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Relationships of brassinosteroid signaling with knox and wab1 genes on maize plant architecture

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Relationships of brassinosteroid signaling with knox and wab1 genes on maize plant architecture

by

Andree Sunanjaya Kusnandar

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Genetics

Program of Study Committee:
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Thomas Peterson

Iowa State University
Ames, Iowa
2016

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ABSTRACT

Improving yield through plant architecture manipulation might be an answer to resolve food and fuel problems. Brassinosteroid (BR) is a plant steroid hormone involved in plant architecture. However, the BR pathway is not well known in maize. In this study, we are interested to analyze the relationship among BR, knox, and wab1 in maize. knotted1-like homeobox (knox) genes encode homeodomain-containing transcription factors that play roles in regulating BR and plant architecture. wavy auricle in blade1 (wab1) encodes a TCP transcription factor (teosinte branched1, cycloidea, and PCF) that controls leaf architecture. In Arabidopsis, TCP1 also regulates BR. Phenotype and gene expression was analyzed for single and double mutants among maize brassinosteroid insensitive2 (bin2)-RNAi, zea mays brassinosteroid insensitive1 (zmbri1)-RNAi, Knotted1-O (Kn1-O), and Wab1-R lines. Phenotype and gene expression analysis by using qRT-PCR showed suppressed Kn1-O phenotype in Kn1-O/+; bin2-RNAi/+, while enhanced in Kn1-O/+; zmbri1-RNAi, indicating BR signaling suppression to kn1. However, the mechanism was still unclear because there were no significant results from BR genes expression analysis. Similarly, phenotype and gene expression analysis showed suppressed Wab1-R phenotype in Wab1-R/+; bin2-RNAi/+, while enhanced in Wab1-R/+; zmbri1-RNAi, indicating BR signaling suppression to wab1. Interestingly, internode phenotype and brassinosteroid dependent1 (brd1) gene expression analysis also indicate wab1 might regulate BR through brd1, which is similar to BR regulation by TCP1 in Arabidopsis through DWARF4 (DWF4). Gene expression analysis of knox genes and BR catabolism genes showed no significant results. In summary, BR signaling might inhibit kn1, however the mechanism is still unclear and other gene might play a role on the phenotype. BR signaling might inhibit wab1. While, wab1 might also regulate BR through
brd1, kn1 and wab1 might not be directly related. wab1 and BR catabolism genes also might not be directly related.
CHAPTER I. GENERAL INTRODUCTION

I.1. Maize and the importance of plant architecture

Food is one of the most important human basic needs. However, the increasing of human population caused a major issue. According to U.S. Census Bureau (www.census.gov), the world population was around 7 billion in 2010 and it is predicted that it will rise to more than 9 billion in 2050. Thus, it is a challenge for scientists to increase the food production. Some crops, for example rice, wheat, and maize, are consumed as a staple food by a large part of the world population (http://faostat.fao.org). Thus, increasing the yield of these major crops might be one of the answers for the world’s food problem.

Maize or corn (Zea mays) is a grain plant from the poaceae (also called gramineae or grasses) family which was originally a domestication of teosinte by the Mexican around 9,000 years ago (Matsuoka et al., 2002). With more than 170 million hectares of plantation area and more than 870 million tons of total production worldwide in 2012 (http://faostat.fao.org), maize is one of the most important crops in the world. Maize is mainly used for livestock feed, human consumption, and biofuel.

Plant architecture is one of the most important agronomic traits because it might affect plant cultivation, efficiency, and yield. Plant architecture is defined as the three dimensional organisation of the plant, including branching pattern, size, shape, and position of plant organs. In the Green Revolution, plant architecture modification played an important role on increasing the productivity (Reinhardt and Kuhlemeier, 2002).

One of the success stories during the Green Revolution was the development of new semi dwarf varieties of rice and wheat with enhanced yield (Athwal, 1971). These new varieties
of wheat were gibberellin abnormal varieties with shorter height and less biomass, but had increased grain yield. These new varieties were also more resistant to wind and rain damage (Hedden, 2003; Peng et al., 1999). Similarly, a semi dwarf rice phenotype also contributed to the significant increase of rice production during the Green Revolution. Later, it was known that the semi dwarf phenotype was caused by sd-1 gene, which encodes gibberellin 20-oxidase, an important enzyme in the gibberellin biosynthesis pathway. Even though, this variety has a semi dwarf phenotype, this variety has higher yield, increased harvest index, improved lodging resistance, and better response to fertilizer (Monna et al., 2002).

Other than for food and livestock feed, maize can also be used to produce biofuel. Due to the increasing world’s human population, expanding industries, and depleting fossil fuel, the world is facing a global fuel crisis. Biofuel from plants is one of the solutions to overcome this crisis.

Even though, there is a food versus fuel problem and harder to convert to biofuel compared to sugar, maize is the main crop used for producing biofuel in the United States (Service, 2007). Ethanol for biofuel can be produced from starch and lignocellulosic biomass. However, ethanol production from lignocellulosic biomass is more efficient because it has higher net energy gain and lower production costs (Yuan et al., 2008). Plant height is highly correlated to biomass yield. Modification of plant height, which is one of the components of plant architecture, might be a potential way to increase lignocellulosic biomass for biofuel production (Salas-Fernandez et al., 2009). Thus, understanding the mechanism of plant architecture might be useful not only to increase yield, but also to overcome other challenges.

There are many factors responsible for plant architecture. Environment factors, such as light, temperature, humidity, nutrition, and plant density, can affect plant architecture.
However, the plant’s genetic is the main factor that determine plant architecture (Wang and Li, 2008). During the latest few decades, our knowledge on the genetics of plant architecture has been greatly increased due to the abundance of studies on several model plants, such as *Arabidopsis thaliana*, rice, and maize, by many scientists (Reinhardt and Kuhlemeier, 2002; Wang and Li, 2008).

Genetic modification of plant growth regulators can potentially affect plant architecture, such as plant height, root architecture, and leaf arrangement, and is an excellent way to increase crop yields (Sakamoto *et al*., 2006). There are some plant growth regulators that are known related to plant architecture, including auxin, brassinosteroid (BR), and gibberellin (Lu *et al*., 2015; Salas-Fernandez *et al*., 2009). Some transcription factors genes, such as *knotted-like homeobox* (*knox*) and *wavy auricle in blade1* (*wab1*) genes are also important for plant development (Hake *et al*., 2004; Hay & Hake, 2004).

Lu *et al*. (2015) showed that modulating auxin related gene expression in rice can improve rice’s plant architecture by increasing the amount of tiller, enhancing the root system, and lengthening panicles, thus increasing the yield potential. As mentioned before, gibberellin abnormal varieties with dwarf phenotypes are one of the success stories of the Green Revolution, proving the importance of gibberellin. However, in this study, we are going to focus on BRs, *knox*, and *wab1* genes. Understanding the mechanism of these genes might be useful for plant architecture modification.
I.2. Brassinosteroids

Brassinosteroids (BRs) are steroid hormones in plants that regulate growth and development (Fujioka and Yokota, 2003). BRs are the sixth class of plant hormones other than abscisic acid, auxins, cytokinins, ethylene, and gibberellins (Taiz and Zeiger, 2010). BRs are similar to animal steroids and share many common features on the biosynthetic pathways (Clouse, 2002). However, unlike animal steroids, which bind to the nuclear receptor of transcription factors to regulate gene expression, BRs in plants bind to cell surface receptors (Thummel & Chory, 2002)

BRs are involved in many aspects of plant growth, development, and stress tolerance. Seed dormancy and germination, which are important for crop production efficiency and weed control, are positively regulated by BRs (Finch-Savage and Leubner-Metzger, 2006; Vriet et al., 2012). BRs are also involved in seed development and filling (Baud et al., 2009; Choe et al., 2001; Finch-Savage and Leubner-Metzger, 2006; Fitzgerald et al. 2009; Li et al., 2007; Reuzeau et al., 2005; Steber and McCourt, 2001; Tanaka et al., 2009; Wu et al., 2008). BRs are involved in primary root elongation and lateral root formation (Bao et al., 2004; Huang et al., 2010; Mussig et al., 2003).

BRs are also involved in fruit ripening, flowering time, fertility, photosynthesis, senescence, and photomorphogenesis (Crocco et al., 2011; Domagalska et al., 2007; Kozuka et al., 2010; Li et al., 2010; Nole-Wilson et al., 2010; Oh et al., 2011; Sorin et al., 2009; Vardhini and Rao, 2002; Ye et al., 2010). In maize, BRs plays a role in sex determination (Hartwig et al., 2011). Other than developmental roles, BRs are also important on biotic and abiotic stress tolerance, such as salt, drought, thermotolerance, oxidative stress, heavy metals,
herbicide, pesticide, and pathogen (Bajguz and Hayat, 2009; Gomes, 2011; Krishna, 2003; Vriet et al. 2012).

BRs are also playing an important role on plant architecture and biomass. BR deficient and insensitive mutants in *Arabidopsis*, pea, tomato, and rice generally showing dwarf phenotypes (Bishop and Koncz, 2002; Nakamura et al., 2006; Vriet et al., 2012). Plant height is one of the most important factors that might affect biomass yield (Salas-Fernandez et al., 2009), thus BRs play an important role in biomass. In rice, the Osdwf4 mutant shows erect leaves and a semi dwarf phenotype. Leaf angle is also an important trait because it allows higher density planting which will increase grain and biomass yield per hectare. Sakamoto et al. (2006) showed that Osdwf4 mutant can increase grain yield even without extra fertilizer.

As mentioned before, BRs are playing important roles in plants. Two strategies with promising results have been employed to increase yield by using BR. The first strategy is by using exogenous application and the second strategy is by using genetic manipulation (Vriet et al., 2012). However, producing synthetic BR molecules requires a lot of money and showed inconsistent results (Gomes, 2011; Khripach et al., 2000). On the other hand, genetic engineering strategy to manipulate the BRs shows an efficient, uniform, and predictable result (Divi and Krishna, 2009). However, with the exception of rice, not much is known about the BRs biosynthetic and signaling pathways in monocots, including maize (Vriet et al., 2012). Thus, understanding BRs mechanism in maize will be useful on maize modification to get plants with desired phenotypes.
I.2.1. Brassinosteroid signaling

Previous studies on BR biosynthesis or signaling mutants showed the importance of BR in many phases of plant development. Important advances have been made to make the BR signalling pathway as one of the best understood signal transduction pathways in plants (Zhu et al., 2013), even though not much is known in maize (Kir et al., 2015; Vriet et al., 2012).

**BRASSINOSTEROID INSENSITIVE1** (*BRI1*) is the first important BR signal transduction gene in *Arabidopsis* (Clouse et al., 1996). *BRI1* encodes a leucine rich repeat receptor kinase in the plasma membrane in *Arabidopsis* (Belkhadir & Chory, 2006). BR binds to *BRI1* to start the BR signaling pathway (Wang et al., 2012) by activating the *BRI1* kinase activity (Hothorn et al., 2011). As reviewed by Zhu et al. (2013), there are 3 factors that are involved in the activation of *BRI1*, the recruitment of co-receptor **BRI1-ASSOCIATED RECEPTOR KINASE1** (*BAK1*) (Gou et al., 2012), separation of inhibitor **BRI1 KINASE INHIBITOR1** (*BKI1*) (Jaillais et al., 2011), and sequential transphosphorylation between the kinase domains of *BRI1* and *BAK1* (Clouse, 2011). *BRI1* then phosphorylates **BRASSINOSTEROID-SIGNALLING KINASE1** (*BSK1*) and **CONSTITUTIVE DIFFERENTIAL GROWTH1** (*CDG1*), which are plasma membrane cytoplasmic kinases, to promotes their binding to **BRI1-SUPPRESSOR1** (*BSU1*), which is a phosphatase (Kim et al., 2011; Tang et al., 2008). The binding of *CDG1* and *BSK1* phosphorylates and activates *BSU1* to inactivates the GSK3-like kinase **BRASSINOSTEROID INSENSITIVE2** (*BIN2*) by dephosphorylating *BIN2* Tyr200 (Kim and Wang, 2010).

*BIN2* also plays an important role in BR signaling pathway by acting as the negative regulator of BR signaling (Tong et al., 2012). When BR concentration is low, *BIN2* is active and phosphorylates transcription factors **BRASSINAZOLE RESISTANT1** (*BZR1*) and *BZR2*
(also known as *BRI1-EMS-SUPPRESSOR1 (BES1)*) (He et al., 2002; Wang et al., 2002; Yin et al. 2002). Phosphorylation of *BZR1* and *BZR2* causes the elimination of their DNA binding activity and their cytoplasmic retention by 14-3-3 proteins (Bai et al., 2007). When BR concentration is high, *BSU1* inactivates *BIN2*, which then degraded by proteasome (Peng et al., 2008). Because *BIN2* is inactivated, *BZR1* and *BZR2* can become dephosphorylated and activated by *PROTEIN PHOSPHATASE 2A (PP2A)* (Tang et al., 2011).

Unphosphorylated *BZR1* and *BZR2* then move to nucleus and bind to the promoters to regulate BR biosynthetic genes that are involved in plant development and also involved in feedback regulation (He et al., 2005; Sun et al., 2010; Yu et al., 2011). *BZR1* targets transcription factors involved in light, gibberellin, and auxin signalling pathways (Sun et al., 2010).

There are some BR signaling mutants that have been studied. In *Arabidopsis*, *bri1* mutant shows a dwarf phenotype with reduced apical dominance and male sterility (Clouse et al., 1996). The rice *OsBRI1* mutant shows a similar phenotype, dwarf with shortened internodes and erect leaves (Yamamuro et al., 2000). Similarly, in maize, *zmbr1*-RNAi shows a dwarf phenotype with shortened internodes, reduced ligule, and auricle area (Kir et al., 2015). Ligule and auricle growth and differentiation are related to leaf angle, which is an important agronomic trait as explained before (Duvick, 2005).

In *Arabidopsis*, overexpressed *BIN2* mutant showed semidwarf phenotype with stunted growth, late flowering, and insensitive to exogenous brassinolide (BL), which is the most active BR in *Arabidopsis* (Li et al., 2001; Li and Nam, 2002). While, suppressed *BIN2* *Arabidopsis* mutant showed elongated and wavy petioles, narrow leaves, and increased BR signaling (Li and Nam, 2002; Yan et al., 2009). In rice, overexpressed *BIN2* mutant also
showed dwarf phenotype with erect leaves, shorter blade, shorter sheath, and BR insensitivity (Tong et al., 2012). Suppressed BIN2 rice mutant showed elongated but narrowed leaves, longer seeds, and increased BR signaling (Tong et al., 2012). In maize, bin2-RNAi showed novel phenotypes with shorter phenotype compared to the wild type due to shorter internodes, elongated leaf blades, elongated leaf sheathes, elongated male inflorescence, and expanded auricles (Kir et al., unpublished).

In Arabidopsis, bzr1-1D mutant, with increased BZR1 expression, can recover the BR insensitive BRI1 and BIN2 mutants and showed increased cell elongation when grown in the dark or in the absence of the upstream BR signaling. However, when grown in the light, bzr1-1D mutant showed reduced cell elongation and a weak dwarf phenotype due to BZR1 roles in regulating BR biosynthesis and downstream growth responses in Arabidopsis (He et al., 2005). BZR1 binds to the promoters of CPD and DWF4 genes, which are BR biosynthetic genes, and negatively regulates their expression (He et al., 2005). In rice, OsBZR1, with suppressed BZR1, showed dwarf phenotype with erect leaves and reduced BR sensitivity, indicating BZR1 as a positive regulator of BR signaling (Bai et al., 2007). Similar to Arabidopsis, D2, D11, and BRD1, which are BR biosynthetic genes in rice, are feedback regulated by BZR1 (Bai et al., 2007).

I.2.2. Brassinosteroids biosynthesis

As mentioned, previous studies on BR biosynthesis or signalling mutants showed the importance of BR in many phases of plant development. Gas chromatography-mass spectrometry (GC-MS) to identify metabolic products and comparison studies between BR
mutants and wild type have been very useful to understand BR biosynthetic pathway (Choi et al. 1997; Fujioka et al., 2000; Kwon and Choe, 2005; Noguchi et al., 2000).

As reviewed by Zhao and Li (2012), there are several steps in the known BR biosynthetic pathway. First, BR specific biosynthetic precursor campesterol (CR) is converted to campestanol (CN), before being converted to BL through the early and late C-6 oxidation pathways. In the early C-6 oxidation pathway, CN can be converted to 6-oxocampestanol (6-oxoCN) or hydroxylated at C-22 to form 6-deoxocathasterone (6-deoxoCT), which leads to the late C-6 oxidation pathway. If the 6-oxoCN was formed, then, it will be converted to, respectively, cathasterone (CT), teasterone (TE), 3-deydroteaserone (3DT), typhasterol (TY), and castasterone (CS). But, if 6-deoxoCT was formed, then, it will undergo similar conversion steps but in C-6 deoxy forms. Both pathways converge at CS. Finally, CS will be catalyzed to BL.

There are several known genes in the BR biosynthetic pathways. In Arabidopsis, T-DNA tagged CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) mutant showed dwarf phenotype and male sterility if grown in the light, while de-etiolated if grown in the dark (Szekeres et al., 1996). CPD encodes a cytochrome P450 (CYP90) and plays a role in catalyzing a C-3 oxidation of the early BR biosynthetic intermediates (Ohnishi et al., 2012; Szekeres et al., 1996). CPD is negatively regulated by BR signaling (Bancos et al., 2002).

DWARF4 (DWF4) is another gene encoding a cytochrome P450. DWF4 gene shares 43% sequence identity with CPD. dwf4 mutants can only be rescued by BRs but not by other phytohormones, indicating DWF4 is also involved in BR biosynthesis (Azpiroz et al., 1998; Choe et al., 1998). Later, it was found that DWF4 encodes a 22-hydroxylase and is involved in the multiple C-22 oxidation pathway by catalyzing CR to 22-OHCR, 4-en-3-one to 22-OH-4-
en-3-one, and 3-one to 22-OH-3-one (Fujioka et al., 2002; Fujita et al., 2006). In Arabidopsis and tobacco, overexpression of DWF4 increases vegetative growth and seed yield (Choe et al., 2001). In rice, a null DWF4 mutant, osdwarf4-1, showed slight dwarfism with erect leaves and without abnormality on the leaf, flower, and grain (Sakamoto et al., 2006). Arabidopsis mutant with overexpressed DWF4 produced more seeds compared to the wild type and increased the seed weight per plant by approximately 60% (Choe et al., 2001). In rice, overexpression of DWF4/CYP regulated by a promoter which is active in stems, leaves, and roots, but not in seeds, also resulted in larger and higher number seeds compared to the wild type, thus increasing grain yield per plant by 15 to 44% (Wu et al., 2008). However, reduced growth and seed production was observed in the rice with overexpression of the DWF4 gene controlled by a medium constitutive promoter (Reuzeau et al., 2005). DWF4 is negatively feedback regulated by BR signaling (Choe et al., 1998).

In rice, BRASSINOSTEROID DEPENDENT1 (BRD1) mutant showed very short leaf sheaths, short and curled leaf blades, and sterile phenotype when grown under normal growth condition. brd1 is a BR deficient mutant which has a 0.2-kb deletion in the genomic region of OsBR6ox, which encodes a cytochrome P450, resulting in the defective of the BR-6-oxidase. Exogenous brassinolide treatment to the mutant can help recover the mutant to normal phenotype (Mori et al., 2002). In maize, brd1-m1 mutant showed no internode elongation and no etiolation response when germinated in the dark condition, alteration in leaf and floral morphology. This mutant can also be rescued by giving exogenous brassinolide treatment (Makarevitch et al., 2012). This gene is negatively feedback regulated by BRs (Bai et al., 2007; Je et al., 2010).
PHYB ACTIVATION-TAGGED SUPPRESSOR1 (BAS1) encodes a cytochrome P450, CYP734A1 (was known as CYP72B1) (Neff et al., 1999; Nelson et al., 2004). BAS1 is a C-26 hydroxylase which converts CS and BL to their C-26 hydroxylated derivatives (Turk et al., 2003). In Arabidopsis, BAS1 mutant, bas1-D, showed suppression to the long hypocotyl phenotype of photoreceptor phytochrome B (phyB) mutant, dwarf phenotype similar to BR biosynthetic and signaling mutants, reduced BR levels and accumulation of 26-hydroxybrassinolide. BAS1 also plays a role in seedling growth, reduced BAS1 expression in seedlings increased the response to BRs under light, but decreased the sensitivity to light under some conditions, suggesting BAS1 plays a role as the control point between photoreceptor systems and the BR signaling pathway by far red light dependent modulation of BR levels (Neff et al., 1999; Turk et al., 2003).

1.3. knotted1-like homeobox (knox)

knoted-like homeobox (knox) genes encode homeodomain-containing transcription factors which play important roles in regulating development (Hake et al., 2004). The homeobox was initially found in Drosophila in genes that encode gene regulatory proteins which bind to specific DNA sequences and can produce homeotic phenotype when mutated (Gehring, 1987; Hake et al., 2004). Similar sequences can be found from insects and vertebrates, indicating the genetic control of development mechanisms might be similar (Gehring, 1987). In maize, the knotted1 (kn1) gene was isolated using transposon tagging from a mutant which showed knotted phenotype on the leaves (Vollbrecht et al., 1991). This gene was revealed to encode a member of the homeodomain superfamily of transcription factors which indicate a similar mechanism of developmental gene regulation in plants (Hake et al.,
In maize, \textit{knl} was revealed to target more than 5000 loci by using chromatin immunoprecipitation and next generation sequencing (ChiP-seq) (Bolduc et al., 2012).

In maize, the phenotype of \textit{knl} gene mutant was first observed in the dominant \textit{Kn1-O} mutant (Bryan & Sass, 1941; Hake et al., 2004). Later, this mutant was found to have a 17-kb tandem duplication of coding and non-coding regions of \textit{knl} (Veit et al., 1990). \textit{Kn1-O} mutants showed knotted phenotypes on the leaves, resulting in ectopic sheath, auricle, and ligule tissues, compared to normal maize leaves which have a proximal sheath and distal blade, separated by the ligule and auricle (Bryan & Sass, 1941; Hake et al., 2004). \textit{Kn1-O} phenotype can be rescued by the insertion of Mu transposons in the promoter area of \textit{knl} gene (Lowe et al., 1992). A \textit{knl} loss-of-function mutant showed reduced shoots formation and abnormal reproductive development (Kerstetter et al., 1997; Vollbrecht et al., 2000).

\textit{knox} proteins contain three extra amino acids between helix 1 and helix 2 (Bertolino et al., 1995), indicating \textit{knox} genes belong to the TALE (three amino acid loop extension) family which can be found in all green plant lineages (Hake et al., 2004; Hay & Tsiantis, 2010). Based on the sequence similarity in the homeodomain, expression, intron, and phylogenetic analysis, \textit{knox} genes can be categorized into two classes (Kerstetter et al., 1994, Reiser et al., 2000; Mukherjee et al., 2009). Class 1 \textit{knox} genes, in which \textit{knl} gene is included, are expressed in the shoot apical meristem (SAM) of monocot and eudicot plants, while the expression of class 2 \textit{knox} genes are more diverse and not much are known about the functions (Hake et al., 2004; Jackson et al., 1994; Kerstetter et al., 1994; Serikawa et al. 1997; Zhong et al., 2008). Overexpression of class 1 \textit{knox} genes can alter leaf morphology and development, resulting in shorter plants, darker leaves color, reduced leaf size, and isodiametric cells (Hake et al., 2004).
In maize and *Arabidopsis*, class 1 *knox* genes are expressed in the shoot meristem and subtending stem, but is not expressed in the leaf, even though, in maize, *kn1* protein was detected in the proximal leaf base which might affect the proximal-distal patterning of leaves (Hake et al., 2004; Jackson, 2004; Kim et al., 2005). The SAM is a pluripotent stem cell population on which plant organs, such as leaves, stems, and flowers, initiate. After leaves initiate, *knox* genes maintain normal development by balancing the organ initiation and meristem renewal by modulating a balance between different plant hormones (Bolduc et al., 2012).

A study by Bolduc et al. (2012) by using ChiP-seq to analyze differentially expressed genes between normal and *kn1* mutants indicates *kn1* genes as positive regulator of auxin and directly regulating genes involved in auxin synthesis, transport, and signaling. Tobacco and lettuce mutants with overexpressed *knox* genes showed an increase in cytokinin concentration and phenotype similar to plants with increased cytokinin, indicating *knox* and cytokinin have similar roles in senescence repression and apical dominance (Frugis et al., 2001; Tamaoki et al., 1997). The *Arabidopsis*, *SHOOT MERISTEMLESS* (*STM*), which is a *knox* gene, mutant can be recovered by giving exogenous cytokinin and expression of *IPT*, a cytokinin biosynthesis gene, through *STM* promoter. Activation of three different *KNOXI* proteins in *Arabidopsis* is also increasing the expression of *ISOPENTENYL TRANSFERASE 7* (*AtIPT7*), a cytokinin biosynthesis gene, and *ARR5*, a cytokinin response factor. These indicate, in *Arabidopsis*, *KNOXI* genes positively regulate cytokinin biosynthesis (Yanai et al., 2005).

In relation with gibberellin, in maize, *knox* genes negatively regulating gibberellin biosynthesis through *ga2ox1*, which encodes gibberellin inactivation enzyme. In overexpressed *knox* mutant, *ga2ox1* was found to be overexpressed in immature leaves, while
in *knox* mutant with null allele, *ga2ox1* was found to be down regulated in reproductive meristem (Bolduc & Hake, 2009). In tobacco, *NICOTIANA TABACUM HOMEobox 15 (NTH15)*, a *knox* gene, suppressed GA 20-oxidase, a gibberellin biosynthetic gene, resulting in the decrease of gibberellin (Sakamoto et al., 2001).

In rice, overexpression of *ORYZA SATIVA HOMEobox 1 (OSH1)*, a *knox* gene, showed insensitivity to BR, while loss of function of *OSH1* showed increasing BR production. *OSH1* induction can increase the expression of *CYP734A2, CYP734A4*, and *CYP734A6*, which are BR catabolism genes and homologous of *Arabidopsis BAS1. CYP734A RNA interference (RNAi) mutant showed similar phenotype to *osh1* and suppressed SAM growth. These suggests *OSH1* gene plays a role in negatively regulating BR through the activation of BR catabolism genes (Tsuda et al., 2014). In maize, *rough sheath1 (rs1)*, which is a *knox* gene, mutant with gain of function allele, *Rs1-O*, showed a similar phenotype to *zmbril*-RNAi (Kir et al., 2015)

**I.4. wavy auricle in blade1 (wab1)**

*wavy auricle in blade1 (wab1)* encodes a TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factor. *wab1* gene is expressed in the boundary between lateral organs and the meristem and plays a role in regulating tassel branch angle. *wab1* is normally not expressed in leaves (Lewis et al., 2014).

In maize, *Wab1-R* mutant, which is a dominant mutant, showed ectopic ligule, ectopic auricle, and narrower leaves compared to normal leaves. While, *wab1* loss of function mutant showed decreased tassel branches and angle, suggesting the expression in the boundary is required for branch initiation. Even though the disruptions of the proximal distal patterning in
Wab1-R mutant is similar to knox mutants, wab1 is not a knox gene (Lewis et al., 2014; Hay and Hake, 2004).

The wab1 gene regulates liguleless1 (lg1) and acts upstream of lg1 in tassel branch angle regulation (Lewis et al., 2014). lg1 encodes a squamosal promoter binding protein and important for ligule and auricle development. In maize, ligule and auricle were not developed in loss of function lg1 mutant resulting in more erect leaves (Moreno et al., 1997). In maize leaves, wab1 is not required for lg1 gene expression, but Wab1 mutant, with increased wab1 gene expression, showed an increase on the lg1 gene expression (Lewis et al., 2014).

In rice, lg1 regulates pulvinus growth (Ishii et al., 2013). CYP734A RNAi showed derepression of pulvinus growth consistent with BR function in this process (Tsuda et al., 2011). In Arabidopsis, TCP1 regulates BR biosynthesis through DWF4 regulation (Guo et al., 2010). These results indicate that there might be a connection among BR, lg1, and wab1. In rice, CYP734A, which is a homolog of BAS1 in Arabidopsis, expression is also regulated by OSH1, a knox gene (Tsuda et al., 2014). This also indicate a connection of knox gene to the pathway.


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CHAPTER II. GENE EXPRESSION ANALYSIS OF BRASSINOSTEROID MUTANTS, BRI1-RNAI AND BIN2-RNAI, IN RELATION TO KN1-O, A KNOX MUTANT

II.1. Abstract

Growing human population is a challenge due to increasing food and fuel demands. As one of the most important source of food, livestock feed, and biofuel, increasing maize productivity through architecture manipulation might be an answer for this challenge. Brassinosteroid (BR) is a plant hormone that plays an important role in plant development including plant architecture. *knox* genes encode homeodomain-containing transcription factors that play roles in negatively regulating BR by activating BR catabolism genes in rice. In this study, we are interested to analyze the relationship between BR and *knox* genes in maize. A *knox* dominant mutant, *Knotted1-O* (*Kn1-O*), and BR RNAi lines were used to produce double mutants of *Kn1-O/+; bin2-RNAi/+* and *Kn1-O/+; zmbril-RNAi/+*. *Kn1-O/+; bin2-RNAi/+* showed similar phenotype to *bin2-RNAi/+* with shorter phenotype, twisted and wavy leaves, and knots from *Kn1-O/+*. However, the knots in *Kn1-O/+; bin2-RNAi/+* were reduced compared to *Kn1-O/+* indicating suppression of *Kn1-O/+* phenotype. *Kn1-O/+; zmbril-RNAi/+* also showed enhanced *Kn1-O/+* phenotype with dwarf phenotype, reduced ligule and auricle area, also knots all around the leaves. Gene expression analysis of *kn1* showed consistent results with the phenotypic observations. Compared to in *Kn1-O/+* single mutant, *kn1* gene expression in *Kn1-O/+; bin2-RNAi/+* was decreased, while, increased in *Kn1-O/+; zmbril-RNAi/+*. However, the gene expression analysis results were not statistically significant, thus, the mechanism is still unclear.
II.2. Introduction

Growing human population in the world makes a challenge for scientists due to increasing food and fuel demands. Maize is one of the most important crops in the world and mainly used for human consumption, livestock feed, and biofuel. New varieties of rice and wheat with modified plant architecture are some of the success stories during the Green Revolution. These new varieties had dwarf phenotype due to gibberellin abnormalities but increased the grain yield and also more resistant to wind and rain damage (Athwayl, 1971; Hedden, 2003; Monna et al., 2002; Peng et al., 1999). In this study, we would like to analyze some genes related to maize architecture.

One of the factors that can affect plant architecture is brassinosteroid (BR). BRs are steroid hormones, which are similar and share many common features with animal steroids, that can be found throughout the plant kingdom and are important on plants growth and development (Clouse, 2002; Fujioka and Yokota, 2003). BR deficient and insensitive mutants are generally showing dwarf phenotype (Bishop and Koncz, 2002; Nakamura et al., 2006; Vriet et al., 2012). In rice, erect leaves phenotype has also been reported on the Osdwarf4 mutant (Sakamoto et al., 2006). Leaf angle is also an important agronomic trait because it allows higher density planting on the field, which will increase grain and biomass yield per hectare.

BR signaling and biosynthesis pathways are well known in Arabidopsis and rice, however not much is known in maize (Kir et al., 2015; Vriet et al., 2012). In recent publication, we reported the BRASSINOSTEROID INSENSITIVE1 (BRI1) RNA interference (RNAi) mutant, zmbri1-RNAi (Kir et al., 2015). BRI1 encodes a leucine rich repeat receptor kinase in plasma membrane and important for BR signaling pathway induction (Belkhadir & Chory, 2006). BR binds to BRI1 to start the BR signaling pathway (Wang et al., 2012) by activating
the *BRI1* kinase activity (Hothorn et al., 2011). In *zmbr1*-RNAi, the *BRI1* genes expression were suppressed resulting in dwarf phenotype with shortened internodes and reduced ligule and auricle area (Kir et al., 2015). Similar phenotype was observed in other *bri1* mutants. In *Arabidopsis*, *bri1* mutants showed dwarf phenotype with reduced apical dominance and sterile male inflorescence (Clouse et al., 1996). In rice, *OsBRI1* mutant showed dwarf phenotype with shortened internodes and erect leaves (Yamamuro et al., 2000).

**BRASSINOSTEROID INSENSITIVE2** (*BIN2*) RNAi line, *bin2*-RNAi, has also been constructed in maize (Kir et al., unpublished). *BIN2* is a GSK3-like kinase, which is also important in BR signaling pathway due to its role as the negative regulator of BR signaling pathway (Tong et al., 2012). When BR concentration is low, *BIN2* inhibits **BRASSINAZOLE RESISTANT1** (*BZR1*) and *BZR2* (also known as **BRII-EMS-SUPPRESSOR1** (*BES1*)), which are transcription factors and important for the regulation of many downstream genes, including genes related to BR biosynthesis. When, BR concentration is high, *BIN2* is deactivated by **BRII-SUPPRESSOR1** (*BSU1*), resulting in the activation of *BZR1* and *BZR2* (He et al., 2002; He et al., 2005; Peng et al., 2008; Sun et al., 2010; Wang et al., 2002; Yin et al. 2002; Yu et al., 2011).

In maize, *bin2*-RNAi showed a shorter phenotypes compared to the wild type due to shorter internodes, which is unexpected. *bin2*-RNAi also showed elongated leaf blades, elongated leaf sheathes, elongated male inflorescence, and expanded auricles (Kir et al., unpublished). In *Arabidopsis*, mutant with overexpressed *BIN2* showed semi-dwarf phenotype, late flowering, and insensitive to exogenous brassinolide (BL) treatment (Li et al., 2001; Li and Nam, 2002). While, mutant with suppressed *BIN2* showed elongated and wavy petioles, narrow leaves, and increased BR signaling (Li and Nam, 2002; Yan et al., 2009). In rice, mutant
with overexpressed BIN2 also showed dwarf phenotype with erect leaves, shorter blade, shorter sheath, and insensitive to BR. While, mutant with suppressed BIN2 showed elongated but narrowed leaves, longer seeds, and increased BR signaling (Tong et al., 2012).

**BRASSINOSTEROID DEPENDENT1 (BRD1)**, **CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD)**, and **DWARF4 (DWF4)**, encode cytochrome P450 and play a role as BR biosynthetic genes (Azpiroz et al., 1998; Choe et al., 1998; Mori et al., 2002; Ohnishi et al., 2012; Szekeres et al., 1996). These genes are negatively regulated by BR signaling (Bai et al., 2007; Bancos et al., 2002; Choe et al., 1998; Je et al., 2010). Mutation on these genes can also lead to disruption on plant architecture, including dwarf phenotype and abnormal leaf and flower (Makarevitch et al., 2012; Sakamoto et al., 2006; Szekeres et al., 1996). **PHYB ACTIVATION-TAGGED SUPPRESSOR1 (BAS1)** is another gene related to BR biosynthesis. BAS1 encodes a cytochrome P450, **CYP734A1** (was known as **CYP72B1**), and plays a role as C-26 hydroxylase which converts CS and BL to their C-26 hydroxylated derivatives (Neff et al., 1999; Nelson et al., 2004; Turk et al., 2003). In Arabidopsis, bas1-D showed dwarf phenotype and reduced BRs level, similar to BR biosynthetic and signaling mutants.

In rice, **CYP734A2**, **CYP734A4**, and **CYP734A6**, which are the homologs of BAS1, genes expression is regulated by **ORYZA SATIVA HOMEBOX1 (OSH1)**, a member of **knotted-like homebox (knox)** genes. Overexpression of OSH1 decreases BR sensitivity, while decreasing OSH1 expression can increase BR production. This indicates that OSH1 plays a role in negatively regulating BR through the activation of BR catabolism genes (Tsuda et al., 2014). In maize, another knox mutant with a dominant allele, **Rough sheath1-O (Rs1-O)**, showed a similar phenotype to zmbr1-RNAi (Kir et al., 2015).
*knox* encodes homeodomain-containing transcription factor and important in regulating development (Hake et al., 2004). *knox* genes regulate some plant hormones, including auxin, cytokinin, gibberellin, and brassinosteroid (Bolduc et al., 2012; Bolduc & Hake, 2009; Sakamoto et al., 2001; Tsuda et al., 2014; Yanai et al., 2005). In maize, *knotted1* (*kn1*) dominant mutant, *Kn1-O*, showed knotted phenotype on the leaves, resulting in ectopic sheath, auricle, and ligule tissues (Hake et al., 2004). While, *kn1* loss of function mutant showed reduced shoots formation and abnormal reproductive development (Kerstetter et al., 1997; Vollbrecht et al., 2000). In this study, we are interested to analyze the relationship between BR and *knox* genes through gene expression analysis of *Kn1-O* and BRs RNAi line mutants.

**II.3. Materials and methods**

**Genetic stocks**

*Kn1-O* was obtained from the maize genetic stocks. *zmbr1*-RNAi was obtained from Dr. Philip W. Becraft and constructed by Gokhan Kir (Kir et al., 2015). *bin2*-RNAi was also obtained from Dr. Philip W. Becraft and constructed by Gokhan Kir (Kir et al., unpublished). *Kn1-O* was backcrossed to B73 background. *zmbr1*-RNAi was backcrossed to W22 and B73 background. *bin2*-RNAi was backcrossed to W22 background. *Kn1-O/+; zmbr1*-RNAi/+ double mutants was obtained from crossing the *Kn1-O/+* with *zmbr1*-RNAi/+. Similarly, the *Kn1-O/+; bin2*-RNAi double mutants was obtained from crossing the *Kn1-O/+* with *bin2*-RNAi/+.
RNA preparation

The segregating Kn1-O/+; zmbri1-RNAi/+ and Kn1-O/+; bin2-RNAi lines were planted at Iowa State University Woodruff Farm in Ames, IA. Samples were screened by using Liberty™ herbicide and genotyped by using herbicide bialaphos (bar) resistance gene primers GGCACAGGGCTTCAAGAG and AGTTCCCCGTGCTTGAGC. Primers specific for the bin2 RNAi, GTTTGCCACAGGGATGTGAAACCA and GATCGATCTGAATAAGGGGAAC, were also used to genotype Kn1-O/+; bin2-RNAi segregating line.

Plants were grown until they reach around ten-leaf seedling stage. Samples for RNA purification were collected from tissues approximately 2 mm around the ligule and auricle area. Tissues from plastochron 6 to 9 were used. Tissues were dissected under RNAlater™ and preserved also by using RNAlater™. Tissues were stored at -20°C before long term storage at -80°C or used for RNA purification.

qRT-PCR

RNA purifications were done by using QIAgen RNeasy Mini Kit according to the manufacturer’s protocol. RNA concentrations were measured by using NanoDrop ND-1000 spectrophotometer. Approximately 1 to 2 µg of RNA was used for each samples. RNA was treated with RQ1 RNase-free DNase (Promega). A total of 1 µL of RQ1 DNase and 1 µL of RQ1 buffer were used. Volumes were adjusted to 10 µL using nuclease-free water. Samples were incubated at 37°C for 30 min. The reaction was stopped by adding 1 µL of RQ1 DNase stop solution and incubated at 65°C for 10 min. A total of 8 µL from each samples were used for reverse transcription PCR (RT-PCR). RT-PCR was performed by using ThermoFisher
Scientific SuperScript® III First-Strand Synthesis System according to the manufacturer’s protocol. To check whether the cDNA construction successful, 1 µL of the cDNA was used for confirmation PCR using a ubiquitin-conjugating enzyme gene (GRMZM2G027378) primers, AACATCCTAACCCAGCTCAAG and CTGTTGGATCCCATGACGG.

qRT-PCR was performed by using iQ SYBR Green Supermix (Bio-Rad) and gene specific primers on a Stratagene MX4000 instrument at the Iowa State University DNA facility. A ubiquitin-conjugating enzyme gene (GRMZM2G027378) was used as the reference gene. A minimum of three biological and two technical replicates were used for each analysis. Gene expression level was analyzed by using a relative quantification strategy, delta delta Ct.

\[
\Delta Ct_1 = Ct \text{ (gene of interest in sample with trait of interest or mutant)} - Ct \text{ (reference gene in sample with trait of interest or mutant)}
\]

\[
\Delta Ct_2 = Ct \text{ (gene of interest in wild type or control)} - Ct \text{ (reference gene in wild type or control)}
\]

\[
\Delta \Delta Ct = \Delta Ct_1 \text{ (sample with trait of interest or mutant)} - \Delta Ct_2 \text{ (wild type or control)}
\]

Normalized target gene expression level = \(2^{-\Delta \Delta Ct}\)

Statistical comparisons two tailed T-Test assuming equal variance to gain the p-value were made using JMP 10.0 statistical software.
II.4. Results and discussion

*bin2-RNAi suppresses the Kn1-O phenotype*

From the cross between *Kn1-O/+* and *bin2-RNAi/+*, we got a segregation of 1 : 1 : 1 : 1 for wild type, *bin2-RNAi/+*, *Kn1-O/+*, and *Kn1-O/+; bin2-RNAi/+*. We observed around 70 plants of this line. *bin2-RNAi/+* showed shorter phenotype compared to wild type, which was unexpected because we expect increased BR signaling in *bin2-RNAi* and larger phenotype. We also observed twisted and wavy leaves, elongated sheath, and expanded auricles, consistent with previous observation by Kir et al. (unpublished). *Kn1-O/+* in B73 background showed knotted phenotype on the leaves, consistent with previous publication by Hake et al., (2004). *Kn1-O/+; bin2-RNAi/+* in W22 background showed similar phenotype to *bin2-RNAi/+*, with shorter phenotype, twisted and wavy leaves, and expanded auricles. Knots were also observed in *Kn1-O/+; bin2-RNAi/+*, however the amount of knots on *Kn1-O/+; bin2-RNAi/+* were reduced as shown in the comparison of leaf 9 from representative plants (Figure 2). These suggest *bin2-RNAi* suppresses *Kn1-O* phenotype (Figure 1 and Figure 2).

*zmbri1-RNAi enhances Kn1-O phenotype*

We also observed around 70 plants of the *Kn1-O/+; zmbri1-RNAi/+* line. *zmbri1-RNAi/+* in W22 and B73 mixed background showed dwarf phenotype with reduced ligule and auricle area, consistent with previous publication by Kir et al. (2015). Similar to *zmbri1-RNAi/+*, *Kn1-O/+; zmbri1-RNAi/+* also showed dwarf phenotype with knots formation on leaf blades. *Kn1-O/+; zmbri1-RNAi/+* also showed reduced ligule and auricle area, which is similar to *zmbri1-RNAi/+*, when the area was not covered by knots, while the ligule and auricle area of *Kn1-O/+* was similar to wild type when the area was not covered by knots (Figure 3 and
Figure 4). However, Kn1-O/+; zmbri1-RNAi/+ has a shorter phenotype and more ectopic sheath tissue on the leaf blades compared to Kn1-O/+. The observation indicates that zmbri1-RNAi enhances Kn1-O phenotype.

**BR signaling might inhibit kn1**

From the phenotype observation, there is an indication that BR signaling might inhibit kn1. In Kn1-O/+; bin2-RNAi/+ with increased BR signaling, the phenotype of Kn1-O/+ was suppressed. While, in Kn1-O/+; zmbri1-RNAi/+ with decreased BR signaling, the phenotype of Kn1-O/+ was enhanced. These indicate that there might a connection between BR signaling and kn1. kn1 might regulate BR signaling or BR signaling might regulate kn1. To test the hypotheses, expression analysis was performed using qRT-PCR.

In Kn1-O/+; bin2-RNAi+/, kn1 expression level was only slightly decreased and not statistically significant compared to kn1 expression in Kn1-O/++. This result is unexpected because the phenotype observation showed that the Kn1-O phenotype was strongly inhibited by bin2-RNAi+. In Kn1-O/+; zmbri1-RNAi+/, kn1 expression level was increased, even though also not statistically significant, compared to kn1 expression in Kn1-O/+ (Figure 5A and Figure 6A). Even though the kn1 expression analysis was a bit unexpected, the results were still consistent with the observed phenotype, which indicates BR signaling might inhibit kn1.

Another knox gene, rough sheath1 (rs1), was also tested (Figure 5B and Figure 6B). rs1 expression was not significantly changed in any mutant. In previous publication by Kir et al. (2015), rs1 dominant mutant, Rs1-O, showed similar phenotype to zmbri-RNAi. qRT-PCR analysis of Rs1-O/+ showed that dwf4, which is a BR biosynthetic gene, expression was significantly increased, while the bri1b expression was significantly decreased. This also
indicates the relationship between BR and \textit{knox} genes in maize (Figure S 1). However, \textit{dwf4} expression was not significantly changed in \textit{Kn1-O/+, Kn1-O/+; bin2-RNAi/+}, and \textit{Kn1-O/+; zmbri1-RNAi/+}. Another BR biosynthetic gene, \textit{brd1}, was also not showing any significant difference in \textit{Kn1-O/+; Kn1-O/+; bin2-RNAi/+}, and \textit{Kn1-O/+; zmbri1-RNAi/+}. \textit{cpd}, which is also a BR biosynthetic gene, showed significant decrease in \textit{Kn1-O/+ from Kn1-O/+; bin2-RNAi/+} line. However, \textit{cpd} did not show any significant change in \textit{Kn1-O/+ from Kn1-O/+; zmbri1-RNAi/+} line. \textit{cpd} also did not show any significant change in \textit{Kn1-O/+; bin2-RNAi/+} and \textit{Kn1-O/+; zmbri1-RNAi/+} (Figure 5C and Figure 6C).

In rice, a \textit{knox} gene, \textit{OSH1}, negatively regulates BR by activating \textit{CYP734A2}, \textit{CYP734A4}, and \textit{CYP734A6}, which are BR catabolism genes and homologs of \textit{Arabidopsis BAS1}. \textit{OSH1} overexpression can decrease BR sensitivity, while suppressed \textit{OSH1} can increase BR production (Tsuda et al., 2014). Thus, we tested \textit{BAS1} homologs, \textit{bas1}, \textit{bas2}, and \textit{bas3}. However, there is no significant difference observed from the \textit{BAS1} homologs gene expression analysis (Figure 5C and Figure 6C).

From the phenotype observations, there is an indication that BR signaling might inhibit \textit{kn1}, however the mechanism is still unclear.
II.5. Figures and tables

Figure 1. Kn1-0/++; bin2-RNAi/+ showed similar phenotype to bin2-RNAi/+. (A) From left to right: Kn1-0/++; bin2-RNAi/+, bin2-RNAi/+, Kn1-0/+, wild type. (B) bin2-RNAi on the field. (C) Kn1-0/++; bin2-RNAi/+ on the field. Similar to bin2-RNAi/+, Kn1-0/++; bin2-RNAi/+ showed shorter phenotype, twisted and wavy leaves.
**Figure 2.** *Kn1-O* and *bin2*-RNAi leaves phenotype comparison.

*Kn1-O/+; bin2-RNAi/+* looks similar to *bin2-RNAi/+* but with knots, similar to *Kn1-O/+*. Knots on *Kn1-O/+; bin2-RNAi/+* are reduced compared to *Kn1-O/+*. (A) Ligule and auricle comparison, from left to right: *Kn1-O/+; bin2-RNAi/+*, *bin2-RNAi/+*, *Kn1-O/+*, wild type. Similar to *bin2-RNAi/+*, *Kn1-O/+; bin2-RNAi/+* showed expanded auricle and ligule compared to wild type. The auricle and ligule area of *Kn1-O/+* was difficult to observed due to the ectopic phenotype caused by the knots. (B) Blade comparison, from left to right: *Kn1-O/+; bin2-RNAi/+*, *bin2-RNAi/+*, *Kn1-O/+*, wild type. *Kn1-O/+; bin2-RNAi/+* showed wavy leaf, similar to *bin2-RNAi/+*. Knots were also observed on the *Kn1-O/+; bin2-RNAi/+* leaf, but greatly reduced. Leaves number 9 were used on the comparison.
Figure 3. *Kn1-O/+; zmbri1-RNAi/+* showed enhanced phenotype. *Kn1-O/+; zmbri1-RNAi/+* and *zmbri1-RNAi/+* showed dwarf phenotype. *Kn1-O/+; zmbri1-RNAi/+* also showed knots similar to *Kn1-O/+*. From left to right: *Kn1-O/+; zmbri1-RNAi/+*, *zmbri1-RNAi/+*, *Kn1-O/+*, wild type.
Figure 4. Kn1-O and zmbri1-RNAi leaves phenotype comparison. 
Kn1-O/+; zmbri1-RNAi/+ looks similar to zmbri1-RNAi/+. (A) Ligule and auricle comparison, from left to right: Kn1-O/+; zmbri1-RNAi/+, zmbri1-RNAi/+, Kn1-O/+, wild type. (B) Ligule and auricle comparison from different plants, from left to right: Kn1-O/+; zmbri1-RNAi/+, zmbri1-RNAi/+, Kn1-O/+, wild type. Similar to zmbri1-RNAi/+, Kn1-O/+; zmbri1-RNAi/+ showed reduced ligule and auricle area.
Figure 5. qRT-PCR analysis of the *Kn1-O/l*; *bin2-RNAi/+* line using ∆∆Ct method. (A) *kn1* gene expression analysis showed *kn1* expression in *Kn1-O/l*; *bin2-RNAi/+* is decreased compared to the *Kn1-O/l* but not statistically significant. (B) *rs1* gene expression was slightly decreased. However, the gene expression was very small, thus, any change in the gene expression might be caused by technical error. (C) BR catabolism gene homologs, *bas1*, *bas2*, and *bas3* were differently expressed but not statistically significant. BR biosynthesis genes, *brd1*, *cpd*, and *dwf4*, expression analysis showed *cpd* gene expression was significantly decreased in *Kn1-O/l+. Y* axis indicates fold change in gene expression level relative to the wild type. Student’s t test was performed assuming equal variances.
Figure 6. qRT-PCR analysis of the Kn1-OI+; zmbr1-RNAi/+ line using ΔΔCt method. (A) kn1 gene expression analysis showed kn1 expression in Kn1-OI+; zmbr1-RNAi/+ is increased compared to the Kn1-OI+ but not statistically significant. (B) rs1 gene expression are slightly decreased. However, the gene expression was very small, thus, any change in the gene expression might be caused by technical error. (C) BR catabolism gene homologs, bas1, bas2, and bas3 were differently expressed but not statistically significant. BR biosynthesis genes, brd1, cpd, and dwf4, were also differently expressed but not statistically significant. Y axis indicates fold change in gene expression level relative to the wild type. Student's t test was performed assuming equal variances.
References


CHAPTER III. GENE EXPRESSION ANALYSIS OF BRASSINOSTEROID MUTANTS, 
BRI1-RNAi AND BIN2-RNAi, IN RELATION TO TCP TRANSCRIPTION FACTOR, 
WAB1, GENE

III.1. Abstract

Plant architecture is one of the most important agronomic traits. Plant architecture modification in other plants have showed successful results on increasing yield and resistance to abiotic factors, such as wind and rain damage. Brassinosteroid (BR), which is a plant steroid hormone, is one of the factors that is involved in plant architecture. Wavy auricle in blade1 (wab1) encodes a TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factor. In Arabidopsis, TCP1 regulates BR biosynthesis through DWF4 regulation. This indicates that there might be a connection between wab1 and BR pathway. In this study, we are interested to analyze the relationship between wab1 and BR in maize. To answer this question, we used a wab1 dominant mutant, Wab1-R, and BR RNAi mutants, zmbri1-RNAi and bin2-RNAi to get the double mutants of Wab1-R/+; zmbri1-RNAi/+ and Wab1-R/+; bin2-RNAi/+. The phenotypes were observed and analyzed further by using qRT-PCR. Wab1-R/+; bin2-RNAi/+ showed slightly rescued Wab1-R phenotype with slightly wider blade width and reduced knot-like structures on the leaves. While, Wab1-R/+; zmbri1-RNAi/+ showed enhanced Wab1-R phenotype with the formation of a new structure and prong, which were not observed on Wab1-R/+ and zmbri1-RNAi/+. Gene expression analysis using qRT-PCR supported the observations with reduced wab1 expression in Wab1-R/+; bin2-RNAi/+ and increased wab1 expression in Wab1-R/+; zmbri1-RNAi/+ compared to Wab1-R/++. This indicates that BR signaling inhibits wab1.
III.2. Introduction

Plant architecture is one of the most important agronomic traits. Plant architecture modification was a major factor on the success of the Green Revolution (Reinhardt and Kuhlemeier, 2002). New rice and wheat varieties with dwarf phenotype and erect leaves can increase the grain yield because it can increase the plant density on the field. These new varieties also shown to be more resistant to wind and rain damage (Athwal, 1971; Hedden, 2003; Monna et al., 2002; Peng et al., 1999; Sakamoto et al.; 2006).

Brassinosteroids (BRs) are plants steroid hormones that are important in many plants’ growth and development processes including plant architecture (Fujioka and Yokota, 2003; Vriet et al., 2012). In rice, OsDWF4 mutant shows a semi dwarf phenotype and erect leaves, which is also an important agronomical trait because it allows higher plant density on the field (Sakamoto et al., 2006). Previous studies on BR signaling and biosynthesis mutants have shown the importance of BR in many plant development processes and important advance on understanding BR signaling and biosynthesis pathways (Zhu et al., 2013). However, not much is known about BR signaling and biosynthesis pathways in maize (Kir et al., 2015).

BRASSINOSTEROID INSENSITIVE1 (BRII) is an important gene, which encodes a leucine rich repeat receptor kinase in the plasma membrane, to start the BR signaling pathway (Belkadir & Chory, 2006; Clouse et al., 1996). In Arabidopsis, rice, and maize, mutants with decreased BRII expression showed dwarf phenotype (Clouse et al., 1996; Kir et al., 2015; Yamamuro et al., 2000). In maize, zmbril-RNAi also showed reduced ligule and auricle area, which are related to leaf angle (Duvick, 2005; Kir et al., 2015).

BRASSINOSTEROID INSENSITIVE2 (BIN2), a GSK3-like kinase gene, is another important gene in BR signaling pathway by acting as the negative regulator of BR signaling
When BR concentration is low, \textit{BIN2} is active and phosphorylates transcription factors \textit{BRASSINAZOLE RESISTANT1 (BZR1)} and \textit{BZR2} (also known as \textit{BR11-EMS-SUPPRESSOR1 (BES1)}) leading to their cytoplasmic retention by 14-3-3 proteins (Bai et al., 2007; He et al., 2002; Wang et al., 2002; Yin et al., 2002). When BR concentration is high, \textit{BIN2} is deactivated by BSU1, allowing the activation of \textit{BZR1} and \textit{BZR2}, resulting in the regulation processes of many downstream genes (He et al., 2005; Peng et al., 2008; Sun et al., 2010; Tang et al., 2011; Yu et al., 2011).

In \textit{Arabidopsis} and rice, overexpressed \textit{BIN2} mutant showed dwarf phenotype and stunted growth, and insensitivity to exogenous brassinolide treatment (Li and Nam, 2002; Tong et al., 2012), while suppressed \textit{BIN2} showed increased BR signaling and elongated but narrower leaves (Li and Nam, 2002; Tong et al., 2012; Yan et al., 2009). In maize, \textit{bin2-RNAi} showed different phenotypes compared to \textit{Arabidopsis} and rice, with shorter plant height compared to the wild type due to shorter internodes. However, the mutant also showed elongated leaf blades, elongated leaf sheathes, elongated male inflorescence, and expanded auricles (Kir et al., unpublished).

\textit{BRASSINOSTEROID DEPENDENT1 (BRD1)}, \textit{CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD)}, and \textit{DWARF4 (DWF4)}, are known BR biosynthetic genes. \textit{BRD1} encodes brassinosteroid BR-6-oxidase (Mori et al., 2002). \textit{CPD} plays a role in catalyzing a C-3 oxidation of the early BR biosynthetic intermediates (Ohnishi et al., 2012; Szekeres et al., 1996). While, \textit{DWF4} encodes brassinosteroid 22-hydrolylase which is involved in the multiple C-22 oxidation pathway by catalyzing CR to 22-OHCR, 4-en-3-one to 22-OH-4-en-3-one, and 3-one to 22-OH-3-one (Fujioka et al., 2002; Fujita et al., 2006). \textit{BRD1}, \textit{CPD}, and \textit{DWF4} are negatively regulated by BR signaling (Bai et al., 2007; Tong et al., 2012).
Bancos et al., 2002; Choe et al., 1998; Je et al., 2010). **PHYB ACTIVATION-TAGGED SUPPRESSOR1 (BAS1)** is another gene related to BR biosynthesis. **BAS1** is a C-26 hydroxylase which converts CS and BL to their C-26 hydroxylated derivatives (Turk et al., 2003).

**Wavy auricle in blade1** (*wab1*) is not a BR gene, but plays a role in maize development. *wab1* encodes a TEOSINTE BRANCHED1, CYCLOIDEA, and PCF (TCP) transcription factor important in regulating tassel branches (Lewis et al., 2014). In maize, *Wab1* dominant mutant showed ectopic ligule, ectopic auricle, and narrower leaves compared to normal (Hay and Hake, 2004). *wab1* is normally not expressed in leaves (Lewis et al., 2014). *wab1* mutant with decreased gene expression showed decreased tassel branches and angle (Lewis et al., 2014).

In maize, *wab1* is known to regulate the **liguleless1** (*lg1*) gene, which encodes a squamosal promoter binding protein important for ligule and auricle development (Lewis et al., 2014; Moreno et al., 1997). In maize, loss-of-function *lg1* mutants showed undeveloped ligule and auricle area, resulting in more erect leaves (Moreno et al., 1997). In rice, *lg1* regulates pulvinus growth, while a BR catabolism mutant, **CYP734A RNAi**, showed derepression of pulvinus growth (Ishii et al., 2013; Tsuda et al., 2011). **CYP734A** genes, which are homologous to *Arabidopsis BAS1*, are regulated by **ORYZA SATIVA HOMEOBOX1 (OSH1)**, a knox gene (Tsuda et al., 2014). In *Arabidopsis*, **TCP1** regulates BR biosynthesis through **DWF4** regulation (Guo et al., 2010). These indicates that there might be a connection among BRs, *lg1*, *wab1*, and knox. In this study, we are interested to analyze the relationship among BRs, *wab1*, and knox through gene expression analysis of **Wab1-R** and BRs RNAi line mutants.
III.3. Materials and methods

Genetic stocks

*Wab1-R* was obtained from the Maize Cooperation Genetic Stock Center. *zmbril*-RNAi was obtained from Dr. Philip W. Becraft and constructed by Gokhan Kir (Kir et al., 2015). *bin2*-RNAi was also obtained from Dr. Philip W. Becraft and constructed by Gokhan Kir (Kir et al., unpublished). *Wab1-R* was backcrossed 3 times to B73 background. *zmbril*-RNAi was backcrossed 5 times to B73 background. *bin2*-RNAi was first crossed to W22 background, then crossed 2 times to B73 background. *Wab1-R/+; zmbril*-RNAi/+ double mutants was obtained from crossing the *Wab1-R/+* (B73 background) with *zmbril*-RNAi (B73). Similarly, the *Wab1-R/+; bin2*-RNAi double mutants was obtained from crossing the *Wab1-R/+* (B73) with *bin2*-RNAi/+ (B73/W22 background).

RNA preparation

The segregating *Wab1-R/+; zmbril*-RNAi/+ and *Wab1-R/+; bin2*-RNAi individuals were planted in the Molecular Biology Building greenhouse, Iowa State University. Plants were screened by using liberty herbicide and genotyped by using herbicide bialaphos (*bar*) resistance gene primers GGCACAGGGCTTCAAGAG and AGTTCCCGTGCTTGAAGC. Primers specific for the *bin2* RNAi, GTTTGCCACAGGGATGTGAAACCA and GATCGATCTGAATAAGAGGGGAAAC, were also used to genotype *Wab1-R/+; bin2*-RNAi segregating line.

Plants were grown until they reach around ten-leaf seedling stage. Samples for RNA purification were collected from tissues approximately 2 mm around the ligule and auricle area. Tissues from plastochron 6 to 9 were used. Tissues were dissected under RNALater and
preserved also by using RNAlater. Tissues were stored at -20°C before long term storage at -80°C or used for RNA purification.

**Leaf measurements**

Leaves number six, seven, and eight were removed and measured from each plant. Sheath length was measured from the point where the leaf was connected to the meristem to the ligule, which is a boundary between blade and sheath. Blade length was measured from the ligule to the distal tip of the blade. Auricle width was measured from the ligule to the distal tip of the auricle area. Blade width is defined as the maximum measurement from left to right of the leaf blade. A minimum of five biological replications were used for each analysis.

**qRT-PCR**

RNA purifications were done by using QIAgen RNeasy Mini Kit according to the manufacturer’s protocol. RNA concentrations were measured by using NanoDrop ND-1000 spectrophotometer. Approximately 1 to 2 µg of RNA was used for each sample. RNA was treated with RQ1 RNase-free DNase (Promega). A total of 1 µL of RQ1 DNase and 1 µL of RQ1 buffer were used. Volumes were adjusted to 10 µL using nuclease-free water. Samples were incubated at 37°C for 30 min. The reaction was stopped by adding 1 µL of RQ1 DNase stop solution and incubated at 65°C for 10 min. A total of 8 µL from each samples were used for reverse transcription PCR (RT-PCR). RT-PCR was performed by using ThermoFisher Scientific SuperScript® III First-Strand Synthesis System according to the manufacturer’s protocol. To check whether the cDNA construction successful, 1 µL of the cDNA was used
for confirmation PCR using a ubiquitin-conjugating enzyme gene (GRMZM2G027378) primers, AACATCCTAACCCAGCTCAAG and CTGTTGGATCCCATGACGG.

qRT-PCR was performed by using iQ SYBR Green Supermix (Bio-Rad) and gene specific primers on an Applied Biosystems StepOnePlus™ instrument at the Iowa State University DNA facility. A ubiquitin-conjugating enzyme gene (GRMZM2G027378) was used as the reference gene. A minimum of three biological and two technical replicates were used for each analysis. Gene expression level was analyzed by using a relative quantification strategy, delta delta Ct.

\[ \Delta Ct_1 = Ct \text{ (gene of interest in sample with trait of interest or mutant)} - Ct \text{ (reference gene in sample with trait of interest or mutant)} \]

\[ \Delta Ct_2 = Ct \text{ (gene of interest in wild type or control)} - Ct \text{ (reference gene in wild type or control)} \]

\[ \Delta \Delta Ct = \Delta Ct_1 \text{ (sample with trait of interest or mutant)} - \Delta Ct_2 \text{ (wild type or control)} \]

Normalized target gene expression level = \( 2^{-\Delta \Delta Ct} \)

Statistical comparisons two tailed T-Test assuming equal variance to gain the p-value were made using JMP 10.0 statistical software.
III.4. Results and discussion

**bin2-RNAi suppresses Wab1-R phenotype**

In order to analyze the relationship between BR and *wab1*, we crossed *Wab1-R/+* with *bin2-RNAi/+* to get an expected ratio of 1 : 1 : 1 : 1 for WT : *Wab1-R/+* : *bin2-RNAi/+* : *Wab1-R/+*; *bin2-RNAi*, respectively. We observed approximately 60 plants of this line. According to Kir et al. (unpublished) and our previous observation, maize *bin2-RNAi/+* showed a shorter plant height phenotype, twisted and wavy leaves, elongated sheath, and expanded auricles compared to the wild type. *Wab1-R/+* showed ectopic auricle and ligule formation in the blade and narrower leaves compared to the wild type. From the *Wab1-R/+*; *bin2-RNAi/+*, we observed similar phenotype to the *Wab1-R/+* with ectopic auricle and ligule area and narrower leaves (Figure 7 and Figure 8). However, from the observations to at least 3 plants for each phenotype, *Wab1-R/+*; *bin2-RNAi/+* also showed wavy leaves similar to *bin2-RNAi/+* and slightly rescued *Wab1-R* phenotype with wider and reduced amount of knot-like structures on leaves.

Shorter phenotype on *bin2-RNAi* was unexpected because in *bin2-RNAi*, we expect higher concentration of BR signaling which might lead to enhanced growth. This shorter phenotype was caused by reduced internodes growth (Kir et al., unpublished) (Figure 9B). Interestingly, the double mutants of *Wab1-R/+*; *bin2-RNAi/+* showed long internodes, which indicates that on the internode, *wab1* rescued *bin2-RNAi* phenotype (Figure 9C). However, to the author's knowledge, there is no report regarding *wab1* roles in internode or plant height. Our observations on *Wab1-R/+* also showed no indication on *wab1* roles in internode. Further study might be needed to answer this question.
**zmbr1-RNAi enhances Wab1-R phenotype**

To test interactions between *Wab1-R* and *zmbr1-RNAi*, a cross was made between *Wab1-R/+* and *zmbr1-RNAi/+*. *zmbr1-RNAi* is a mutant with decreased BR signaling. A dwarf phenotype with reduced ligule and auricle area was observed from the *zmbr1-RNAi*, consistent to the phenotype observed by Kir et al. (2015). From the double mutants of *Wab1-R/+; zmbr1-RNAi/+*, similar phenotype to the combination of *Wab1-R/+* and *zmbr1-RNAi/+* was observed. *Wab1-R/+; zmbr1-RNAi/+* showed a dwarf phenotype with shortened internodes, ectopic auricle, and narrower leaves (Figure 9D-E, Figure 10, Figure 11). Similar to *zmbr1-RNAi*, *Wab1-R/+; zmbr1-RNAi/+* also showed shorter sheath and similar reduced ligule (Figure 12). However, *Wab1-R/+; zmbr1-RNAi/+* also showed enhanced phenotypes. *Wab1-R/+; zmbr1-RNAi/+* showed knot like structures, formation of prongs, and novel structures on the auricle area starting at around leaf number 9 (Figure 13). With the exception of the knot-like structure formation, these phenotypes were not observed on *Wab1-R/+* and *zmbr1-RNAi/+*. This suggests that *zmbr1-RNAi* enhances *Wab1-R* phenotype.

**Wab1-R/+; bin2-RNAi/+ leaf measurement**

To study further about the interactions between BR and *wab1*, sheath length, blade length, auricle width, and blade width were measured from at least 5 plants for each plant type of *Wab1-R/+; bin2-RNAi/+* line. Sheath length of *Wab1-R/+*, *bin2-RNAi/+*, and *Wab1-R/+; bin2-RNAi/+* were longer than wild type. Elongated *bin2-RNAi* sheath phenotype is consistent with the observation from Kir et al. (unpublished). This also indicates that *wab1* might play a role in sheath development when overexpressed, even though, *wab1* is normally not expressed
in leaves (Lewis et al., 2014). The blade length of Wab1-R/+ and Wab1-R/+; bin2-RNAi/+ were shorter compared to wild type and bin2-RNAi. Wab1-R/+ and Wab1-R/+; bin2-RNAi/+ have narrower leaves compared to wild type, but the auricle width was similar to wild type due to ectopic phenotype on the auricle area resulting in larger auricle area. bin2-RNAi auricle width was wider, consistent with previous observation by Kir et al. (unpublished). Wab1-R/+; bin2-RNAi/+ blade width was slightly wider than Wab1-R/+ indicating that bin2-RNAi slightly rescued the Wab1-R phenotype (Figure 14).

Wab1-R/+; zmbril-RNAi/+ leaf measurement

As mentioned before, the sheath length of Wab1-R/+; zmbril-RNAi was shorter compared to wild type, similar to zmbril-RNAi. Interestingly, Wab1-R/+ and Wab1-R/+; bin2-RNAi showed elongated sheath length, similar to bin2-RNAi. Blade length of Wab1-R/+; zmbril-RNAi was also shortened compared to wild type, similar to Wab1-R/+ and Wab1-R/+; bin2-RNAi/+. The auricle width of Wab1-R/+; zmbril-RNAi was significantly enlarged compared to Wab1-R/+; zmbril-RNAi was significantly enlarged compared to Wab1-R/+; zmbril-RNAi was significantly enlarged compared to Wab1-R/+; zmbril-RNAi was significantly enlarged compared to Wab1-R/+; zmbril-RNAi. However, there was no significant differences observed on the blade width between Wab1-R/+ and Wab1-R/+; zmbril-RNAi (Figure 15).

BR signaling might regulate wab1

bin2, bri1a, and bri1b expression were tested as positive controls (Figure 16A and Figure 17A). As expected, the expression of bin2 in bin2-RNAi/+ and Wab1-R/+; bin2-RNAi/+ were significantly decreased compared to the wild type. Expression of bin2 in Wab1-R/+ was not changed compared to the wild type. In zmbril-RNAi/+ and Wab1-R/+; zmbril-RNAi/+
brila and brilb expression were also significantly decreased compared to the wild type, but not changed in the Wab1-R/+.

wab1 expression in bin2-RNAi/+ and zmbril-RNAi/+ were not changed relative to the wild type (Figure 16B and Figure 17B). In Wab1-R/+; bin2-RNAi/+, wab1 expression was not significantly decreased compared to Wab1-R/+; zmbril-RNAi, wab1 expression was increased significantly compared to wab1 expression in Wab1-R/+. These results are consistent with the observations that increased BR signaling inhibits the Wab1-R phenotype and decreased BR signaling enhances Wab1-R phenotype, suggesting BR signaling might play a role as the negative regulator of wab1.

**wab1 might also regulate BR**

We also tested brd1, cpd, and dwf4 genes expression (Figure 16D and Figure 17D). brd1, cpd, and dwf4 are BR biosynthesis genes which are negatively regulated by BR signaling (Bai et al., 2007; Bancos et al., 2002; Choe et al., 1998; Je et al., 2010). brd1 gene expression was decreased significantly in bin2-RNAi/+ and Wab1-R/+; bin2-RNAi/+ compared to the wild type. brd1 gene expression was also decreased if compared between bin2-RNAi/+ and Wab1-R/+; bin2-RNAi/+ but not significantly. In zmbril-RNAi/+ and Wab1-R/+; zmbril-RNAi/+, brd1 gene expression was significantly increased compared to the wild type. brd1 expression was also increased significantly when compared between zmbril-RNAi/+ and Wab1-R/+; zmbril-RNAi/+. cpd gene expression was also increased significantly in Wab1-R/+; zmbril-RNAi/+ but there were no significant differences observed in other mutants.

Interestingly, brilb gene expression analysis in Wab1-R/+ from Wab1-R/+; zmbril-RNAi/+ line showed significant decrease of gene expression level compared to the wild type.
However, *bri1b* gene expression in *Wab1-R/+; zmbril-RNAi/+* was also significantly decreased compared to *zmbril-RNAi/+*. This result is unexpected, because *bri1b* gene expression is decreased in both *Wab1-R/+* and *zmbril-RNAi/+* single mutant, so in the double mutants, we expect synergistic effect on the *bri1b* gene expression. Interestingly, *bin2* gene expression in *Wab1-R/+; bin2-RNAi/+* was also significantly decreased compared to *bin2-RNAi/+*. *bri1b* significant decrease in *Wab1-R/+* compared to wild type is consistent with *brd1* significant increase in *Wab1-R/+; zmbril-RNAi/+* compared to *zmbril-RNAi/+*. While, *bri1b* significant increase in *Wab1-R/+; zmbril-RNAi/+* compared to *zmbril-RNAi/+* is consistent with *bin2* significant decrease in *Wab1-R/+; bin2-RNAi/+* compared to *bin2-RNAi/+*. These two results do not support each other. However, these results indicate that *wab1* regulate BR, even though the mechanism is still unclear.

In *Arabidopsis*, *TCP1* regulates BR biosynthesis through positive regulation of *DWF4* (An et al., 2011; Guo et al., 2010). From the qRT-PCR results, *dwf4* gene expression was significantly decreased in the *Wab1-R/+* from *Wab1-R/+; zmbril-RNAi* line, but not significant in the *Wab1-R/+* from *Wab1-R/+; bin2-RNAi/+* line. *dwf4* gene expression analysis also showed no significant result in the other mutants. These indicates that in maize, *wab1*, which also encodes TCP transcription factor, might regulate BR biosynthesis through *brd1* but not through *dwf4*. However, further confirmation is needed on these results.

**wab1** is not directly related to **knox**

To study the relationship between *wab1* and *knox*, gene expression of *knotted1* (*kn1*) and *rough sheath1* (*rs1*), which are *knox* genes, were analyzed using qRT-PCR. There were differences in expression level of *kn1* and *rs1* in all mutants relative to the wild type (as shown
in Figure 16C and Figure 17C), but the \textit{kn1} and \textit{rs1} gene expressions were very low and in some samples or replications were not detected, making measurement unreliable. This result was consistent with previous study by Hay and Hake (2004), which showed that \textit{wab1} is independent from \textit{knox} pathway.

**\textit{wab1} might not be directly related to \textit{BAS1} homologs**

In maize, \textit{wab1} regulates \textit{lg1} (Lewis et al., 2014; Moreno et al., 1997), while in rice, \textit{lg1} regulates pulvinus growth, which is repressed by \textit{CYP734A}, a BR catabolism gene and homolog of Arabidopsis \textit{BAS1} (Ishii et al., 2013; Tsuda et al., 2011; Tsuda et al., 2014). This indicates that there might be a connection between \textit{wab1} and BR catabolism. To answer this question, we also analyzed the gene expression of three \textit{BAS1} homologs in maize, \textit{bas1}, \textit{bas2}, and \textit{bas3} (Figure 16E and Figure 17E).

In \textit{Wab1-R/+} and \textit{bin2-RNAi}, there was no significant difference observed from the \textit{BAS1} homologs. In \textit{Wab1-R/+; bin2-RNAi}, \textit{bas2} was significantly increased, while in \textit{bri1-RNAi/+} and \textit{Wab1-R/+; zmbri1-RNAi}, all \textit{BAS1} homologs were significantly decreased. These indicate that \textit{wab1} might not be directly related to \textit{BAS1} homologs.
III.5. Figures and tables

Figure 7. *Wab1-R/+; bin2-RNAi/+* showed similar phenotype to *Wab1-R/+*. From left to right: *Wab1-R/+; bin2-RNAi/+*, *Wab1-R/+*, and Wild type.
Figure 8. **Wab1-R/+; bin2-RNAi/+** line leaves phenotype comparison. (A) Ligule and auricle area comparison, from left to right: Wab1-R/+; bin2-RNAi/+, Wab1-R/+, and wild type. Wab1-R/+; bin2-RNAi/+ looks similar to Wab1-R/+ with ectopic auricle and ligule area. (B) Wab1-R/+; bin2-RNAi/+ showed wavy leaves phenotype, similar to bin2-RNAi/+ (C) Blade comparison, from top to bottom: Wild type, Wab1-R/+, and Wab1-R/+; bin2-RNAi+. Wab1-R/+; bin2-RNAi/+ looks similar to Wab1-R/+ with narrower blade. But, Wab1-R/+; bin2-RNAi/+ blade is slightly wider than Wab1-R/+. The knot-like structures on Wab1-R/+; bin2-RNAi/+ also seems to be reduced compared to Wab1-R/+. Leaves number 9 were used for the comparison.
Figure 9. Internode comparison.
(A) Wab1-Rl+. (B) bin2-RNAi+. (C) Wab1-Rl+; bin2-RNAi+. (D) zmbr1-RNAi+. (E) Wab1-Rl+; zmbr1-RNAi+. (F) Wild type
Figure 10. *Wab1-R/++; zmbril-RNAi/+* and *zmbril-RNAi/+* showed dwarf phenotype. From left to right: *Wab1-R/++; zmbril-RNAi/+, zmbril-RNAi/+, Wab1-R/+, Wild type
Figure 11. *Wab1-R/+; zmbril-RNAi/+* line leaves phenotype comparison. *Wab1-R/+; zmbril-RNAi/+* looks similar to *Wab1-R/+* with ectopic auricle and ligule area and narrower blade. (A) Ligule and auricle area comparison, from left to right: Wild type, *Wab1-R/+; zmbril-RNAi/+*, *zmbril-RNAi/+*, and *Wab1-R/+*. (B) Blade comparison, from left to right: Wild type, *Wab1-R/+; zmbril-RNAi/+*, *zmbril-RNAi/+*, and *Wab1-R/+*. Leaves number 9 were used for the comparison.
Figure 12. Sheath and ligule phenotype of Wab1-R/+; zmbr1-RNAi/+.
(A) Sheath comparison, 1: Wab1-R/+; 2: zmbr1-RNAi/+; 3: Wab1-R/+; zmbr1-RNAi/+; 4: wild type. (B) Ligule comparison, 1: zmbr1-RNAi/+; 2: Wab1-R/+; zmbr1-RNAi/+; 3: wild type.
Figure 13. *Wab1-R/+; zmbril-RNAi/+* showed enhanced phenotype.

(A) Novel structure around ligule and auricle area. (B) Prong formation on the leaf. (C) Knot-like structures also observed on the leaf.
Figure 14. Leaf measurements of the Wab1-R+/; bin2-RNAi/+ line from leaves number 6, 7, and 8.
(A) Sheath length. (B) Blade length. (C) Auricle width. (D) Blade width. Y axis indicates the measurement results in cm. Error bar indicates standard deviation. * indicates Student’s t test p-value < 0.05. ** indicates Student’s t test p-value < 0.01. Student’s t test was performed by comparing to Wab1-R+/; bin2-RNAi/+ measurement results.
Figure 15. Leaf measurements of the Wab1-R/+; zmbri1-RNAi/+ line from leaves number 6, 7, and 8. (A) Sheath length. (B) Blade length. (C) Auricle width. (D) Blade width. Y axis indicates the measurement results in cm. Error bar indicates standard deviation. * indicates Student’s t test p-value < 0.05. ** indicates Student’s t test p-value < 0.01. Student’s t test was performed by comparing to Wab1-R/+; zmbri1-RNAi/+ measurement results.
Figure 16. qRT-PCR analysis of the Wab1-R/+; bin2-RNAi/+ line using ΔΔCt method.
(A) bin2 gene expression analysis as the positive control of the RNAi mutant. (B) wab1 gene expression analysis showed wab1 expression in Wab1-R/+; bin2-RNAi/+ is decreased compared to in Wab1-R/+ but not statistically significant. (C) knox genes expression, including kn1 and rs1, are decreased in all mutants but the genes expression are very low. (D) BR biosynthesis genes, brd1, cpd, and dwf4, expression analysis showed brd1 gene expression is significantly decreased in bin2-RNAi/+ and Wab1-R/+; bin2-RNAi/+. (E) BR catabolism gene homologs, bas1, bas2, and bas3, expression analysis showed bas2 gene expression is significantly increased in Wab1-R/+; bin2-RNAi+. Y axis indicates fold change in gene expression level relative to the wild type. Student’s t test was performed assuming equal variances.
Figure 17. qRT-PCR analysis of the Wab1-R/+; zmbril1-RNAi/+ line using ΔΔCt method.
(A) bri1a and bri1b genes expression analysis as the positive controls of the RNAi mutant. (B) wab1 gene expression analysis showed wab1 expression in Wab1-R/+, zmbril1-RNAi/+ is significantly increased compared to in Wab1-R/+. (C) knox genes expression, including kn1 and rs1, are increased in zmbril1-RNAi/+ but the genes expression are very low. (D) BR biosynthesis genes, brd1, cpd, and dwf4, expression analysis showed brd1 gene expression is significantly increased in zmbril1-RNAi/+ and Wab1-R/+. zmbril1-RNAi/+ cpd is increased significantly in Wab1-R/+. zmbril1-RNAi/+ dwf4 is decreased significantly in Wab1-R/+.
(E) BR catabolism gene homologs, bas1, bas2, and bas3, expression analysis showed the genes expression are significantly decreased in zmbril1-RNAi/+ and Wab1-R/+. zmbril1-RNAi/+. Y axis indicates fold change in gene expression level relative to the wild type. Student’s t test was performed assuming equal variances.
References


CHAPTER IV. GENERAL CONCLUSION

Plant architecture is one of the most important agronomical traits, because it might affect yield. BR is a plant steroid hormone which plays important roles in plant development including plant architecture. *knox* genes encode homeodomain-containing transcription factors. In rice, *OSH1*, a *knox* gene, regulates *CYP734A* genes, which are homologous to *Arabidopsis BAS1*, a BR catabolism gene. Overexpression of *OSH1* showed insensitivity to BR, while loss of function of *OSH1* showed increasing BR production, indicating *OSH1* gene plays a role in negatively regulating BR through the activation of BR catabolism genes (Tsuda et al., 2014). This indicates a relationship between *knox* and BR. In rice, *CYP734A* RNAi showed derepression of pulvinus growth, while *lg1* regulates pulvinus growth (Ishii et al., 2013; Tsuda et al., 2011). In maize, *lg1* is regulated by *wab1*, which encodes TCP transcription factor (Lewis et al., 2014; Moreno et al., 1997). In *Arabidopsis*, *TCP1* regulates BR biosynthesis by positively regulates *DWF4* (An et al., 2011; Guo et al., 2010). These indicate that there might be a connection between *wab1* and BR. In maize, *Rsl-O*, a *knox* dominant mutant, showed similar phenotype to *zmbri1-RNAi* (Kir et al., 2015). qRT-PCR results from *Rsl-O/+* showed significant decrease of *bri1b* expression and significant increase of *dwf4* expression. These indicate a connection between *rs1* and BR in maize, even though the mechanism is still unclear. From these previous publications, there is an indication that there might be a connection among BR, *knox*, and *wab1*. The connections from previous publications is summarized on Figure 18. In this study, we are interested to analyze the relationship.

In chapter II, we analyzed the relationship between *knox* and BR by analyzing the phenotype of double mutants *Kn1-O/+; bin2-RNAi/+* and *Kn1-O/+; zmbri1-RNAi/+* compared
to wild type and single mutants, \textit{Kn1-O/+}, \textit{bin2-RNAi/+} and \textit{zmbril-RNAi/+}. Phenotype analysis showed strong interaction between \textit{kn1} and BR. \textit{Kn1-O/+; bin2-RNAi/+} showed suppressed \textit{Kn1-O} phenotype by \textit{bin2-RNAi}, while \textit{Kn1-O/+; zmbril-RNAi/+} showed enhanced \textit{Kn1-O} phenotype by \textit{zmbril-RNAi}. In \textit{bin2-RNAi} mutant, we expect increased BR signaling level because \textit{bin2-RNAi} suppressed \textit{bin2}, which is a negative regulator of BR signaling pathway. While, in \textit{zmbril-RNAi} mutant, we expect decreased BR signaling level because \textit{zmbril-RNAi} suppressed \textit{bri1}, which encodes receptor kinase and important for BR signal transduction. This suggests BR signaling might inhibit \textit{kn1}. Gene expression analysis by using qRT-PCR also showed reduced \textit{kn1} expression in \textit{Kn1-O/+; bin2-RNAi/+} and increased \textit{kn1} expression in \textit{Kn1-O/+; zmbril-RNAi/+} compared to \textit{Kn1-O/+}. These results support the phenotype observation. However, \textit{kn1} gene expressions were only slightly changed and not statistically significant. Gene expression analysis on \textit{rs1, brd1, cpd, dwf4, bas1, bas2}, and \textit{bas3} also showed no significant results. Even though the \textit{cpd} gene expression was significantly decreased in \textit{Kn1-O/+} from \textit{Kn1-O/+; bin2-RNAi/+} line, the result is not consistent because the \textit{cpd} gene expression in another line (\textit{Kn1-O/+; zmbril-RNAi/+}) was not significantly different. This might indicate that there is another gene that is responsible for the phenotype.

In chapter III, we analyzed the relationship between \textit{wab1} and BR. We used similar approaches with chapter II. \textit{Wab1-R/+; bin2-RNAi/+} showed slightly suppressed \textit{Wab1-R} phenotype by \textit{bin2-RNAi}. While, \textit{Wab1-R/+; zmbril-RNAi/+} showed enhanced \textit{Wab1-R} phenotype by \textit{zmbril-RNAi}. Leaves measurement also supported this observation. These also indicate BR signaling might inhibit \textit{wab1}. However, interestingly, short internode phenotype in \textit{bin2-RNAi} was rescued by \textit{Wab1-R}. This indicates \textit{wab1} might also regulate BR. Gene
expression analysis by using qRT-PCR showed *wab1* expression is decreased in *Wab1-R/+; bin2-RNAi/+*, even though not significantly, and increased significantly in *Wab1-R/+; zmbri1-RNAi/+* compared to *Wab1-R/+*. These support the observation that BR signaling might inhibit *wab1*. Interestingly, gene expression analysis of *brd1* also showed significant results. *brd1* was not differently expressed in *Wab1-R/+* but significantly decreased in *bin2-RNAi* and *Wab1-R/+; bin2-RNAi/+* compared to the wild type. While, in *zmbri1-RNAi* and *Wab1-R/+; zmbri1-RNAi/+*, *brd1* were significantly increased compared to the wild type. *brd1* were also decreased in *Wab1-R/+; bin2-RNAi/+* even though not significantly compared to *Wab1-R/+*, and significantly increased in *Wab1-R/+; zmbri1-RNAi/+* compared to *Wab1-R/+*. In *Arabidopsis*, *TCP1* regulates BR biosynthesis by positively regulates *DWF4* (An et al., 2011; Guo et al., 2010). qRT-PCR results did not show significant results in *dwf4* and *cpd* expressions. These indicate, in maize, *wab1* might regulate BR through *brd1* even though further investigation is still needed. We observed no significant results on *kn1* and *rs1* gene expression analysis, indicating *wab1* might not be directly related to *knox* genes, which is consistent to previous study by Hay and Hake (2004). We observed significant results from *BAS1* homologs in *zmbri1-RNAi/+* and *Wab1-R/+; zmbri1-RNAi/+*. However, the results were not consistent when compared among the mutants, which might indicate that *wab1* is not directly related to *BAS1* homologs.
IV.1. Figures

Figure 18. Known relationship among BR, knox, and wab1.
References


## Table S1. List of primers used

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APPENDIX B. Rs1-O/+ qRT-PCR results

Figure S 1. qRT-PCR analysis of the Rs1-O/+ line using ΔΔCt method. (A) rs1 genes expression analysis as the positive controls of Rs1-O/+ mutant. (B) bri1a and bri1b gene expressions analysis showed slightly but significant decrease on bri1b gene expression in Rs1-O/+ mutant. (C) BR catabolism gene homologs, bas1, bas2, and bas3, and BR biosynthesis genes, brd1, cpd, and dwf4, expression analysis showed the dwf4 expression is significantly increased in Rs1-O/+ line. Y axis indicates fold change in gene expression level relative to the wild type. * indicates Student’s t test p-value < 0.05. ** indicates Student’s t test p-value < 0.01. Student’s t test was performed by comparing to the wild type.