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The molecular mechanisms of brassinosteroid-regulated drought stress response in Arabidopsis thaliana

Buyun Tang
Iowa State University

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The molecular mechanisms of brassinosteroid-regulated drought stress response in *Arabidopsis thaliana*

by

Buyun Tang

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
Yanhai Yin, Major Professor
Steven Rodermel
Thomas Peterson

Iowa State University

Ames, Iowa

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Brassinosteroid Signaling Pathway

Plant steroid hormones, Brassinosteroids (BRs), are ubiquitously distributed throughout plant kingdom. The role of BRs in plants was clarified in the mid-1990s through BR-deficient mutants in *Arabidopsis thaliana*. BR-deficient mutants displayed dwarf phenotype with curly leaves, which can be rescued by exogenous brassinolide (BL), the most active form of BR (Li et al., 1996). For the past decade, BRs have been identified as essential growth-promoting hormones involved in a variety of developmental processes including cell expansion and division, photomorphogenesis, senescence and stress response (Clouse et al., 1996; Mandava, 1988; Krishna, 2003).

The BR signaling pathway has been well established (Figure 1). BRs signal through a membrane-localized leucine-rich repeat receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Li and Chory, 1997; Hothorn et al., 2011; She et al., 2011). In the absence of BRs, SBI1 (Wu et al., 2011), BKI1 (Wang et al. 2011), BIK1 (Lin et al., 2013) and BRI1 tyrosine phosphorylation (Oh et al., 2012) function to inhibit BRI1 functions. Upon BR binding, BRI1 dissociates with its repressor BRI1 KINASE INHIBITOR1 (BKI1) (Wang et al., 2011) and heterodimerizes with another receptor kinase BRI1 ASSOCIATED KINASE1 (BAK1) (Li et al., 2002; Nam and Li, 2002). This leads to the phosphorylation of BR SIGNALING KINASES (BSKs) (Tang et al., 2008) and consequently activates BRI1 SUPPRESSOR1 (BSU1) (Mora-García et al., 2004; Sreeramulu et al., 2013). The activated BSU1 inhibits a glycogen synthase kinase 3 (GSK3) kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) (Kim et al., 2011).
Subsequently, two central transcription factors BRI1 EMS SUPPRESSOR1 (BES1) (Yin et al., 2002) and BRASSINAZOLE RESISTANT 1 (BZR1) (Wang et al., 2002) accumulates and are translocated into nucleus to regulate downstream gene expression.

BES1 and BZR1 are atypical basic Helix-Loop-Helix (bHLH)-like transcription factors, which are regulated by BIN2. In the absence of BRs, BIN2 phosphorylates BES1/BZR1 and inhibits their function through several mechanisms including protein degradation, cytoplasmic retention and reduced DNA binding capacity (Li and Jin, 2007). In the presence of BRs, BIN2’s activity is inhibited, BES1/BZR1 become dephosphorylated form and accumulate in the nucleus. BES1/BZR1 can bind to either E-BOX (CANNTG) or BRRE (CGTGT/CG) promoter elements to induce or repress target gene expression (Yu et al., 2011). BES1 also interacts with many other transcriptional regulators such as IWS1, MYBL2, HAT1 and SDG8 to modulate BR target gene expression (Li et al., 2010; Ye et al., 2012; Zhang et al., 2013; Wang et al., 2014).

Several genome-wide analysis of gene expression in *Arabidopsis* identified around 5000 BR-regulated genes (Yin et al., 2002; Müssig et al., 2002; Goda et al., 2002). Furthermore, chromatin immunoprecipitation coupled with *Arabidopsis* tiling arrays (ChIP-chip) and gene expression studies identified about 1600 target genes regulated by BES1/BZR1, including many cell wall organization enzymes, transcription factors, and stress responsive genes (Yu et al., 2011). However, BES1 downstream regulatory mechanisms by which BES1 controls different target gene expression in different organs, developmental stages and environmental conditions remain to be fully defined.
1.2 Drought and Abscisic Acid Signaling Pathway

Being sessile organisms, plants are constantly exposed to diverse and changing biotic and abiotic environmental conditions. Therefore, plants have to integrate and interpret different environmental cues to generate appropriate physiological responses to coordinate growth and development (Farrant et al., 2015). Among abiotic stresses, drought, high salinity, and low temperature are considered three major adverse environmental conditions that prevent plants from fully realizing their genetic or agricultural potential (Zhu, 2002). The problem of drought, in particular, is most pervasive and economically damaging. Therefore, understanding the physiological or biochemical responses of drought stress and gene regulatory network of drought signaling pathway is essential for plant biologists to develop crop plants with enhanced tolerance to drought stress (Valliyodan and Nguyen, 2006).

Drought stress signaling can be divided into three functional categories: ionic and osmotic stress signaling pathway to reestablish cellular homeostasis, detoxification signaling pathway to control and repair stress damages, and signaling to inhibit cell expansion and division (Zhu, 2001; Zhu, 2002). Transcriptome analysis of plants under stress conditions identified a large variety of cis-acting elements and stress-inducible genes. The promoter of typical stress-inducible genes such as RESPONSIVE TO DESSICATION 29A (RD29A), COLD REGULATED 78 (COR78), and LOW TEMPERATURE INDUCED 78 (LTI78) have been analyzed and two major cis-acting elements associated in stress-induced gene expression are revealed: the ABA-responsive element (ABRE) and the dehydration responsive element (DRE)/C-repeat (CRT)
ABRE and DRE/CRT motifs function in ABA-dependent and ABA-independent gene expression in response to abiotic stresses, respectively.

The products of drought-induced genes contain many transcription factors, enabling us to analyze the transcriptional regulatory network of abiotic stress signaling pathways. At least six signaling transduction pathways have been identified in drought, high salinity and cold-stress responses: three are ABA dependent signaling pathways including MYB/MYC, NAC and AREB/ABF families of transcription factors; and three are ABA-independent signaling pathways including NAC/HD-ZIP, DREB2 and DREB1/CBF families of transcription factors (Shinozaki and Yamaguchi-Shinozaki, 2006) (Figure 2).

ABA belongs to a group of metabolites known as isoprenoids or terpenoids, which is produced by multiple organisms, including fungi, plants and metazoans (Oritani and Kiyota, 2003; Nambara and Marion-Poll, 2005). ABA regulates a wide range of developmental processes such as seed development, dormancy, germination, and seedling growth. Moreover, ABA mediates physiological responses to environmental stresses, including drought, salinity, hypoxia, cold stress, wound stress, and pathogen invasion (Finkelstein et al., 2002). Many regulatory components of ABA signaling pathway have been well characterized, including protein kinases, phosphatases, transcription factors and a myriad of ABA-responsive genes (Weiner et al., 2010).

Protein receptors in Arabidopsis that directly bind to ABA and trigger signaling transduction pathway have been identified by two research groups. Sean Cutler’s group applied synthetic chemical pyrabactin as a selective ABA agonist and identified several
mutants insensitive to this synthetic growth inhibitor (Park et al., 2009). They characterized \textit{PYRABACTIN RESISTANCE 1} (\textit{PYRI}) and several \textit{PYR1}-related homologs (PYLs) as high affinity ABA-binding receptors, which in turn bind to and inhibit PP2Cs. PP2Cs are group A type 2C protein phosphatases, which negatively regulate ABA signaling and repress ABA responses at an early step of the pathway (Allen et al., 1999). Prototypes of these PP2Cs are \textit{ABI1} (Abscisic Acid Insensitive 1) and its homologues \textit{ABI2} (Abscisic Acid Insensitive 2) and \textit{HAB1} (Hypersensitive to ABA 1) (Merlot et al., 2001; Saez et al., 2006). Erwin Grill’s group applied yeast two-hybrid screening for \textit{ABI1} and \textit{ABI2} interactors and identified Regulatory Component of ABA Receptors (RCARs) (Ma et al., 2009). They showed RCARs bind ABA with high affinity and mediate ABA-dependent inactivation of \textit{ABI1} or \textit{ABI2}. The PYR/PYL/RCAR family of ABA receptors contains 14-member gene family in \textit{Arabidopsis}, nearly all of which function in ABA perception (Fujii et al., 2009).

ABA signaling is activated through the formation of a ternary PYR/PYL-ABA-PP2C complex (Melcher et al., 2009). PYR/PYL proteins contain a central hydrophobic pocket flanked by two mobile loops called “latch” and “gate”. ABA binding to the pocket causes the closure of the gate, creating a binding surface for PP2C, which docks on the closed structure. The docking site blocks the magnesium ion of PP2Cs and thus PP2C catalytic activity is inhibited. This revealed a gate-latch-lock mechanism of ligand sensing and signaling transduction of ABA.

A plant specific protein kinases called subfamily 2 SNF1-related kinases (SnRK2s) play central role in positively regulating ABA signaling (Mustilli et al.,
2002; Yoshida et al., 2002; Fujii et al., 2007). In the absence of ABA, the phosphatase activity of PP2C inhibits the kinase activity of SnRKs. However, ABA binding to receptors triggers the inactivation of PP2Cs. As a consequence, the protein kinases SnRK2s are released from inhibition and directly phosphorylate and regulate key transcription factors in the nucleus, such as ABI5 and related ABA Responsive Factors (ABFs). Phosphorylated ABFs bind to ABA-responsive cis-acting element (ABRE), in concert with other transcriptional regulators, providing ABA-responsive transcriptional activation (Figure 3) (Raghavendra et al., 2010; Klingler et al., 2010).

1.3 Objectives and Significance

BRs are involved in diverse developmental processes such as cell elongation, vascular differentiation, senescence and stress responses. The mechanisms and regulatory networks of BR-regulated plant growth and development has been well described for the past decade with the identification of receptors, kinases and central transcription factors involved in BR signaling. Recent studies revealed BRs also extensively participated in plant responses to environmental stresses (Kagale et al., 2007; Bajguz and Hayat, 2009). However, the molecular mechanisms of BR-regulated stress response is largely unknown.

Coordination of plant growth and stress response requires integration of multiple signaling outputs through hormonal crosstalk. Studies of BR signaling pathway and BR-mediated physiological responses indicate there are intensive interactions between BRs and other phytohormones such as auxin, abscisic acid, jasmonic acid and ethylene (Bao et al., 2004; Zhang et al., 2009; Grauwe et al., 2005).
This study aims to unravel the function and regulatory mechanisms of BRs in abiotic stresses, particularly drought stress, through investigation of the crosstalk between BR and drought/ABA signaling pathways. The knowledge generated from this study could provide insights into understanding how plants modulate signaling outputs to coordinate growth and defense through hormonal crosstalk. Moreover, our studies of stress-inducible transcription factor can be utilized by plant breeders to engineer staple crops with higher resistance to adverse environments.

1.4 Thesis Organization

To characterize BR-regulated drought stress response in *Arabidopsis thaliana*, we identified a transcription factor *RESPONSIVE TO DESSICATION 26 (RD26)* as a marker gene through ChIP-chip and microarray to study the interaction between BR and ABA signaling pathways since RD26 gene expression is up-regulated in response to ABA while down-regulated in response to BR. Through genetic, genomic and biochemical assays, we showed RD26 mediates the crosstalk between BR and ABA signaling pathways. Our results are presented in the following chapters.

Chapter 2 characterizes the role of RD26 in BR signaling pathway and the antagonism between drought and BR signaling pathways mediated by RD26-BES1 protein-protein interaction. This project was initiated and mostly performed by Huaxun Ye, a former graduate student in our lab, and I participated in some work. The work I performed is summarized in Chapter 2. The whole manuscript is now under review in *Nature Communications*. 
Chapter 3 details the regulation of RD26 by a GSK3-like kinase BIN2 and the establishment of BIN2 as a converging point on BR and ABA signaling pathways. I performed nearly all of the experiments. We plan to submit the manuscript to *Genes & Development.*

Finally, conclusions and future directions are summarized in chapter 4.

1.5 References


Transcription factor HAT1 is phosphorylated by BIN2 kinase and mediates brassinosteroid repressed gene expression in Arabidopsis. *The Plant Journal*, 77(1), 59-70.


1.6 Figures

**Figure 1. Brassinosteroid (BR) signaling pathway.**

In the absence of BRs, negative regulator BIN2 kinase phosphorylates BES1/BZR1 family transcription factors and inhibits their functions. The binding of BRs to BRI1 leads to the dissociation of BKI1 and the association with co-receptor BAK1. BRI1 and BAK1 transphosphorylate and activate each other, which further phosphorylates downstream kinases (BSK and CDG) to regulate BSU1 phosphatase. The inhibition of BIN2 by BSU1, the action of PP2A phosphatase as well as the interaction of BKI1 with 14-3-3 leading to reduced sequestration of BES1/BZR1 result in the accumulation of unphosphorylated BES1/BZR1 in the nucleus. BES1/BZR1 cooperates with other DNA binding transcription factors and interacts with transcriptional cofactors to regulate thousands of genes for BR-regulated growth and other cellular processes. Adapted from Guo et al., 2013.
Figure 2. Transcriptional regulatory networks of abiotic stress signals and gene expression.

At least six signal transduction pathways exist in drought, high salinity, and cold-stress responses: three are ABA dependent and three are ABA independent. In the ABA-dependent pathway, ABRE functions as a major ABA-responsive element. AREB/ABFs are AP2 transcription factors involved in this process. MYB2 and MYC2 function in ABA-inducible gene expression of the RD22 gene. MYC2 also functions in JA-inducible gene expression. The RD26 NAC transcription factor is involved in ABA- and JA-responsive gene expression in stress responses. These MYC2 and NAC transcription factors may function in crosstalk during abiotic-stress and wound-stress responses. In one of the ABA-independent pathways, DRE is mainly involved in the regulation of genes not only by drought and salt but also by cold stress. DREB1/CBFs are involved in cold-responsive gene expression. DREB2s are important transcription factors in dehydration and high salinity stress-responsive gene expression. Another ABA-independent pathway is controlled by drought and salt, but not by cold. The NAC and HD-ZIP transcription factors are involved in ERD1 gene expression. Adapted from Shinozaki and Yamaguchi-Shinozaki, 2007.
Figure 3. The core PYR/PYL–PP2C–SnRK2 signaling pathway.
In the absence of ABA, PP2C activity is high, and the PP2Cs prevent accumulation of phosphorylated SnRK2 kinases by directly dephosphorylating them. In the presence of ABA, PYR/PYL proteins bind to and inhibit PP2Cs, which leads to the accumulation of phosphorylated and active SnRK2s. Once activated by phosphorylation, the SnRK2s can then directly phosphorylate downstream targets, such as the ABFs, SLAC, KAT1. Adapted from Weiner et al., 2010.
CHAPTER 2. THE INTERACTION BETWEEN BES1 AND RD26 MEDIATES THE CROSSTALK BETWEEN BRASSINOSTEROID AND DROUGHT SIGNALING PATHWAYS

Modified from a manuscript submitted to *Nature Communications* with the data I contributed shown

Huaxun Ye\(^1\), Sanzhen Liu\(^2\), Buyun Tang\(^1\), Maneesha Aluru\(^3\), Srinivas Aluru\(^3\), Patrick Schnable\(^2\) & Yanhai Yin\(^1\)*

\(^1\)Department of Genetics, Development and Cell Biology

\(^2\)Department of Agronomy

\(^3\)Department of Electrical and Computer Engineering

Iowa State University, Ames, IA 50011

2.1 Abstract

A group of steroid hormones, Brassinosteroids (BRs), play crucial roles in regulating plant growth, development and response to stresses. BRs signal through membrane localized receptor BRI1 and several intermediates to activate central transcription factors BES1/BZR1, which regulate thousands of BR-responsive genes. The function and signaling pathway for BR-regulated stem elongation and plant growth have been well established for the past decade. However, how BRs regulate plant response to environmental stresses is largely elusive. Here we show that RD26, a NAC family transcription factor, interacts with BES1 to mediate the crosstalk between BR and drought
signaling pathways. Our ChIP-chip data identified RD26 as a BES1 direct target. RD26 expression is repressed by BL and BES1. In addition, RD26 overexpressors displayed dwarf phenotype and are less resistant to brassinozole (BRZ), a BR biosynthetic inhibitor, while RD26 quadruple mutants are more resistant to BRZ, compared with wild type. Moreover, RD26 interacts with BES1 in vitro and in vivo through GST pull-down, split luciferase complementation assay and co-immunoprecipitation assay. Furthermore, drought tolerance assay revealed an antagonism between BR and drought signaling pathways. Our results established a novel molecular mechanism that balance plant growth and stress response through the crosstalk between BR and drought signaling pathways.

2.2 Introduction

Brassinosteroids (BRs) are a group of plant phytohormones that regulate plant growth, development and stress responses (Clouse et al., 1996; Krishna, 2003; Bajguz and Hayat, 2009). The BR signaling pathway has been well described since mid-1990s. Many BR signaling components have been identified and characterized since the past decade, including the identification of receptors, phosphatases, protein kinases, transcription factors and BR target genes (Belkhadir and Chory, 2006; Yu et al., 2011; Guo et al., 2013). BES1 and BZR1 were discovered as two main transcription factors regulating BR-responsive genes (Yin et al., 2002; Yin et al., 2005; Wang et al., 2002; He et al., 2005).

Plants respond to biotic and abiotic stresses in environment. These factors include dehydration, high salinity, extreme temperature, wound stress, pathogen and pest
attack. Abiotic stresses lead to a wide range of plant morphological, physiological, biochemical and molecular changes. Several studies indicated BRs promote stress resistance (Krishna, 2003; Kagale et al., 2007; Sairam, 1994). However, recent studies revealed that defective mutants in either BR biosynthetic pathway or BR signaling pathway showed high sensitivity to stresses (Zhang et al., 2009; Hu and Yu, 2014; Chung et al., 2014). These studies suggest complicated BR function in stress responses.

RD26 (RESPONSIVE TO DESSICATION 26), a NAC (NAM, ATAF and CUC) transcription factor, was firstly identified as a transcription activator in ABA-dependent stress signaling pathway (Fujita et al., 2004). RD26 expression was induced by drought, NaCl, ABA and JA. Microarray analysis showed RD26 overexpressors upregulate a large portion of stress-inducible genes while RD26 quadruple mutants down-regulate a large portion of stress-inducible genes, suggesting the positive role of RD26 in stress responses. Interestingly, RD26 and several of its homologs, including ANAC019, ANAC055, ANAC102, were identified as BES1/BZR1 targeted transcription factors (Yu et al., 2009; Sun et al., 2010). Since RD26 function in both BR and stress signaling pathways, it is intriguing to investigate how RD26 and its homologs are involved in BR signaling pathway.

In our study, we showed that RD26 is a direct target of BES1. RD26 negatively regulates BR signaling pathway. RNA-Seq analysis suggests RD26 affect BR-regulated genes globally. The antagonism of RD26 to BR signaling pathway is mediated by the interaction between RD26 and BES1. BES1 and RD26 form heterodimer and inhibit each other’s function. Additionally, compared with wild type, BR loss-of-function mutants
displayed higher drought tolerance, while gain-of-function mutants showed lower resistance to drought stress. These results suggest that RD26 inhibit BR-mediated plant growth, and BR pathway also negatively regulate drought stress response. These results establish a molecular mechanism that coordinate plant growth and stress response through the coordination between BR and drought signaling pathways. In this part, I will describe my work in partial fulfillment of the project.

2.3 Results

**RD26 inhibits plant growth and is a negative regulator in BR signaling pathway**

To determine RD26’s function in plant growth and development, we generated RD26 overexpression transgenic plants in *Arabidopsis thaliana*. Consistent with previous study, RD26 overexpressors (*RD26OX*) displayed slightly chlorotic leaves and a stunted growth phenotype (Figure 1A) (Fujita et al., 2004). Previous ChIP-chip studies indicated that RD26 was a direct target of BES1/BZR1 (Yu et al., 2011; Sun et al., 2010). *Bes1-D* mutant, a monogenic semidominant mutation of BES1, displays constitutive BR responses (Yin et al., 2002). Microarray analysis of *bes1-D* showed RD26 expression was repressed by BL and BES1 (Figure 1B).

Our previous results indicated that RD26 loss-of-function mutants had a small increase in BR response than wild type (Yu et al., 2011), suggesting that RD26 functions to inhibit BR response. To confirm that RD26 is related to reduced BR response, we used *RD26OX* and RD26 quadruple mutants *rd26 anac019 anac055 anac102* to determine their sensitivity to BL, the most active form of BR, and BRZ, a BR biosynthetic inhibitor.
The sensitivity to either BL or BRZ was indicated by the relative fold change of hypocotyl length in seedlings. Compared with wild type Col-0, *RD26OX* were less responsive while *rd26 anac019 anac055 anac102* were more responsive to BL. Consistently, in BRZ responsive assay, *RD26OX* were hypersensitive while the quadruple mutants displayed less sensitivity to BRZ (Figure 2). All these results demonstrate that RD26 plays a negative role in the BR signaling pathway.

**RD26 interacts with BES1 through corresponding DNA binding/dimerization domains**

Our previous gel mobility shift results demonstrated that RD26 and BES1 can form heterodimer on their DNA binding motifs including both E-box (CANNTG) and BRRE (CGTGT/CG) sites (Ye et al., unpublished). We want to understand if the heterodimer formed by RD26 and BES1 was mediated through their physical interaction. To examine the interaction between RD26 and BES1, we expressed full-length or truncated BES1 protein with MBP, and full-length or truncated RD26 protein with GST, respectively (Figure 3A). GST pull-down assays revealed a direct interaction between full length RD26 and full length BES1 (Figure 3B). Moreover, to determine whether RD26 and BES1 form heterodimer through their DNA binding domains/dimerization domains, we tested their interaction using truncated RD26 and BES1 proteins. Interestingly, the domains involved in DNA binding/dimerization of BES1(aa. 1-89) and RD26 (aa. 1-140) are sufficient for the interaction (Figure 3C). Split Luciferase (LUC) assay was used to test if RD26 and BES1 interact in plants. RD26 was fused with N-terminal part of LUC
(NLUC) and BES1 was fused with C-terminal part of LUC (CLUC). Co-infiltration of RD26-NLUC and BES1-CLUC in tobacco leaves led to increased LUC activity, while co-expression of controls (RD26-NLUC/CLUC or NLUC/BES1-CLUC) only produced background level activities (Figure 3D). Furthermore, co-expression of RD26 and BES1 in tobacco leaves confirmed that RD26 binds with BES1 in vivo (Figure 3E). These results indicated that BES1 and RD26 can interact with each other through corresponding DNA binding/dimerization domains to modulate target gene expression (Figure 3F).

**Altered drought stress response in BR signaling mutants**

Since BRs function through BES1/BZR1 to repress the expression of RD26, we determined whether BR pathway affects plant drought response mediated by RD26. Previous data showed that the expression of RD26 was induced by drought (Fujita et al., 2004). Drought induces over 2500 genes and represses around 3000 genes (Kim and Wang, 2010). Analysis of genes affected in **RD26OX** and drought-regulated genes revealed that RD26 mediates a large portion of both drought-induced and drought-repressed genes, indicating that RD26 is a major regulator of plant drought tolerance.

If BR signaling indeed inhibits drought response, we expect that BR loss-of-function mutants have increased and gain-of-function mutants have decreased drought tolerance. We thus tested drought tolerance of BR gain-of-function mutant **bes1-D** and loss-of-function mutant **bri1-5** (Figure 4). Interestingly, gain-of-function mutant in BR pathway, **bes1-D**, showed less drought tolerance compared with wild type
EN2. On the other hand, BR loss-of-function mutant, bri1-5, a BR receptor mutant, displayed higher resistance to drought stress compared with wild type WS. Furthermore, we tested drought tolerance of double mutants RD26OX bes1-D and bes1-D. While bes1-D were hypersensitive to drought stress, RD26OX bes1-D restored plant resistance to drought stress since the double mutants showed higher survival rate than bes1-D. These results demonstrate that RD26 plays a positive role in drought tolerance by antagonizing BR signaling. In addition, our results also indicate that BR signaling pathway inhibits drought stress response, partially through the inhibition of RD26.

2.4 Discussion

In this study, we found that BR signaling pathway negatively regulates a drought-responsive transcription factor RD26. RD26 is a direct target of BES1, and posses a BES1 repressed binding motif BRRE in the promoter region (data not shown). RD26 expression is repressed by BL and BES1. In addition, RD26 antagonizes BR signaling pathway. RD26OX displayed retarded growth phenotype. Moreover, RD26OX are hypersensitive to BRZ and less responsive to BL, while rd26 anac019 anac055 anac102 were more resistant to BRZ and more responsive to BL. Based on my results and previous results (Ye et al., unpublished), we constructed a regulatory network to explain the coordination between plant growth and stress response mediated by the crosstalk between drought and BR signaling pathways (Figure 5).

Our genetic, genomic and biochemical studies demonstrate RD26 plays a crucial role in the coordination of BR and drought signaling pathways. RD26 is antagonistically
regulated by BR and ABA, as RD26 is upregulated by ABA but down-regulated by BRs (Nemhauser et al., 2006). Recently a research group generated RD26pro:GUS Arabidopsis plants and conducted histochemical analysis (Chung et al., 2014). They found the induction of GUS expression after NaCl treatment was suppressed by co-treatment with BR, but enhanced by co-treatment with BR biosynthetic inhibitor, further confirm the ABA and BR act antagonistically on their target genes such as RD26.

Global gene expression studies with RD26 mutants in the absence or presence of BL using high throughput RNA-sequencing (RNA-Seq) further support that RD26 negatively regulates BR signaling pathway. From RNA-seq data, we found among 2678 BR-induced genes in wild type, 43% were down-regulated in RD26OX and their induction by BRs is compromised in RD26OX. On the other hand, among 2376 BR-repressed genes, 25% were upregulated in RD26OX and their repression by BRs is also compensated in RD26OX (data not shown). Although there are overlapping genes that are synergistically regulated by both BR and RD26, our data pointed out RD26 negatively regulates a large portion of BR responsive genes.

This antagonistic interaction between RD26 and BES1 ensures plant coordination between growth and defense. Under normal growth conditions, BRs signal through BES1 to inhibit RD26 expression and turn off drought signaling. On the other hand, when plants are under drought stress, RD26 is upregulated to inhibit BR signaling. As a consequence, stress responses are triggered and plant growth is compromised. Our results thus reveal the important functions of BES1-RD26 interaction in coordinating plant growth and stress response.
2.5 Materials and Methods

GST pull-down assays

For purification of GST or MBP fused proteins, GST-RD26, GST-RD26-N, GST-RD26-ΔN, MBP-BES1, MBP-BES1-N, MBP-BES1-ΔN were expressed in *Escherichia coli* BL21. Bacterial cells were incubated in 500mL terrific broth at 16°C induced with 1M isopropyl β-D-thiogalactoside (IPTG). The GST-tagged and MBP-tagged recombinant proteins were purified using glutathione beads (Sigma) and amylose resin (NEB), respectively. GST pull-down assays were performed as described previously (Yin et al., 2002).

Hypocotyl sensitivity assays

Seeds were sterilized by 70% (v/v) ethanol and 0.1% (v/v) Triton X-100 and grown on half-strength medium supplied with 0.1% phytoagar. After three days incubation at 4°C, seeds were put in light conditions for five hours to promote germination and then incubated in either dark or light conditions. BL and BRZ were added to the half-strength medium during the assays.

Split luciferase complementation assay

The Split Luciferase Complementation Assay was performed as described by Jian-Min Zhou’s lab based on published methods. The coding region of RD26 and BES1 were inserted into pCAMBIA1300-nLUC construct and pCAMBIA1300-cLUC construct, respectively. Tobacco leaf transient assay was used to examine luciferase activity in the
presence or absence of RD26 and/or BES1. Equal amount of Agrobacterium cells (measured by OD., 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 systems (Progema). The luciferase levels were normalized by the total protein from each sample.

Co-IP Assay

Tobacco leaves were homogenized in protein lysis buffer (1 mM EDTA, 10% glycerol, 75 mM NaCl, 0.05% SDS, 100 mM Tris-HCl, pH 7.4, 0.1% Triton X-100 and 1 × complete cocktail protease inhibitors). After protein extraction, anti-GFP antibody was added to total proteins. After incubation with gentle mixing for 1 h at 4°C, 200 uL fresh 50% slurry of protein A beads (Trisacryl Immobilized Protein A-20338, Thermo Sci) were added, and incubation was continued for 1h. Protein A beads were pelleted by centrifugation at 2000 rpm for 1 min, and the supernatant was removed. The precipitated beads were washed at least 4 times with the protein extraction buffer and then eluted by 2 × SDS protein loading buffer with boiling for 5 minutes.

2.6 Acknowledgments

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2.7 References


2.8 Figures

Figure 1. RD26 inhibits plant growth and is a negative regulator in BR signaling pathway. (A) The phenotype of 4-week-old RD26 overexpression plants. Western-blotting showed the expression of RD26-MYC protein. (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.
Figure 2. RD26 overexpressors and quadruple mutants displayed opposite sensitivity to BL or BRZ.

(A) Compared with wild type, RD26OX is less responsive to BL while rd26 anac019 anac055 anac102 is more responsive to BL. (B) Compared with wild type, RD26OX is less resistant to BRZ while rd26 anac019 anac055 anac102 is more resistant to BRZ.
Figure 3. RD26 interacts with BES1 through corresponding DNA binding/dimerization domains.

(A) Schematic representation of BES1 and RD26 proteins. Full-length (FL) or domains involved DNA binding/dimerization of BES1 (aa 1-89) or RD26 (aa 1-140) are shown. (B) BES1 interacts with RD26 in GST pull-down assays, GST-RD26, but not GST, pulled down BES1. (C) The DNA binding and dimerization domains of BES1 (1-89) and RD26 (1-140) interacts with each other. MBP-BES1 was detected by anti-MBP antibody. (D) RD26 and BES1 interact with each other in vivo. RD26-NLUC and CLUC-BES1 as well as indicated controls were co-expressed in tobacco leaves and LUC activities were measured and normalized against total protein. (E) BES1 interacts with BES1 through co-immunoprecipitation assay. (F) A model for RD26 and BES1 protein interaction on EBOX/BRRE sites.
Figure 4. Altered drought stress response in BR signaling mutants. (A) BR gain-of-function mutants (bes1-D) have decreased drought tolerance. (B) BR loss-of-function mutants (bri1-5) have increased drought tolerance. (C) Transgenic plants bes1-D RD26OX displayed higher drought tolerance than bes1-D.
Figure 5. A model for crosstalk between BR and drought signaling pathways. Drought stress induces the expression of RD26 to mediate plant drought response. Increased RD26 expression inhibit BES1 activity. On the other side, BR signaling repress RD26 expression through BES1 and other drought-related genes.
CHAPTER 3. A GSK3-LIKE KINASE BIN2 PHOSPHORYLATES AND INTERACTS WITH RD26 TO POTENTIATE DROUGHT SIGNALING IN ARABIDOPSIS

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Buyun Tang, Huaxun Ye, Yanhai Yin
Department of Genetics, Development and Cell Biology
Iowa State University, Ames, IA 50011

3.1 Abstract

Plant steroid hormones Brassinosteroids (BRs) have emerged as crucial regulator in plant growth and stress response. The extensive crosstalk between BR and Abscisic Acid (ABA) signaling pathways modulates various developmental processes in Arabidopsis thaliana. Recent studies revealed an antagonistic relationship between BR and ABA pathways. It is suggested that ABA could regulate BR signaling through unknown components downstream of BRI1 and upstream of BIN2, although the molecular mechanisms remain largely unknown. Here, we show that BIN2 serves as a node to integrate BR and ABA signaling output by phosphorylating and interacting with RD26 transcription factor during drought stress response. RD26 protein is differentially regulated by BR and ABA. While BR reduces RD26 protein level, ABA induces its accumulation. Both BR and ABA-mediated RD26 regulation is through BIN2 kinase. Mechanistic studies revealed that BIN2 physically interacts with RD26 through RD26
DNA binding domain. In addition, RD26 exists as phosphorylated form in plants and could be directly phosphorylated and sequentially stabilized by BIN2. Additionally, BIN2-mediated RD26 phosphorylation on RD26 PEST domain is required for RD26 transcriptional activity. RD26 target gene analysis and reporter gene assays further demonstrate BIN2 and RD26 function cooperatively in drought signaling. Consistent with these findings, BIN2 gain-of-function mutants, bin2-D, displayed high resistance to drought stress. Moreover, a negative phosphatase in ABA signaling pathway, ABI1, serves as a positive regulator in BR signaling, by dephosphorylating BIN2 and inactivating RD26 activity. In conclusion, our data suggests that BIN2 positively regulates drought stress response by a phosphorylation-dependent regulation of RD26. However, ABI1 functions to attenuate drought signaling output by repressing BIN2-RD26 phosphorylation, adding another layer of regulation during stress response. Our study established a novel molecular mechanism that coordinate plant growth and stress response through hormonal crosstalk.

### 3.2 Introduction

As sessile organisms, plants are constantly exposed to diverse environmental conditions. Drought is one of the major problems that prevent plants from fully developing its genetic or agricultural potential (Zhu, 2002; Shinozaki et al., 2003). Plants have demonstrated extensive physiological, biochemical, cellular and molecular adaptation to drought stress, thus enabling them to survive (Shinozaki and Yamaguchi-Shinozaki, 2006). In the model systems *Arabidopsis thaliana*, a myriad of
molecular candidates that mediate drought stress response have been identified through microarray analysis. They include proteins that function in stress tolerance and stress response, and various regulatory transcription factors, both ABA-dependent and ABA-independent, such as MYC, NAC (NAM, ATAF1/2 and CUC2), bZIP (basic region/leucine zipper), DREB2 family transcription factors (Shinozaki et al., 2006). Among these, RESPONSIVE TO DESSICATION 26 (RD26), encoding a NAC protein, has been identified as an ABA-inducible transcriptional activator under abiotic stresses such as drought and high salinity (Fujita et al., 2004). While RD26 activates ABA signaling, it negatively regulates plant growth and development by inhibiting BR signaling (Chung et al., 2014; Ye et al., unpublished). Such dual regulatory roles suggest RD26 plays important role in coordinating plant growth and stress response.

Effectively integrating multiple signaling pathways is quite important for plants to coordinate many biological processes (Depuydt et al., 2011; Bai et al., 2012). Plant steroid hormones BRs are key regulators in modulating cell elongation, vascular differentiation, senescence and stress responses in plants (Belkhadir et al., 2006; Ye et al., 2011; Yu et al., 2011; Ryu et al., 2013). BRs also control a wide range of physiological and developmental processes by coupling with other phytohormones such as auxin, jasmonic acid and ABA and other plant signaling pathways (Choudhary et al, 2012). For instance, the interplay between BR and PRR-triggered immunity (PTI) signaling pathway ensures a fine-tuning between plant growth and immunity trade-off (Lozano-Durán et al., 2014). Additionally, BR interacts with auxin to promote lateral root development by increasing auxin transport in *Arabidopsis* (Bao et al., 2004). As a stress hormone, ABA
has been reported to interact with BR in various biological and developmental processes, including seed germination, lateral root elongation, stomatal closure and responses to environmental stresses (Steber et al., 2001; Haubrick, 2006; Kagale et al, 2007; Serna et al., 2014).

The BR signaling pathway has been well established (Figure 1). BRs signal through a membrane-localized leucine-rich repeat receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Li and Chory, 1997; Hothorn et al., 2011; She et al., 2011). In the absence of BRs, SBI1 (Wu et al., 2011), BKI1 (Wang et al. 2011), BIK1 (Lin et al., 2013) and BRI1 tyrosine phosphorylation (Oh et al., 2012) function to inhibit BRI1 functions. Upon BR binding, BRI1 dissociates with its repressor BRI1 KINASE INHIBITOR1 (BKI1) (Wang et al., 2011) and heterodimerizes with another receptor kinase BRI1 ASSOCIATED KINASE1 (BAK1) (Li et al., 2002; Nam and Li, 2002). This leads to the phosphorylation of BR SIGNALING KINASES (BSKs) (Tang et al., 2008) and consequently activates BRI1 SUPPRESSOR1 (BSU1) (Mora-García et al., 2004; Sreeramulu et al., 2013). The activated BSU1 inhibits a glycogen synthase kinase 3 (GSK3) kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) (Kim et al., 2011). Subsequently, two central transcription factors BRI1 EMS SUPPRESSOR1 (BES1) (Yin et al., 2002) and BRASSINAZOLE RESISTANT 1 (BZR1) (Wang et al., 2002) accumulates and are translocated into nucleus to regulate downstream gene expression. Unlike BR signaling pathway, ABA signaling pathway involves several receptors including PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCARs) family (Park et al., 2009; Ma et al.,
These receptors function as allosteric switches and triggers the formation of PYR/PYL-ABA-PP2C ternary complex in response to ABA, which masks PP2C catalytic sites and inhibits its function (Melcher et al., 2009). The inactivation of PP2C launches SNF1-type kinase action, leading to ABA-dependent gene expression and activation of ion channels (Raghavendra et al., 2010; Weiner et al., 2010).

GLYCOGEN SYNTHASE KINASE (GSK3)/SHAGGY kinases are involved in diverse developmental signaling pathways in plants, including flower development, hormone signaling and stress responses (Jonak et al., 2002). In Arabidopsis, a GSK3-like kinase BIN2 is firstly identified as a negative regulator in BR signaling pathway (Li et al., 2002; Yan et al., 2009). BIN2 has been reported to function in a wide range of developmental processes in plants, including seed germination, diurnal hypocotyl growth and stomatal development (Kim et al., 2012; Bernardo-García et al., 2014; Hu et al., 2014). As a highly conserved Ser/Thr kinase, BIN2 phosphorylates consensus sequence Ser/Thr-X-X-X-Ser/Thr (Doble et al., 2001). Based on recent reports, BIN2 phosphorylation on its substrates leads to at least two consequences. On the one hand, BIN2 could phosphorylates its substrates and target them to protein degradation. The light-regulated PIF4 (phytochrome-interacting factor 4) is a phosphorylated by BIN2, which targets it to proteasomal degradation (Bernardo-García et al., 2014). In addition, BIN2 kinase phosphorylates and inactivate YDA during stomatal production pathway (Kim et al., 2012). On the other hand, BIN2 phosphorylation on its substrates could enhance their activity and potentiate the signaling outputs. For instance, BIN2-mediated phosphorylation of ARF7 and ARF19 suppresses its interaction with AUX/IAAs and
subsequently enhance ARF7/19 transcriptional activities to increase the expression of target genes LBD16 and LBD29 during auxin mediated lateral root development (Cho et al., 2014). In addition to the involvement in auxin signaling, BIN2 has been implicated in the regulation of ABA signaling. A gain-of-function mutant, bin2-1, displayed hypersensitivity to ABA during root elongation (Hu et al., 2014), and BIN2 seemed to enhance ABA signaling through phosphorylating SnRK2s (Cai et al., 2014), a positive regulator in ABA signaling. However, it is largely unknown how BIN2 action is integrated into drought signaling pathways during plant growth and development.

In this study, we showed that BIN2 positively modulates drought stress signaling by controlling RD26 protein activity. RD26 was differently regulated by BR and ABA. BIN2 was found to physically interact with RD26. Moreover, BIN2 phosphorylates, stabilizes and enhances RD26 transcriptional activity on its target gene GLYI7 (AT1G80160). Gene expression analysis suggested BIN2 and RD26 function in the same signaling cascade during drought stress. Consistently, BIN2 gain-of-function mutants and loss-of-function mutants displayed different sensitivity to drought stress. Finally, we found that the negative regulation of ABI1 on BIN2-RD26 module adds another layer of crosstalk between BR and ABA signaling pathways.

3.3 Results

**RD26 is differentially regulated by BR and ABA**

Our previous study indicated that RD26 is a negative regulator in BR signaling pathway. RD26 overexpressors (RD26OX) display dwarf phenotype, and RD26OX
bes1-D double mutants suppress the constitutive growth of bes1-D plants (Fujita et al., 2004; Ye et al., unpublished). We found RD26 represses BR signaling by inhibiting unphosphorylated BES1 (Figure S2), the active form of BES1 in plants. To investigate the regulation of RD26 by BR, MYC-tagged RD26 transgenic plants were treated with either BL (brassinolide, the most active BR) or BRZ (brassinozole, a BR biosynthetic inhibitor). RD26 protein level was examined by Western blot. We found that in the presence of BL, RD26 protein level was significantly reduced after eight hours BL treatment, while remained mostly constant upon BRZ treatment (Figure 1A, upper panels). As a negative regulator in BR pathway, BIN2 serves to repress BR signaling by phosphorylating BES1/BZR1 and targeting them to protein degradation (Wang et al., 2002; Yin et al., 2005). Thus, we were interested to examine whether BIN2 is involved in RD26 regulation. To test this possibility, BIN2 inhibitor bikinin (Rybel et al., 2009) was applied to examine RD26 protein stability. Compared with mock treatment, RD26 protein became unstable and decreased significantly after eight hours of bikinin treatment (Figure 1B, top panels), suggesting BIN2’s activity is required to maintain RD26 protein stability. As previously described, BL and bikinin treatments led to the accumulation of unphosphorylated BES1 while BRZ treatment led to phosphorylated BES1 (Figure 1A and 1B, middle panels; Mora-García et al., 2005; Yan et al., 2009). Taken together, these results demonstrated that BR negatively regulates RD26 protein through the inhibition of BIN2.

RD26 involved in a novel ABA-dependent stress signaling pathway, and Northern blot revealed RD26 transcription is induced by drought, high salinity and ABA (Figure S1; Fujita et al., 2006). We wondered whether RD26 protein is posttranslationally
regulated by ABA. Therefore, we examined RD26 protein level after treating the transgenic plants with ABA. We observed that ABA could significantly induce RD26 protein accumulation within two hours (Figure 1C). This observation confirms that RD26 acts as a transcriptional activator in ABA signaling. To further understand whether ABA-induced RD26 was regulated through BIN2, we transformed RD26 overexpression constructs into bin2-3 bil1 bil2 triple mutants and named the transgenic plants RD26 bin2-3 bil1 bil2. We treated the transgenic plants with ABA. Interestingly, ABA failed to induce RD26 accumulation in RD26 bin2-3 bil1 bil2 transgenic plants (Figure 1D), suggesting ABA-induced RD26 expression is dependent on BIN2.

To understand how RD26 was degraded, a proteasome inhibitor, MG132, was applied to examine BR-induced RD26 reduction. When the plant samples were treated with BL, RD26 protein was indeed significantly decreased. However, MG132 treatment resulted in highly accumulation of RD26 protein (Figure S3), indicating RD26 protein degradation was regulated through proteasome-mediated pathway. Our results thus showed a differential regulation of RD26 protein by BR and ABA.

**BIN2 physically interacts with RD26 in vitro and in vivo**

To understand how BR and ABA modulate RD26 protein level, we examined whether there is physical interaction between RD26 and BIN2 proteins. Firstly, an *in vitro* GST pull-down assay was performed using full length RD26 and BIN2. GST-RD26, but not GST, pulled down significant amount of MBP-BIN2 protein, suggesting a direct interaction between RD26 and BIN2 (Figure 2A). RD26 contains a NAM (No Apical
Meristem) DNA binding domain, which consists of the first 140 amino acids, and a C-terminal protein regulatory domain spanned from amino acid 141 to 297. We thus truncated RD26 protein into N-terminal domain (aa. 1-140) and C-terminal domain (aa. 141-297) and performed pull down assay with MBP-BIN2. Specifically, the N-terminal NAM DNA binding domain, but not the C-terminal regulatory domain, interacts with BIN2 (Figure 2B).

We next investigated whether BIN2 could interact with RD26 in vivo by conducting Bimolecular Fluorescence Complementation (BiFC) assay. RD26 was fused to N-terminal YFP (RD26-nYFP) and BIN2 was fused to C-terminal YFP (BIN2-cYFP). When RD26-nYFP and BIN2-cYFP were co-infiltrated into Nicotiana benthamiana leaves, strong fluorescent signals were observed in the nucleus (Figure 2C, upper panel). In contrast, neither BIN2-cYFP/nYFP nor RD26-nYFP/cYFP co-transformation showed any fluorescent signal (Figure 2B, middle and bottom panel). Taken together, our results indicate BIN2 interacts with RD26 in vitro and in vivo.

Next, a genetic study was conducted to examine the epistasis of RD26 and BIN2. Previous studies revealed that BIN2 loss-of-function triple mutants bin2-3 bil1 bil2 display constitutive growth phenotype (Yan et al., 2009). We wondered if RD26 functions downstream of BIN2 and reverses the growth phenotype of bin2-3 bil1 bil2 mutants. To test the hypothesis, we transformed MYC-tagged RD26 overexpression constructs into bin2-3 bil1 bil2 mutants and two transgenic lines were characterized by Western blot with MYC antibody. Compared with bin2-3 bil1 bil2 mutants, RD26 bin2-3 bil1 bil2 transgenic plants displayed shorter petiole length. Moreover, RD26 bin2-3 bil1 bil2
showed slightly chlorotic leaves and retarded growth, resembling the phenotype of *RD26OX* (Figure 2D). Those results suggest RD26 functions downstream of BIN2 in BR signaling pathway.

**BIN2 phosphorylates and stabilizes RD26**

BIN2 regulates a wide range of developmental processes through phosphorylating various transcriptional regulators and modulating their transcriptional activities (Kim et al., 2012; Cho et al., 2014; Bernardo-Garcia et al., 2014; Hu et al., 2014). We examined RD26 protein and found there are 14 BIN2 phosphorylation residues. The involvement of BIN2 in RD26 regulation and the direct interaction between these two proteins promoted us to test whether RD26 is a substrate of BIN2 kinase. Therefore, we performed a semi *in vivo* kinase assay to see if BIN2 phosphorylates RD26. In this assay, RD26 protein was firstly immunoprecipitated from transgenic plants using MYC antibody. BIN2-MBP, extracted from *Escherichia coli*, was then added to the precipitated RD26-MYC protein. The protein was then run on a phos-tag SDS-PAGE gel. Phos-tag reagent will bind to phosphorylated groups and reduce protein mobility (see Materials and Methods). Compared with untreated sample, BIN2-treated sample led to a significant protein accumulation and shift (Figure 3A), indicating RD26 protein was phosphorylated and increased after BIN2 treatment.

Then, an *in vitro* kinase assay was performed with MBP-BIN2 and GST-RD26. BIN2 can phosphorylate GST-RD26, but not GST alone (Figure 3B). Furthermore, a substrate-concentration dependent kinase assay was performed. We found
BIN2-phosphorylated RD26 was enhanced when more MBP-RD26 substrates were supplied (Figure 3C), indicating the phosphorylation was indeed mediated by BIN2. Overall these results demonstrate BIN2 can directly phosphorylate and stabilize RD26.

If RD26 is phosphorylated by BIN2, we would expect RD26 existed as phosphorylated form in plants. To test the idea, RD26 protein was firstly immunoprecipitated from transgenic plants and then subjected to phosphatase (CIP) treatment. Followed CIP treatment, RD26 protein was run on a phos-tag SDS-PAGE gel. Significant protein band shift was observed before and after CIP treatment, indicating RD26 protein was transformed from phosphorylated form to unphosphorylated form, and supporting the idea that phosphorylated RD26 is the form detected in plants (Figure 3D). To confirm that RD26 phosphorylation is mediated by BIN2, we grew RD26 transgenic plants on half-strength MS media containing BL or BRZ for two weeks. Consistently, RD26 protein was decreased on BL plate while accumulated on BRZ plate (Figure 3E, middle panel). Surprisingly, on phos-tag gel, BRZ treatment led to two strong slow-migrating bands, which correspond to hyperphosphorylated RD26, while BL treatment induced one weak fast-migrating band corresponding to hypophosphorylated RD26 form (Figure 3E, upper panel). These observations suggest that RD26 protein becomes dephosphorylated in the presence of BR, and the unphosphorylated RD26 protein is targeted to protein degradation. In contrast, RD26 protein is phosphorylated and stabilized when BR signaling is inhibited. Taken together, our in vivo and in vitro results strengthen the evidence that BIN2 plays a key role in phosphorylating and stabilizing RD26 protein.
**BIN2-mediated RD26 phosphorylation is required for RD26 transcriptional activity**

There are 14 BIN2 phosphorylation sites in predicted RD26 protein. Among these phosphorylation sites, 8 are clustered in the predicted domain of RD26, a domain implicated in protein degradation. Other sites are located either within amino acid 141 to 182, or in C-terminal domain (Figure 4A). To map the BIN2 phosphorylation domain on RD26, we performed *in vitro* kinase assay using truncated GST-RD26 proteins. In accordance with the predicted sites, BIN2 showed no phosphorylation on RD26 DNA binding domain (aa. 1-140) and very weak phosphorylation on the first 182 amino acids (aa. 1-182). However, strong phosphorylation signal was observed when PEST domain is included in RD26 truncated protein (aa. 1-204). Surprisingly, full length RD26 protein (aa. 1-297) showed a faint phosphorylation band, indicating there might be a repression domain within the C-terminal region (Figure 4B). We thus conclude that BIN2-mediated RD26 phosphorylation is mainly at the PEST domain.

To further characterize the function of BIN2-mediated RD26 phosphorylation, we examined the phosphorylation-dependent RD26 transcriptional activity through luciferase transient assay. As previously described, RD26 protein activates the promoter of its target gene glyoxalase I family protein 7 (GLYI7) (Fujita et al., 2006). This is confirmed by our gene expression studies. We found GLYI7 transcription is up-regulated by around 100-fold in *RD26OX* (Figure S4A). Then, we fused *GLYI7* gene promoter to luciferase (*GLYI7:LUC*) and used luciferase as a reporter to test RD26 transcriptional activity. Compared with vector control, BIN2 induced *GLYI7::LUC* expression by 2-fold, while RD26 activates luciferase expression by 60-fold. Moreover, co-expression of RD26
with BIN2 further induced luciferase activity to 100-fold (Figure 4D). These data indicates BIN2 enhances RD26-mediated transcription activity.

We next investigated whether increased RD26 transcriptional activity was due to BIN2 phosphorylation. Compared with wild type RD26, RD26MA, in which putative BIN2 phosphorylated S/T residues are mutated to A, failed to induce luciferase activity. However, constitutive luciferase expression was observed when phosphorylation mimicking form, RD26ME (S/T replaced with E) was introduced to plants. Moreover, when RD26MA and RD26ME were transformed together with BIN2 into Nicotiana benthamiana leaves, only RD26ME could further induce reporter gene activity (Figure 4C & D). These results suggest that BIN2 phosphorylation on RD26 PEST domain is required to activate RD26 transcription activity on RD26 target genes.

**BIN2 positively regulate drought stress response**

According to the RNA-seq data of RD26 overexpressors (RD26OX) and quadruple mutants (RD26Q, rd26 anac019 anac055 anac102) (Ye et al., unpublished), we characterized one group of genes that are up-regulated in RD26OX and down-regulated in RD26Q (Figure S4A) and another group of genes that are down-regulated in RD26OX while up-regulated in RD26Q (Figure S4B). Close examination revealed that the first group of genes are mainly stress responsive genes, including RD26, AT1G29395, AT1G22400, AT4G18010, GLYI7, and the second group of genes are mainly growth responsive genes, including AT4G00360, PRP4, AAP6, CIR1, RVE1. To test our hypothesis that BIN2 is involved in RD26-mediated drought signaling pathway, the
expression level of several RD26 induced and repressed genes was examined in Col-0, bin2-D and bin2-3 bfl1 bfl2 plants. Quantitative RT-PCR (qRT-PCR) results showed that the expression of most of these stress responsive genes was increased in bin2-D and decreased in bin2-3 bfl1 bfl2 (Figure 5A), while the expression of all growth responsive genes was down-regulated in bin2-D mutant and up-regulated in bin2-3 bfl1 bfl2 mutants (Figure 5B). These results raised a possibility that BIN2 and RD26 function in the same signaling cascade to regulate drought stress response. To further test this possibility, BIN2 mutants were subjected to drought sensitivity assay. Consistent with gene expression results, bin2-D, BIN2 gain-of-function mutants, displayed high resistance to drought stress. However, loss-of-function mutants bin2-3 bfl1 bfl2 exhibited high sensitivity to drought stress, although bin2-3 bfl1 bfl2 were less sensitive than wild type (Figure 5C). Likewise, RD26OX are highly resistant to drought stress but not RD26Q (Figure S5A&B). Taken together, our results suggest that BIN2 functions positively during drought stress response.

**ABI1 is a positive regulator in BR signaling and dephosphorylates BIN2**

The antagonism of ABA on BR signaling output is possibly mediated through two PP2C phosphatases ABI1 and ABI2 (Zhang et al, 2009). Here we characterized the function and mechanism of ABI1 on BR signaling pathway. Firstly, we investigated the role of ABI1 in BR signaling pathway. We obtained abi1-1, gain-of-function mutants of ABI1. Altered BR responses were observed in abi1-1 through the measurement of hypocotyl length in seedlings in the presence of BL or BRZ. In the absence of BL, no
obvious difference was observed between wild type ler-0 and abil-1 in terms of hypocotyl length. However, after 100nM BL treatment for 2 weeks, abil-1 mutants displayed significantly higher hypocotyl length compared with ler-0, suggesting abil-1 are more sensitive to BR (Figure 6A).

In the dark, plants undergo etiolated growth with elongated hypocotyl, folded cotyledon and apical hook in the shoot apical meristem. It has been shown that BR plays a positive role in etiolated growth signaling by promoting PIF4 transcription regulator (Bernardo-García et al., 2014). To confirm its role in BR signaling pathway, abil-1 were then subjected to BRZ response in the dark. Compared with ler-0, abil-1 mutants had slightly shorter hypocotyl length in the dark grown seedlings. However, after grew on medium supplied with 2000nM BRZ for two weeks, abil-1 mutants showed longer hypocotyl length, indicating the abil-1 mutants were more resistant to the BR biosynthetic inhibitor (Figure 6B). Together with BL sensitivity assay, these results demonstrate ABI1 plays a positive role in the BR signaling pathway.

To further characterize the mechanism of ABI1 in the BR signaling pathway, we then seek to test whether there is regulation between ABI1 phosphatase and BIN2 kinase. BIN2 has been shown to be involved in ABA signaling (Zhang et al., 2009; Hu and Yu, 2014). The involvement of BIN2 in ABA signaling promoted us to examine whether ABI1 could dephosphorylate BIN2. Interestingly, in vitro kinase assay showed MBP-ABI1 was directly phosphorylated by BIN2 when MBP-ABI1 was added together with MBP-BIN2 (Figure 6C, lane 5; Figure S6B, lane 4). Moreover, the autophosphorylation of BIN2 was also decreased in the presence of ABI1 (Figure 6C,
lane 5; Figure S6B, lane 4), suggesting ABI1 can directly dephosphorylate BIN2. However, when ABI1 is supplied together with BIN2 and RD26, we observed decreased phosphorylation on both BIN2 and RD26 (Figure 6C; Figure S6A). We also observed decreased phosphorylation of both BIN2 and BES1 when ABI1 is added together BIN2 and BES1 (Figure S6A). Band intensity quantification showed the dephosphorylation of BIN2 was much more significant compared with that of RD26 (Figure 6C), suggesting that reduced RD26 phosphorylation was due to inactive BIN2 kinase activity rather than direct ABI1 dephosphorylation. To confirm this idea, preincubated BIN2 and RD26 were made followed by the addition of BIN2 inhibitor bikinin. Surprisingly, both autophosphorylated BIN2 and phosphorylated RD26 were completely abolished by bikinin treatment for one hour (Figure 6, lane 3 & 4), indicating BIN2 activity is required to maintain and stabilize RD26 phosphorylation.

Since BIN2 could phosphorylate ABI1 and ABI1 could inversely dephosphorylate BIN2, we tested whether phosphorylated ABI1 is more efficient in dephosphorylating BIN2 than unphosphorylated ABI1. We used GST-RD26 (in beads) and preincubated GST-RD26 with MBP-BIN2 (in solution) in the absence of ABI1 (Figure 6D, lane 1). Followed this incubation, ATP maintained in the solution was removed. GST-RD26 beads were saved followed by the addition of either phosphorylated or unphosphorylated ABI1. Phosphorylated ABI1 was generated using non-radio labeled ATP in kinase buffer. When ABI1 was added to the preincubated samples, BIN2 lost its kinase activity in the absence of ATP and cannot phosphorylate ABI1 (data not shown). After treatment for one hour with phosphorylated ABI1, autophosphorylated BIN2
decreased more compared with samples treated with unphosphorylated ABI1 (Figure 6D, lane 3&4), suggesting that the effect of phosphorylated ABI1 is more effective in dephosphorylating BIN2. Taken together, these results suggest ABI1 plays a positive role in BR signaling pathway by dephosphorylating BIN2.

3.4 Discussion

The plant steroid hormones BRs are key regulators in growth and development. BRs function through a well established signaling pathway in which repression of BIN2 and activation of BES1/BZR1 upon BR perception triggers expression changes of large number of genes. However, the mechanisms underlying BR-mediated stress response are not well understood. Our study revealed a previously unknown regulatory module involving phosphorylation-dependent regulation of transcriptional activity during drought stress response.

A GSK3-like kinase, BIN2, positively regulates drought stress response through phosphorylating its substrate RD26 (Figure 7). This enhances RD26 protein accumulation, likely by preventing RD26 from protein degradation, and eventually increases its transcriptional activity. Several lines of evidence support the function of BIN2-mediated RD26 phosphorylation during drought stress response. First, BIN2 inhibitor bikinin treatment leads to decreased RD26 protein level. In addition, RD26 protein exists as phosphorylated form in plants, and BRZ treatment enhances phosphorylated form of RD26, suggesting the involvement of a kinase or kinases in RD26 phosphorylation. Moreover, kinase assays point out a direct role of BIN2 in phosphorylating and stabilizing
Finally, luciferase transient assay demonstrates that BIN2 phosphorylated RD26 is required for RD26 transcriptional activity.

The dual roles of RD26 in up-regulating stress response and down-regulating growth requires it to be tightly monitored by different environmental or hormonal signals. In addition to the regulation by BIN2 kinase, RD26 is regulated by BR signaling through BES1 transcription factor. RD26 and its close homologs are inhibited by BR signal. Moreover, RD26 is a direct target of BES1. BES1 heterodimerizes with RD26 to inhibit RD26 transcriptional activities on BR-regulated genes, which leads to RD26 repression during plant growth and development (Ye et al., unpublished). RD26 has been shown to be induced by drought, high salinity and ABA at both transcriptional and translational level (Fujita et al., 2006; Figure 1C). This extensive involvement in stress response suggests other regulatory mechanisms on RD26. As crucial positive regulators in ABA signaling pathway, subgroup III Snf1-related kinase 2s (SnRK2s) phosphorylates and activates many transcription factors, such as ABA Responsive Element Binding Factor (ABFs), to modulate ABA responsive gene expression (Fujii et al., 2007; Fujii et al., 2009; Fujita et al., 2009). Interestingly, GSK3-like kinases could directly phosphorylate SnRK2s to potentiate ABA signaling (Cai et al., 2014). These studies suggest a complex regulatory mechanisms between BIN2 kinase and downstream responsive genes in ABA signaling pathway.

In *Arabidopsis*, the ABI1 gene encodes a member of the type 2C class of protein serine/threonine phosphatases (PP2C) (Leung et al., 1994; Gosti et al., 1999). As a negative regulator in ABA signaling pathway, ABI1 dephosphorylates and inactivates
SnRK2s, leading to the inhibition of downstream transcriptional factors required for ABA response (Umezawa et al., 2009; Rodrigues et al., 2013). Here we report ABI1 could dephosphorylate BIN2 to attenuate drought signaling. Our kinase assays revealed that the autophosphorylation of BIN2 is significantly decreased in the presence of ABI1 (Figure 6C&D; Figure S6A&B). As a consequence, the phosphorylation of RD26 is impeded following BIN2 dephosphorylation by ABI1 (Figure 6C&D; Figure S6A&B). We think that RD26 dephosphorylation was caused by the inactivity of BIN2, instead of the direct dephosphorylation from ABI1. This is confirmed by our phosphatase assay, as ABI1 treatment failed to produce fast-migrating band of RD26 (Figure S7). Interestingly, we found BIN2 could directly phosphorylate ABI1, and phosphorylated ABI1 is more efficient in dephosphorylating BIN2 compared with unphosphorylated ABI1 (Figure 6D). Consistently, ABI1 appears to be a positive regulator in BR signaling (Figure 6 A&B). In summary, the feedback regulatory mechanism of ABI1 on BIN2-RD26 module ensures a fine-tuning of signaling outputs during drought stress response.

BR and ABA act antagonistically during various physiological responses such as seed germination and stress response (Steber et al., 2001; Zhang et al., 2009; Chung et al., 2014). The antagonism between these two pathways is critical for plants to regulate the trade-off between growth and defense. For example, ABA represses seed germination and post-germination growth while BRs antagonize ABA-mediated inhibition and promote these processes. Hu and Yu demonstrated BIN2 interacts with ABSCISIC ACID INSENSITIVE5 (ABI5) to inhibit the function of BR during seed germination (Hu and Yu, 2014). Intriguingly, our proposed BIN2-RD26 signaling cascade during drought stress
response shared similarities to the BIN2-ABI5 module in seed germination. While BIN2 phosphorylates a bZIP transcription factor ABI5 to antagonize BR-inhibited seed germination and early seedling growth, BIN2 phosphorylates RD26 to potentiate BR-repressed drought signaling. These studies demonstrate a pervasive role of BIN2 in mediating the interactions between BR and ABA pathways to modulate various physiological responses.

We also examined BIN2 and RD26 expression profiles under various abiotic stress conditions using Arabidopsis e-FP browser (Figure S8). Under drought condition, high BIN2 expression is observed among all tissues in seedling within a quarter hour, followed by high RD26 expression among all tissues within one hour. Then, both BIN2 and RD26 are highly expressed after three hours of drought treatment. This strengthens our model that BIN2 functions upstream of RD26 and RD26 needs to be activated by BIN2 during drought condition. However, similar expression patterns of BIN2 and RD26 in other abiotic stress conditions such as cold stress, osmotic stress and salt stress were not observed. The regulation of RD26 by BIN2 possibly does not apply to other stress conditions besides drought.

In conclusion, our studies showed that BIN2-RD26 regulatory module plays an important role in drought stress response, uncovering a mechanism through which multiple signaling pathways are integrated. Future work to characterize the mechanisms of ABI1 regulation on BIN2 and the crosstalk between BR and ABA signaling pathways will offer in-depth understanding of the regulation and coordination between plant growth and drought stress response.
3.5 Materials and Methods

Plant materials and growth condition

T-DNA insertion mutants, \textit{rd26} (AT4G27410, SALK\textunderscore 063576), \textit{abi1-1} (AT4G26080 ,CS22) were obtained from ABRC (Arabidopsis Biological Resource Center). All plants were grown on half-strength MS plates and/or in soil under long day conditions (16h light/ 8h dark) at 22°C. For the BRZ mediated hypocotyl elongation assay, seeds were sterilized and planted in half-strength and kept at 4°C in the dark for 4 days, and then exposed to light for 5 hours before kept in the dark at room temperature for 5 days. Hypocotyls were measured from the root of the seedlings to base of the cotyledons.

Plasmid constructs

For transgenic plants, RD26 genomic sequence including 5’ UTR was cloned from wild type genomic DNA and fused with MYC tag and CaMV 35S promoter into pZP211 vector (Hajdukiewicz et al., 1994). For luciferase assay, RD26 cDNA was cloned into 2×MYC tag flanked by the BRI1 promoter. Phosphorylation mutaion constructs were performed using PCR Mutagenesis Techniques and RTS (Roche, http://www.5prime.com/media/434208/app_13.pdf). For recombinant protein and GST pull-down assay, the coding regions of RD26, BIN2 and ABI1 were amplified from Col-0 cDNA and cloned into either pET42a(+) or pETMALc-H vector. For the BiFC assay, BIN2 coding region was fused to C-terminal YFP and RD26 coding region was incorporated into N-terminal YFP. All the primers are listed in Table S1.
Transgenic plants

The construct of RD26-MYC driven by 35S promoter was transformed into Agrobacterium tumefaciens (Strain GV3101). The recombinant strains were then transformed into *bin2-3* *bil1* *bil2* mutants through floral dip method. Transgenic lines were selected on half-strength MS containing 150 ug/ml gentamycin. Transgene expression was detected by Western Blotting.

Protein-protein interaction assays

For purification of GST or MBP fused proteins, GST-RD26, GST-RD26-N, GST-RD26-C, BIN2-MBP were expressed in *Escherichia coli* BL21. Bacterial cells were incubated in 500mL terrific broth at 16 induced with 1M isopropyl β-D-thiogalactoside (IPTG). The GST-tagged and MBP-tagged recombinant proteins were purified using glutathione beads (Sigma) and amylose resin (NEB), respectively. GST pull-down assays were performed as described previously (Yin et al., 2002). For biomolecular

Mobility shift detection of phosphorylated proteins

Phos-tag reagent (NARD institute) was used to determine the phospho-protein mobility shift of RD26 protein. Proteins were separated in a 10% SDS-PAGE gel containing 5mM Phos-tag and 10mM MnCl2. The gel was soaked in transfer buffer containing 1 mmol/L EDTA for 10 minutes, and again soaked in transfer buffer without EDTA for 10 minutes with gentle agitation. After proteins were transferred to a nitrocellulose membrane, MYC-tagged RD26 was detected using anti-myc antibody.
**Luciferase transient assay**

In luciferase transient assay, the promoter of GLYI7 (AT1G80160) was fused to luciferase gene as the reporter. MYC-tagged RD26, RD26MA, RD26ME and BIN2 were used as effectors. Those constructs were transformed into Agrobacterium and equal amount of Agrobacterium cells (measured by O.D600, adjusted to same with vector containing strain) were infiltrated into *Nicotiana benthamiana*. Total proteins were extracted from quintic samples after two-day infiltration and luciferase activities were measured using Berthold Centro LB960 luminometer with luciferase assay system (Promega). The relative levels of luciferase activities were normalized by the total proteins. This assays has been repeated for more than three times with similar results.

**In vivo and in vitro kinasy assay**

For *in vivo* kinase assay, RD26 protein was immunoprecipitated from MYC-tagged transgenic plants using anti-myc antibody. BIN2-MBP was added to RD26-MYC protein and incubated at 37 °C for 60 min. They samples were ran on Phos-tag SDS-PAGE gel to detect phospho-protein shift. *In vitro* kinase assay was performed as described previously (Yin et al., 2002). GST, GST-RD26, MBP-ABI1 (20ng) each were incubated with MBP-BIN2 kinase (200ng) each in 20 ul of kinase buffer [20 mm Tris (pH 7.5), 100 mm NaCl, and 12 mm MgCl2] and 10 μCi 32P-ATP. After incubation at 37 °C for 60 min, the reactions were stopped by adding 20 μl of 2 × sodium dodecyl sulfate (SDS) buffer and boiling for 5 min. Proteins were resolved by polyacrylamide gel electrophoresis (PAGE) and phosphorylation was detected by
exposing the dried gel to an X-ray film. The assays were repeated three times with similar results.

**Gene expression analysis by Real-Time qRT-PCR**

For drought-responsive and growth-responsive gene expression, total RNAs were extracted and purified from 2-week-old plants from *Col-0, bin2-d* and *bin2-3 bil1 bil2* 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.

**Drought tolerance assay**

Drought stress tolerance experiments were carried out as described previously (Tran et al., 2004). 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°C, 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 plants. The number of plants, which survived and continued to grow were counted, was counted after rewatering for 7 d.

3.6 References

Academy of Sciences, 106(11), 4543-4548.
redundantly with other Arabidopsis GSK3-like kinases to regulate brassinosteroid signaling. Plant Physiology, 150(2), 710-721.


3.7 Figures

Figure 1. Differential regulation of RD26 by BR and ABA.
(A) BL induced destabilization of the RD26 protein. Two-week-old RD26-MYC transgenic plants were treated with 1 μM BL or 1 μM BRZ or for indicated period of time and total protein extracts were used to detect RD26 (top), BES1 (middle) and a control protein (bottom). (B) Bikinin treatment leads to the degradation of RD26 protein. Two-week-old RD26-MYC transgenic plants were treated with 100 μM bikinin for the indicated times and total proteins extracts were used to detect RD26 (top), BES1 (middle) and a control protein (bottom). (C) ABA induced RD26 protein accumulation. Two-week-old RD26-MYC transgenic plants were treated with 1 μM ABA for indicated times and total protein extracts were used to detect RD26 (top) and a control protein (bottom). (D) ABA failed to induce RD26 protein in RD26 bin2-3bil1bil2 transgenic plants. Two transgenic lines of RD26 bin2-3bil1bil2 were used and treated with 1 μM ABA for 4 hours. The RD26 proteins were detected using anti-MYC antibody.
Figure 2. BIN2 interacts with RD26 in vitro and in vivo.

(A) In vitro pull down assay for BIN2/RD26 interaction. GST and GST-RD26 were incubated with purified MBP-BIN2 proteins and pull down with glutathione-sepharose beads. GST or GST-RD26 bound proteins were detected with anti-MBP antibody. (B) BIN2 interacts with RD26 through its DNA binding domain. Only RD26 N-terminal domain (aa. 1-140) showed interaction with MBP-BIN2. (C) BIN2 interacts with RD26 in BiFC assay. BIN2-cYFP and RD26-nYFP constructs were coinfiltrated in N. benthamiana leaves. Nuclear YFP signals were observed in cells with these two constructs but not in cells infiltrated with BIN2-cYFP and nYFP or cYFP and RD26-nYFP, used as negative controls. (D) Genetic interaction between BIN2 and RD26. RD26 suppress bin2-3 bil1 bil2 growth phenotype. Four-week-old plants of wild type Col-0, bin2-3 bil1 bil2, RD26 bin2-3 bil1 bil2 were shown. RD26 bin2-3 bil1 bil2 transgenic plants were confirmed by Western blotting using anti-MYC antibody.
Figure 3. RD26 is stabilized by BIN2 phosphorylation.

(A) BIN2 phosphorylates and stabilizes RD26 proteins. Equal amount of immunoprecipitated RD26-MYC proteins were treated with or without purified MBP-BIN2 for 1 hour. The samples were ran on Phos-Tag-SDS-PAGE followed by MYC immunoblot (Kinoshita, 2009). (B) BIN2 phosphorylates GST-RD26, but not GST, in the in vitro kinase assay. ● indicates autophosphorylated BIN2 and ★ indicates phosphorylated RD26. (C) Dose-dependent phosphorylation of RD26 by BIN2 kinase. (D) RD26 is phosphorylated in vivo. Immunoprecipitated RD26 proteins were treated with calf alkaline phosphatase (CIP), and separated on Phos-Tag-SDS-PAGE gel. CIP treatment led to one fast-migrating band and and one slow-migrating band, corresponding to unphosphorylated and phosphorylated RD26 proteins, respectively. A regular SDS-PAGE gel is shown on the bottom. (E) BL leads to dephosphorylated RD26 protein. RD26-MYC seedlings were grown on half-strength MS medium supplied with 1 μ M BL or 1 μ M BRZ for two weeks. Total proteins were extracted from seedlings and run on Phos-Tag gel (top) or regular SDS-PAGE gel (middle).
Figure 4. RD26 phosphorylation mediated transcription activation.

(A) The full-length RD26 structure (aa 1-297), including the NAM DNA binding domain, PEST motif and C-terminal domain, and truncated RD26 structure are shown. The * indicates potential BIN2 phosphorylation sites. (B) BIN2 mainly phosphorylates RD26 on its PEST domain. In vitro kinase assay using GST, GST-RD26 (1-140/1-182/1-204/1-297) and MBP-BIN2 was performed. The strongest phosphorylation band was observed when RD26 PEST motif is incorporated. (C) The structure of RD26 phosphorylation dead form (RD26MA) and phosphorylation mimicking form (RD26ME). (D) The phosphorylation of RD26 is required for its transcriptional activity. Luciferase transient assays were performed in N. benthamiana leaves with GLY17-LUC reporter, coinfiltrated with RD26, RD26MA, RD26ME, BIN2 effectors via Agrobacterium. The relative expression were normalized with total protein. The average and standard deviations were from quintic biological replicates. This assay has been repeated more than three times. The significance was determined base on student’s t-Test (*p<0.05, **p<0.01, n=5).
Figure 5. BIN2 positively regulate drought stress response.

(A) Drought-repressed genes were down-regulated in bin2-D mutants and up-regulated in bin2-3 bil1 bil2 mutants. Quantitative RT-PCR was performed using RNA from two-week-old seedlings. (B) Drought-induced genes were up-regulated in bin2-D mutants and down-regulated in bin2-3 bil1 bil2 mutants. (C) BIN2 gain-of-function mutants bin2-D displayed higher resistance to drought stress. Survival rate of Col-0, bin2-D and bin2-3 bil1 bil2 mutants plants were counted after drought treatment for three weeks and re-watered for seven days. (D) The survival rate of Col-0, bin2-D and bin2-3 bil1 bil2.
Figure 6. ABI1 is a positive regulator in BR signaling and dephosphorylates BIN2.
(A) Hypocotyl length of two-week-old light-grown seedlings grown in the absence and presence of BL of 100 nM were measured. (B) Hypocotyl length of two-week-old dark-grown seedlings grown in the absence and presence of BRZ of 2000 nM were measured. (C) ABI1 is a substrate of BIN2 and dephosphorylates BIN2. GST-RD26 and MBP-BIN2 proteins are incubated in the absence or presence of MBP-ABI1 (lane 2 & 5). Bikinin pre-incubation inhibits BIN2 autophosphorylation and RD26 phosphorylation (lane 3 & 4). (D) Phosphorylated ABI1 is more effective in dephosphorylating BIN2 compared with unphosphorylated ABI1. MBP-BIN2 and GST-RD26 proteins are pre-incubated in kinase buffer for one hour. After the incubation, GST-RD26 beads were saved before the adding of ABI1. Phosphorylated and unphosphorylated GST-ABI1 proteins were made in kinase buffer with BIN2 in the presence or absence of 1mM ATP.
Figure 7. RD26 regulation by BR and drought signaling through BIN2.
BR signaling negatively regulate BIN2, leading to RD26 destabilization. However, RD26 is induced by ABA signaling triggered by drought stress through the inactivation of ABI1 and activation of BIN2. BIN2 phosphorylates and stabilizes RD26 protein, resulting in RD26 transcriptional activity on its target genes. The BIN2 and RD26 module is likely to undergo crosstalk with other ABA signaling components.
3.8 Supplemental Materials

Figure S1. RD26 plays a positive role in seed germination under high salinity. (A) The germination rate of Col-0, RD26OX and RD26Q under salt stress condition. (B) Relative root length of Col-0, RD26OX and RD26Q under salt stress condition.

Figure S2. RD26 inhibits unphosphorylated BES1 protein in RD26OX.
Figure S3. Proteasome-mediated RD26 protein degradation.
RD26 transgenic plants 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 RD26 (top), BES1 (middle) and a control protein (bottom).

Figure S4. Stress and growth responsive gene expression study on RD26OX and rd26 anac019 anac055 anac102 quadruple mutant.
Figure S5. Drought stress response in BR and ABA signaling mutants.

Figure S6. ABI1 is a substrate of BIN2, but negatively dephosphorylates BIN2 and RD26 phosphorylation.
Figure S7. CIP, ABI1 and BIN2 treatment of immunoprecipitated RD26 protein.
Figure S8. BIN2 and RD26 exhibit similar expression profiles under drought condition.

The figure was generated using Arabidopsis e-FP browser at http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al., 2007. PLoS One 2(8): e718). The color scale at the left represents the relative expression of BIN2 and RD26: red means high BIN2 expression level, blue indicates high RD26 expression level, and yellow suggests similar expression levels between BIN2 and RD26.
Table S1 The primers used in this study.

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CHAPTER 4. GENERAL CONCLUSIONS AND PROSPECTIVE

Plants integrates multiple signaling outputs to coordinate growth, development and stress response through the crosstalk between phytohormones. An intriguing question for plant biologists to answer is how plants coordinate the trade-off between growth and defense. In this thesis, we investigated the interplay between growth-promoting hormone BRs and drought signaling pathways and revealed a molecular mechanism that coordinate plant growth and stress response.

Through genome-wide microarray experiments and ChIP-chip (Chromatin Immunoprecipitation followed by genomic tiling arrays) in Arabidopsis, we identified RD26 as a direct target of BES1, and found RD26 expression is repressed by BR and bes1-D. The fact that RD26 is up-regulated by abiotic stresses while down-regulated by BR makes it an ideal molecular candidate to investigate the crosstalk between BR and drought signaling pathways. Through genetic, genomic and biochemical studies, we discovered an antagonistic relationship between plant growth and defense mediated by RD26 and BES1 transcription factors. The antagonism is mediated by RD26 and BES1 protein interaction, which is confirmed by in vitro pull-down assay and in vivo split luciferase assay and co-immunoprecipitation assay. We showed RD26 and BES1 interact with each through their DNA binding/dimerization domains, supporting the idea that RD26 and BES1 form a heterodimer and inhibit each other’s function. Plant growth phenotype and stress response confirmed the antagonism between BR and drought signaling pathways. On the one hand, RD26 overexpression lines displayed stunted growth phenotype and suppressed bes1-D constitutive growth. RD26 overexpressors
appeared to be more sensitive to BRZ and less responsive to BL, while quadruple mutants rd26 anac019 anac055 anac102 were more resistant to BRZ and more responsive to BL. On the other hand, BR signaling mutants displayed opposite drought tolerance. Compared with wild type, bri1-5 is more resistant while bes1-D is more sensitive to drought stress. Furthermore, RNA-seq analysis revealed a large portion of BR-induced genes were repressed in RD26 overexpressors, and a large portion of BR-repressed genes were up-regulated in RD26 overexpressors.

We also examined if RD26 is regulated through an upstream kinase BIN2. As a well-established signaling kinase in BR pathway, BIN2 is recently reported to function in diverse biological processes including stomatal development, lateral root elongation, etiolated growth, seed germination, etc (Youn and Kim, 2014). Through our study, we demonstrated BIN2-dependent phosphorylation of RD26 is required for drought signaling. The interaction between BIN2 and RD26 is confirmed by in vitro GST pull-down, yeast two hybrid and in vivo BiFC assay. In addition, BIN2 directly phosphorylates RD26 through RD26 PEST domain. The phosphorylation results in RD26 protein stability and transcriptional activity. Our genetic study revealed that RD26 functions downstream of BIN2 in drought signaling pathway, as RD26 bin2-3 bill bil2 transgenic plants resemble the phenotype of RD26 overexpressors and restore plant drought tolerance. The positive role of BIN2 in drought signaling is strengthened based on the fact that bin2-D displayed higher resistance to drought stress compared with wild type. Furthermore, gene expression studies revealed drought-responsive genes are up-regulated in bin2-D while down-regulated in bin2-3 bill bil2. However, growth-responsive genes behave in an
opposite manner. Furthermore, we examined the interaction between BR and ABA signaling pathway. Interestingly, a regulatory phosphatase in ABA signaling pathway, ABI1, functions as a positive regulator in BR signaling pathway. *In vitro* kinase assay suggested BIN2 phosphorylates ABI1 and enhances ABI1 dephosphorylation activity, ABI1 in turn dephosphorylates BIN2 and inactivates RD26 activity. This delicate regulatory feedback mechanism ensures plants to fine-tune the strength of drought signaling outputs to reach a balance between plant growth and stress response.

Our studies thus established RD26 as a mediator between BR and drought signaling pathways coordinating plant growth and stress response. A model has been presented to demonstrate the molecular mechanisms of BR regulated stress response in *Arabidopsis*. In order to further understand the function, mechanism and regulatory network of BR in stress response, future work should center on:

1) Examining the potential regulation of SnRKS on RD26. Studies have revealed that ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis* (Yoshida et al., 2002; Geiger et al., 2009). Interestingly, a research group showed BIN2 positively regulates ABA signaling by phosphorylating SnRK2s (Cai et al., 2014). This raised the possibility that SnRK2s may also be involved in phosphorylating and regulating RD26 during drought signaling.

2) Understanding the feedback regulatory mechanism between ABI1 and BIN2. Our preliminary results showed BIN2 can phosphorylate ABI1 and ABI1 could dephosphorylate BIN2, revealing a complex regulatory mechanism between BIN2 kinase and ABI1 phosphatase. To better understand how the regulation is achieved and the
biological function of it, protein-protein interaction, phosphorylation site/domain mapping, in vivo phosphorylation assay between ABI1 and BIN2 need to be performed.

3) Characterizing the crosstalk between BRs with other hormones such as jasmonic acid and ethylene. Some studies suggested BRs promote stress resistance (Krishna, 2003; Kagale et al., 2007; Zhang et al., 2014), suggesting the complex function and regulatory mechanisms of BR in stress responses. The intricate roles of BRs in stress responses indicate the crosstalk with other phytohormones through different stress-responsive transcription factors. Analysis of bes1-D microarray data will identify other BES1 targeted transcription factors associated in other phytohormone signaling pathways.

4.1 References

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