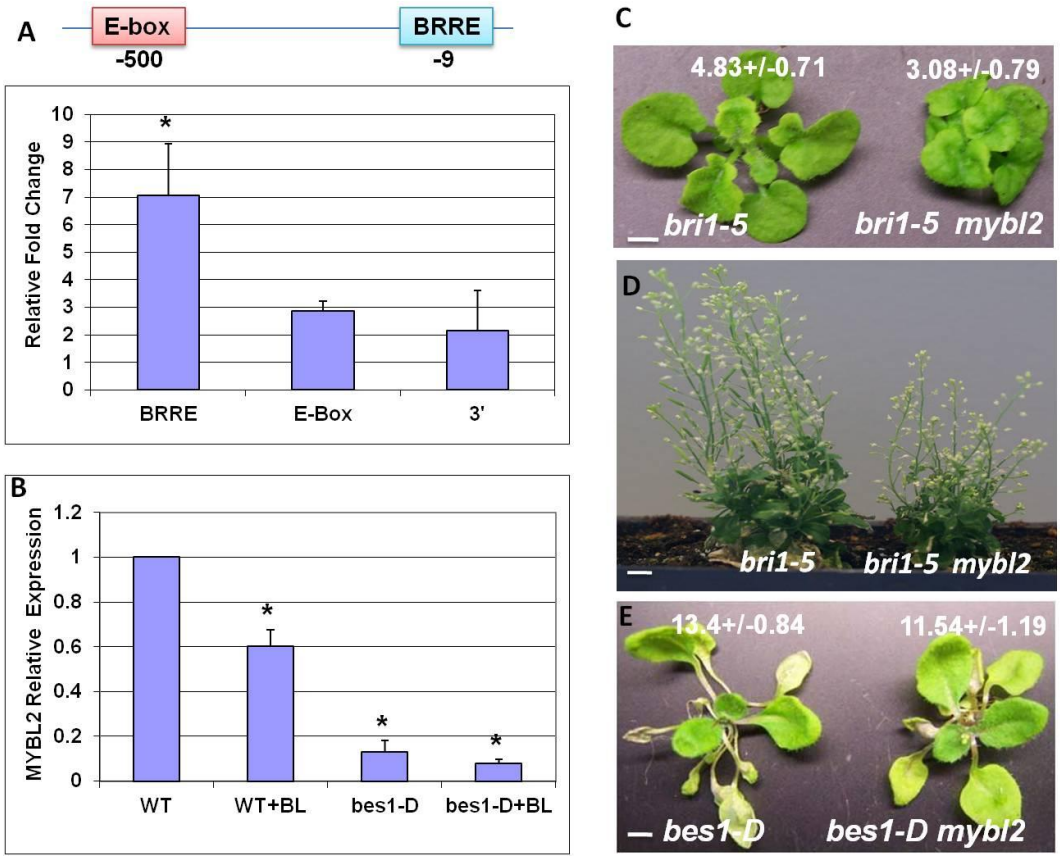

Fig. 1. MYBL2 is repressed by BES1 and acts as a positive regulator in the BR pathway. (A) BES1 targets the BRRE site on MYBL2 promoter. ChIP was performed with anti-BES1 antibody in WT seedlings. The bindings of BES1 at BRRE site (-9bp), E-box (-500bp) and 3' region of the *MYBL2* gene were examined by qPCR. The 5s rRNA was used as internal control. (B) The expression of *MYBL2* was examined by quantitative RT-PCR in 2-week-old WT and *bes1-D* seedlings with or without 1,000 nM BL treatment for 2.5 hr. (C, D) The phenotypes of *bril-5* mutants and *bril-5 mybl2* double mutants at different growth stages. Bars represent 2.5 cm (C) and 1 cm (D), respectively. (E) The phenotype of 4-week-old *bes1-D* mutant and *bes1-D mybl2* double mutant. Bar represents 5 cm. The average petiole lengths of the longest (fifth) leaf for each genotype are indicated (C and E). The average and standard deviations were from 10 plants. The difference was significant as analyzed by Student's t-Test (*<0.05).

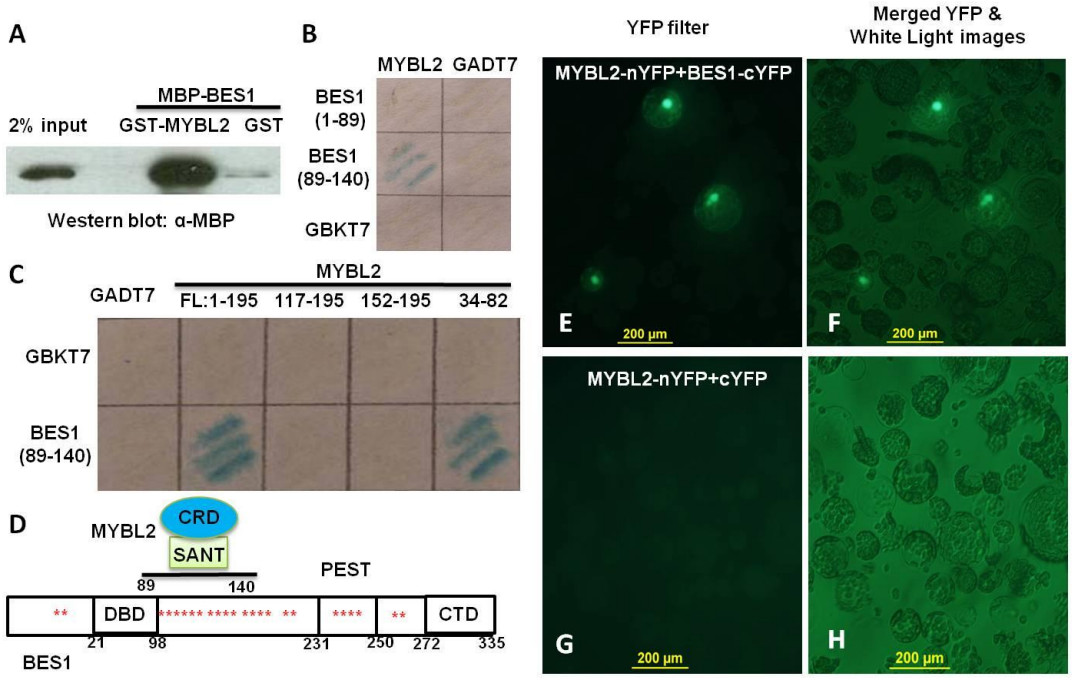

Fig. 2 BES1 interacts with MYBL2 in vitro and in vivo. (A) GST pull-down using GST, GST-MYBL2 and MBP-BES1. BES1 was detected by Western Blotting with anti-MBP antibody. (B) MYBL2 interacts with BES1 (aa 89-140) in yeast as detected by β-galactosidase (LacZ) activity. (C) MYBL2 interacts with the phosphorylation domain of BES1 (aa 89-140) through its SANT domain (aa 34-82). (D) A model shows BES1 protein structure and MYBL2's corresponding domain involved in the interaction. DBD: BES1 DNA binding domain; CTD: BES1 C-terminal domain. CRD: MYBL2 C-terminal Repression Domain. The "*" indicate BIN2 phosphorylation sites. (E-H) MYBL2 interacts with BES1 in vivo by BiFC. Cotransformation of MYBL2-nYFP and BES1-cYFP led to the reconstitution of YFP activity in Arabidopsis protoplasts (E, under YFP filter and F, merged microscopic images from YFP and white light), while co-expression of MYBL2-nYFP and cYFP did not produce any positive YFP signal (G and H).

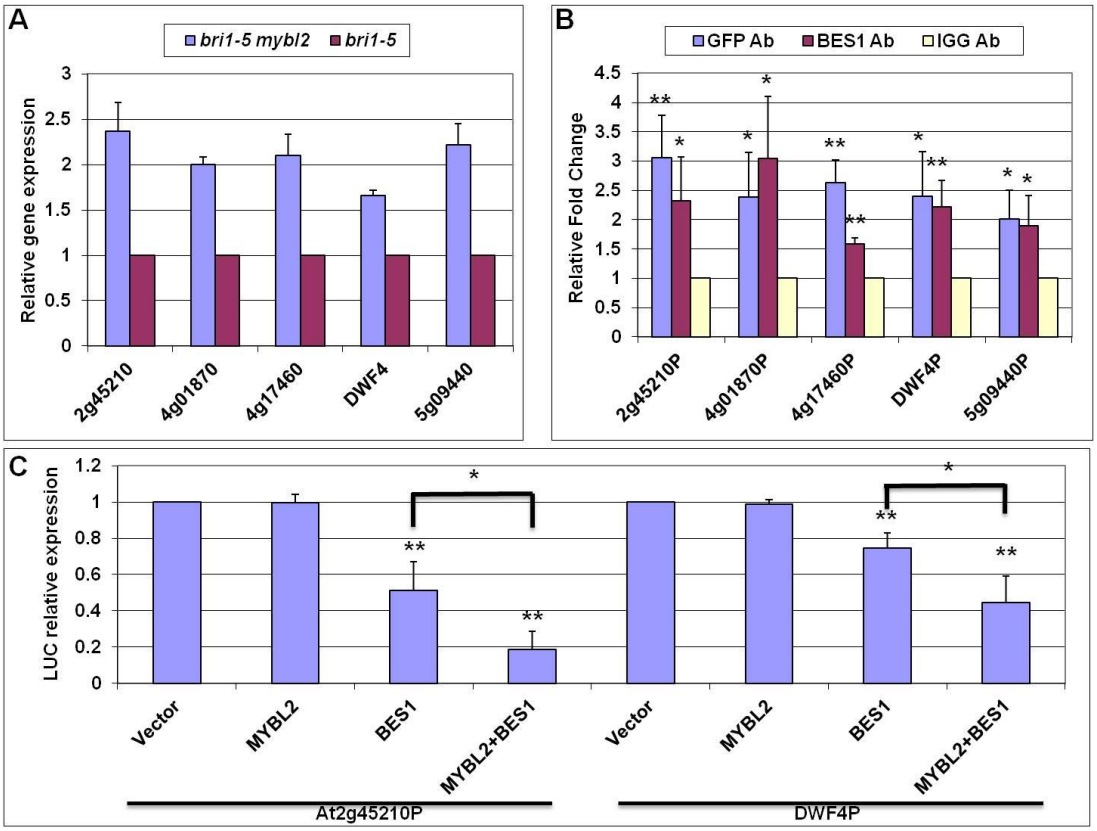

Fig. 3. MYBL2 cooperates with BES1 to inhibit BR-repressed gene expression. (A) BR-repressed genes were increased in *bri1-5 mybl2* double mutant compared to *bri1-5*. Quantitative RT-PCR was performed with RNA from 4-week-old plants. (B) MYBL2 is targeted to BR-repressed genes in vivo. ChIP using MYBL2-GFP transgenic plants with anti-GFP antibody, anti-BES1 antibody and normal IgG control. The bindings of MYBL2 and BES1 to indicated gene promoters are detected by qRT-PCR flanking the BRRE sites. The 5s rRNA was used as internal control. (C) Transient gene expression assays were performed in tobacco leaves with At2g45210-LUC and DWF4-LUC reporter genes co-transfected with BES1 and/or MYBL2 via Agrobacterium. The relative expression levels were normalized with total protein. The average and standard deviations were from three biological repeats. The significant difference was analyzed by Student's t-Test (*<0.05, **<0.01).

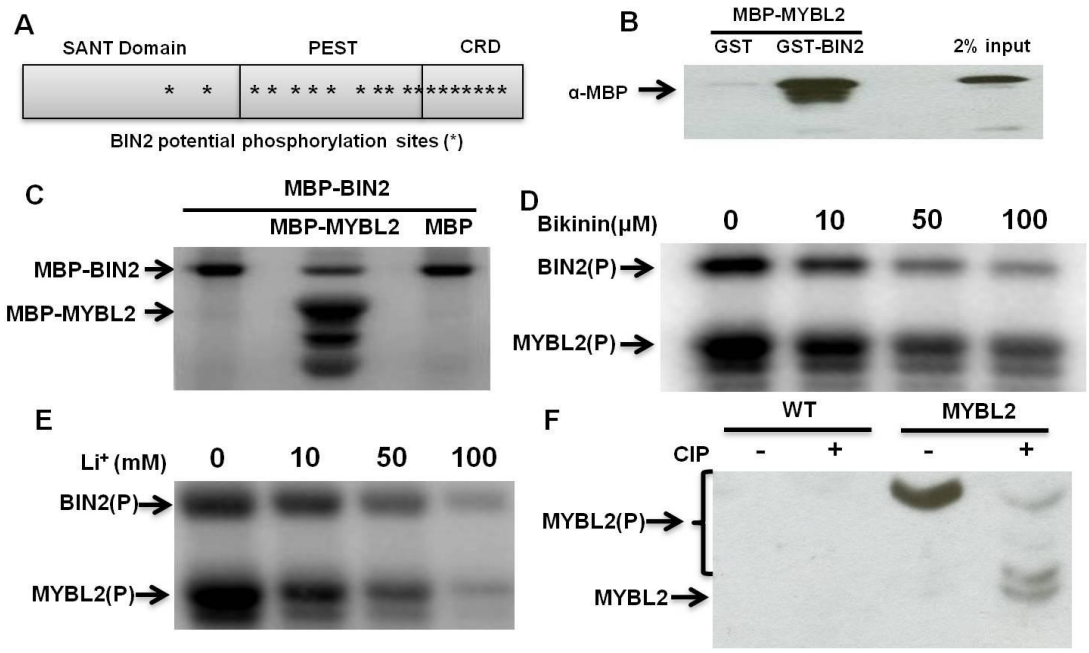

Fig. 4. MYBL2 is a substrate of BIN2 kinase in BR signaling. (A) The structure of MYBL2. The * indicates potential BIN2 phosphorylation sites. The SANT domain, putative PEST domain and C-terminal repression domain (CRD) in MYBL2 are indicated. (B) GST pull-down experiments using GST, GST-BIN2 and MBP-MYBL2. MYBL2 was detected by Western Blotting with anti-MBP antibody. (C) BIN2 phosphorylates MBP-MYBL2 but not MBP in the in vitro kinase assay. Arrows indicate phosphorylated-MYBL2 or autophosphorylated-BIN2. (D) BIN2 phosphorylation of MYBL2 is inhibited by Bikinin in kinase assay in vitro. (E) The phosphorylation of MYBL2 by BIN2 was inhibited by LiCl in kinase assay in vitro. (F) MYBL2 is phosphorylated in vivo. MYBL2 immunoprecipitated from transgenic plants was treated with calf alkaline phosphatase (CIP), and separated on SDS-PAGE gel containing Phos-tag reagent (NARD Institute). CIP treatment produced several fast-migrating bands. Arrows indicate the phosphorylated- or unphosphorylated- MYBL2.

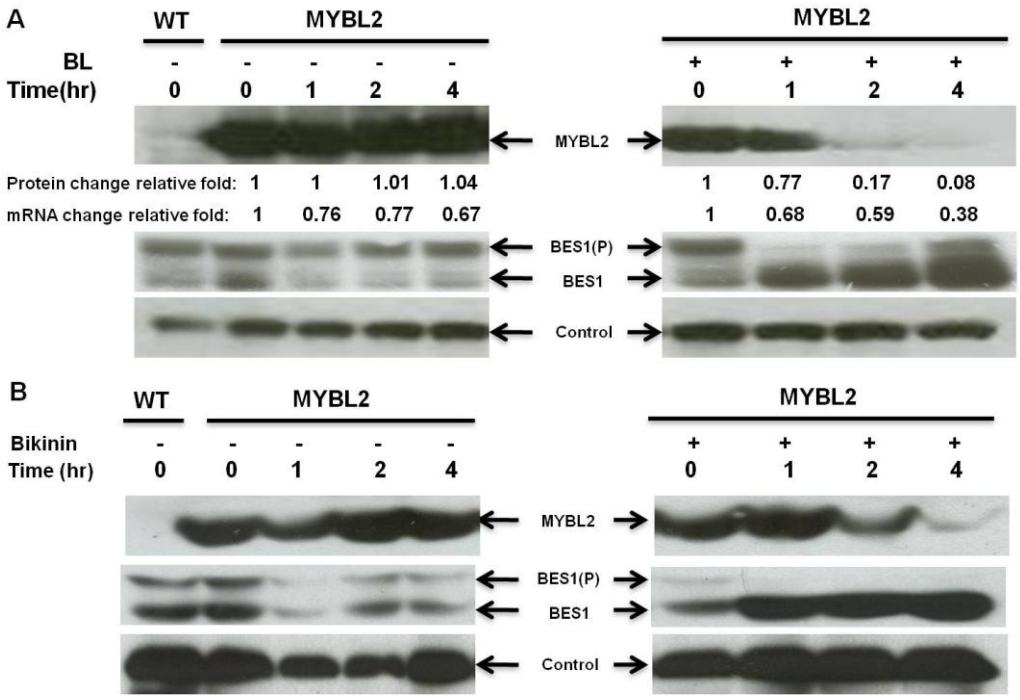

Fig. 5: BL and BIN2 inhibitors destabilize MYBL2 protein. (A) BL treatment destabilizes MYBL2 protein. Four-week-old MYBL2-GFP transgenic plants were treated with or without 1 mM BL for indicated periods of time and used to prepare protein to detect MYBL2 (top), BES1 (middle) and a control protein (bottom). MYBL2 protein levels were quantified using Alphalmger 3400 and MYBL2 mRNA levels were quantified by qRT-PCR. (B) Bikinin treatment induces the degradation of MYBL2 protein. MYBL2-GFP transgenic plants were treated with or without 100 mM Bikinin for indicated periods of time and used to prepare protein to detect MYBL2 (top), BES1 (middle) and a control protein (bottom).

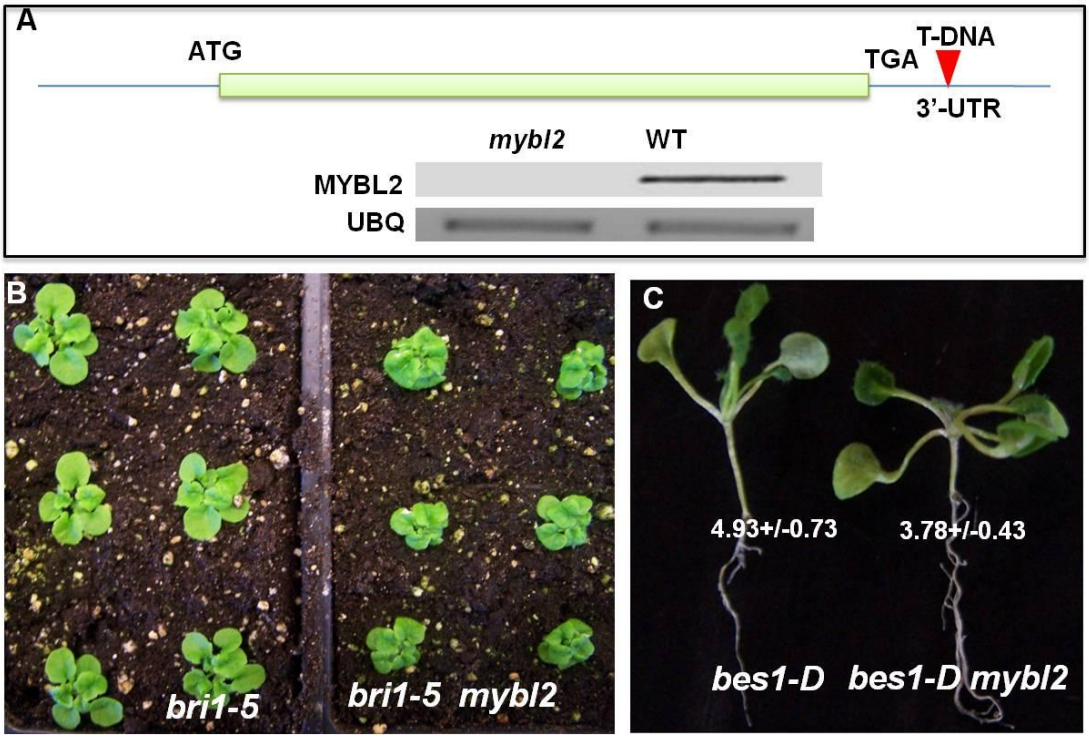

Fig. S1. MYBL2 T-DNA insertion mutant.
A. Schematic representation of T-DNA knockout allele of *MYBL2 gene*. *MYBL2* expression is not detected by RT-PCR in the T-DNA mutant.
B. *mybl2* suppressed *bes1-D* phenotype at seedling stage.
Two-week-old seedlings of *bes1-D* and *bes1-D mybl2* double mutants are shown. The average hypocotyl length and standard deviations are indicated (n=10).

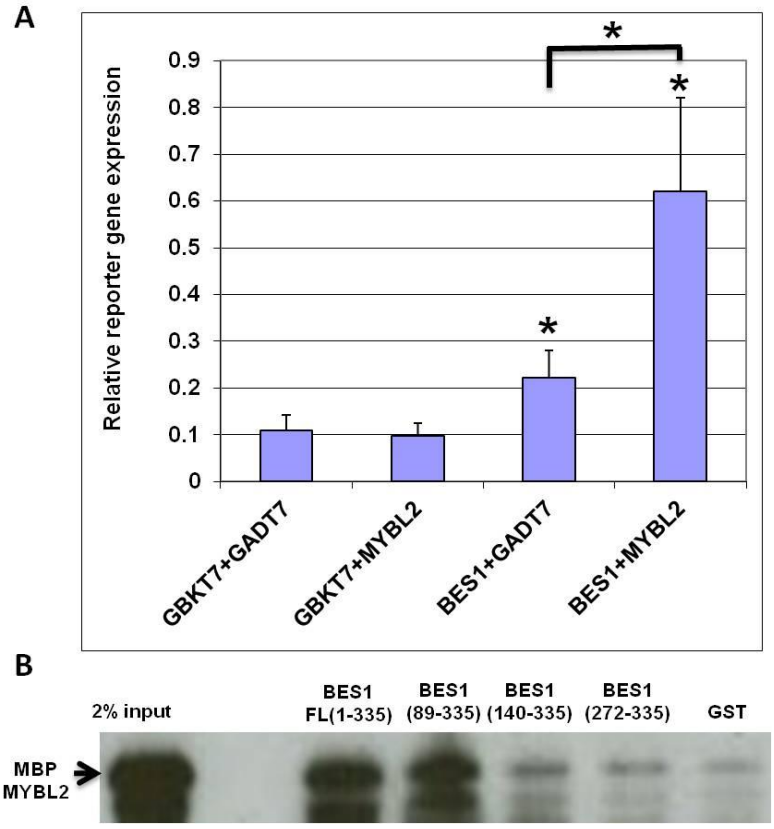

Fig. S2. BES1 interacts with MYBL2 in yeast and in vitro.
A: β-galactosidase(LacZ) activity was detected with ONPG in a quantitative liquid-culture assay to test the protein-protein interaction in yeast two-hybrid experiment.
B: GST pull-down experiments using MBP-MYBL2 and GST tagged full-length or different truncated BES1. MYBL2 was detected by Western Blotting with anti-MBP antibody.

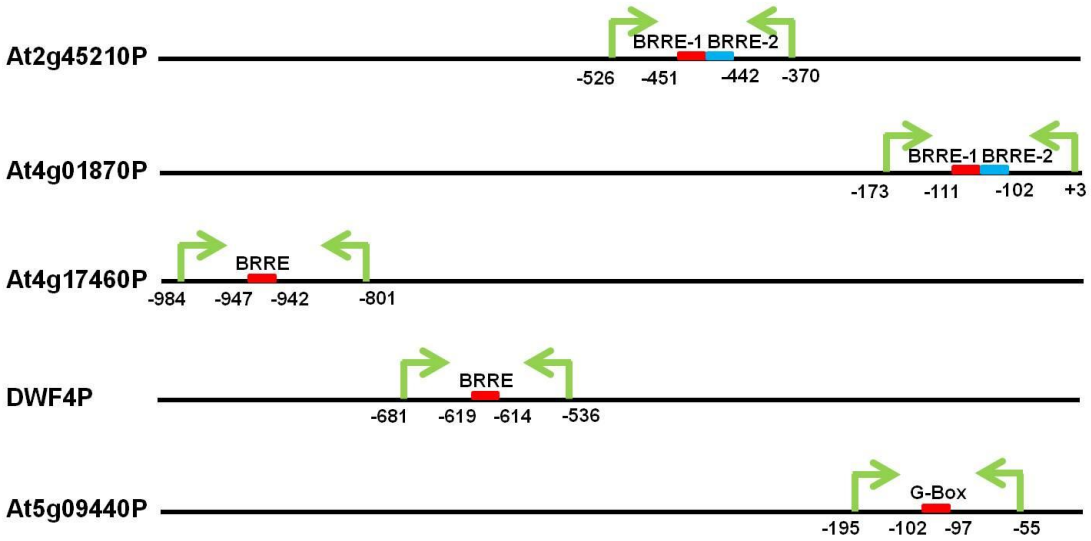

Fig. S3. BRRE or G-box in BR-repressed BES1 target gene promoters.
The BRRE and G-box, both are enriched in BR-repressed BES1/BZR1 target genes, are shown in the promoters used for ChIP-PCR analysis with BES1 and MYBL2. The numbers indicate nucleotide positions relative to transcription start sites.

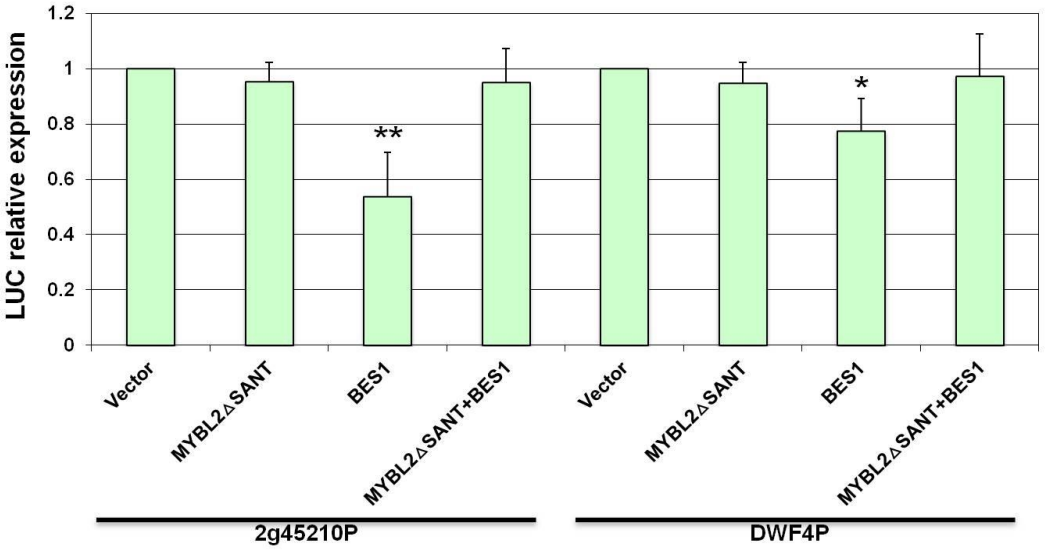

Fig.S4: BES1 and MYBL2ΔSANT did not have synergistic repression effect on BR-repressed genes.

Transient gene expression assays were performed in tobacco leaves with At2g45210-LUC and DWF4-LUC reporter genes co-transfected with BES1 and/or MYBL2ΔSANT via Agrobacterium. The relative expression levels were normalized with total protein. The average and standard deviations were from three biological repeats. The significant difference was analyzed by Student's t-Test (*<0.05, **<0.01).

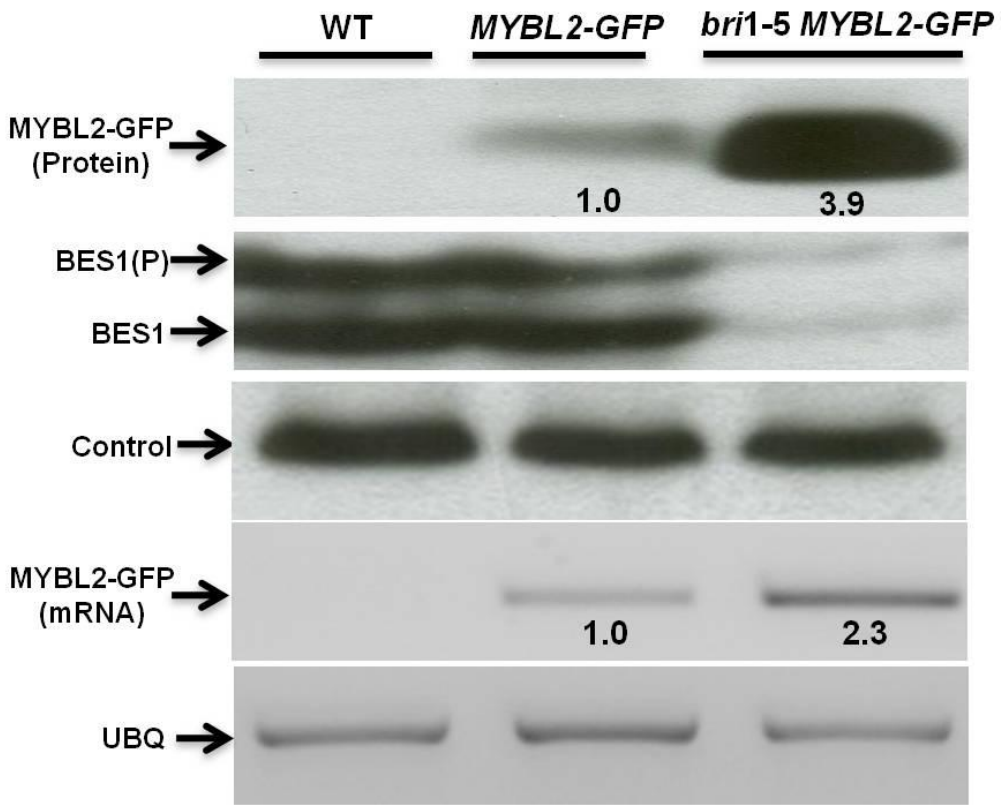

Fig. S5: MYBL2 protein accumulated in *bri1*-5 background.
MYBL2-GFP protein and mRNA level were examined in WT and *bri1*-5 background. The numbers indicated the amount of MYBL2-GFP protein or transcripts quantified using Alphalmger 3400.

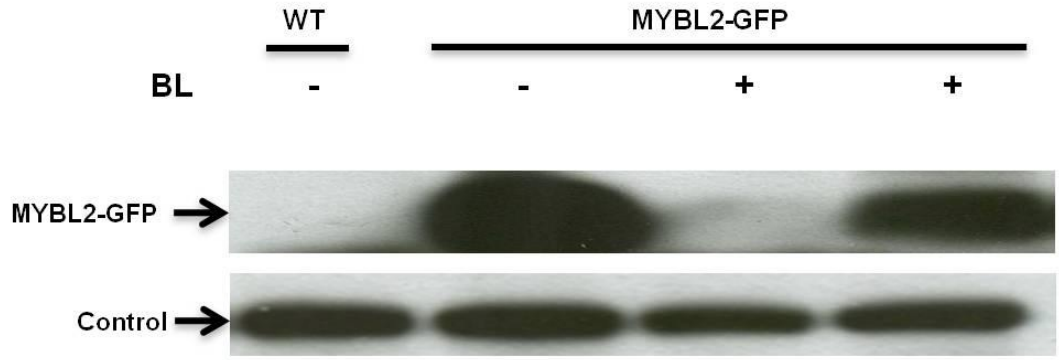

Fig. S6: The reduced MYBL2 protein level caused by BL was reversed by proteasome inhibitor MG132.

The plant samples were treated without BL, with 1 mM BL and with both BL and MG132 (30 μM) for 4 hours and used to prepare protein to detect MYBL2 (top) and a control protein (bottom).

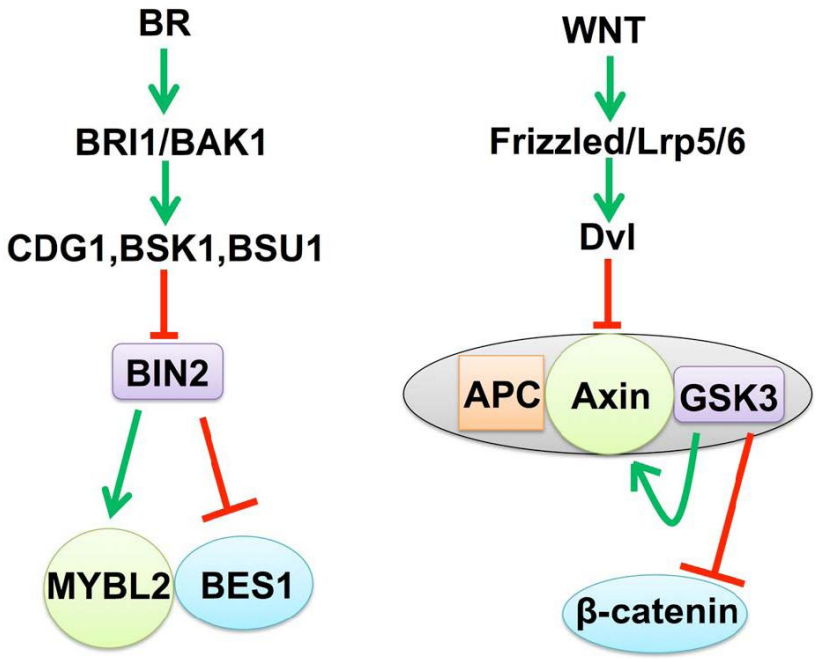

Fig. S7. BR and WNT signaling pathways.
The regulation of BES1 and MYBL2 by BIN2 phosphorylation in the BR pathway is similar to the regulation of b-catenin and Axin by GSK3 kinase in the WNT pathway, despite the fact that the substrates in each pathway do not have any similarities in protein sequences.

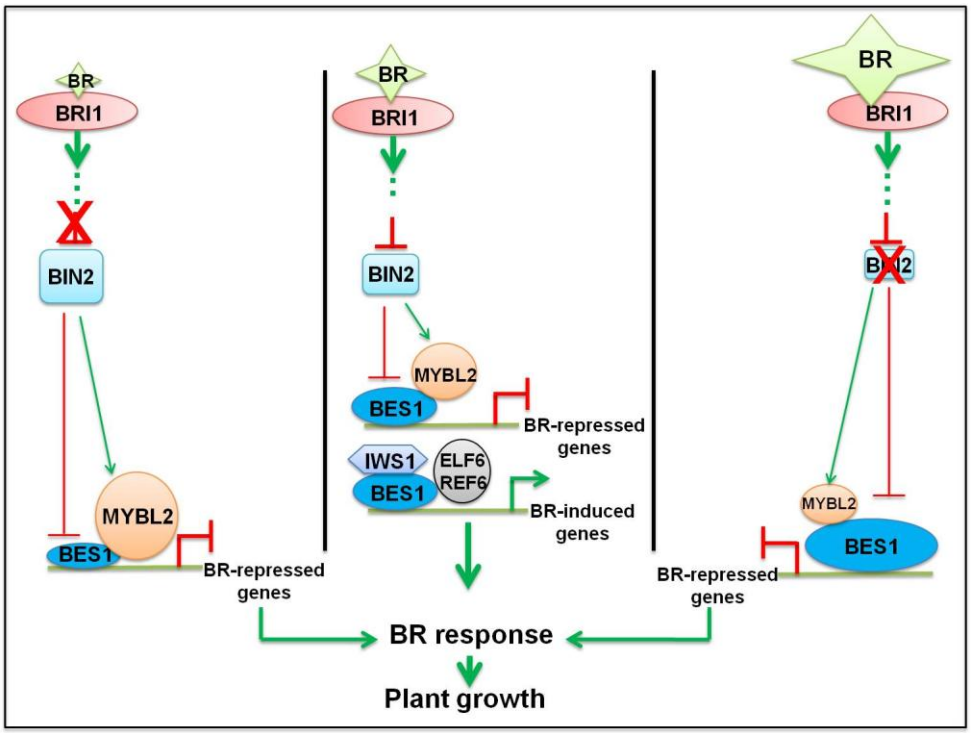

Fig. S8: A model for MYBL2 function in the BR pathway.
Under optimal conditions, the plants respond to BR signaling and maintain balanced levels of BES1 and MYBL2 to regulate BR-repressed genes (middle). However, when BR level and signaling is reduced, BES1 function will be reduced by increased BIN2 phosphorylation (left). In the meantime, MYBL2 protein level is increased due to the increased MYBL2 transcript by reduced BES1 and stabilization of MYBL2 by BIN2 phosphorylation. The increased MYBL2 protein likely compensates for the reduced BES1 in BR-repressed gene expression. On the other hand, if the concentration of BR is increased in plants (right), BES1 protein will accumulate and MYBL2 will be reduced to balance the BR-repressed gene expression. The increased BES1 leads to decreased MYBL2 transcript level and dephosphorylation of MYBL2 destabilizes the protein. The reduced MYBL2 protein level will alleviate the transcription repression caused by increased BES1.

Table S1: Primer sequences used in the study.

mybl2LP	GAGATGTCGATTGAGAGGTCG	Genotyping
mybl2RP	GCTGTATTAGCTATAATTTCTTACAG	
gMYBL2NAsp718	CGCGGTACCTCTTATATGATTTTGGAGTA GATGGTAAGTGAG	Transgenic plant
gMYBL2CSalI	CGCGTCGACTCGGAATAGAAGAAGCGTT TCTTGACCTG	
MYBL2AD7NEcoR1	GCGGAATTCATGAACAAAACCCGCCTTC GTGC	Yeast two hybrid
MYBL2AD7CXho1	GCGCTCGAGTCATCGGAATAGAAGAAGC GT	
MYBL2AD7D1NEcoR1	GCGGAATTCAAGATTATTAGTGATCAATC	
MYBL2AD7D2NEcoR1	GCGGAATTCAGTCATTTGCCTGACCTAAA CA	
MYBL2BDNEcoR1	CGCGAATTCAAACAACGCAACTTCTCAA AAGATG	
MYBL2BDCSal1	CGCGTCGACTCACCTTTTTAGGTAAGTTT CCCAAT	
MYBL2MBPNEcoR1	GCCGAATTCGATGAACAAAACCCGCCTT CGTGC	Protein expression
MYBL2MBPMBPCAsp718	CGCGGTACCTCATCGGAATAGAAGAAGC GT	
cMYBL2NAsp718	CGCGGTACCTCAACCCACCAGTCCAAGT CAAACCTCCTC	BiFC
gMYBL2CSalI	CGCGTCGACTCGGAATAGAAGAAGCGTT TCTTGACCTG	
MYBL2RTF	ATAGTACTAGTACCGGACGAAGTC	Gene expression
MYBL2RTR	CAAACATCGTTATACCATCTCTCTAGTG	
2G45210RTF	CTGTCATAGAGTTTTGGTACCCATC	
2G45210RTR	CGAAATCTGAATACAGACAAGGAAT	
4G01870RTF	CATGTGAGTTTCAATAAAGATGGTG	
4G01870RTR	CGTCTAATTTCACAACGTACAAATC	
4G17460RTF	AGAAGCTAGGTTTAACAGCAAGACA	
4G17460RTR	CTTCCGTTAATTTCTCAACACATCT	
5G09440RTF	ATTATAAACATCGCGACTCTTCTTG	
5G09440RTR	CACTCGTCTTGTCTACGAGAACC	
DWF4RTF	GAAATGTAGTTAGGTTTTTGCATCG	
DWF4RTR	GAGATTAGGTTGGTCATAACGAGAA	
MYBL2Chip1F	TGTGGGACCAATTAACAAGG	ChIP qPCR
MYBL2Chip1R	GATGGCTTGAGGAGGTTTGA	
MYBL2Chip2F	ATGGTAAGTGAGATAGGGAAGTGG	
MYBL2Chip2R	GGTTCAGGAACAGATAAGGGAGA	
MYBL2Chip3F	CGATAACCGCTGCTTATTTGA	
MYBL2Chip3R	TGTAGTTTGGAGGAAAATGAACA	

2g45210PFBamH1	CGCGGATCCtcatcaacgtacacaagtaacgcaactag	Transient expression
2g45210PRHind3	CGCAAGCTTCTTCTTATAGCTAACTTTAAAAAACAG	
DWF4FBamH1	CGCGGATCCtggaatggaagtagtaatatacattaagc	
DWF4REcoR1	CGCGAATTCGGAGCTAGTTTCTCTCTCTCTCTCACTCAC	
MYBL2DENAsp	CGCGGTACCatgAAGATTATTAGTGATCAATC	
MYBL2DECSal	CGCGTCGACTCATCGGAATAGAAGAAGCGT	

CHAPTER III.

RD26 mediates the crosstalk between drought and Brassinosteriod signaling pathways

A paper to be submitted to Nature Genetics

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Abstract

Brassinosteroids (BRs) play important roles in plant growth, development and plant responses to both abiotic and biotic stresses. BR signal through receptor BRI1 and BAK1 as well as a series signaling intermediates to control the activities of BES1/BZR1 family transcription factors, which control the expression of thousands of genes for various BR responses. BRs are known to be involved in drought response, but the mechanism of interactions between these two pathways remains to be established. Here we show that NAC family transcription factor RD26 and its close homologs mediate crosstalk between drought and BR signaling pathways. We found that *RD26* is a BES1 target genes and functions to inhibit BR-regulated growth as overexpression of *RD26* gene leads to decreased plant growth and knockout of *RD26* and its close homologs results in increased BR response. Gene expression studies revealed that RD26 modulates BR-regulated gene expression in a complex way. RD26 represses many BR-induced genes including those implicated in cell elongation and activates many BR-repressed genes, thereby inhibiting BR functions. On the other hand, BR signaling also inhibits drought responses. The reciprocal inhibitory effects of BES1 and RD26 are mediated by their interactions on different promoter elements. This mechanism ensures that BR-induced plant growth is inhibited under drought condition, which induces *RD26* expression. The mechanism also prevents unnecessary activation of drought response when plants undergo BR-induced growth, during which BES1 accumulates. Our results thus revealed a previously unknown mechanism coordinating plant growth and drought tolerance.

Introduction

Brassinosteroids is a group of plant steroid hormones regulating plant growth, development, and responses to abiotic stresses [1-3]. Recently the main components in BR signaling pathway have been identified and characterized [4-7]. BR signaling leads to the accumulation of BES1/BZR1 in nucleus to control the expression of targets for growth and other BR responses [8-13]. Several studies indicated that treatment of exogenous BR could enhance the tolerance of plants to drought [1,14,15]. BR-deficient mutants not only display strong dwarf phenotype, but also has an enhanced tolerance to drought [16,17]. Several transcription factors, including drought-induced transcription factor RD26 and several of its close homologs have been identified as the direct targets of BES1 and BZR1 [8,9]. These studies indicate that BR pathway have a strong relationship with drought tolerance.

RD26 belongs to NAC (petunia NAM and Arabidopsis ATAF1, ATAF2 and CUC2) family transcription factors, which are induced by drought, abscisic acid (ABA), NaCl and jasmonic acid (JA)

[18-20]. RD26 and its homologs function to promote drought responsive gene expression and increase plant drought tolerance [19]. Recent results showed that RD26 and two of its homologs, ANAC019 and ANAC055 are involved in plant bacterial pathogenesis [21]. These three genes function to promote bacterial infection as they down-regulate salicylic acid (SA) level by repressing SA biosynthesis and activating SA degradation pathways. Yeast-one-hybrid and DNA binding experiments showed that RD26 could bind to CATGTG and CACG core sequence [19], which are either an E-box (CANNTG) or partial sequence of BRRE site (CGTGT/CG), well established binding sites for BES1/BZR1[8,9]. The potential overlapping binding sites of RD26 and BES1/BZR1 indicates that there might be a crosstalk between drought and BR signaling pathways through BES1/BZR1 and RD26.

In this paper, we confirmed that *RD26* is a direct target of BES1 and negatively regulates BR signaling pathway. RD26 and BES1 bind to the same DNA-binding site to block each other's function. RNA-Seq data confirmed that RD26 could function as both activator and repressor on the different genes. Drought stress experiment showed that the loss-of-function mutants in BR signaling pathway had higher drought tolerance, while gain-of-function mutant in the BR pathway (*bes1-D*) exhibited lower drought tolerance compared to wild type control. These results suggest that RD26 inhibits BR pathway and BR pathway also negatively regulates drought tolerance, establishing a mechanism for crosstalk between these two important pathways for plant growth and stress responses.

Results

RD26 is a direct target of BES1 and is a negative regulator of the BR signaling pathway.

Our previous data indicated that RD26 was a direct target of BES1 and was repressed by BL (brassinolide, the most active BR) and/or BES1 and BZR1 [8,9]. Since, BES1 and BZR1 can bind to BRRE to repress gene expression, we examined *RD26* gene promoter and revealed a BRRE site around -851 relative to transcriptional start site. ChIP experiments with anti-BES1 antibody showed that BES1 was more enriched at BRRE site compared to control site (Fig. 1A), suggesting BES1 binds to this site to repress the expression of *RD26*. Indeed, *RD26* expression was reduced by BL in WT (Wild-Type) (Fig 1B). Moreover, *RD26* expression was repressed significantly in *bes1-D*, in which BES1 protein accumulates and *RD26* was further reduced by BL treatment. These results confirm that *RD26* is a direct target of BES1 and is repressed by BL through BES1.

Our previous result indicated that loss-of-function *RD26* mutant has a small increase in BR response[8], suggesting that RD26 functions with its homologs to inhibit BR response. To confirm this hypothesis, we generated *RD26* overexpression lines. *RD26* overexpressing plants (*RD26OX*) displayed a stunted growth phenotype, the severities of which correspond well with RD26 protein

levels (Fig 2A). Moreover, *RD26OX* transgenic line could suppress the phenotype of *bes1-D*, a gain-of-function mutant in the BR pathway (Fig 2B). Western blot result indicated that the BES1 protein levels and forms (phosphorylated and unphosphorylated) did not change significantly in *bes1-D RD26OX* double mutant. The results suggest that RD26 functions downstream of BES1. To confirm that *RD26OX* phenotype is due to reduced BR response, we determined the mutant responses to BR biosynthesis inhibitor brassinazole (BRZ), which reduces endogenous BR levels. *RD26OX* seedlings had shorter hypocotyls and were more sensitive to BRZ compared to WT (Fig 2C). Several RD26 homologous genes, *ANAC019*, *ANAC055* and *ANAC102*, are also regulated by BR and likely function redundantly in BR responses [8,9]. We generated quadruple mutant of *rd26 anac019 anac055 anac102*. The quadruple mutant showed more resistant to BRZ compared to WT, although they did not exhibit any obvious growth phenotype under normal condition (Fig. 2A). All the genetic evidences demonstrate that RD26 plays a negative role in the BR signaling pathway.

RD26 negatively regulates the expression of BR-responsive genes.

To understand how RD26 negatively regulate BR response, we performed gene expression studies with *RD26* mutants in the absence or presence of BR by RNA-seq. We used 4-week-old adult plants for gene expression studies because the *RD26OX* plants display clearest phenotype at this stage. In WT, 2678 genes are induced and 2376 genes are repressed by BR, among around 22,000 genes analyzed (Fig3, Fig S1, S2). Consistent with the strong phenotype of *RD26OX* plants, 3246 genes are up-regulated and 5479 genes are down-regulated in the transgenic plants, respectively (Fig3, Fig S1, & FigS2). RD26 and its homologs modulate BR-responsive genes in complex ways (Fig 3, Fig S1 & Fig S2). Consistent with the negative role of RD26 in BR response, 43% (1141, Group I) BR-induced genes are down-regulated in *RD26OX* mutant and their induction by BRs are reduced but not abolished (Fig 3A and B). In contrast, only 20% (539, Group III) of BR-induced genes are up-regulated in *RD26OX*. We examined the expression of 95 Arabidopsis genes implicated in cell elongation, including those encoding expansin, xyloglucan endotransglycosylases (XTHs) and pectinlyase. Thirty-five of these cell wall-modifying genes were induced by BL, and 30 of them (86%) are down-regulated in *RD26OX* plant, while only 1 gene was up-regulated in the mutant (Table S1). The reduced expression of these genes may account for the reduced growth and BR response of *RD26OX* transgenic plants.

On the other hand, among 2376 BR-repressed genes, 595 (25%, Group II) are up-regulated and 823 (35%, Group IV) are down-regulated and in RD26OX (Fig 3C, D and Fig S1-2). While Group III and Group VI genes suggest positive role of RD26 in BR response (i.e. BR-induced genes are up-

regulated and BR-repressed genes are down-regulated in RD26OX), Group I and Group II genes demonstrated a negative role of RD26 in BR response (BR-induced genes are down-regulated and BR-repressed genes are up-regulated in RD26OX). Since *RD26OX* displayed a negative role in BR response (Fig 2), we focus on the Group I and Group II genes to determine the mechanisms by which RD26 regulates BR responses.

Consistent with the relative weaker BR-response phenotype of the *rd26 anac019 anac055 anac102* mutant, only 405 genes are up-regulated and 378 are down-regulated in *rd26 anac019 anac055 anac102* quadruple mutant (Fig S3A and B). BR-induced genes that are down-regulated in *RD26OX* and up-regulated in the quadruple mutant (Group C), BR-induced genes that are up-regulated in *RD26OX* and down-regulated in the quadruple mutant (Group E), BR-repressed genes that are up-regulated in RD26OX and down-regulated in the quadruple mutant (Group D), and BR-repressed genes that are down-regulated in RD26OX and up-regulated in the quadruple mutant (Group F) are listed in Table S2. Clustering analysis indicated that most of the genes are affected in opposite ways in the RD26 and *rd26 anac019 anac055 anac102* mutant and *RD26OX* (Fig S3). The results support the conclusion that RD26 and its homologs act to modulate BR responses.

RD26 and BES1 bind to E-box and BRRE site simultaneously and cancel each other's transcriptional activities

To reveal how RD26 inhibits Group I BR-induced genes and activates Group II BR-repressed genes, we chose promoter of one representative gene from each group for mechanistic studies. A BR-induced gene At4G00360 was chosen to represent Group I gene as it is down-regulated in RD26OX and its promoter contains a CATGTG E-box known to be BES1 binding site. Likewise, a BR-repressed gene, AT4G18010, was chosen to represent Group II genes because it is up-regulated in RD26OX and its promoter contains BRRE site, a well established promoter element enriched in BR-repressed genes.

First, the promoter of AT4G00360 and AT4G18010 were fused with *luciferase* (*LUC*) gene, to generate reporter constructs. *BES1*, *RD26* and *BES1+RD26* were expressed together with the reporter construct and the reporter gene expression was determined. While BES1 activated and RD26 repressed the expression of AT4G00360P-LUC, the reporter gene expression was in between when both *BES1* and *RD26* were co-expressed (Fig. 4A). In contrast, BES1 activated and RD26 repressed AT4G18010P-LUC reporter gene and the expression was in the middle when both *RD26* and *BES1*, are coexpressed (Fig. 4B). These results indicated BES1 and RD26 act antagonistically to regulate BR regulated gene expression.

To confirm if such antagonistic interactions happen in vivo, we then examined the expression of these two genes in *bes1-D, RD26OX* and *bes1-D RD26OX*, in which BES1, RD26 or both of them are increased, respectively. As shown in Fig.4 C, the expression of AT4G00360 was much higher in *bes1-D* compared to *bes1-D RD26OX*, while its expression was significantly repressed in *RD26OX*. In contrast, expression of AT4G18010 was down-regulated and up-regulated in *bes1-D* mutant and *RD26OX* transgenic plant, respectively, but this gene's expression level was between *bes1-D* mutant and *RD26OX* in *bes1-D RD26OX* double mutant (Fig. 4D).

Previous DNA binding experiments with RD26 indicated that RD26 can bind sequences with a CACG core as well as CATGTG E-box sequence [19]. Interestingly, CACG core overlaps with BES1/BZR1 BRRE (CACGT/CG) enriched in BR-repressed genes and E-boxes including CACTTG enriched in BR-induced genes [8,9]. These results suggest that BES1 and RD26 could bind to the same site to modulate BR-regulated gene expression. To reveal the biochemical interaction between BES1 and RD26, Electrophoretic Mobility Shift Assay (EMSA) experiments were performed with recombinant BES1 and RD26 proteins using DNA probes containing BRRE and CATGTG E-box (Fig. 4E-F). While either RD26 or BES1 can each bind to E-box (CATGTG), they together can bind to the probe more strongly, which is greatly reduced when the E-box is mutated (Fig. 4E). These results suggest that BES1 and RD26 can bind to E-box synergistically, presumably as heterodimer. Similar results were obtained with probe containing BRRE sites. While either RD26 or BES1 can bind BRRE site separately, RD26 and BES1 synergistically bind to WT but not mutant probe in which BRRE site is mutated (Fig. 4F). These binding results suggest that RD26 and BES1 inhibit each other function likely by forming inactive heterdimers on either E-box or BRRE site.

BR signaling pathway inhibits drought response

Since BES1 and RD26 appear to inhibit each other's activities, we determined if BR pathway affects plant drought response mediated by RD26 and its homologs. Previous data showed that the expression of RD26 was induced by drought [14,15,19]. Drought induces 2503 and represses 2862 genes (combination of 2-day and 3-day drought treatment data) [22]. Analysis of genes affected in RD26OX and Drought-regulated genes revealed that RD26 mediated 38% (963) of drought-induced genes and 45% (1299) of drought-repressed genes (Fig. S5), indicating that RD26 is a major regulator of plant drought tolerance.

If BR signaling indeed inhibits drought response, we expect that loss-of-function BR mutants have increased and gain-of-function mutants have decreased drought tolerance. BR loss-of-function mutant, *bri1-5*, a BR receptor mutant, was exposed to drought stress. After drought stress and

recovery, 50% of *bri1-5* mutants survived compared to 16% for WT. On the other hand, gain-of-function mutant in BR pathway, *bes1-D*, showed less drought tolerance. Only 22% of *bes1-D* mutants survived, but all of WT controls survived in the drought stress experiment (Fig. 5B). To confirm our hypothesis that BR signaling pathway inhibits drought response through repressing RD26, the expression of several RD26-mediated drought-induced or drought-related genes were examined in *bri1-5* mutant and *bes1-D* mutant. Transgenic plants overexpressing *RD26/ANAC072, ANAC019 or ANAC055* could enhance the tolerance to drought stress, suggesting that RD26 and its homologs ANAC019 and ANAC055 are definitely involved in drought response[19]. RT-qPCR results showed that the expression of all the three genes are increased in *bri1-5* mutant and decreased in *bes1-D* mutant. We also examined 5 other genes involved in drought tolerance [23]. All 5 genes are up-regulated in *bri1-5* and down-regulated in *bes1-D*. The gene expression results are consistent with drought stress phenotype of BR mutants, confirming that BR signaling pathway inhibits drought response, likely by repressing the expression of *RD26* and its homologs as well as by forming BES1/RD26 heterodimer, which interferes with drought response gene expression.

Discussion

In this paper, we found that drought responsive transcription factor *RD26* is a target of BES1 and functions to inhibit BR responses. Gene expression studies revealed that RD26 and BES1 act antagonistically in the regulation of a majority of BR response genes. The antagonistic interactions happen at multiple levels: *BES1* and *RD26* inhibit the expression of each other (*BES1* is down-regulated in *RD26OX* by about 20%) and the corresponding proteins appear to form inactive heterodimers on some of the target genes. Our results thus established a molecular link between BR and drought response pathways (Fig S6).

RD26 is induced by drought, promotes drought regulated gene expression and confers drought tolerance when overexpressed[19,20]. Our genetic studies demonstrate that RD26 is a negative regulator of BR pathway as overexpression of *RD26* leads to reduced plant growth and BR response and knockout of RD26 and three of its homologs have increased BR response. Several possibilities can explain the relative weak phenotype of *rd26 anac019 anac055 anac102* mutant. First, additional family members likely function redundantly in the inhibition of BR response. The existence of additional family members supports the hypothesis (Fig S7). Second, other family members may be up-regulated in the *rd26 anac019 anac055 anac102* mutant. We have established a transcriptional network with RD26 and its close homologs and found that many of the homologous genes can regulate each other [24] (Fig S8). Indeed, while some of the homologs are down-regulated, others are

up-regulated in the *rd26 anac019 anac055 anac102* mutant (Table S3). The result suggests that RD26 and its homologs have evolved to be a highly redundant and complex network to confer drought tolerance and to inhibit plant growth during drought stress. The fact that a fewer number of genes affected in *rd26 anac019 anac055 anac102* mutant compared to RD26OX transgenic plants supports this hypothesis.

RD26 is able to both activate and repress gene expression as a large number of genes are either up- or down-regulated in *RD26OX* transgenic plants. Comparison of genes affected in *RD26OX* plants and BR-regulated genes revealed that RD26 function to modulate BR response gene expression in a complex way, i.e. RD26 can either activate or repress both BR-induced and BR-repressed genes. A large number of BR-induced genes (1141 or 43% of BR-induced genes identified in the experiment) are significantly down-regulated in *RD26 OX* (Group I, Fig3A and B). The Group I genes include many genes involved in cell elongation, which explains the reduced growth phenotype of the *RD26OX* plants. Our molecular and biochemical studies suggest that RD26 affects Group I gene expression by binding to the BES1 target site (E-box) and neutralizing BES1's activation function, likely by forming inactive heterodimer, Likewise, 595 (or 25%) BR-repressed genes are up-regulated in RD26 OX, suggesting that BR and RD26 have opposite function on these genes. Indeed, the molecular and biochemical evidence suggest that while BES1 binds to BRRE to repress gene expression, RD26 binds to the same site to neutralize BES1's repression function. A common theme from Group I and Group II gene is that RD26 and BES1 can bind to same site (E-box or BRRE), have opposite transcriptional activities and therefore cancel each other's functions. Our findings reveal a novel mechanism that two signaling pathways converge on the same promoter element and thus differentially regulate two biological processes.

First, the antagonistic interaction between BES1 and RD26 likely ensure that plant growth is reduced when plants are under drought stress, under which RD26 and its homologs are up-regulated to inhibit BR-induced growth thus allowing more resources to deal with drought stress. The reduced growth phenotype of RD26OX plants supports the hypothesis. Second, under normal growth condition when there is no drought stress, BR signaling pathway keeps basal expression of *RD26* and its homologs at low level. The increased drought tolerance and expression of drought-related genes in BR-loss-of-function mutant and decreased drought tolerance in gain-of-function BR mutant (Fig 5) provided strong support for the possibility.

It's worthy noting that RD26 and BES1 don't seem to act antagonistically all the time. For example, 539 BR-induced genes (20%, Group III) are up-regulated, and 823 BR-repressed genes (35%, Group IV) are down-regulated genes in *RD26OX* transgenic plants (Fig S2), indicating that

RD26 and BES1 act in a similar way on these two group of genes. It's possible that RD26 and BES1 target to different promoter elements to achieve the positive interactions between RD26 and BES1. It has been suggested that at least under some conditions, exogenously applied BR can improve plant drought tolerance [25]. It's possible that under certain circumstances, the Group II and Group IV genes play dominant roles over Group I and Group II genes, which can potentially make BR to activate some drought-induced genes and repress BR-repressed genes and thus promote drought tolerance. Full understanding the interaction between RD26 and BES1 on Group III and IV genes is needed to test the possibilities.

Materials and Methods

Plant materials and growth condition

T-DNA insertion mutants, *rd26* (AT4G27410, SALK_063576), *anac019* (AT1G52890, SALK_096295), *anac055* (AT3G15500, SALK_014331), and *anac102* (AT5G63790, SALK_030702) were obtained from ABRC (Arabidopsis Biological Resource Center). All plants were grown on 1/2MS plates and/or in soil under long day conditions (16h light/ 8h dark) at 22 ℃. BRZ response experiments were carried out as previous described [26].

Plasmid constructs

For MYC-tagged transgenic plants, RD26 genomic sequence including its 5' UTR was cloned from wild type and fused with MYC tag into pZP211 vector [27]. For recombinant protein purification, *RD26* and *BES1* coding region were cloned into pETMALc-H vector, respectively.

Transgenic plants

The construct of RD26-MYC driven by 35S promoter was transformed into Agrobacterium tumefaciens (stain GV3101) which were used to transform plants by the floral dip method [28]. Transgenic lines were selected on 1/2 MS medium plus 60ug/ml gentamycin. Transgene expression was analyzed by western blotting.

Gene expression analysis and Chromatin Immunoprecipitation (ChIP)

For RD26, 4G00360 and AT4G18010 gene expression, total RNA was extracted and purified from 2-week-old plants of different genotypes using RNeasy Mini Kit (Qiagen). Mx4000 multiplex quantitative PCR system (Stratagene) and SYBR GREEN PCR Master Mix (Applied Biosystems) were used in quantitative real-time PCR analysis. For transient expression, AT4G00360

promoer(1552 inculding 5'UTR) and AT4G18010 promoter (1515bp including 5'UTR) were cloned and used to drive luciferase reporter gene expression. BES1 coding region driven by CaMV 35S promoter were cloned into pZP211 vector, while RD26-MYC construct used in transgenic plant generation was also used in transient experiment. Tobacco leaf transient assay [29] was used to examine the effect of RD26 and BES1 on reporter gene expression either in individual protein or in combination of BES1 and RD26. Equal amount of Agrobacterium cells(measured by $O.D_{600}$, adjusted to the same with vector-containing strain) were injected to the leaves of tobacco. The activities of luciferase were measured from total protein extracts from triplicate sample and measured using Berthold Centro LB960 luminometer with luciferase assay system followed by instruction (Promega). The relative levels of luciferase were normalized by the total protein from each sample.

For global gene expression, total RNA were extracted and purified from 4-week-old plants of different genotypes using RNeasy Mini Kit (Qiagen). RNA samples were analyzed by RNA-seq method in facility of Iowa state university. Raw RNA-seq reads were subjected to quality checking and trimming and then aligned to the Arabidopsis reference genome (TAIR10) using Genomic Short-read Nucleotide Alignment Program (GSNAP) [30]. The alignment coordinates of uniquely aligned reads for each sample were used to independently calculate the read depth of each annotated gene. These values were compared between WT and mutant samples treated with or without BR using the Poisson generalized linear model (GLM) [31] and controlling the false discovery rate (FDR) [32]. For heatmap plotting, average reads per million (RPM) for each gene were used and RPM data were scaled to the same level between genes.

Chromatin immunoprecipitation was performed as previously described [8]. BES1 antibody was used to precipitate chromatin from WT plant. BES1 enrichment was examined at BRRE site (-851) and control site (-115), respectively.

EMSA experiment

EMS experiments were carried out as described previously [10]. After synthesize and annealed, Oligonucleotide probes were labeled with P32-γ-ATP by using T4 nucleotide kinase. About 0.2ng probe and indicated amount of proteins purified from E. coli were reacted in 20μl binding buffer (25 mM HEPES-KOH [pH 8.0], 1 mM DTT, 50 mM KCL, and 10% glycerol). After 40min incubation on ice, the reactions were resolved by 5% native polyacrylamide gels with 1×TGE buffer (6.6 g/l Tris, 28.6g/l glycine, 0.78 g/l EDTA [pH 8.7]).

Drought stress tolerance of BR signaling mutants

Drought stress tolerance experiments were carried out as described previously [19] with minor modification: different genotype plants were grown on 1/2 MS medium in Petri dishes for 2 weeks, then transferred to soil, and grown for one more week in growth chamber (22℃, 60% relative humidity, long day conditions) before exposure to drought stress. Drought stress was imposed by withholding water until the lethal effect of dehydration was observed on wild type control or bes1D plants. The number of plants, which survived and continued to grow were counted, was counted after rewatering for 7 d.

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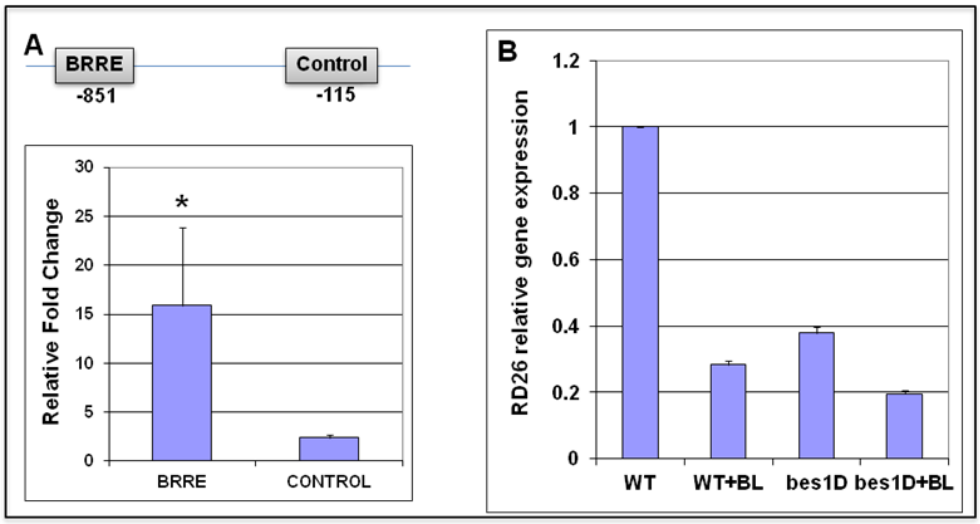

Fig. 1. RD26 is direct target of BES1 and is repressed by BL and/or BES1.
(A) BES1 targets the BRRE site on RD26 promoter. ChIP was performed with anti-BES1 antibody in WT seedlings. The bindings of BES1 at BRRE site (-851) and control site (-115) of the *RD26* gene promoter were examined by qPCR. The 5s rRNA was used as internal control.
(B) The expression of RD26 was examined by quantitative RT-PCR in 2-week-old WT and bes1-D seedlings with or without 1,000 nM BL treatment for 2.5 hr.

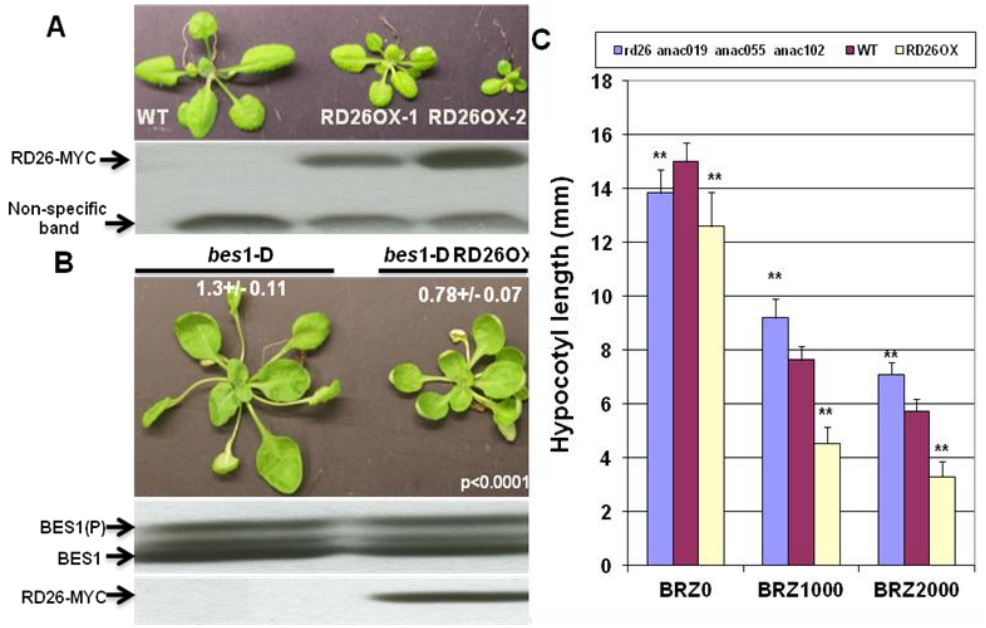

Fig. 2. RD26 functions as a negative regulator in BR signaling pathway.
(A) The phenotype of 4-week-old RD26 overexpression plants. The stunted growth phenotype of RD26OX plant (upper) is correlated with the protein level of RD26 trangene (lower panel) examined by Western blot.
(B) RD26OX suppressed *bes1-D* phenotype. 4-week-old plants of *bes1-D* and *bes1-D RD26OX* double mutants are shown. The average petiole length and standard deviations are indicated (n=10). BES1 protein levles and forms (phosphorylated and unphosphorylated) did not change in *bes1-D RD26OX* double mutant in Western blot test (middle panel) and RD26-MYC gene only expressed in *bes1-D RD26OX* double mutant (lower panel).
(C) The hypocotyl lengths of 5-day-old dark-grown seedlings in the absence or presence of different concentration (0uM, 1uM and 2uM) BRZ. Averages and standard deviations were calculated from 15-20 seedlings. The difference was significant as analyzed by Student's t-Test (**<0.01).

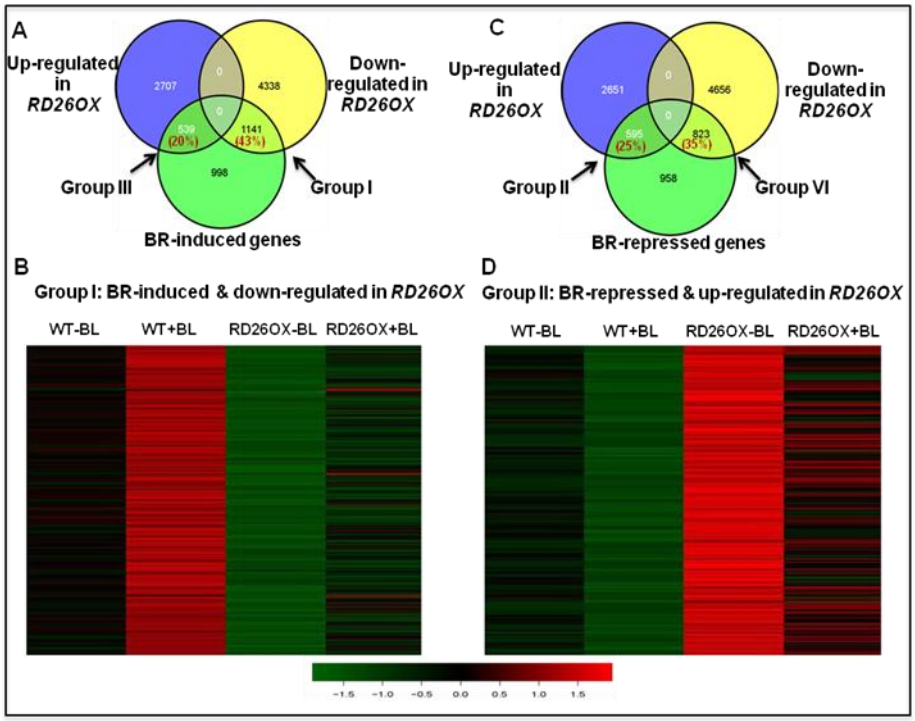

Fig. 3. RD26 negatively regulatesd the expression of some BR-responsive genes.
(A) Venn diagram shows the overlap genes between BR-induced genes and RD26OX-regualted genes.
(B) Clustering analysis of Group I genes. 1141 BR-induced genes are down-regulated in *RD26OX* plants.
(C) Venn diagram shows the overlap between BR-repressed genes and genes affected in *RD26OX*.
(D) Clustering analysis of Group II genes. 595 BR-repressed genes are up-regulated in *RD26OX* plant.

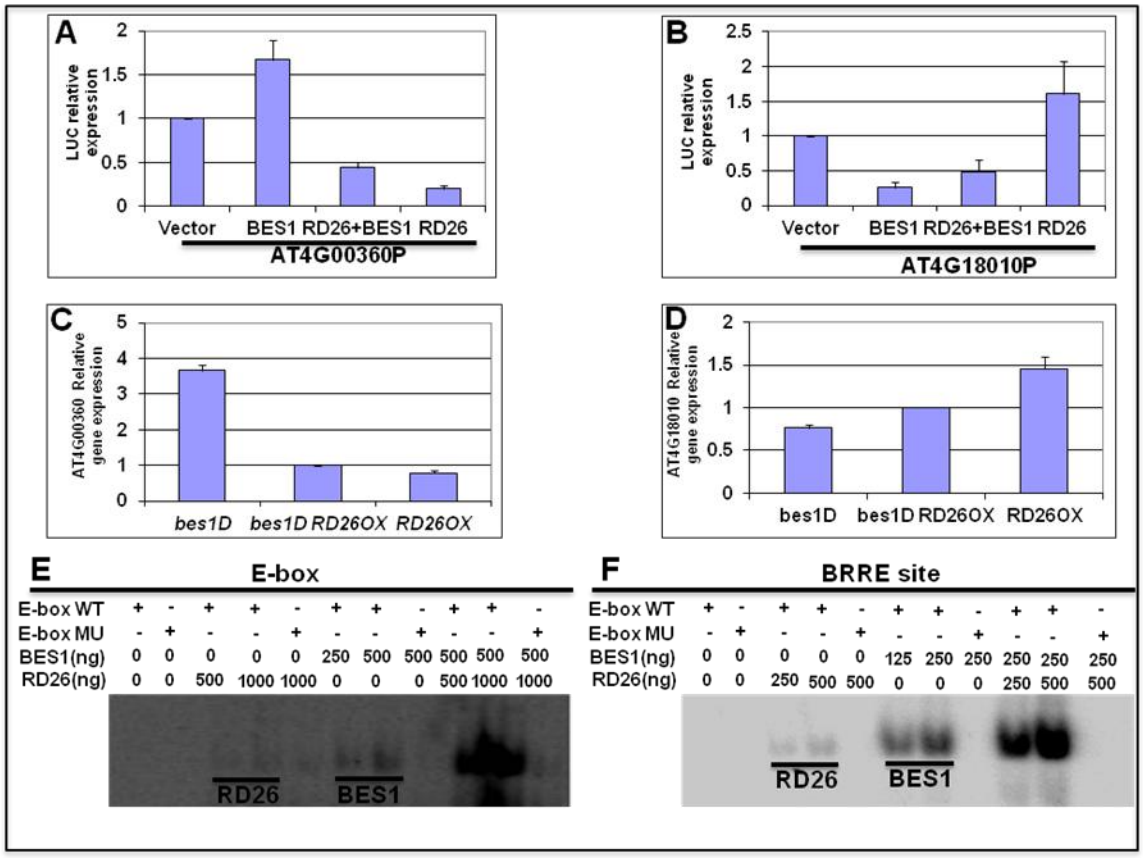

Fig.4 BES1 and RD26 bind to E-box and BRRE site simultaneously and block each other's transcriptional activities.

(A,B) Transient gene expression assays were performed in tobacco leaves with *At4g00360-LUC* and *At4g18010-LUC* reporter genes co-transfected with BES1 and/or RD26 via Agrobacterium. The relative expression levels were normalized with total protein. The average and standard deviations were from three biological repeats.

(C,D) The expression of At4g00360 and At4g18010 were examined in *bes1-D*, *bes1-D RD26OX* double mutant and *RD26OX* mutant, by qPCR.

(E,F) BES1 or RD26 individually binds to E-box and BRRE site, respectively, but displayed strong synergistic binding abilities on both of these two sites. WT and mutant (MU) probes containing E-box or BRRE were labeled with 32P-ATP and used in binding with indicated amount of recombinant proteins.

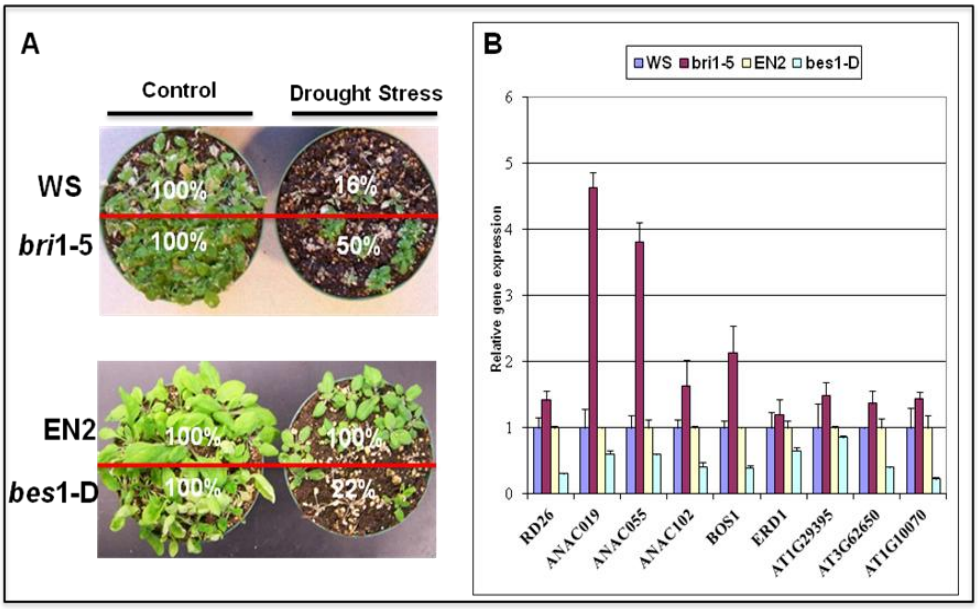

Fig. 5. BR signaling pathway inhibits drought response.
(A) Survival rates of WS (wild-type), *bri1-5* mutant, *EN2* (wild-type) and *bes1-D* mutant plants after withholding water for 14-20 d (drought stress) and rehydration for 7 d (Rehydration). The survival rate is indicated in the picture. This experiment was repeated three times with similar results.
(B) Gene expression of drought-induced genes was examined by RT-qPCR.

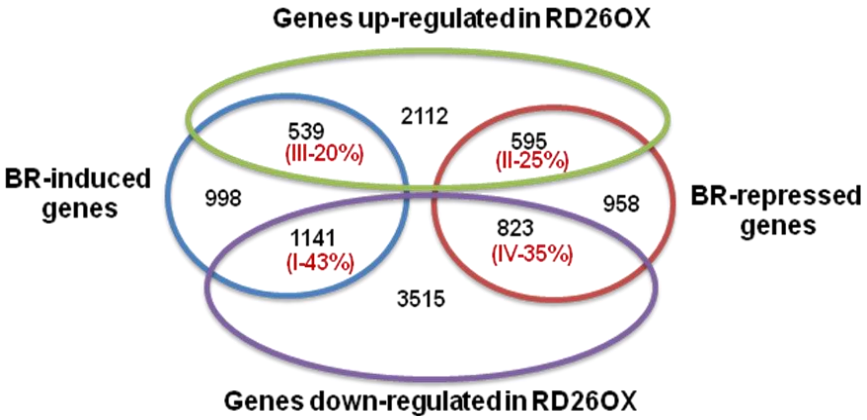

Fig. S1. Overlap between BR-regulated genes and genes affected in RD26OX transgenic plants. Overlapped genes were divided into four groups: (I) BR-induced and down-regulated in *RD26OX* mutant; (II) BR-repressed and up-regulated in *RD26OX* mutant; (III) BR-induced and up-regulated in *RD26OX* mutant; (IV) BR-repressed and down-regulated in *RD26OX* mutant.

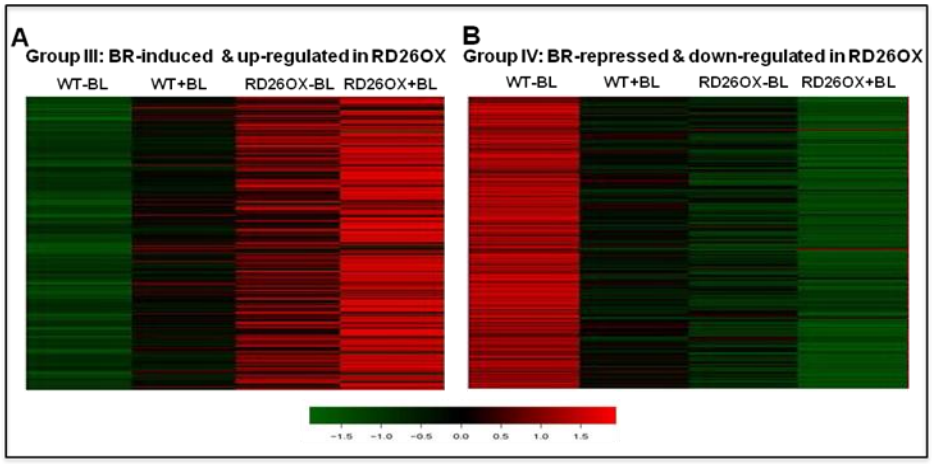

Fig. S2. Clustering analysis of Group III and Group IV genes.
(A) Group III genes expression in WT with or without BL treatment (lane 1 and lane 2) and in *RD26OX* transgenic plant with or without BL treatment (lane 3 and lane 4).
(B) Group IV genes expression in WT with or without BL treatment (lane1 and lane 2) and in *RD26OX* transgenic plant with or without BL treatment (lane 3 and lane 4).

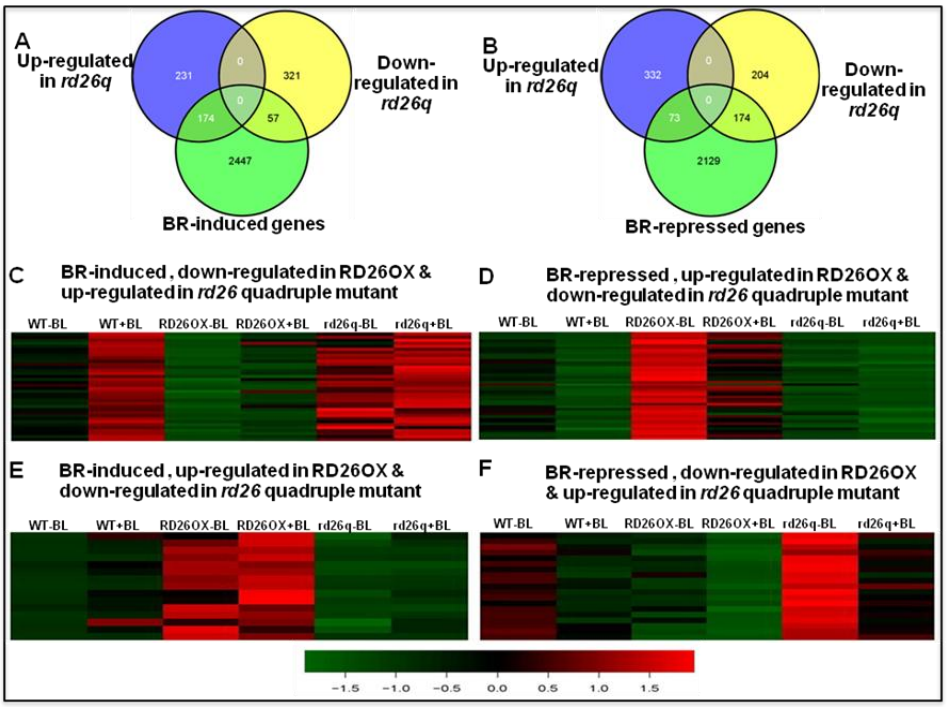

Fig. S3. Overlap between BR-regulated genes and genes affected in *rd26 anac019 anac055 anac102* quadruple mutant.

(A, B) Venn diagram shows the overlap genes between BR-regulated genes and *rd26 anac019 anac055 anac102* quadruple mutant-regulated genes.

(C, D, E, F) Clustering analysis of BR-regulated genes affected in opposite ways in RD26OX and *rd26 anac019 anac055 anac102* quadruple mutant.

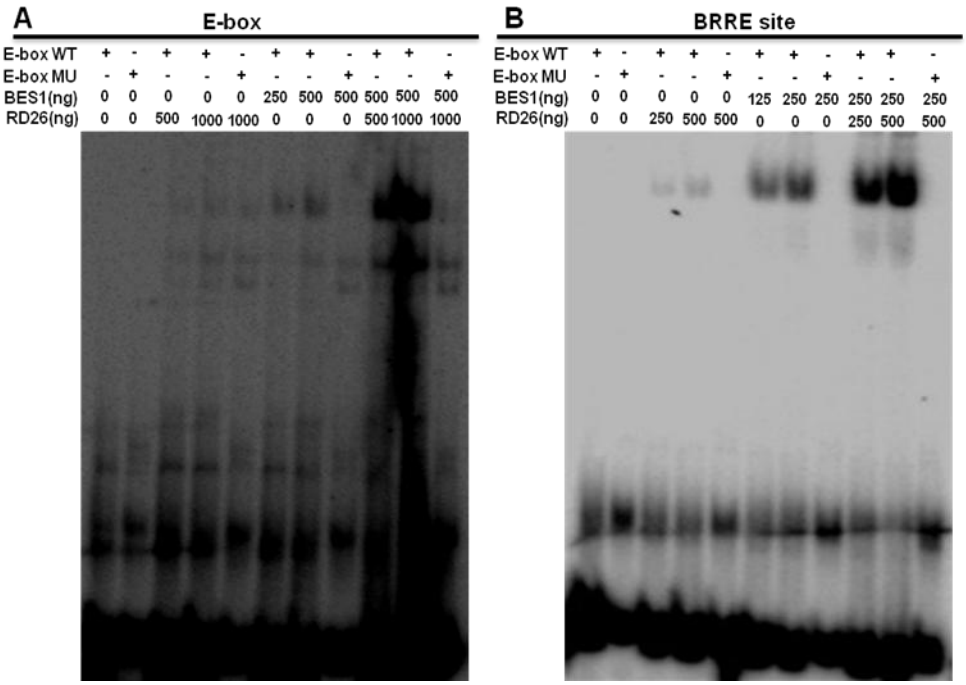

Fig. S4. BES1 and RD26 individually binds to E-box and BRRE site, respectively, but displayed strong synergistic binding abilities on both of these two sites. The full images for Fig.4 E-F are shown.

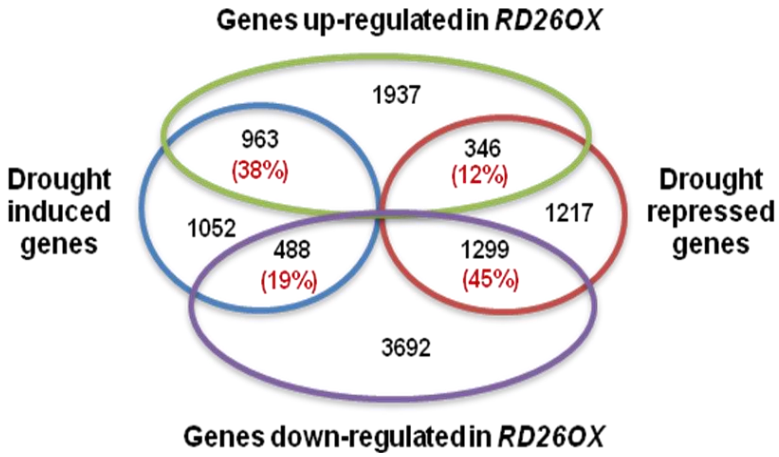

Fig. S5. RD26 mediates large portions of drought-responsive genes expression.

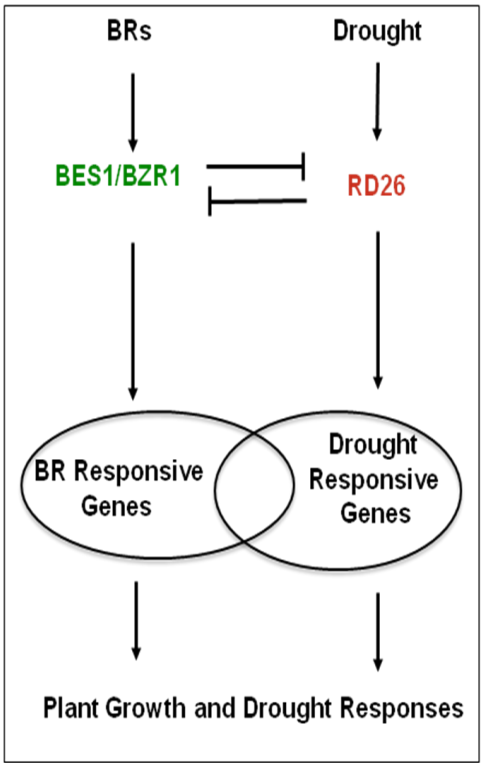

Fig. S6. A model of crosstalk between BR and drought response pathways.
Drought stress induces the expression of RD26 to mediate the response of plant to drought. Upon the increase expression, RD26 not only inhibits the expression of BES1 on mRNA level, but also binds to E-box and BRRE site to inhibit BES1's functions in mediating BR-regulated gene expression (Group I and II genes), which results in the inhibition of BR regulated growth. On the other hand, BR signaling represses the expression of *RD26* through BES1 and also directly inhibits the expression of other drought-related genes to inhibit drought response.

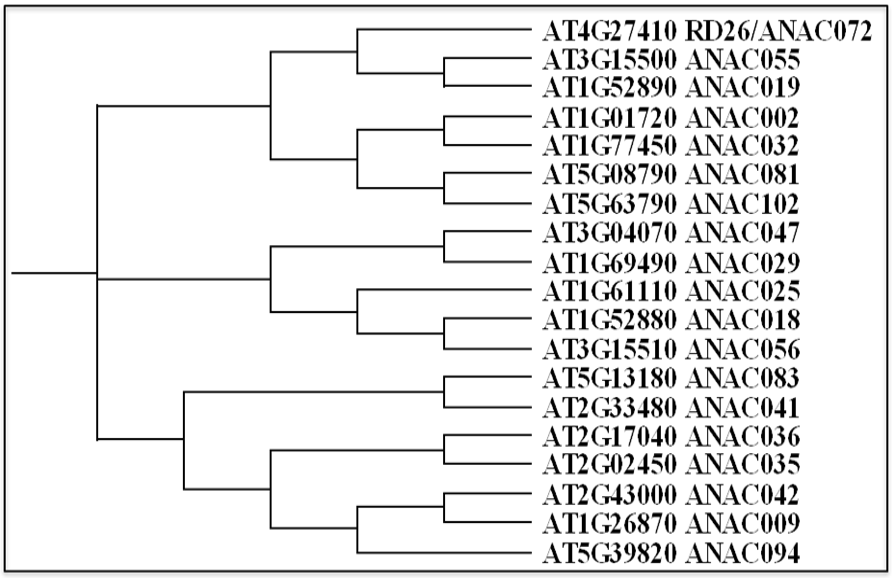

Fig. S7. Phylogenetic tree of RD26 and its homologs. The protein sequence of all family members was downloaded from NCBI and put into Cluster software to generate phylogenetic tree.

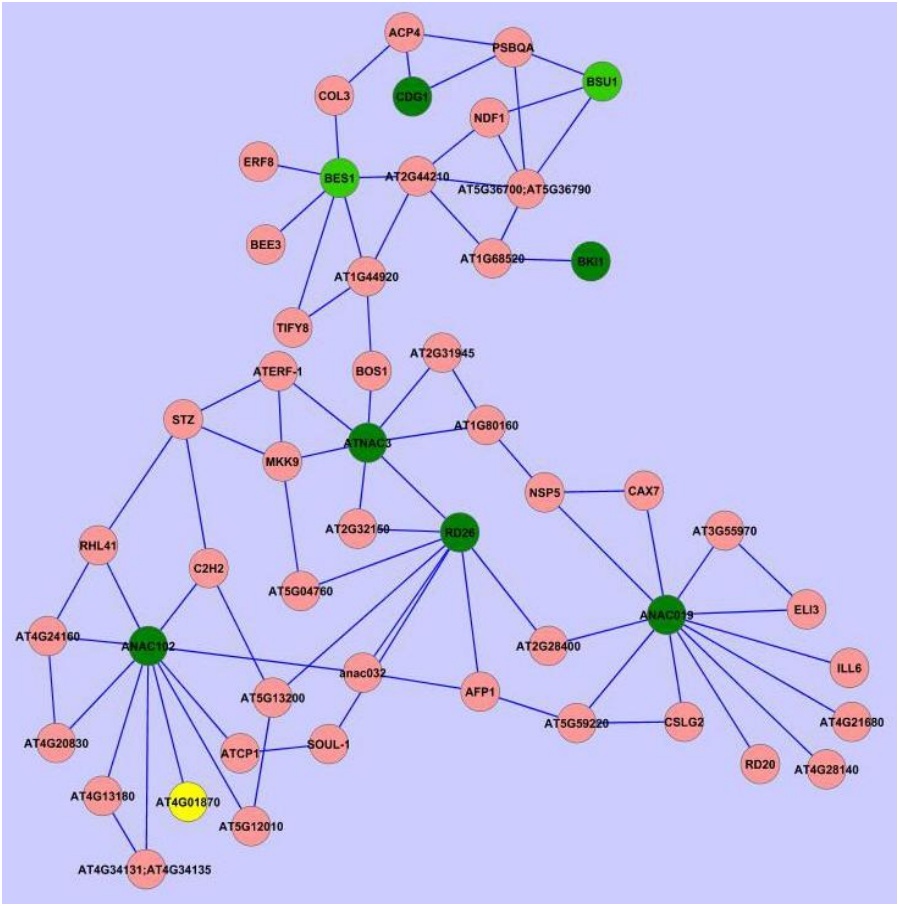

Fig. S8. GRN (Gene Regulatory Network) between RD26 and its homologs-regulated genes and BR-regulated genes. The GRN was inferred by ARACNe(Algorithm for the Reconstruction of Accurate Cellular Networks) algorithm.

Table S1. The expression of 35 cell elongation genes in WT and RD26OX plant with or without BL treatment.

		Annotation	WT-BL	WT+BL	RD26OX-BL	RD26OX+BL
BR-induced(5)	AT4G30290	XTHs	5.47	31.16	15.00325	129.74325
	AT3G27400	Pectinlyase	11.84	19.26	11.7687	16.33625
	AT4G13710	Pectinlyase	5.172	7.65	5.5543	6.72335
	AT3G45970	Expasin	25.32235	36.3283	23.2261	36.0245
	AT3G53190	Pectinlyase	37.5	48.17	36.55495	46.1981
BR-induced&RD26OX-downregulated(30)	AT1G65310	XTHs	2.014	6.622	0.10985	0.52035
	AT3G44990	XTHs	11.21	15.88	1.0057	2.2879
	AT1G10550	XTHs	1.76	4.372	0.2122	0.20285
	AT2G20750	Expasin	1.238	2.324	0.1728	0.74725
	AT3G15370	Expasin	0.444	1.226	0.08635	0
	AT4G37800	XTHs	102.4	147.2	20.13065	34.96855
	AT4G28250	Expasin	32.28	41.13	7.7075	9.59765
	AT4G38400	Expasin	8.628	18.93	2.24655	5.59605
	AT3G29030	Expasin	82.37	116.1	24.014	40.46875
	AT1G67750	Pectinlyase	28.91	66.07	11.71375	39.3672
	AT5G48900	Pectinlyase	30.69	45.78	13.28355	26.71975
	AT3G07010	Pectinlyase	48.2	83.31	21.18045	46.1361
	AT3G45960	Expasin	0.997	2.554	0.43995	1.36465
	AT1G11545	XTHs	24.74	49.98	12.2086	28.7697
	AT4G03210	XTHs	93.51	119.8	46.34625	66.76695
	AT4G25810	XTHs	13.45	16.79	6.82485	8.99955
	AT3G23730	XTHs	36.09	46.43	19.68215	25.1925
	AT2G37640	Expasin	48.85	83.75	26.80395	74.36785
	AT2G06850	XTHs	189.7	379	105.39005	192.97225
	AT5G63180	Pectinlyase	105.2	125.8	60.522	99.66275
	AT4G30280	XTHs	25.52	47.21	15.7012	26.08125
	AT5G57560	XTHs	81.32	164.8	50.47035	205.32555
	AT1G04680	Pectinlyase	122.5	222.2	79.00165	136.75605
	AT2G40610	Expasin	25.69	61.91	17.0787	74.2948
	AT3G09540	Pectinlyase	9.846	16.06	7.0148	8.6908
	AT4G24780	Pectinlyase	121.7	199.4	97.6874	169.80625
	AT3G55500	Expasin	7.024	13.17	5.7734	12.037
	AT2G03090	Expasin	18.73	30.39	16.458	28.4276
	AT1G20190	Expasin	19.65	36.18	17.2835	25.81695
	AT1G69530	Expasin	391.2	465.5	356.1768	677.0837

Table S2. Expression of genes in Fig S3 in WT, *RD26OX* and *rd26 anac019 anac055 anac102* quadruple mutant with or without BL treatment.

	WT-BL	**WT+BL**	***RD26*OX-BL**	***RD26*OX+BL**	***rd26q*-BL**	***rd26q*+BL**
GroupA (36)						
AT1G13650	23.09725	28.85945	14.15135	17.3653	38.6383	27.43585
AT1G14250	166.6151	194.00215	65.18615	72.79095	208.9811	205.36595
AT1G30360	255.0864	350.8197	168.55605	196.3652	308.2065	352.6094
AT1G35230	20.56295	42.0358	16.01865	17.78275	27.57505	44.12355
AT1G56510	35.76665	42.05585	25.15355	20.52375	46.95615	47.6455
AT1G60950	716.9099	923.94735	492.71435	571.84265	939.8871	840.47925
AT1G61340	15.1847	28.085	9.92035	23.81795	26.8278	35.4707
AT1G65490	45.2857	80.4395	14.80125	12.9503	57.22575	77.75055
AT1G77450	16.29695	26.2864	12.49175	12.7667	28.71705	32.2811
AT2G17880	10.96815	17.458	6.31685	9.20825	18.1397	18.56445
AT2G20880	43.683	119.07375	28.01465	42.75135	73.7454	129.4358
AT2G21790	55.30795	77.48275	40.2374	72.1414	68.67635	76.38615
AT2G37170	213.4747	422.69585	133.53435	258.4417	290.2612	549.48565
AT3G19720	84.55195	115.8625	47.55195	64.01595	100.825	125.95825
AT3G22310	36.092	49.54185	28.3818	41.82695	44.86805	48.39895
AT3G24518	18.13405	28.71695	6.1269	11.4038	29.4009	32.9105
AT3G24520	18.1711	29.7442	6.0016	11.3038	29.0404	32.915
AT3G28740	10.29175	20.6498	4.39015	4.54595	29.5511	24.3906
AT3G48460	27.66305	47.25865	21.40755	26.74605	37.7181	46.99795
AT4G00360	305.3788	515.2095	193.7143	265.9568	375.67105	497.5998
AT4G14365	125.7212	186.36405	66.68225	46.2431	157.2402	255.1737
AT4G17340	307.0009	689.30175	134.46665	303.58155	392.8377	745.77495
AT4G27520	210.2423	269.3349	166.8826	192.03385	269.87825	265.11655
AT4G29740	24.94345	42.4492	8.71045	10.4461	34.94745	47.7664
AT4G38770	323.1254	428.70975	161.2939	227.7022	401.03265	375.112
AT4G39330	201.8816	422.37535	170.72275	389.0989	245.23865	409.3909
AT5G02490	82.56045	126.25165	29.895	34.2833	106.4945	138.4249
AT5G15970	126.3341	174.84505	83.1332	123.4281	219.8792	218.2869
AT5G17300	44.1949	60.6481	16.9134	25.5299	62.93165	58.8049
AT5G22390	42.2034	52.59005	7.66755	8.6745	56.1679	53.265
AT5G28030	14.3147	24.07455	9.27675	13.42275	22.0298	27.45515
AT5G37260	30.58275	46.49445	21.15535	25.88185	39.44695	51.2774
AT5G48490	5.52735	9.0351	1.35125	1.581	13.9246	6.99835
AT5G49630	67.4041	103.6471	29.49155	33.7296	88.21985	92.6924
AT5G51720	19.4384	25.90565	4.7139	5.2084	32.44795	23.78535

Table S2 continued

AT5G52310	177.486	353.91265	69.1172	106.89715	370.9196	432.148
Group B (44)						
AT1G02850	14.145	10.8913	110.4668	149.376	8.77705	5.8264
AT1G12780	103.8864	77.3277	196.59325	189.3167	85.27235	78.004
AT1G14520	7.3184	4.29425	12.6273	7.66005	3.71705	3.9879
AT1G22400	62.97735	41.5616	220.23395	171.044	42.6289	35.5143
AT1G44100	51.9614	45.3014	72.7958	63.93055	36.27805	42.4114
AT1G53570	61.5957	53.99855	110.08945	115.15585	47.4416	49.54845
AT1G59700	32.22065	27.3441	83.6537	83.2838	22.93745	19.71385
AT1G79110	33.69215	27.18385	43.05995	29.5175	22.6023	18.814
AT2G23170	56.3798	21.47795	257.4839	90.9052	37.88165	17.6107
AT2G30140	48.8382	40.6907	210.41155	156.83545	36.68685	28.94345
AT2G38240	40.7815	26.6069	71.4781	38.20305	30.7938	27.96135
AT2G39970	66.71595	52.8367	103.87275	98.9038	52.52565	56.6648
AT3G01420	10.68895	7.6144	58.0988	40.07055	1.44215	0.74885
AT3G03470	55.14885	43.24495	250.3425	211.45315	38.66425	32.845
AT3G04000	10.58425	6.10725	36.9394	27.1302	6.1663	4.7741
AT3G09260	70.1968	34.53795	161.8094	96.1616	56.69075	41.1186
AT3G21690	103.4795	89.1742	150.3803	133.7993	80.7841	71.85265
AT3G47420	68.80045	52.8204	86.9726	65.17425	43.8483	37.8941
AT3G51860	18.9786	12.99845	31.10935	19.70405	11.8099	7.97425
AT3G56310	87.98905	67.74525	120.47375	100.13165	72.7904	72.09615
AT3G60130	44.1692	29.5497	139.9902	118.35935	30.14805	26.47755
AT3G60690	33.69535	16.81835	63.34695	31.80775	24.87675	13.4197
AT3G63380	31.35325	10.4707	47.32835	10.404	21.10915	9.442
AT4G12290	161.8283	80.8355	358.6627	252.77725	120.86775	66.48715
AT4G15530	119.1553	100.71495	336.26435	297.91005	99.0729	90.05545
AT4G16260	40.70915	32.09765	81.8287	40.6722	26.8778	25.80135
AT4G18010	54.6719	30.20245	90.167	60.8832	44.4949	28.32655
AT4G21680	19.9087	8.31335	32.0643	20.89095	13.377	4.8459
AT4G30270	136.8270	52.72215	594.0671	223.4223	86.6755	41.24805
AT4G37520	90.75255	54.0323	328.0711	245.64985	66.38665	47.59505
AT4G37530	35.91655	23.48295	51.70825	41.79365	24.44135	23.6358
AT4G37980	62.4763	28.9009	536.65365	286.39525	47.35405	21.3818
AT5G07010	32.7024	10.4309	175.39105	107.6497	18.49955	6.6066
AT5G13180	65.0979	53.66345	420.52295	313.1494	49.6042	37.31735

Table S2 continued

AT5G13330	10.3486	5.99875	150.29035	96.9258	6.89975	3.9281
AT5G13550	58.62975	31.94225	106.31835	68.6888	44.4693	36.537
AT5G13740	91.74205	19.69885	116.78865	38.8111	68.16355	29.3696
AT5G17860	33.22175	17.78805	102.08685	57.7311	22.96175	12.2597
AT5G20250	362.6615	125.0508	532.96465	296.3969	298.0346	179.52905
AT5G47370	52.7844	22.5558	104.5691	48.0112	35.64325	23.07555
AT5G55930	92.04095	56.97515	197.2187	83.29145	70.5647	57.7581
AT5G64370	68.49965	49.00855	84.36955	63.5219	54.98735	50.09985
AT5G64570	278.1016	167.0494	532.53105	316.38745	203.4692	131.11295
AT5G64572	180.0868	108.552	341.61885	209.526	133.5989	87.482
Group C (14)						
AT1G13360	54.39665	67.23655	105.3281	104.59515	41.2874	46.4246
AT1G47510	9.06995	15.5016	104.12305	118.8581	5.2091	8.2627
AT1G51780	12.3373	25.97625	40.42865	91.87355	4.56265	9.01725
AT1G71880	274.5567	324.4686	501.65005	378.99775	217.5256	287.6734
AT1G78230	29.47715	42.38355	45.997	78.1543	21.09705	32.00055
AT2G02990	13.3936	21.08755	107.9196	131.947	6.6388	10.67375
AT2G17500	8.8797	14.303	45.82445	40.71025	5.2835	9.3718
AT2G29350	62.9913	78.4611	1178.66505	887.43195	43.03995	43.65275
AT3G11480	10.033	13.84995	246.12295	308.22055	2.08885	4.62915
AT3G48520	16.11485	21.53675	53.29795	40.4524	7.38445	5.92645
AT3G51670	86.36045	99.862	159.4063	194.82745	67.1469	85.30665
AT4G03400	104.6444	148.53325	136.2703	187.73345	81.21965	117.6537
AT4G37990	43.2109	76.9383	309.2599	313.9178	18.2379	17.9409
AT5G41400	18.94645	31.02795	26.8561	30.69155	12.09695	20.4195
Group D (19)						
AT1G01470	140.0829	102.6991	107.07845	82.39745	190.11995	107.9534
AT1G13260	72.16335	42.1623	40.28545	23.4958	105.1514	54.04115
AT1G15125	249.8335	190.71875	66.4088	27.5032	307.2472	198.36895
AT1G24145	21.43295	13.84815	16.91225	5.4125	28.91725	18.0799
AT1G25560	130.8327	114.23485	104.0547	76.854	194.89915	148.9607
AT1G68840	70.1039	41.2577	48.00675	29.14445	116.24085	70.54965
AT1G69490	45.1965	37.65655	27.00765	17.04485	64.75965	54.24485
AT1G71030	103.8781	45.89525	92.56005	34.0997	147.71955	57.91165
AT2G15080	120.195	102.99435	39.15855	23.6583	142.3865	116.08735

Table S2 continued

AT2G26190	63.1404	47.8205	52.31795	28.26215	96.7473	65.54915
AT2G34430	1086.803	804.54285	249.3455	233.7147	1403.9932	1113.5056
AT2G34620	44.8309	35.31575	36.9736	31.4528	57.6234	39.42315
AT3G27690	367.7227	304.4575	130.56095	108.60675	490.56435	315.3156
AT3G61060	28.50005	20.32545	20.29035	14.3986	50.05195	31.93795
AT4G26530	147.062	89.0123	49.5151	20.9816	174.4479	90.65965
AT5G01900	6.55635	3.76885	0.9033	0.2327	9.90845	5.44675
AT5G14120	350.9463	229.904	287.99695	184.21185	453.3459	294.5224
AT5G19190	54.08205	40.35595	31.93135	26.2374	70.428	44.3752
AT5G59080	39.1931	27.07125	24.80745	17.056	52.1026	34.26215

Table S3. The expression levels of other RD26 homologs in WT, *rd26 anac019 anac055 anac102* quadruple mutant and *RD26OX* transgenic plants with and without BL treatment.

Gene	WT-BL	WT+BL	rd26quadruple-BL	rd26quadruple+BL	RD26OX-BL	RD26+BL
AT1G01720	34.0317	41.5302	57.19825	46.8501	41.59855	37.15
AT1G77450	16.29695	26.2864	28.71705	32.2811	12.49175	12.7667
AT5G08790	133.9362	40.94885	141.45105	52.9303	97.1285	31.48555
AT3G04070	8.9497	4.89535	8.97595	3.66655	29.16145	14.83185
AT1G69490	45.1965	37.65655	64.75965	54.24485	27.00765	17.04485
AT1G61110	0.2562	0.29475	0.21135	0.26605	0.49485	0.4742
AT1G52880	14.62855	14.0124	11.77325	12.31055	8.0285	7.3928
AT3G15510	22.51135	17.09175	24.6287	19.5151	0.7151	0.8665
AT5G13180	65.0979	53.66345	49.6042	37.31735	420.52295	313.1494
AT2G33480	28.3402	29.86735	17.93935	20.0173	54.31555	62.99565
AT2G17040	32.24455	56.0541	35.7052	67.13055	23.45155	26.61095
AT2G02450	13.29315	21.687	21.246	21.5807	13.92885	13.42275
AT2G43000	4.83695	5.61835	4.37605	4.40935	11.854	8.1225
AT5G39820	0	0.10505	0.0746	0.0897	0.0549	0
AT1G26870	0	0	0	0	0	0

Table S4 The primers used in this study

RD26LP	AGTGATCGAGTGCTTCAGGAC	GENOTYPING
RD26RP	ACTCGTGCATAATCCAGTTGG	
ANAC019LP	TCAATGAACTCAAGGGATTGC	
ANAC019RP	ATGCGGTTTGGGTTAGAAAAC	
ANAC055LP	TAAACGATGAGCGATAGCGAG	
ANAC055RP	AAAGGAACCAAAACCAATTGG	
ANAC102LP	TAATCGTATGACCCGACTTGG	
ANAC102RP	TCTATCTTTGCCGGAGATGTG	
RD26NBAMH1	CGCGGATCCATGGGTGTTAGAGAGAAAGATCCGTTAG	PROTEIN EXPRESSION
RD26CSAC1	GCCGAGCTCTCATTGCCTAAACTCGAATGTTTGACCCG	
gRD26NASP718	CGCGGTACCATCTCTCTGTGAACAAGAATTCTCCACGTTCAC	TRANSGENIC PLANT
gRD26CSAL1	CGCGTCGACTTGCCTAAACTCGAATGTTTGACCCGAAACACC	
RD26CHIP1F	TCCCAACACGTGTACAATTCA	ChIP ASSAY
RD26CHIP1R	AAAACAAATGGCACTAAGACGTT	
RD26CHIPCF	TTGTCCAAAAGATCGACGAA	
RD26CHIPCR	CTTCGATTCCTCAGCAACCA	
RD26RTF	GGCACTAAAACCAACTGGATTATGCACGAG	GENE EXPRESSION
RD26RTR	GGAGTAACAGCTTGTCTCTGAGATCCAG	
ANACO19RTF	GCTCCTAAAGGTACTAAAACCAATTGGATC	
ANAC019RTR	CCATTATCGTAAACTTGTTTTTGTGCAC	
ANAC055RTF	TTGGATTATGCATGAGTACCGTCTCATCG	
ANAC055RTR	CCATTGTTGCTGTATTCACGACCACTCG	
ANAC102RTF	CGAGTATCGTCTCGCTAATGTCGATCGATC	
ANAC102RTR	ACGTACTCATCTTTTCCGTCGGTTTCTCAG	
BOS1RTF	TTCATGAATTACGACTACAACAACAA	
BOS1RTR	AGAACCAGAATTCTTCATCAGTTTCT	
ERD1RTF	ATTGATCATAATGACCTCTAATGTCG	
ERD1RTR	ATCTTCAACAATCTCTGTGACAGTTC	
AT1G29395RTF	CAGAAACCATTCCTCTCTCTTAAACT	
AT1G29395RTR	ATACACCATACTCTCCCTTAATCCAG	
AT3G62650RTF	GGAGAGGATACGAGAAGCTTGAT	
AT3G62650RTR	CACCATCAGTATCGACTTGTAAATCT	
AT1G10070RTF	GTCTATGCATCTCCAGTTGGTAACTA	
AT1G10070RTR	GCCTTCTCTACTACCTGATAACCTTG	
4G18010FBH1	CGCGGATCCTTGTGAATCAAACTAATTTATTTAAGTAGC	TRANSIENT EXPRESSION
4G18010RHD3	CGCAAGCTTCTTCTTAGATCTCAGAAAAAGATTTTGTTTC	
4G00360FBH1	CGCGGATCCTCTCTTGATACAATGCATATAGAAACTGAC	
4G00360RHD3	CGCAAGCTTATCAATGAATATGAAATGATACTAAAATGG	
4G18010BDF	AAACCGAAAACACGTGTGAGAAAGAAGAAA	EMSA EXPERIMENT
4G18010BDR	TTTCTTCTTTCTCACACGTGTTTTCGGTTT	
4G18010BDMF	AAACCGAAAACATTTTTTAGAAAGAAGAAA	
4G18010BDMR	TTTCTTCTTTCTAAAAAATGTTTTCGGTTT	
4G00360BDF	ATTACCTAACTATA**CATGTG**TAATGTGTTC	
4G00360BDR	GAACACATTA**CACATG**TATAGTTAGGTAAT	
4G00360BDMF	ATTACCTAACTATAAAAAAATAATGTGTTC	
4G00360BDMR	GAACACATTATTTTTTTATAGTTAGGTAAT	

CHAPTER IV
CONCLUSION AND PROSPECTIVE

In this thesis, two transcription factors with novel functions in BR signaling pathway have been identified and characterized. Major components from receptor of BR to downstream targets in BR signaling pathway have been well studied in the past few years. Gene expression analysis indicated that BRs could activate and repress about equal number of genes expression and BES1/BZR1 play a major role in mediating the expression of BR-targets. Previous data showed that BES1 could bind to E-box (CANNTG) and BRRE (CGTGT/CG) promoter elements, to activate and repress target genes, respectively. On E-box, BES1 could activate the expression of BR-induced targets by itself or in cooperation with its coactivators, such as transcription factors (BIM1, MYB30), chromatin-modification enzymes (REF6/ELF6 histone demethylase) and transcription elongation factor (IWS1). However, how BES1 represses the gene expression at BRRE site is not well understood.. The proposed model is that BES1 recruits one or more corepressors to repress the expression of BR-repressed targets. In chapter I of this thesis, the first corepressor of BES1, MYBL2, was identified from ChIP-chip assay and microarray data. Our genetic evidences that *mybl2* mutant could enhance the dwarf phenotype of *bri1-5* and suppress the phenotype of *bes1-D* indicated that MYBL2 functioned as a positive regulator in BR signaling pathway. Yeast two hybrid experiment and BiFC experiment demonstrated that MYBL2 and BES1 could interact with each other in vivo. Further analysis by GST pull-down deletion experiment showed that “SANT” domain of MYBL2 interact the phosphorylation domain of BES1. Gene expression studies, ChIP-qPCR and transient experiment results demonstrated that BES1 and MYBL2 could form a complex to inhibit the expression of BR-repressed genes, indicating that MYBL2 was a corepressor of BES1. Moreover, MYBL2 is phosphorylated by BIN2 kinase, a negative regulator in BR pathway. Interestingly, BIN2 phosphorylation of MYBL2 stabilizes the protein. In this section, our studies identified the first corepressor of BES1 and revealed a novel function of BIN2 phosphorylation in BR signaling pathway.

Although several studies about MYBL2 have been finished and a preliminary model was also presented, there are many work need to be done in the future.

1) *mybl2 bri1-5* double mutant and *mybl2 bes1-D* double mutant displayed obvious phenotypes compared to *bri1-5* and *bes1-D*, but *mybl2* single mutant did not exhibit any clear growth phenotype. There are two possible explanations: A) there are other MYBL2 homologs playing similar role as MYBL2 in plants. Previous studies showed that MYBL2 was a small MYB transcription factor in Arabidopsis and there was no close homologs in Arabidopsis. B) MYBL2 is responsible for only a

branch of BR-repressed genes, there must be other co-repressors. So future work about how BES1 represses BR-repressed targets should focus on the transcription factors which contains repression domain. The phenotype of loss-of-function mutants of these repressor candidates should be dwarf (loss-of-function BR mutant phenotype) and the gain-of-function mutant of these candidates should display long hypocotyl, long petiole lengths or curl leaves (gain-of-function BR mutant phenotype).

2) In this thesis, we proposed that MYBL2 acted as corepressor of BES1 and examined several BR-repressed genes expression in *bri1-5 mybl2* double mutant and *bri1-5* single mutant. However, global gene expression in *mybl2* single mutant, *bri1-5* single mutant and *bri1-5 mybl2* double mutant should be performed to confirm our hypothesis and to determine how many BR-repressed targets are regulated through the repression effect of MYBL2.

3) Our data strongly indicated that MYBL2 and BES1 interacted with each other in vitro and in vivo and both of them were regulated by BR signaling. However, we have not tested if BR treatment could affect the interaction between MYBL2 and BES1, so co-immunoprecipitation experiment after BL treatment should be performed or protein-protein interaction between MYBL2 and unphosphorylated- or phosphorylated- BES1 should be investigated.

In addition to MYBL2, RD26, another transcription factor, was identified as a negative regulator in BR signaling pathway. Similar to MYBL2, RD26 was also found to be direct target of BES1 from our ChIP-chip data and microarray data. ChIP-qPCR and gene expression analysis confirmed that RD26 was direct target of BES1 and was repressed by BL and/or BES1. While *rd26 anac019 anac055 anac102* quadruple mutant showed increased BR responses, *RD26OX* transgenic plants led to dwarf plants that are hypersensitive to BRZ. In addition, *RD26OX* plant could suppress the long petiole length phenotype of *bes1-D*. These genetic evidences strongly indicated that RD26 was a negative regulator in BR signaling pathway. Further global gene expression analysis demonstrated that many BR-induced genes including 30 cell elongation genes were down-regulated and many BR-repressed genes were up-regulated in *RD26OX* plants, which is consistent with the negative function of RD26 in the BR pathway. Like the negative regulation of BR signaling by RD26, BR signaling also poses negative regulation on drought response. The loss-of-function mutant, *bri1-5*, in BR pathway had higher tolerance to drought stress, while gain-of-function mutant, *bes1-D* displayed less tolerance to drought stress. Gene expression studies suggested that RD26 and BES1 regulated the expression of target in opposite ways. DNA binding experiments indicated that RD26 and BES1 could bind to the same binding sites (E-box and BRRE site) likely as inactive heterodimer. Our results thus identified a molecular link between drought response and BR signaling. Through the antagonistic interaction between BES1 and RD26, plants could adjust growth processes under normal condition or

drought stress. In the future, there are several experiments need to be done to clearly understand how RD26 negatively regulated BR signaling pathway and how BR signaling pathway and Drought response affected each other.

1) *rd26 ana019 anac055 anac102* quadruple mutant does not display any clear growth phenotype, suggesting that there are other function redundant homologs. There are six close homologs of RD26 in the same branch (Fig S7), it is likely that all of them are involved in BR signaling pathway. Knocking out all the seven genes may generate the mutant with obvious growth phenotype..

2) In addition to two groups (Group I and II) of common targets oppositely regulated by BR and RD26, there are other two overlapped groups (Group III and VI) of genes on which BES1 and RD26 act in the similar ways. How BR and RD26 regulate these genes need to be further analyzed.

3) Our drought stress experiments showed that loss-of-function mutants of BR signaling pathway could enhance the drought tolerance, while gain-of-function mutant of BR signaling pathway decreased the drought tolerance, which might be used in crops, to enhance their tolerance to drought. The loss-of-function BR mutants displayed higher drought tolerance, but the growth of these mutant are reduced, which might affect the yield production. Manipulating the level of BR signaling in plant should be deeply studied to generate the best crops with highest tolerance to drought with maximum yield production.

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