Brassinosteroid regulation of plant height in maize

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Brassinosteroid regulation of plant height in maize

by

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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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2010, December

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To my parents
## TABLE OF CONTENTS

### CHAPTER 1. GENERAL INTRODUCTION

1.1. BRASSINOSTEROIDS ................................................................. 2
1.2. GIBBERELLINS ................................................................. 11
1.3. AUXIN ................................................................. 16
1.4. REFERENCES ................................................................. 18

### CHAPTER 2. IDENTIFICATION OF NOVEL MAIZE DWARF MUTANTS

2.1. ABSTRACT ................................................................. 24
2.2. INTRODUCTION ................................................................. 24
2.3. RESULTS ................................................................. 27
2.4. DISCUSSION ................................................................. 35
2.5. MATERIALS AND METHODS ................................................................. 40
2.6. FIGURES AND TABLES ................................................................. 43
2.7. REFERENCES ................................................................. 59

### CHAPTER 3. TRANSGENIC ANALYSIS OF BRASSINOSTEROID (BR)
MEDIATED GROWTH AND DEVELOPMENT IN MAIZE

3.1. ABSTRACT ................................................................. 63
3.2. INTRODUCTION ................................................................. 63
3.3. RESULTS AND DISCUSSION ................................................................. 64
3.4. MATERIALS AND METHODS ................................................................. 68
3.5. FIGURES AND TABLES ................................................................. 72
3.6. REFERENCES ................................................................. 85

### CHAPTER 4. GENERAL CONCLUSION

ACKNOWLEDGEMENTS ................................................................. 89
CHAPTER 1. GENERAL INTRODUCTION

Increasing world population is a major problem. Besides other demands, supplying food for this growing population is a major concern for international organizations and the scientific community. Since most countries mainly depend on cereals such as wheat, corn, and rice, (http://faostat.fao.org/site/339/default.aspx) the basic goal of supplying food requires increased yield and biomass from these crops. For that purpose, scientists have been working to increase crops’ disease resistance and stress tolerance including biotic and abiotic stresses, especially in light of climate change.

Even though the Green Revolution helped to increase yield from agriculturally important crops (EVENSON and GOLLIN 2003; SAKAMOTO and Matsuoka 2004; SALAMINI 2003), the nearly 3 billion increase in population after the Green Revolution and predicted 9-10 billion world population by 2050 (U.S. Census Bureau, International Data Base, June 2010 Update) causes concerns for feeding this huge population.

In addition to feeding the world’s population, plants are key to overcoming the global fuel crisis. The world has depended on fossil fuel for many years and fossil fuel reserves will not meet increasing population demands. These tough situations led countries to search for alternative fuel sources, one of which is biofuels (JACOBSON 2009). Today, research on improving biomass yield from plants such as maize, sorghum and switchgrass, which are good candidates for biofuel production, is the focus of many laboratories (SOMERVILLE et al. 2010). Among these plants, maize is currently the most widely used plant for ethanol production in the U.S. (SERVICE 2007) and it is the best model to understand any C4 system for biofuel production (LAWRENCE and WALBOT 2007).
Plant height is an important agronomic trait that affects grain and biomass yield, and is one of the foci of agricultural sciences (SALAS-FERNANDEZ et al. 2009). It is possible to manipulate this trait with breeding and genetics. Identifying genes related to plant height would enable genetic manipulations and mutations that cause dwarf plants identify such genes (ASHIKARI et al. 1999; CHOE et al. 1998). Dwarf plants have shorter stature compared to the wild type (HONG et al. 2003). Studying dwarves in different species such as maize, pea, rice, and Arabidopsis has advanced our understanding about what causes dwarfism. It is well known that mutations in the biosynthesis or signaling pathways of some hormones such as gibberellin (GA), brassinosteroid (BR), and auxin cause dwarfism (reviewed in SALAS-FERNANDEZ et al. 2009). However, many questions remain regarding the genetic mechanisms that cause dwarfism (SUNOHARA et al. 2009). If the mechanisms behind dwarfism could be well understood, then it would be easier to manipulate plant height to get more biomass and yield. In addition, dwarfism itself is an agricultural advantage in some cases. As seen in the Green Revolution, using semi-dwarf crops contributed to increased yields in wheat and rice (KHUSH 2001). The resistance of semi-dwarf plants to extreme conditions such as strong wind, rain, and lodging, as well as improved harvest index from reallocation of carbon from vegetative tissues to grain yields was the underlying basis of the Green Revolution (HEDDEN 2003).

1.1. BRASSINOSTEROIDS

Brassinosteroids (BRs) are a type of hormone that is found throughout the plant kingdom. They are steroid hormones similar to those found in animals (FUJIOKA and YOKOTA 2003). They promote cell growth, even if at low concentrations, by regulating cell
divisions and elongation (CLouse 1996a; Mandava 1988). BRs also have roles in enhancing tracheary element differentiation, stimulating ATPase activity, controlling microtubule orientation, changing the mechanical structure of the cell wall, controlling flowering time, fertility, and leaf development (CLouse 1996a). In addition to these developmental roles, BRs also play positive roles in resistance to both biotic and abiotic stresses such as extreme temperatures, drought, and pathogen attacks (Krisha 2003).

The amount of BRs in the cell varies by the developmental stage of the plant, type of the tissue, and organ. In younger tissues the level of BRs are higher than older ones (CLouse 2002). In Arabidopsis, the most active of member of BRs is brassinolide (BL) and it is found widely in the plant kingdom (Fujioka and Yokota 2003).

Despite BRs’ roles stated above and their broad effects on the physiological and developmental processes of plants, they were not widely recognized as plant hormones until the mid 1990s (CLouse 1996a; Divi and Krisha 2009). With the identification of BR deficient mutants and subsequently mutants disrupted in BR signaling, the importance of BRs for the plant development was realized.

1.1.1. Brassinosteroid Biosynthesis

As mentioned, the most active BR identified thus far is brassinolide (BL) (Fujioka and Yokota 2003; Kim et al. 2006). BL is derived from campesterol (CR) through several enzymatic reactions including hydroxylations, reductions, epimerization, and a Baeyer-Villiger-type oxidation (Choe et al. 1998). Briefly, BR biosynthesis is as follows: first, campesterol is converted to campestanol, and then castasterone (CS) is formed from campestanol either via early C-6 oxidation or late C-6 oxidation. Finally, CS is catalyzed to
BL (FUJIOKA and YOKOTA 2003). There are two parallel pathways for BL biosynthesis named the late or early C-6 oxidation pathways according to the oxidation state of C-6 (CHOE et al. 1998; FUJIOKA et al. 1997). These pathways are not completely independent from each other because several enzymes have required functions in both (FUJIOKA and YOKOTA 2003).

Genes functioning in the BR biosynthesis pathways have been identified in several species, including Arabidopsis and rice, by the availability of dwarf mutants. BR deficient mutants can be recognized by the ability of exogenous BR to rescue the dwarf phenotype. The Arabidopsis DET2 is the first gene identified in the biosynthetic pathway of BRs. A dwarf phenotype, reduced male fertility, and dark green leaves are some of the phenotypic characteristics of det2 mutants. This shows that BRs are critical compounds for the development of the plants. DET2 in Arabidopsis encodes a 5α-reductase which catalyzes 5α-reduction of (24R)-ergost-4-en-3-one to (24R)-5α-ergostan-3-one and (22S, 24R)-22-hydroxyergost-4-en-3-one to (22S, 24R)-22-hydroxy-5α-ergostan-3-one in BR biosynthesis (FUJIOKA et al. 1997; FUJIOKA and YOKOTA 2003; LI et al. 1996).

Arabidopsis DWF4 (DWF4) encodes cytochrome P450 monooxygenase (CYP90B1), which is a key enzyme in the biosynthesis of BRs (KIM et al. 2006). DWF4 catalyzes a C-22 hydroxylation of the steroid side chain (CHOE et al. 1998). In Arabidopsis, this is a rate-limiting step of BR biosynthesis and it is feedback regulated by BR signaling (CHOE et al. 1998). A disruption in BR signaling causes increased levels of DWF4 transcript accumulation. Overexpression of DWF4 in Arabidopsis caused increased BR levels, prolonged flowering and increased seed yield (CHOE et al. 2001).

Another P450 gene in BR biosynthesis is CPD. cpd mutants have defects in cell elongation (SZEKERES et al. 1996). Arabidopsis CPD (CYP90A1) acts just downstream of
*DWF4* in the Arabidopsis BR biosynthesis pathway and encodes a C-23 steroid hydroxylase. The *CPD* gene is also negatively regulated by BR signaling (BANCOS et al. 2002; MATHUR et al. 1998).

The Arabidopsis *brassinosteroid-6-oxidase* (AtBR6ox) genes encode members of the CYP85 cytochrome P450 family. CYP85A1 and CYP85A2 catalyze the formation of teasterone from 6-deoxoteasterone, 3-dehydroteasterone from 3-dehydro-6-deoxoteasterone, typhasterol from 6-deoxotyphasterol, and castasterone from 6-deoxocastasterone (NOMURA et al. 2005; SHIMADA et al. 2001). CYP85A2 has also been shown to have BL synthase activity, catalyzing a Baeyer-Villiger oxidation of castasterone to form BL (NOMURA et al. 2005). In Arabidopsis, these enzymes are genetically redundant; they show largely overlapping expression patterns and single mutants have nearly normal phenotypes. In contrast, double mutants show a severe dwarf phenotype that can be rescued with exogenous BL but not CS (CASTLE et al. 2005; NOMURA et al. 2005; SHIMADA et al. 2001).

Several additional enzymes that function in the synthesis of campesterol also are required for BR production, and result in dwarfism when their functions are impaired. The Arabidopsis *DWF7* gene catalyzes Δ⁷ sterol C-5 desaturase steps in the formation of 5-Dehydroavenasterol from Avenasterol and 5-Dehydroepisterol from Episterol. Defects in this step inhibit campesterol production, and so BL synthesis (CHOE et al. 1999). Another gene found to be involved in sterol biosynthesis before campesterol is *DWF5*. *DWF5* is a sterol Δ⁷ reductase gene involved in the formation of 24-Methylenecolesterol from 5-Dehydroepisterol. *dwf5* mutants are dwarf due to the lack of BRs (CHOE et al. 2000). Arabidopsis DIM/DWF1 is an integral membrane protein that forms campesterol from 24-methylenecolesterol in the early steps of BR synthesis. DIM/DWF1 is not a rate-limiting
step (Klahre et al. 1998). And finally, the Arabidopsis *ROTUNDIFOLIA3* (*ROT3*) gene encodes a cytochrome P450 enzyme (CYP90C1) resembling steroid hydroxylases. *ROT3* has a role in the polar elongation of Arabidopsis cells (Kim et al. 1998). Even though *rot3* mutants do not show characteristic BR mutant phenotypes such as photomorphogenesis in the dark, it is thought they are involved in steroid synthesis (Kim et al. 1998).

Rice shows many similarities as well as some differences with Arabidopsis. The BR related rice mutants show short leaf sheaths and blades, less tillering, curled leaf blades, reduced lamina joint bending and dwarfism (Bai et al. 2007; Mori et al. 2002; Sakamoto et al. 2006). Erect leaf habit could be an important agricultural trait because it increases the light captured by leaves for photosynthesis and nitrogen use efficiency for grain yield (Sinclair and Sheehy 1999). Although BL is the most active form of BR in Arabidopsis, its presence has not been shown in rice. This is probably because OsCYP85A1 has CS synthase activity but not BL synthase, suggesting that CS instead of BL may be the active BR in rice (Kim et al. 2008).

In rice, it has been shown that OsDWARF4/CYP90B2 encodes a cytochrome P450 that catalyzes C-22 hydroxylation, which is also a rate-limiting step for the pathway, and that OsDWARF4/CYP90B1 is also feedback regulated by BRs (Sakamoto et al. 2006). However, there is a difference between Arabidopsis and rice at this step. While there is one enzyme catalyzing C-22 hydroxylation in Arabidopsis, in rice there is redundancy for this step between D11/CYP724B1 and OsDWARF4 (Sakamoto et al. 2006).

*D2* in rice encodes a cytochrome P450 (CYP90D) that functions in BR C-3 oxidation reactions such as the conversion of 3-dehydro-6-deoxoteasterone from 6-deoxoteasterone and 3-dehydroteasterone from teasterone. *D2* is feedback regulated by endogenous BRs (Hong
et al. 2003). No Arabidopsis mutant has been reported for this step. Rice brd1 is a BR deficient mutant that has defects in the OsBR6ox gene, which encodes a cytochrome P450 (Mori et al. 2002). This is also in contrast to Arabidopsis where no mutant is known for this step. In rice, the BR biosynthetic genes D2, D11, and BRD1 are feedback regulated by BRs (Bai et al. 2007)

1.1.2. Brassinosteroid Signaling

With the discovery of BR biosynthetic genes, intensive work helped to identify other genes related to the BRs signaling. The first gene found to be important for BR signal transduction was BRI1 in Arabidopsis. Arabidopsis bri1 mutant plants are dwarved, have reduced apical dominance and male-sterility. Hormone application studies showed that bri1 plants have reduced sensitivity to BRs, which led to the finding that these plants had defects in BR signal transduction (Clouse et al. 1996). BRI1 is a leucine rich repeat kinase which is the BR receptor (Li and Chory 1997). The BRI1 receptor is located on the cell surface, which makes plant steroid hormone signaling different than animals which receive steroids by nuclear receptors (Li and Jin 2007). BRI1 binds BR and transduces the signaling to the downstream components by the activity of its kinase domain. In rice, OsBRI1 is similar to the Arabidopsis BRI1 gene (Yamamura et al. 2000). Mutations in this gene prevent BR signaling to reach downstream components. Some phenotypes of OsBRI1 rice mutants are erect leaves, photomorphogenesis in the dark, and shorter internodes (Yamamura et al. 2000).

With the availability of a signaling mutant, subsequent studies concentrated on uncovering the downstream network. Another component that plays a role in BR signaling is BKII. This plasma membrane associated protein is a negative regulator of BR signal
transduction. It interacts with \textit{BRI1} through its kinase domain. The overexpression of this protein causes dwarfism (Li and Jin 2007; Wang and Chory 2006). BKI1, TRANSTHYRETIN-LIKE (TTL) protein, TGFβ-RECEPTOR-INTERACTING PROTEIN 1 (TRIP-1), and BR SIGNALING KINASES (BSKs) are proteins phosphorylated by BRI1 (Kim and Wang 2010). In the absence of BR, BKI1 binds to BRI1 and prevents the BRI1-BAK1 interaction (Wang and Chory 2006). When BR is present, BRI1 phosphorylates BKI1, which leads to its accumulation in the cytosol. Dissociation of BKI1 from BRI1 allows the dimerization of BRI1 and BAK1 (Gendron and Wang 2007; Li and Jin 2007). For the complete activity of the BRI1 kinase, BAK1 is a required element (Kim and Wang 2010). BAK1 was identified through a yeast two hybrid screen for interactors with BRI1. BAK1 is a leucine-rich repeat receptor-like kinase (LRR-RLK) which is located on the cell surface. This protein interacts with BRI1 on the cell surface and they trans-phosphorylate each other. This interaction enhances BRI1 kinase function and BR signaling to downstream elements. Genetic experiments showed that a loss-of-function mutation in the \textit{bak1} gene resembles a weak \textit{bri1}-like plant phenotype (Nam and Li 2002).

After the activation of BRI1, BSK1, which is a plasma membrane localized protein kinase, is phosphorylated by BRI1 (Kim \textit{et al.} 2009). Phosphorylation of BSK1 causes its dissociation from BRI1. BSK1 then binds to the BSU1 phosphatase and activates it (Tang \textit{et al.} 2008). BSU1, which promotes cell elongation, is a positive regulator of the pathway (Mora-Garcia \textit{et al.} 2004). BSU1 has an N-terminal kelch-repeat domain and a C-terminal phosphatase domain (Mora-Garcia \textit{et al.} 2004). This Ser/Thr protein phosphatase has a role between BSK1 and BIN2. Following the binding of BSK1 to BSU1, activated BSU1
inhibits BIN2 activity by dephosphorylating a conserved phospho-tyrosine residue (pTyr 200) of BIN2 (Kim et al. 2009).

BIN2 was the second gene found in the BR signal transduction pathway after BRI1. Plants with the semi-dominant bin2 mutation showed a semi-dwarf phenotype that could not be rescued by exogenously applied BR. Further studies showed that BIN2 was a negative regulator of the pathway (Li et al. 2001). The BIN2 gene encodes a GSK3/SHAGGY-like kinase (Li and Nam 2002). In animal systems, GSK3 kinases have been shown to negatively regulate many pathways by phosphorylation (Cohen and Frame 2001).

A genetic suppressor screen on a bri1 mutant resulted in the isolation of BES1 (Yin et al. 2002). At nearly the same time, a screen for mutants that were insensitive to the brassinosteroid biosynthetic inhibitor, brassinozole, identified BZR1 (Wang et al. 2002). Studies showed that both genes are positive regulators of the BR pathway that act downstream of BRI1 and BIN2 (Wang et al. 2002). These two genes’ products have 88% amino acid sequence identity (Wang et al. 2002).

In the presence of BRs, BES1 and BZR1 accumulate in the nucleus. In the absence of BR, they are phosphorylated by active BIN2, which inhibits their DNA binding activity, prevents their entering the nucleus, and leads to their 26S proteasome-mediated degradation (Vert and Chory 2006; Wang et al. 2002; Yin et al. 2002). Cytoplasmic retention of phosphorylated BES1/BZR1 is mediated by 14-3-3 proteins (Gampala et al. 2007).

Since BES1 and BZR1 proteins are highly related and accumulate in the nucleus, it may be expected that they are responsible for the same biological functions. However, the phenotypic differences between light grown bes1 and bzr1 mutant plants show that the roles of these proteins are not identical. While BZR1 has effects on the feedback regulation of BR
biosynthesis, BES1 does not (Wang et al. 2002). BZR1 binds to the promoters of BR biosynthetic genes *CPD* and *DWF4* leading to their inhibition (He et al. 2005). A rice ortholog of *AtBZR1* called *OsBZR1* is also a positive regulator of BR signaling (Bai et al. 2007). Similar to Arabidopsis, the rice BR biosynthetic genes *D2*, *D11*, and *BRD1* are feedback regulated by BRs via the action of *OsBZR1* (Bai et al. 2007; Tong et al. 2009).

After BES1 accumulates in the nucleus, it recruits other transcriptional modulators to regulate the further processes (Kim and Wang 2010). BES1 interacts with BIM1 which is a basic helix-loop-helix (bHLH) protein and they together bind to the E-box sequences which leads to the regulation of BR-induced genes (Yin et al. 2005).

In rice, another transcription factor is a positive effector of BR responses. Mutants in *dwarf and low-tillering* (*dlt*) show typical BR deficient phenotypes, and insensitivity to applied BR (Tong et al. 2009). DLT is a GRAS family member that promotes tillering, and other BR responses, and is involved in feedback inhibition of BR biosynthetic genes (Tong et al. 2009). Furthermore, *DLT* itself is feedback regulated by BR through the binding of *OsBZR1* to its promoter.

In rice, it has been shown that a short vegetative phase (SVP)-group MADS-box member, *OsMDP1*, is a negative regulator of BR signaling (Duan et al. 2006). This protein is mainly involved in the BR control of rice lamina joint bending and coleoptile elongation (Duan et al. 2006). Another study showed that, two other SVP-group members, *OsMADS55* and *OsMADS22*, also act as negative regulators of BR responses (Lee et al. 2008). While *OsMADS55* is the main repressor, the presence of the *OsMADS22* increases *OsMADS55* activity (Lee et al. 2008).
1.2. GIBBERELLINS

1.2.1 Gibberellin Biosynthesis

Gibberellins (GAs) constitute a large family of plant growth compounds (LANGE and LANGE 2006). They belong to the family of tetracyclic diterpenoid phytohormones (SAKAMOTO et al. 2004). GA has extensive roles in the development of plants as has been shown by the availability of GA mutants (WINKLER and HELENTJARIS 1995). GAs function in the development of flowers, leaf expansion, trichome development, fruit set, stem elongation, seed germination, and the control of fertilization (DELLAPORTA and CALDERONURREA 1994; LANGE and LANGE 2006; OIKAWA et al. 2004; SASAKI et al. 2002; ZHANG et al. 2008).

Since GA promotes plant height, mutations in the signaling and biosynthesis of this hormone causes dwarfism (SASAKI et al. 2002). GA mutants are also often male sterile and late flowering (LANGE and LANGE 2006). Dwarfism is an important agricultural trait; semi-dwarf GA mutants were used in the Green Revolution to allow increased grain yields from several crops such as wheat and rice (SASAKI et al. 2002). This was due to decreased plant lodging and increased allocation of carbon to grain production instead of vegetative tissues. For example, the Green Revolution semi-dwarf IR8 rice was a GA deficient mutant with a mutation in the GA20ox-2 gene.

The production of active GAs occurs in three places in the cells of higher plants: ent-kaurene is produced in proplastids, ent-kaurene is converted to GA12 in the endoplasmic reticulum, and lastly 2-oxoglutarate-dependent dioxygenases form active GAs in the cytosol (OIKAWA et al. 2004). As reviewed in (SAKAMOTO et al. 2004), the steps leading to full hormonal activity are as follows: the first committed step in GA biosynthesis is the diterpene
cyclization of geranylgeranyl diphosphate (GGDP) to ent-copalyl diphosphate (CDP) by the action of copalyl diphosphate synthase (CPS). This is followed by a second cyclization by ent-kaurene synthase (KS) to form the tetracyclic diterpenoid compound ent-kaurene. Through the action of three cytochrome P450 mono-oxygenases, ent-kaurene 19-oxidase (EKO), ent-kaurenoic acid 7β-hydroxylase, and GA12-aldehyde synthase, GA12-aldehyde is produced. This is then oxidized to GA12 by GA 7-oxidase (GA7ox) followed by a further oxidation reaction, catalyzed by GA20-oxidase (GA20ox), that results in the production of C19-GAs. Finally, by the action of GA3-oxidase (GA3ox) bioactive GAs (e.g., GA4 and GA1) are produced from C19-GAs. Any disruption in this pathway causes dwarfism (SAKAMOTO et al. 2004).

GA2-oxidases (GA2ox) play a role in catabolism. They catalyze GA1, GA4, GA20, GA9, and C20 GAs to permanently inactive forms such as GA29 or GA8. In the homeostasis of GA, GA20ox and GA3ox are feedback regulated, whereas GA2ox is feedforward regulated.

The rice EUI gene, which is a cytochrome P450 mono-oxygenase, also has a role in the catabolism of GAs. An increase in the amount of EUI causes a reduction in GA levels, and consequently leads to dwarfism (ZHANG et al. 2008).

1.2.2. Gibberellin Signaling

Important steps of the GA signaling system have been identified by intensive work over the last decade. Key components of the pathway have been identified in plants such as Arabidopsis, rice, maize, and others. It was shown in rice that GID1 is the GA receptor (UEGUCHI-TANAKA et al. 2005). GID1 encodes a protein that localizes in the nucleus and has similarities with HSLs (Hormone Sensitive Lipases) (UEGUCHI-TANAKA et al. 2005; UEGUCHI-TANAKA et al. 2007). Different GAs have different affinities to GID1 and the one
with the highest affinity is GA4, the major bioactive GA in rice (Ueguchi-Tanaka et al. 2007).

*gid1-1* rice mutants have dark-green leaf blades with a dwarf stature resembling GA related mutants, and show greatly reduced sensitivity to applied GA (Ueguchi-Tanaka et al. 2005). The role of GID1 in Arabidopsis is the same as in rice. However, while rice has one *GID1* gene, Arabidopsis has three; At*GID1a*, At*GID1b*, and At*GID1c* (Nakajima et al. 2006). Even though single or double knock down mutants for these receptors do not cause strong phenotypes, the triple mutant of Arabidopsis GID1 receptors displays a severe dwarf phenotype (Griffiths et al. 2006).

The GID1 receptor interacts with DELLA proteins in the presence of GAs. In the absence of GA, DELLA proteins accumulate in the nucleus and negatively regulate GA responsive gene expression (De Lucas et al. 2008). In the presence of GA, DELLA proteins are degraded by 26S proteasome-mediated proteolysis, which in turn derepresses GA responsive gene transcription. In Arabidopsis, interactions between DELLAs and GID1a happen via N-Terminal DELLA and VHYNP motifs of DELLA proteins (Griffiths et al. 2006).

In rice, GID1 interacts with SLENDER RICE1 (SLR1), a DELLA protein (Ueguchi-Tanaka et al. 2005). GA concentration determines the strength of the GID1-SLR1 interaction (Ueguchi-Tanaka et al. 2007). In *gid1* mutants, SLR1 accumulates to high levels and is not degraded by GAs. Thus, *gid1* mutants show a GA insensitive dwarf phenotype. Conversely, *slr1* mutants show a constitutive elongated plant phenotype that is independent of GA (Ikeda et al. 2001).
DELLA proteins are conserved in many plants such as Arabidopsis, maize, rice, and barley (Dill et al. 2004). SLR1 (Rice) is an ortholog of GAI, RGA (Arabidopsis), RHT (Wheat), and D8 (Maize) (Ikeda et al. 2001). RHT-1 is an important Green Revolution gene for increasing yield because of its semi-dwarf phenotype (Peng et al. 1999). While rice has only one DELLA gene, Arabidopsis has five: RGA (REPRESSOR OF GA1-3), GAI (GA-INSENSITIVE), RGL1 (RGA-LIKE1), RGL2, and RGL3 (Ueguchi-Tanaka et al. 2007). All AtGID1 proteins interact with AtDELLA proteins in the presence of GAs. In contrast to AtGID1a and c, the AtGID1b-RGL1 interaction can be seen even at lower GA concentrations, which shows the possibility that this interaction could be independent of GA (Nakajima et al. 2006).

In Arabidopsis, DELLA genes GAI and RGA function redundantly in GA signaling pathway. An active protein of one of these is enough for the normal action even if the other is null (Ikeda et al. 2001). A mutation in the DELLA domain of rga causes a dominant dwarf phenotype in Arabidopsis. This mutation makes rga mutant proteins resistant to GA mediated degradation (Dill et al. 2001). Similar to rga, the gai mutant also remains resistant to GA and keeps showing its effects on the signaling pathway even in the presence of GA (Peng et al. 1997). Mutations in the DELLA domains of D8, D9 (Maize), and Rht1 similarly cause dominant, GA-insensitive dwarf phenotypes (Peng et al. 1999; Winkler and Freeling 1994). Even though RGA and GAI have similar properties in Arabidopsis GA signaling, RGA is a stronger repressor than GAI (Dill et al. 2004).

DELLA proteins are involved in several signaling systems. PIF4 (PHYTOCHROME INTERACTING FACTOR 4) which is a light regulated factor has a positive role in light mediated cell elongation (Lucyshyn and Wigge 2009). This protein localizes in the nucleus.
and leads to cell elongation. However, DELLA proteins interact with PIF4 and prevent this protein’s transcriptional activity, thereby blocking PIF4-mediated cell elongation. Presence of GAs leads to the degradation of DELLA proteins which causes cell elongation by the releasing of PIF4 from DELLA proteins. This shows that DELLA proteins are at a cross-talk point between light and GA signaling (DE LUCAS et al. 2008).

In rice and Arabidopsis, it has been shown that SPINDLY (SPY) encodes an O-linked N-acetylglucosamine transferase which is a negative regulator of GA signaling. SPY functions to promote the suppression activity of DELLA proteins in GA signaling (HARTWECK et al. 2006; SHIMADA et al. 2006).

The interaction of GID1 with DELLAs, in the presence of GA, results in the degradation of the DELLAs. Key components of this mechanism are GIBBERELLIN INSENSITIVE DWARF2 (GID2) in rice and SLEEPY1 (SLY1) in Arabidopsis, which encode F-box proteins that function in the degradation of proteins through the 26S proteasome. GID2 and SLY1 are members of a SCF<sub>SLY1/GID2</sub> E3 ubiquitin ligase complex (DILL et al. 2004; GOMI et al. 2004). The SCF<sub>SLY1/GID2</sub> E3 complex mediates the degradation of DELLA proteins in the presence of GAs (DILL et al. 2004). The SCF complex, consists of Skp1, cullin, and RBX proteins. With one of its domains, GID2 interacts with SCF complex via Skp1 and interacts with phosphorylated SLR1 via another domain (GOMI et al. 2004).

SLY1 and GID2 localize to the nucleus. GID2 interacts with phosphorylated SLR1 (GOMI et al. 2004). In Arabidopsis, SLY1 binds to the GA-GID1-RGA complex and the GA-GID1 interaction increases the interaction of RGA-SLY1. SLY1 interacts with RGA and GAI proteins by a C-terminal GRAS domain (DILL et al. 2004; GOMI et al. 2004).
These interactions lead to the ubiquitination of RGA by the SCF^{SLY1} Ub E3 ligase and result in the degradation of DELLAs by 26S proteasome (GRIFFITHS et al. 2006). The net outcome of these interactions is that the Arabidopsis SLY1 and rice GID2 proteins function as positive regulators of GA signaling.

1.3. AUXIN

Auxin is the first hormone identified in plants (TROMAS and PERROT-RECHENMANN 2010). It is involved in many aspects of plant development and responses to environmental changes. For example, auxin is involved in cell division, cell elongation, vascular tissue formation, floral development, root formation, shade avoidance syndrome, tropisms, embryogenesis, and more (TROMAS and PERROT-RECHENMANN 2010; VANNESTE and FRIML 2009;SAZUKA et al. 2009). Auxin’s distribution in the plant differs from tissue to tissue and auxin transport is important for regulating many cellular patterning and differential growth processes (VANNESTE and FRIML 2009).

Auxin is important for regulating plant height. Mutations in the biosynthesis or polar transport of auxin caused dwarfism in plants such as in maize, rice, Arabidopsis, and sorghum (GEISLER et al. 2003; MULTANI et al. 2003; SAZUKA et al. 2009). The rice tryptophan deficient dwarf1 (tdd1) has a disruption in auxin biosynthesis. TDD1 encodes beta-subunit of anthranilate synthase (OASB1), which is a rate-limiting enzyme for Trp biosynthesis (SAZUKA et al. 2009) and these mutants show decreased levels of Trp and IAA. This mutant shows a dwarf phenotype and also has disrupted leaf morphology.

Mutations disrupting polar auxin transport can also cause dwarfism. Maize brachytic2 (br2) mutants are dwarf plants that are different than GA and BR mutants because the dwarf
phenotype is restricted to the lower stalk internodes, while the rest of the plants look normal. Cell sizes of the stalk are reduced, but the girth of the plant is increased compared to the wild type. Studies on this mutant showed that br2 plants show a disruption in the mechanism of polar auxin transport. The br2 gene encodes a Phospho-glycoprotein (PGP) (MULTANI et al. 2003). PGPs belong to the ABCB subgroup of the ATP-Binding-Cassette (ABC) transporter family (TITAPIWATANAKUN and MURPHY 2009). Most ABC proteins are membrane bound and they mediate transport of different molecules across the membranes (LUSCHNIG 2002; VERRIER et al. 2008). PGPs modulate auxin-mediated growth in plants by functioning in polar auxin movements (MULTANI et al. 2003; NOH et al. 2001). In maize, the expression of BR2 is found in the nodes, not in the internodes (KNOELLER et al. 2010).

The BR2 ortholog in Arabidopsis is ABCB1, which has a role in auxin efflux in the meristamatic regions of shoots and roots (KNOELLER et al. 2010; TITAPIWATANAKUN and MURPHY 2009). Even though maize BR2 and Arabidopsis ABCB1 are similar to each other in amino acid sequence homology, the growth phenotypes of maize br2 and Arabidopsis abcb1 mutants are different than each other. This difference is primarily due to BR2’s role in intercalary and apical meristems, where Arabidopsis ABCB1 does not function (KNOELLER et al. 2010).

Another auxin transport mutant in Arabidopsis is twisted dwarf1 (TWD1), which has reduced cell elongation and disrupted growth. TWD1/FKBP42 encodes a FK506-binding protein (FKBP)-like immunophillin, which is an anchored plasma membrane protein. FKBP2s have roles in inhibiting the efflux of some anticancer drugs, modulating signal transduction pathways, and regulating MDR-like ABC transporters (CARDENAS et al. 1994; GEISLER et al. 2003). Arabidopsis TWD1 physically interacts with AtPGP1 and AtPGP19 and is necessary
for their proper function (Geisler et al. 2003). Like atpgp1, and atpgp19, mutations in twd1 show disrupted polar auxin transport and a dwarf phenotype.

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CHAPTER 2. IDENTIFICATION OF NOVEL MAIZE DWARF MUTANTS

2.1. ABSTRACT

Plant height is an important agricultural trait determined by several factors including gibberellins (GA), brassinosteroids (BRs), and auxin, with BR’s roles in maize the least understood. Mutations in these hormone systems can cause dwarfism. To identify unknown factors, including potential BR-related genes, that control plant height in maize we screened uncharacterized maize dwarf mutants. Complementation tests indicate that many of the mutants were allelic to previously described mutants, while several were potentially new. Dwarf mutants were phenotyped for total plant height, node number, internode lengths, internode epidermal cell lengths, and flowering time. Several novel mutants were identified, including at least one likely candidate for a BR-related mutant. Several mutants were putatively mapped to chromosome locations that suggest they represent previously unreported loci.

2.2. INTRODUCTION

Plant height is an important agricultural trait (SALAS-FERNANDEZ et al. 2009; SAKAMOTO and MATSUOKA 2004; WANG and LI 2006). In the Green Revolution, decreased plant height increased grain yield (EVENSON and GOLLIN 2003; HEDDEN 2003; KHUSH 2001; PENG et al. 1999). Semi-dwarf varieties are particularly important for rice and wheat (KHUSH 2001; PENG et al. 1999). Besides increased grain yield, another reason to manipulate plant height is to increase biomass yield, which could be important for supplying future fuel demands. Unlike grain yield, it is increased plant height that is useful for increasing whole-plant biomass. Maize, switchgrass, and sorghum are some of the plants on which research is
conducted for biofuel production (SOMERVILLE et al. 2010). Among these, the most widely used plant for ethanol production in the U.S. is currently maize (SERVICE 2007). For these and several other reasons, plant height is of interest in agricultural studies (SALAS-FERNANDEZ et al. 2009).

Several plant hormones known to control plant height include gibberellin (GA), brassinosteroids (BRs), and auxin (HONG et al. 2003; MULTANI et al. 2003; UEGUCHI-TANAKA et al. 2005). Mutations that block GA, BR, or auxin biosynthesis or signaling can cause dwarfism in plants. Despite extensive study in several species, the genetic regulation of plant height, particularly in maize, remains poorly understood (SUNOHARA et al. 2009). In maize, the importance of the GA hormone system for controlling plant height is well established, although only a few components have thus far been isolated. Mutants in DELLA proteins demonstrate the importance for GA signaling in controlling normal plant height. DELLA proteins, such as GAI in Arabidopsis, encode negative regulators of GA signaling (WANG and Li 2006). Dwarf8 (D8) and D9 are maize orthologs of GAI (PENG et al. 1999; LAWIT et al. 2010). Plants with dominant D8 or D9 mutations are GA insensitive and show either dwarf or semi-dwarf stature, depending on the gene and the allele. D8 has both dwarf and semi-dwarf alleles (HARBERD and FREELING 1989), while the D9 mutant is semi-dwarf (WINKLER and FREELING 1994; LAWIT et al. 2010). Two genes involved in GA biosynthesis have been isolated in maize. dwarf3 (d3) mutants are GA deficient and show a dwarf phenotype. The d3 gene encodes GA13-hydroxylase (GA13ox), a cytochrome P450 that catalyzes the conversion of GA_{12} to GA_{53} in the biosynthesis of GAs (WINKLER and HELENTJARIS 1995). anther ear1 (an1) mutants show semi-dwarf phenotypes. an1 encodes a cyclase, most likely copalyl pyrophosphate synthase (CPS), which catalyzes the cyclization
of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP) in an early step of GA biosynthesis (BENSEN et al. 1995). The semi-dwarf phenotype (as opposed to fully dwarfed) is thought to be due to partial redundancy in the maize genome.

The importance of auxins for controlling normal plant stature has been demonstrated through the analysis of the brachytic2 (br2) mutant. Homozygous recessive br2 mutants show a semi-dwarf phenotype. Studies on this mutant show that br2 encodes a B subfamily member of the ABC/PGP/MDR (ATP binding cassette/P-glycoprotein/multidrug resistance) type membrane transporters that function in the polar transport of auxin (KNOELLER et al. 2010; MULTANI et al. 2003). br2 mutants show decreased polar auxin transport in the stem, apparently leading to shortened internodes and a semi-dwarf plant phenotype (MULTANI et al. 2003).

BR is well known to be required for normal plant stature in Arabidopsis, rice and other plants (reviewed in BAJGUZ 2007; BISHOP 2003; KIM and WANG 2010). Arabidopsis det2 mutants, which are defective in BR biosynthesis, show a dwarf phenotype, reduced male fertility, and dark green leaves. DET2 encodes a 5α-reductase in the biosynthesis of BRs (FUJIOKA et al. 1997; LI et al. 1996). The DWF4 gene in Arabidopsis catalyzes a rate-limiting step in BR biosynthesis (CHOE et al. 1998). It encodes cytochrome P450 monooxygenase (CYP90B1) catalyzing C-22 hydroxylation of the steroid side chain (CHOE et al. 1998). In Arabidopsis, overexpression of DWF4 caused increased BR levels, prolonged flowering, and increased seed yield (CHOE et al. 2001). BR mutants in rice show short leaf sheaths and blades, less tillering, curled leaf blades, reduced lamina joint bending and dwarfism (BAI et al. 2007; SAKAMOTO et al. 2006). For example, the brdl1 mutant has defects in the OsBR6ox gene and shows these phenotypes (MORI et al. 2002). No Arabidopsis
mutant is known for this step. Although the requirement for BR is well established in several species, no BR-related mutants in maize have been published to date. Therefore, we chose to characterize existing and newly isolated maize dwarf mutants in an effort to identify components of the BR hormone system in maize.

2.3. RESULTS

Complementation Tests

To search for mutants involved in hormone systems other than GA, we searched publically available maize stocks for dwarf mutants annotated as non-responsive to GA or with phenotypic descriptions distinct from GA mutants; we obtained over 40 stocks. Several mutants showed severe developmental defects that suggested the primary function of the gene was more complex than the regulation of plant growth per se. Several other mutants showed phenotypes that were too mild to score with confidence under our conditions. These two groups were not considered further. To determine the allelic relationships among the remaining mutants, and whether they corresponded to already described loci, complementation tests were performed. Pairwise crosses were performed among these mutants and with already described mutants, and the F₁ examined for a dwarf phenotype. Thus far, complementation tests have been completed for the known mutants br₁, br₂, br₃, and nana plant1 (na₁). We have identified one additional mutant allele of br₁, six alleles of br₂, nine alleles of br₃, and two alleles of na₁. Seven additional mutants have complemented all mutants thus far tested and so potentially represent new loci. In addition, genetic mapping results (see below) suggest at least two newly identified mutants, d*-EV2 and d*-EV4, represent unique loci. Results are shown in Table-1.
GA Rescue Experiments

To verify that the mutants obtained were not GA related, their phenotypes were observed and GA rescue experiments performed. Mutants deficient in GA biosynthesis or signaling have distinct phenotypes, described in detail below. Two of the mutants, \( d^*-N2256 \) and \( d^*-64-4156-1 \) showed typical GA-related phenotypes, including andromonoecy. Representative mutants for each complementation group were also tested for responses to GA applied foliarly to growing seedlings. The \( d^*-N2256 \) and \( d^*-64-4156-1 \) mutants both showed rapid growth induction in response to exogenous GA, suggesting they are likely deficient in GA biosynthesis. An example is shown in Figure 1. None of the other mutants were rescued by GA treatments, nor showed anther-ear phenotypes, suggesting their defects are independent of the GA hormone system.

Phenotypic Characterization

To help understand the characteristics of these mutants a phenotypic analysis was performed. Each mutant was backcrossed to the B73 inbred line 2-4 times. In addition to decreased plant height, phenotypic characters often found in different classes of dwarf mutants include broad leaves, upright leaves and dark green leaf color. These phenotypic characters were assessed and the data are presented in Table 2 and Figure 2, while other features of note are described below. For reference, known GA, auxin, and BR mutants were also described.

GA mutants produce distinct “andromonoecious dwarf” phenotypes that have been described (COE et al. 1988; NEUFFER et al. 1997). Briefly, strong GA dwarves show dramatically shortened internodes, compact tassels, and leaves that are short, broad, crinkled, and dark green in color. A distinct characteristic of GA mutants is the anther-ear phenotype,
where male reproductive organs are de-repressed in the ear florets, allowing bisexual flowers to develop in place of female (HARBERD and FREELING 1989 and references therein). These phenotypes were clearly displayed in strongly dwarfed GA-deficient d1 and d3 mutants. Among the GA-insensitive mutants D8-1 also showed a strong dwarf phenotype while D8-Mpl and D9-1 were semi-dwarf (WINKLER and FREELING 1994).

The br2 mutant is defective in auxin polar transport and has shortened internodes, particularly in the lower part of the plant, with increased stalk girth (MULTANI et al. 2003). The growth of other plant parts is not noticeably affected. In addition, we observed that upper leaves of br2 showed an upright habit. Six additional mutants were identified in the current study as members of the br2 complementation group. While some of them resemble br2 plants, some of them are somewhat different. For instance, br2-Singleton-8 alleles produce stronger dwarfism than br2-R. While br2-Singleton-8 mutants are 47% as tall as their wild type (WT) siblings, br2-R mutants are 65%.

The na1 gene has been reported to encode a det2 homolog, believed to be involved in BR biosynthesis (HARTWIG et al. 2010). This mutant has erect, twisted, and dark green leaves. The internodes closer to the bottom of the plant are shortened, causing lower leaves to be clustered together. We observed that a relatively long internode often interspersed the short ones (Figure 3). Also, na1 mutants often have tasselseed phenotypes resulting from female reproductive development in tassel florets (Figure 4). This phenotype is unique among the mutants examined in this study. The br*-SGL635-7 and d*-N282 mutants failed to complement na1-R and were therefore renamed na1-SGL635-7 and na1-N282, respectively. The phenotypes of both mutants resembled na1-R, although na1-SGL635-7 had a weaker
effect on plant height (29% vs. 17% of WT, respectively). Additional phenotypic characteristics of particular complementation groups follow:

\textit{br1}: Stems are thicker than WT.

\textit{br3}: There is variation in the degree of dwarfism among \textit{br3} alleles. \textit{br3-2180} is the strongest allele at about 25% of WT plant height.

\textit{d*-N1168B}: Leaves have a light green color.

\textit{bv*-N2282}: This mutant has thin stalk.

\textit{d*-3047}: This mutant often has a barren inflorescence resulting in male sterility.

Homozygous mutants have not been successfully crossed as females either.

\textit{D10}: This mutant has a strong dwarf stature with short internodes and narrow leaves.

\textit{br*-Osijek-Yugoslavia}: The original \textit{br*-Osijek-Yugoslavia} mutant stock obtained from the Maize Genetics Cooperation Stock Center showed a clearly dwarf stature. After backcrossing this mutant to three different inbred lines, including B73, Mo17 and W22, the expression of the mutant phenotype dwindled. After two backcrosses to B73, the \textit{br*-Osijek-Yugoslavia} mutants had only 2 or 3 a few compressed internodes near the upper ear node. Mutants became indiscernible upon further backcrossing or when we crossed \textit{br*-Osijek-Yugoslavia} mutants to the Mo17 inbred to create an F$_2$ mapping population.

The remaining mutants listed below were recently isolated from an EMS mutagenesis screen and are still in early stages of characterization.

\textit{d*-EV1}: This mutant is a moderate dwarf and has an unusually thin stem.

\textit{d*-EV2}: Plants show a clear dwarf stature. Lower internodes appear most strongly affected.
$d^*-EV4$: This is a semi-dwarf with a unique phenotype among our mutant collection. Leaves are broad and crinkled.

$d^*-EV5$: The stem is thinner than normal.

$d^*-EV7$: This mutant is a semi-dwarf. Leaf habits look normal.

The Morphological Basis of the Dwarf Phenotypes

Morphological characters were measured in each mutant to help determine the physiological functions of each gene and whether different genes appeared to control related processes. The following traits were measured: overall plant height, node number, internode lengths, and epidermal cell lengths of internodes. As shown in Figure 5, the strength of the dwarf phenotypes varied substantially among the mutants. The $na1-N282$ mutant produced the strongest dwarf phenotype with a mean overall plant height only 17% of WT. The weakest mutant in this study was $br^*Osijek$ at 83% of WT mean plant height. Among the unique mutants that complemented known loci, $d^*-N1168B$ was the strongest showing a mean height 36% of WT. $na^*-1519D$ showed a moderately dwarf phenotype at 49% of WT, while $br^*-Brawn219$ was semi-dwarf at 72% of WT height.

To determine the basis of the decreased height in the mutants, nodes were counted, internode lengths measured, and internode epidermal cell lengths measured. The number of nodes was decreased an average of 4 nodes in $na1-N282$ alleles (Figure 6). Several other mutants, including $d^*-N1168B$, $br2-Brawn230$ and $br3-2180$ showed lesser reductions in node number, indicating that this factor contributes variably to the decreased plant height of some mutants. On the other hand, the GA-deficient $d^*-64-4156-1$ mutant had more nodes than the WT. For the most part, decreased node number does not appear to be the primary
factor responsible for the dwarf stature. For example, na1-N282 had over 75% the mean number of nodes as WT but was only 17% as tall.

Internode length measurements are shown in Figures 7 and 8. For all mutants, internode lengths were substantially decreased compared to WT, indicating that this is a major contributing factor to the dwarf habits. Interestingly, different mutants showed various patterns of internodes most strongly affected (Figure 8). For example, br3-2180 showed relatively weak effects on the first couple internodes but the effects gradually increased in later internodes. In contrast, br1-R mutants showed fairly constant effects with the shortening slightly more pronounced in early internodes than late ones. na1 mutants showed an unusual pattern with a couple relatively mildly effected internodes, followed by the majority of internodes strongly shortened, and then another mildly effected (long) internode near the tassel (Figure 9).

Decreased internode lengths can arise from decreased internode cell lengths, from decreased cell numbers, or both. To determine the cellular basis of the internode shortening, epidermal cell lengths in the most strongly affected internodes for each mutant were measured. The epidermis is the primary site of BR-directed growth regulation in Arabidopsis stems (Savaldi-Goldstein et al. 2007). As shown in Figures 10 and 11, decreased epidermal cell lengths were observed for several of the dwarves, including na1-N282 mutants, both GA deficient mutants, and br*-Brawn219-221. Figure 11 illustrates an example of br2-Brawn230 mutant epidermal cells that are clearly shorter than on their WT sibling. On the other hand, no decrease in cell length was observed for br3-R or na*-N1519D, indicating that these mutants must have a decreased number of internode cells. Figure 11 also
shows \textit{d*-N1168B}, which shows no discernable difference in epidermal cell morphology to its normal sibling control.

**Flowering Time**

GAs and BRs regulate flowering time and flower development (Li \textit{et al.} 2010; Mutasa-Gottgens and Hedden 2009). In both cases, hormone biosynthesis or signaling deficient mutants tend to flower late. Furthermore, early flowering is a potential mechanism for producing short plant stature, as has been shown for the \textit{early phase change} mutant (Vega \textit{et al.} 2002). Because of these links between flowering time and plant stature, flowering time was recorded for these dwarf mutants.

To measure flowering times, heterozygous mutants in a B73 background were self pollinated to generate segregating families. Flowering time was measured as days from planting to anthesis for mutant and WT individuals. The mean values for each mutant line are shown in Figure 12. As expected, the presumptive GA deficient mutant, \textit{d*-64-4-4156-1}, and the BR deficient mutant, \textit{na1}, flowered later than WT (Figure 12). The \textit{br2} mutants, defective in auxin polar transport, did not show a significant difference from WT, nor was there a detectable difference in \textit{br1} or \textit{br3} mutants.

Among the remaining mutants, \textit{br*-Brawn219-221} flowered simultaneously with WT and \textit{d*-N1168B} mutants were not significantly late. However, there was a clear late flowering phenotype for \textit{na*-N1519D} and \textit{bv*-N2283} mutants. In addition, the \textit{d*3047} and \textit{d*-N2256} mutants appeared to flower late however recording the dates of anthesis in these mutants was precluded by the barren tassel and lack of anther exertion, respectively.
Identification of Chromosomal Map Locations for Select Mutants

Several mutants with unknown map locations were deemed of interest for further analysis and therefore genetic mapping experiments were undertaken. Two mutants, na*-N1519D and d*-N1168B, in B73 genetic backgrounds were each crossed to the Mo17 inbred and self pollinated to generate segregating F₂ mapping populations. Four additional mutants, designated d*-EV2, d*-EV4, d*-EV6, and d*-EV7 were derived from an EMS mutagenesis performed in a Mo17 background. Each of these mutants was crossed to B73 and an F₂ mapping population generated.

Bulk segregant analysis (BSA) was performed using Sequenom™ technology to assay single nucleotide polymorphisms (SNPs) distributed throughout the genome at an average of 2 cM intervals (Liu et al. 2010). Candidate chromosomes were identified for all 6 mutants (Table 3). For 4 mutants, a single candidate region was returned, while for na*-N1519D and d*-EV7 two candidate chromosomal regions were identified.

Further mapping experiments were performed to verify the chromosomal locations and further define the genetic interval occupied by na*-N1519D and d*-N1168B. MaizeGDB (http://www.maizegdb.org/) was searched for IDP marker loci, PCR-based insertion-deletion polymorphism markers (Fu et al. 2006), in the genomic regions identified by BSA. As shown in Figure 13, na*-N1519D showed tight linkage to IDP4282, which is located at 80 cM on Chromosome 4 of ISU IBM Map7. Marker IDP454, at position 74 cM, showed 2 recombinant chromosomes out of 28 (not shown). Therefore, this mutant gene is located on chromosome 4 near position 80 cM. d*-N1168B was similarly mapped using IDP markers located on chromosome 5. Figure 13 shows that for d*-N1168B, 2 out of 12 chromatids were recombinant with marker IDP4768, which is located at 25.4 cM of Chr 5. Therefore, this
mutant is near this chromosomal region. More precise map locations will require further mapping with larger segregating populations.

2.4. DISCUSSION

We screened a collection of maize dwarf mutants to identify novel genes controlling plant height in maize. Several hormones such as GA, BR, and auxin are important for plant height (Geisler et al. 2003; Hedden 2003; Multani et al. 2003; Oikawa et al. 2004; Szekeres et al. 1996). Disruptions in the biosynthesis, signaling, or transport of these hormones could lead to dwarfism. Among these hormones, BRs are the least studied in maize, so we were particularly interested in mutants associated with this hormone. GA mutants have distinctive andromonoecious dwarf phenotypes, and the phenotype of the auxin polar transport mutant, br2 has been described (Knoeller et al. 2010; Multani et al. 2003; Winkler and Freeling 1994; Winkler and Helentjaris 1995). Therefore, we were interested in dwarf mutants with phenotypes distinct from these known mutants to identify novel genes regulating maize plant growth.

We obtained over 40 uncharacterized dwarves from the Maize Genetics Cooperation Stock Center and identified others in mutagenesis experiments. Complementation tests to date have revealed one new allele of br1, six of br2, nine new alleles of br3 and two of na1. Six mutants have complemented all other mutants thus far tested, suggesting they each represent single isolates of different complementation groups.
Phenotypic analyses identify multiple mechanisms of dwarfism

Each mutant was phenotypically characterized to describe the effects on plant architecture. The primary phenotypic characteristic of interest is plant height. We saw that different mutations cause variable reductions in total plant height (Figure 5). While some mutants show very strong dwarfism, some can be considered as semi-dwarf mutants.

Mutants that show strong dwarf phenotypes represent critical genes for maize plant development. For example, as seen in Figures 3 and 5, na1 alleles show very strong dwarf phenotypes. For semi-dwarf mutants, we may infer either that the mutants are not complete losses-of-function, the mutated genes are partially redundant or that the genes are less critical for controlling normal plant height. For genes such as br2, where multiple alleles all produce semi-dwarf plants, it is likely that this represents the null phenotype for this locus.

To understand the basis of the plant height phenotypes, several characters were examined. The average number of nodes was decreased for most mutants indicating that this was often a contributing factor. However, this did not account for the overall decrease in plant height for any mutant, and a couple, including br1, showed no decrease in node number. All mutants examined showed decreases in internode length. Epidermal cell lengths were measured for each mutant in one of the most strongly affected internodes. Epidermal cells are thought to be key in regulating overall stem elongation (SAVALDI-GOLDSTEIN et al. 2007). Most mutants showed shortened cells, compared to WTs, but some mutants, such as d*-N1168B, showed no difference or even slightly elongated cells. Thus both decreased cell elongation and decreased cell numbers are associated with decreased internode length in various mutants.
A decrease in internode length, without a decrease in cell length, implies one of two things. One possibility is that the rate of cell proliferation is decreased, resulting in fewer internode cells separating nodes. The second possibility is that the duration of proliferation is abbreviated. One way to achieve an abbreviated period of internode growth is to accelerate the rate of leaf (node) initiation. Several accelerated plastochron mutants in rice show dwarfism and shortened internodes (Kawakatsu et al. 2009). The d*-N1168B mutant showed a decreased node number and no significant difference in time to flowering, so clearly an accelerated rate of leaf initiation did not cause the abbreviated internode length. Further studies will be required to directly measure the relative cell division rate in the stems of this mutant.

Interestingly, although most mutants caused a shortened phenotype for all internodes, the strength of the effects showed variable patterns among mutants. Some, such as br2, showed the strongest effects on lower nodes (Multani et al. 2003; Pilu et al. 2007), while others like br3-2180 showed relatively weak effects on lower internodes and stronger on upper internodes. Yet others, including brl, showed fairly uniform effects on all internodes. An unusual pattern was observed in nal mutants, where a single elongated internode was often interspersed among shortened ones.

In summary, it is clear that the regulation of plant height is complex. Both the number and length of internodes are important components of overall plant height. The regulation of internode length involves both the regulation of cell number (presumably proliferation) and cell length (elongation). Different dwarfing genes contribute variously to regulating all these different processes. Furthermore, different genes function most prevalently at different times throughout the growth of the plant, some acting early, some late, and others continually.
The dwarf mutants affect other traits in addition to plant height

Most of these dwarf mutants affected other aspects of plant architecture beside plant height. One common characteristic was erect leaf habit. This could have agricultural implications because upright leaves allow light to penetrate the canopies of crops planted at high density, resulting in increased yields (MORINAKA et al. 2006). Erect leaves are a feature of BR mutants in rice and the \textit{br2} auxin transport mutant of maize (MORINAKA et al. 2006; MULTANI et al. 2003). Additionally, some of the mutants showed broad leaf morphology and many also displayed dark green color. Both these traits are associated with GA mutants in maize (reviewed in COE et al. 1988; NEUFFER et al. 1997) while dark green leaf color is also observed in the \textit{br2} mutant (PILU et al. 2007).

We also recorded the flowering time for these mutants because flowering time is also regulated by GA, BR, and auxin (DELLAPORTA and CALDERONURREA 1994; LEE et al. 2008; SAZUKA et al. 2009). Not surprisingly, several of these mutants also were late flowering. On the other hand, we confirmed that \textit{br2} does not alter flowering time (PILU et al. 2007) nor does \textit{br3} or a potentially novel mutant \textit{br*-Brawn219-221}. Therefore, plant stature and flowering time are not inexorably coupled. It should be noted that one of the \textit{br3} alleles potentially showed late flowering, which remains to be verified.

Genetic mapping of select dwarf mutant loci

Lastly, several mutant loci were mapped to chromosomes by BSA using high-throughput quantitative SNP genotyping. The \textit{br1}, \textit{br2}, \textit{br3}, and \textit{na1} map locations are already known so mutants of these loci were not considered for this analysis. The \textit{d*-N1168B} and \textit{na*-N1519D} mutants complemented all other mutants tested and showed potentially interesting phenotypes. \textit{d*-N1168B} mapped to chromosome 5, where several known
dwarfing genes are located. The \textit{na*-N1519D} mutant mapped to chromosome 4, near where one previously mapped mutant resides.

In addition, 7 dwarf mutants were identified in an EMS mutagenesis screen. The phenotypic characterization for these remains to be completed, but four mutants of preliminary interest were subjected to genetic mapping. Putative locations for three of these mutants were identified on chromosomes 5, 6 and 8, while the fourth mutant returned potential locations on chromosomes 3 and 5.

Further mapping and complementation studies will resolve whether these mutants represent novel loci or alleles of previously described mutants, provide more precise genomic locations, and allow the molecular identification of the underlying genes.
2.5. MATERIALS AND METHODS

Genetic Stocks

Publically available maize genetic stocks were searched at MaizeGDB (http://www.maizegdb.org/) for mutants that were annotated as having phenotypes related to “dwarf plant” or “small plant”, and described as being unresponsive to applied GA or otherwise unrelated to GA (LAWRENCE et al. 2008). Stocks were ordered and received from the Maize Genetics Cooperation Stock Center, University of Illinois, Urbana (SACHS 2005) (http://maizecoop.cropsci.uiuc.edu/). Each mutant was grown and observed under field conditions in Ames, IA. It was ascertained that several mutants showed severely pleiotropic developmental defects that would not be informative for understanding the regulation of plant growth per se, and these were not considered further. Each mutant was backcrossed to the B73 inbred, most 4 times, but some as few as 2 if they were obtained at a later date or a field plot was lost to weather or other factors.

Plant Growth Conditions

Field grown maize plants used in these experiments were grown at the Iowa State University Curtiss Farm during the summers of 2009 and 2010. Greenhouse grown plants were grown in 1-gallon pots containing universal potting mix under 16 hour light period with natural daylight supplemented by sodium-halide artificial lighting.

GA Treatment

GA treatments were performed on soil grown young plants. ~288 µM GA3 (Sigma) dissolved in water with 0.1% Tween-20 was sprayed on the foliage of the growing young plants (2-4 weeks old). This treatment was carried out 2-3 times in a week. Known GA
deficient and GA insensitive plants were used as controls. Since apparent toxicity seen on some GA treated plants, treatment was repeated with 50µM GA3.

**Complementation Tests**

Homozygous dwarf mutants were crossed to each other. The following summer, progeny from these crosses were observed. Mutants were noted as complementing if a WT phenotype was exhibited or as non-complementing, probably allelic, if a dwarf phenotype was observed.

**Phenotypic characterization**

Plant height is measured from the bottom of the plant (the soil surface) to the top of the tassel. Measurements were carried out on fully mature plants. Nodes and internodes were numbered and counted starting from the first seedling leaf node at the bottom of the plant, progressing to the upper parts. Each node length was measured. Impressions of epidermal cells were obtained from internodes by painting the culm surface with clear nail polish. After drying, the nail polish was peeled and examined under an Olympus BX60 microscope using differential interference contrast (DIC). Impressions were digitally photographed with a Jenoptik C5 camera and cell lengths were measured with PROGRES 2.0 image analysis software. Cell sizes were measured at 20X calibration. Flowering time was recorded as the time between planting date and the onset of anthesis.

**Mapping**

For mapping, dwarf mutants in a B73 background were crossed to Mo17, or vice versa, mutants in a Mo17 background were crossed to B73 maize inbreds. F₂ segregating populations from these crosses were grown in the field. Leaf tissues were sampled from
equal numbers of WT and dwarf plants. For each dwarf, WT and mutant samples were pooled separately and DNA extracted according to a modified protocol from (MICHAELS and AMASINO 2001). Extracted DNA samples were submitted to the Iowa State University Genome Technologies Facility. BSA analysis was performed using Sequenom™ technology to assay 2076 SNPs known to be polymorphic between B73 and Mo17 inbreds. These represent 1038 loci distributed throughout the genome at an average of 2 cM intervals (Liu et al. 2010).

Preliminary mapping results were verified for two of the mutants, na*-N1519D and d*-N1168B, using PCR based molecular markers. WT and mutants, from the same populations used for BSA, were sampled individually and DNA prepared. MaizeGDB (http://www.maizegdb.org/) was searched for indel (IDP) markers on chromosomes 4 and 5. Markers IDP4282, which is located at 80 cM on chromosome 4, and IDP454, located at 74 cM, were polymorphic in the family segregating na*-N1519D and so were used for mapping (Figure 13). Similarly, marker IDP4768, which is located at 25.4 cM on chromosome 5, and marker IDP1638, located at 14.3 cM, were polymorphic in the family segregating d*-N1168B.

PCR was performed for these markers using 50-70 ng of maize genomic DNA and the reaction conditions specified at MaizeGDB. Amplification products were electrophoresed through a 2% agarose gel, stained with ethidium bromide and photographed with a UVP® Ultraviolet Transilluminator.
2.6. FIGURES AND TABLES

Figure 1. GA treatment of $d^*-N2256$ mutants. The first two plants on the left are dwarves treated with GA, followed by an untreated WT and mutant, respectively. Note the height difference between GA treated and untreated mutants.
<table>
<thead>
<tr>
<th>$br1$-$R$</th>
<th>$br2$-$R$</th>
<th>$br3$-$R$</th>
<th>$na1$-$R$</th>
<th>Individuals$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$br1$-$384$</td>
<td>$br2$-Singleton1969252</td>
<td>$br3$-Brawn274-275</td>
<td>$na1$-SGL635-7</td>
<td>$br^*$-OsijekYugoslavia</td>
</tr>
<tr>
<td></td>
<td>$br2$-Singleton-8</td>
<td>$br3$-Brawn261-262</td>
<td></td>
<td>$br^*$-Brawn219-221</td>
</tr>
<tr>
<td></td>
<td>$br2$-PI228171</td>
<td>$br3$-Brawn267-268</td>
<td></td>
<td>$na^*$-N1519D</td>
</tr>
<tr>
<td></td>
<td>$br2$-Brawn231-233</td>
<td>$br3$-Brawn263-266</td>
<td></td>
<td>$d^*$-N1168B</td>
</tr>
<tr>
<td></td>
<td>$br2$-Brawn227-229</td>
<td>$br3$-Brawn269-271</td>
<td></td>
<td>$d^*$-3047</td>
</tr>
<tr>
<td></td>
<td>$br2$-Brawn230</td>
<td>$br3$-Brawn235-237</td>
<td></td>
<td>$d^*$-EV2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$br3$-Brawn272-273</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$br3$-Brawn259-260</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$br3$-2180</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Mutants that have produced complementation in all tests thus far performed, or which represent unique loci based on their chromosomal map positions (see below).
Table 2. Phenotypic characteristics of the dwarf mutants.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Erect Leaf</th>
<th>Broad Leaf</th>
<th>Dark Leaf Color</th>
<th>Height; d/wt</th>
</tr>
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<tbody>
<tr>
<td>br1-R</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.44</td>
</tr>
<tr>
<td>br2-R</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.65</td>
</tr>
<tr>
<td>br3-R</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.3</td>
</tr>
<tr>
<td>nal- N282</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0.17</td>
</tr>
<tr>
<td>d*-64-4156-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.68</td>
</tr>
<tr>
<td>d*-N2256</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.31</td>
</tr>
<tr>
<td>d3-Coop</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>br*-Brawn219</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>0.72</td>
</tr>
<tr>
<td>na*-N1519D</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0.49</td>
</tr>
<tr>
<td>d*-N1168B</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0.36</td>
</tr>
<tr>
<td>bv*-N2282</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.53</td>
</tr>
<tr>
<td>bv*-N2283</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0.57</td>
</tr>
<tr>
<td>d*-EV1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>d*-EV2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>d*-EV4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>d*-EV5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>d*-EV6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
<tr>
<td>d*-EV7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>D10</td>
<td>+</td>
<td>-</td>
<td>+?</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>
Figure 2. Some dwarf mutants are shown. $br2-R$, $d3-Coop$, $na1-R$, and $d*-N1168B$ are shown in A, B, C, and D, respectively.
Figure 3. The internode phenotype of *na1* mutants. A. A WT plant and two *na1* mutants. B. A closer view of the center *na1* plant is shown. The long internode is shown with a red arrow and the shortened-clustered internodes with a black arrow. C. The shortened-clustered internodes (8 internodes) are shown with black arrows.
Figure 4. *nal* mutants show tasselseed phenotypes. *nal-N282* is shown in this picture.
Figure 5. Plant height comparison. The strongest alleles of the br1, br2, br3, and na1 mutants are compared to the individual mutants. For br1, br2, br3, and na1 we used br1-R, br2-Singleton-8, br3-2180, and na1-N282 alleles, respectively. Error bars indicate standard deviation. * Mean differences between mutant and WT segregants were statistically significant at the p=0.05 level according to a Student’s t-test.
Figure 6. Numbers of nodes in mutant plants compared to WTs. For, br1, br2, br3, and na1, we used br1-R, br2-Brawn230, br3-2180, na1-N282 alleles, respectively. br*-Brawn219 represents br*-Brawn219-221. Error bars indicate standard deviation. * Mean differences between mutant and WT segregants were statistically significant at the p=0.05 level according to a Student’s t-test.
Figure 7. Internodes that showed the strongest reductions in length for various mutants. Internode numbers are stated above the boxes. Bars indicate the percentage of dwarf internode length to WT (br2 is br2-Brawn230, br3 is br3-Brawn2180, d*64 is d*-64-4156-1, and br*-Brawn219 is br*-Brawn219-221).
Figure 8. The relative internode length distributions of several mutants. Mutant internode lengths are plotted as a percentage of WT. A. The effect of the mutation in br1 mutants is similar on each internode. B. The br2 mutant shows the greatest reduction on the first couple internodes followed by a relatively constant effect on later internodes. C. The first couple internodes are less affected in br3-2180 mutants, while the effect is strong on the rest of the internodes. D. The middle internodes of br*-Brawn219-221 mutant plants were most strongly affected. Since this is a semi-dwarf mutant, reduction in internodes lengths is less compared to other mutants. E. There is a nearly constant effect of the mutation on each internode in na*-N1519D mutants. F. The first several internodes of the d*-N1168B mutants might be slightly less affected compared to the rest of the plant.
Figure 9. In \textit{na1} mutants several internodes were less affected, followed by strongly affected and then another weakly effected.
Figure 10. Measurement of epidermal cell lengths of internodes shown in Figure 5. Columns represent the proportion of the mutants’ internode epidermal cell lengths to WTs in percent scale. * denotes that the cell lengths between dwarf and WT were statistically significant at the p=0.05 level according to a Student’s t-test.
Figure 11. Epidermal cells of WT and mutant internodes. A, B. Epidermal cells are shown from internode 2 of WT and br2-Brawn230, respectively. C, D. Internode 6 epidermal cells are shown for WT and d*-NI168B, respectively. Scale bars are 200µm.
Figure 12. Flowering time of the dwarves compared to WT individuals in segregating families. Reference alleles were used for $br1$, $br2$, and $br3$. $na1$-$SGL635$-7 allele was used for $na1$. * donates that the flowering time between dwarf and WT is statistically significant at the $p=0.05$ level according to a Student’s $t$-test.
Table 3. Putative chromosomal locations of mapped mutants

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>na</em>-N1519D</td>
<td>4</td>
</tr>
<tr>
<td><em>d</em>-N1168B</td>
<td>5</td>
</tr>
<tr>
<td><em>d</em>-EV2</td>
<td>8</td>
</tr>
<tr>
<td><em>d</em>-EV4</td>
<td>6</td>
</tr>
<tr>
<td><em>d</em>-EV6</td>
<td>5</td>
</tr>
<tr>
<td><em>d</em>-EV7</td>
<td>3 or 5</td>
</tr>
</tbody>
</table>
Figure 13. Verification of chromosomal locations for $na^*-N1519D$ and $d^*-N1168B$

using PCR markers. A. Linkage was detected for $na^*-N1519D$ mutants with marker IDP4282, which is located at 80 cM on Chromosome 4 (see Methods). 14 mutants (28 chromatids) are linked to the upper molecular weight allele of this PCR marker, while two polymorphic alleles clearly segregate among the WT segregants. B. Linkage detection for $d^*-N1168B$ with marker IDP4768, which is located at 25.4 cM of Chr 5. There is one recombinant chromatid (individual #4) among 12 for this marker, suggesting the gene is in the vicinity of this marker. 4 out of 5 WT segregants appear heterozygous.
2.7. REFERENCES


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CHAPTER 3. TRANSGENIC ANALYSIS OF BRASSINOSTEROID (BR) MEDIATED GROWTH AND DEVELOPMENT IN MAIZE

3.1. ABSTRACT

Brassinosteroids (BRs) are naturally occurring steroid phytohormones that are involved in many developmental processes throughout the plant kingdom. Work on model species has established pathways for both their biosynthesis and signaling but BR functions in maize are relatively unknown. To contribute to this, we took a transgenic approach to investigate the functions of maize homologs for genes known to be important for BR functions in model species. We generated overexpression transgenic plants for DWF4, BIN2, and BES1, and knock down transgenic plants for DWF4, BIN2, and BRI1 genes. Here we present preliminary results from this transgenic work.

3.2. INTRODUCTION

Brassinosteroids (BRs) are steroid phytohormones widely distributed in the plant kingdom (Clouse 1996a; Fujioka and Yokota 2003; Haubrick and Assmann 2006), (Clouse 1996b; Divi and Krishna 2009; Fujioka and Yokota 2003; Li and Nam 2002; Nomura et al. 2005). The most active BR in Arabidopsis is Brassinolide (BL). BRs have important roles in plant developmental processes including cell elongation, flowering time, leaf development, fertility and microtubule orientation (Clouse 1996a; Kim et al. 2006; Li et al. 1996). BRs are also involved in biotic and abiotic stress responses (Krishna 2003). Thus, BRs impact many agriculturally important traits (Divi and Krishna 2009).

The BR biosynthetic and signaling pathways have been well studied in the model plant Arabidopsis and in rice (reviewed in Bajguz 2007; Kim and Wang 2010). Yet, despite the agricultural importance of maize, these pathways remain relatively unexplored. RNAi
suppression of the maize \textit{dwf4} gene, encoding a key enzyme in BR biosynthesis, resulted in plants with shortened stature and upright leaf habit (TAO et al. 2004). The maize \textit{dwf4} gene was also shown to be functional by its ability to complement Arabidopsis \textit{dwf4} mutants (LIU \textit{et al.} 2007) or by its ability to increase BR regulated processes when overexpressed in rice (WU \textit{et al.} 2008). The \textit{nana plant1 (na1)} gene was recently reported to encode a homolog of the Arabidopsis \textit{DET2} gene, which encodes another BR biosynthetic enzyme (HARTWIG \textit{et al.} 2010). No studies on maize BR signaling have been reported to date.

To address this issue, we took a transgenic approach to generate maize plants altered for different BR related gene functions. Homologs of \textit{DWARF4}, \textit{BRI1}, \textit{BIN2}, and \textit{BES1} were identified in maize and used to generate overexpression and RNAi knockdown lines. Transgenic maize plants were generated with RNAi constructs for \textit{DWF4}, \textit{BRI1} and \textit{BIN2} and overexpression constructs for \textit{BES1}, \textit{DWF4} and \textit{BIN2}.

For this work we collaborated with Dr. Yanhai Yin, Associate Professor at Iowa State University. Huaxun Ye, a member of Yin Lab, was in part of the project. He generated overexpression constructs for \textit{DWF4}, \textit{BIN2}, \textit{BES1}, and RNAi constructs for \textit{BIN2}. He also helped on the screening of the transgenic plants.

3.3. RESULTS AND DISCUSSION

Bioinformatic identification of BR-related homologs in maize

Known BR genes from Arabidopsis or rice were used in BLAST searches of maize databases. A \textit{DWF4} homologous sequence (EU957115) encodes a protein with 69\% identity and 80\% similarity to Arabidopsis \textit{DWF4}. For \textit{BIN2}, a homologous sequence (EU964751) in maize database was found with 88\% protein identity over the full-length Arabidopsis \textit{BIN2} protein. We were not able to amplify \textit{BRI1}, so we got a full-length cDNA from the Arizona
Genomics Institute. We sequenced the clone and it matched with maize c0106G19 BAC clone (AC199011.4). We found ESTs gb|DR808205.1 and gb|DR808206.1 corresponded to the 3’ and 5’ ends of the BRI1 gene within the sequences in this BAC clone. The protein product of this gene shows 55% identity and 69% similarity with Arabidopsis BRI1. A clear maize BES1 homolog was identified (EU969826). This gene encoded a protein with 52% amino acid identity and 64% similarity. Unfortunately, this gene proved to be problematic for PCR amplification or cloning, therefore, the rice BES1 (Os07g39220) was used in further experiments instead.

**dwf4 transgenic lines**

*DWF4* encodes a cytochrome P450 monooxygenase that catalyzes a C-22 hydroxylation, which is a rate-limiting step in BR biosynthesis (CHOET et al. 1998). Therefore, we expected to see reduced stature or dwarf phenotypes in dwf4RNAi plants. As expected, we saw some events that show putative dwarf phenotypes (Figure 1). Interestingly, one event produced plants showing a tasselseed phenotype, where female florets and kernels develop on the tassel. Additionally, several showed a terminal ear phenotype in which an ear formed one node below or directly below the tassel (Figure 1). Such ears were completely fertile and made seeds.

It was previously reported that overexpression of *DWF4* in Arabidopsis caused increased BR levels, demonstrating the importance of *DWF4* in the BR biosynthesis pathway (CHOET et al. 2001). Plant height and seed yield were increased in these overexpression lines, therefore, we expected to see taller plants in our dwf4 overexpression lines. The heights of T0 plants obtained from these constructs were not definitively taller (Figure 2). Analysis of
further generations will be required to determine the effect of this construct on plant architecture.

**bri1 RNAi transgenic lines**

*BRI1* encodes a membrane-localized leucine rich repeat receptor kinase, which is the receptor of the BR signaling pathway (*Li and CHORY 1997; Li and Jin 2007*). Knockout mutations in Arabidopsis and rice cause dwarfism (*Li and CHORY 1997; Nakamura et al. 2006*). Like other BR mutants, *bri1* mutants also cause an upright leaf habit in rice. 13 out of 17 BRI1-RNAi transgenic events showed clear, phenotypes similar to those reported in rice at the seedling stage (Figure 3).

**bin2 transgenic lines**

BIN2 is a negative regulator of the BR signaling pathway in Arabidopsis (*Kim et al. 2009; Vert and Chory 2006*), therefore, we expected RNAi knockdown plants to have a taller phenotype and overexpression plants should be dwarf. Plants obtained from the *bin2*RNAi events showed variable phenotypes. Taller plants looked like wild type, and were fully fertile (Figure 4). Other plants showed decreased stature and unusual leaf morphology (Figure 5). Leaves were unusually long and showed crenulated leaf margins. These plants were sterile and could not be recovered. Sterility has been reported in BR mutants in other plants (*Clouse 1996a; Ye et al. 2010*).

We hypothesized that a dwarf phenotype would result from BIN2 overexpression. However, we did not obtain any dwarves from this construct. It is possible that decreased lamina joint bending was observed, but this remains to be verified in more advanced generations (Figure 6).
**bes1 transgenic lines**

BES1 is a positive regulator of the BR signaling (Yin et al. 2002). BES1 accumulates in the nucleus when BRs are present, leading to BR induced gene expression (Yin et al. 2002). In Arabidopsis, over expression of BES1 increases plant growth; therefore we anticipated that taller plants would result from our transgene. Surprisingly, we obtained putative dwarf as well as tall plants from this construct (Figure 7). Interestingly, the dwarf plants produced ear shoots on lower nodes near the soil surface. This is opposite the terminal ear phenotype observed in several dwf4RNAi lines. One event also showed chlorotic patches suggesting there may have been a disruption in chloroplast development (Figure 8). It has been shown that BRs have role in chloroplast development (Chory et al. 1991).
3.4. MATERIALS AND METHODS

Generation of RNAi constructs:

**DWF4-RNAi construct**: ATGGGCCGCATGATGGCTCCA and TCAGGCCCTCTTGTGCTCTCGGTC primers were used to amplify the \textit{DWF4}. These primers amplified bases from the start codon to stop codon (1521 bp). To fuse the amplified \textit{DWF4} cDNA fragment into the pMCG1005 vector (Figure 9), we added suitable restriction enzyme sites onto the 5’ ends of these primers. \textit{SacI} and \textit{SpeI} enzymes were used to put the \textit{DWF4} between OCS 3’ and rice Waxy-\textit{a} intron, while \textit{AvrII} and \textit{-AscI} enzymes were used to put \textit{DWF4} in opposite orientation between Adhl intron and rice Waxy-\textit{a} intron.

**BRI1-RNAi construct**: TGGACCTCTCCCGGAACAAGAT and TGGTGCAGTTGGAGATTGAC primers were used to amplify \textit{BRI1} for the RNAi construct. These primers amplified bases 611 to 1108 (starting from the start codon) of the cDNA, which codes for the extracellular domain. Suitable enzymes that do not cut the cDNA were used to put it into the pMCG1005 vector. While \textit{AvrII}-\textit{AscI} enzymes were used to put the \textit{BRI1} between Adhl intron and rice Waxy-\textit{a} intron, \textit{XmaI}-\textit{SpeI} enzymes were used to put the \textit{BRI1} in opposite orientation between OCS 3’ and rice Waxy-\textit{a} intron.

**BIN2-RNAi construct**: ATGGGCCGCATGCGGGGTGGGGCCAC and TGGTCCAGTACGGCGGAATAATGTGAG primers were used to amplify \textit{BIN2} for the RNAi construct. These primers amplified bases from the start codon to 1236 bp. Enzymes used for inserting \textit{BIN2} into the pMCG1005 RNAi vector are \textit{StuI}, \textit{AvrII}, \textit{SpeI}, and \textit{XmaI}. While \textit{StuI} and \textit{AvrII} enzymes were used to put the gene between Adhl intron and rice Waxy-\textit{a} intron, \textit{SpeI} and \textit{XmaI} enzymes were used to put the gene in opposite orientation between OCS 3’ and rice Waxy-\textit{a} intron.
**Generation of overexpression constructs**

For overexpression constructs, the pTF101.1 vector (Figure 10) was used. The complete coding regions of each gene of interest were fused in frame at the carboxy terminus with the FLAG epitope tag inserted to the pTF101.1 vector. Genes were put into the vector with the \textit{Bam}HI restriction enzyme. Primers used for each construct are shown in Table 1. \textit{Bam}HI sites were added to the 5’ and 3’ ends of each primer sequence.

**Transformation**

Overexpression and RNAi constructs were transformed into the \textit{Agrobacterium tumefaciens} strain EHA101. Transgenic lines were generated in a Hi-II background by the Iowa State University Plant Transformation Facility using Agrobacterium mediated transformation. Selected seedlings were grown and recovered by crossing to maize inbreds; B73, W22, or H99.

**RT-PCR**

Leaf tissues collected from plants were frozen immediately in liquid nitrogen. Tissues were ground and kept in -50 °C until RNA purification. RNA was purified with a Qiagen RNeasy Mini Kit’s according to the manufacturer’s protocol.

RNA concentrations were measured by a NanoDrop ND-1000 Spectrophotometer. RNA samples were DNase treated (Promega RQ1 RNase-free DNase) and 1-2 µg RNA was used per experiment. 1µl DNase and 1.2 µl RQ1 buffer was used and volumes adjusted to 12 µl with nuclease free water. Samples were incubated at 37°C for 30 minute, after which 1 µl RQ1 DNase stop solution was added and samples kept at 65°C for 10 minutes. 11 µl of this volume was used directly for RT-PCR reactions. For RT-PCR,
Invitrogen SuperScript™ III Reverse Transcriptase protocol was used. From the 20µl cDNA reaction, 1-2 µl was used for further PCR reactions.

**Screening of the Transgenic Plants**

Transgenic seedlings were received from the transformation facility and screened for transgene expression. We used western blot, dot blot, and Elisa methods for screening overexpression transgenic plants using specific antibodies targeted to the Flag epitope tag fused to the protein of interest. For screening of the RNAi knockdown plants, transgenic gene expression was analyzed by RT-PCR. When these results were ambiguous, plants were chosen by the expected phenotype. Some results are shown in Figures 11 and 12.

**Protein Extraction**

For Western Blot, we extracted proteins from leaf tissues. 100 mg leaf tissue was ground in 300 µl protein extraction buffer (100 mM Tris pH 6.8, 200 mM DTT, 4% SDS, 0.7 % bromophenol, and 20% glycerol). Samples were spun down at 12,000 rpm for 2 minutes at 4 °C. Supernatants of the samples were collected for western blot.

**Immunoblotting**

Immunoblots were used to screen the overexpression transgenic plants with specific antibodies targeting the FLAG tag fused to the carboxy terminus of each protein coding region. For this method a modified protocol from [http://www.abcam.com/ps/pdf/protocols/WB-beginner.pdf](http://www.abcam.com/ps/pdf/protocols/WB-beginner.pdf) was used. Protein samples were run by SDS-PAGE using 8 % acrylamide stacking and running gels. Samples were transferred to a Bio-RAD Pure Nitrocellulose membrane (0.45 µm). Sigma ANTI-FLAG® rabbit antibody was used to bind the FLAG tag. ImmunoPure® Goat Anti-Rabbit IgG (H+L)
Peroxidase Conjugated antibody used as the second antibody. Peroxidase was detected using Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate system.
3.5. FIGURES AND TABLES

Figure 1. *dwf4*-RNAi plants. Plants have dwarf phenotypes. There are tasselseed and terminal ear phenotypes on the left plant, indicated by the arrow.
Figure 2. *DWF4* overexpression transgenic plant. Lamina joint bending appears increased.
Figure 3. *bri1*-RNAi plants. Transgenic T0 seedlings show promising phenotypes. The plant at the left is another transgenic plant with normal BRI1 expression. The plants in the middle and right are regenerated *bri1*-RNAi transgenic plantlets. The center plant looks fairly normal while the rightmost plant shows a dwarf stature with dark green color and upright, twisted leaf habit. All plants are at the same age. Pot label stake is 15 cm.
Figure 4. *bin2*-RNAi. The plant appears relatively taller and has healthy reproductive organs. Scale is 80 cm.
Figure 5. *bin2*-RNAi plants which show reduced plant stature and unusual leaf morphology. Leaves appeared long and droopy, with scalloped margins.
Figure 6. BIN2 overexpression plants did not produce conclusive phenotypic alterations. Lamina joint bending might be slightly decreased.
Figure 7. *BES1* overexpression transgenic plants. The plant on the left shows a dwarf phenotype with a basal ear, while the one at the right is relatively taller.
Figure 8. *BES1* overexpression plants showing dwarf phenotypes. Leaves are erect with chlorotic mottling.
Figure 9. pMCG1005 vector used for RNAi transgenic plants.
Figure 10. Vector map of pTF101.1 used for overexpression constructs.
Table 1. Primers used for generating overexpression constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primers (5’-3’)</th>
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<tbody>
<tr>
<td>DWF4ox-F</td>
<td>CGACGAAGTGGGGCGCCTAGCTTCTTAC</td>
</tr>
<tr>
<td>DWF4ox-R</td>
<td>CGCCTCTTTGTGCTCTCGGTCAAAACGCTAC</td>
</tr>
<tr>
<td>BIN2ox-F</td>
<td>CACCGAGGAGAGAGAGACCGGATAC</td>
</tr>
<tr>
<td>BIN2ox-R</td>
<td>CTGGTCCAGTAGCGGGAATAATGTGAG</td>
</tr>
<tr>
<td>BES1ox-F</td>
<td>GCTGGATTCAGAATTCGACGC</td>
</tr>
<tr>
<td>BES1ox-R</td>
<td>ACGCTCGGCCTCGGCGCGAA</td>
</tr>
</tbody>
</table>
Figure 11. RT-PCR screening of *dwf4*-RNAi transgenic plants. Events showing decreased level of *DWF4* expression such as 13 and 14 were selected for further steps.
Figure 12. Screening of BES1 overexpression constructs by immunoblot. BES1 expression of the transgenic plants was measured before and after Brassinolide (BL) treatment. BL treatment increases the accumulation of unphosphorylated BES1. Events showing increased BES1 expression were chosen for the further steps. Arrow shows BES1 bands.
3.6. REFERENCES


NOMURA, T., T. KUSHIRO, T. YOKOTA, Y. KAMIYA, G. J. BISHOP et al., 2005 The last reaction producing brassinolide is catalyzed by cytochrome P-450s, CYP85A3 in tomato and CYP85A2 in Arabidopsis. Journal of Biological Chemistry 280: 17873-17879.


CHAPTER 4. GENERAL CONCLUSION

Despite well known brassinosteroid (BRs) biosynthetic and signaling pathways in model plants such as rice and Arabidopsis, their mechanism in maize is relatively unknown. Since it has been shown that BRs are growth regulators in different plants, it prompted us to study their role in maize, which is an agriculturally and industrially important crop around the world. We followed two approaches; first, since it has been shown that dwarfism is an indicative phenotype of BR related mutants, we characterized over 50 dwarf mutants to identify potential BR mutants. Second, we took a transgenic approach by overexpressing and suppressing some key BR-related genes to see their effect on the growth and development of maize.

In Chapter 2, the results obtained from genetic analyses of the dwarf maize mutants are presented. By complementation tests we were able to categorize the mutants into at least 12 complementation groups. By phenotypic analyses, we found specific features for different mutants. Since it was shown to be related to the BR mutants, flowering time was recorded for the mutants. There are late flowering, and simultaneously flowering mutants compared to WT plants. While some mutants could be considered as strong dwarves, some of them could be as semi-dwarf. We saw that dwarfism in mutants could be either because of decreased node number or shortened internodes, and often both. The shortened internodes could be either associated with decreased cell number or cell length. We genetically mapped some potentially novel mutants to chromosome locations which will help us for future map based cloning efforts.
In Chapter 3, the initial results of transgenic work are shown. The plants transformed with constructs designed to overexpress or RNAi suppress some key BR-related genes were recovered for future analysis. Based on preliminary phenotypic observations, transgenic plants showed some promising results. For example, some of them displayed dwarf phenotypes and some of them were potentially taller, compatible with the expected functions of the transgenes. Some phenotypes observed in transgenic plants showed similarities with the dwarf mutants we analyzed. Analysis of future generations will reveal whether there are any relationships between any of these mutants and transgenic lines.

As we stated above, this part comprises the starting point for future work. When we get the next generation of transgenic plants, they will be analyzed based on their phenotypic characteristics such as plant height, leaf angle, and yield. More molecular and genetic characterization will also be required. For the dwarves, mapping results will be continued. Larger mapping populations will allow more precise map locations to be defined and potential genes to be cloned. This work will contribute to our understanding of BR-mediated growth and development in maize.
ACKNOWLEDGEMENTS

First of all, I would like to thank to my major professor, Dr. Phil Becraft, for his guidance and assistance during these past two years to conduct my research and write this thesis.

I also want to express my pleasure to my POS committee; Dr. Carolyn Lawrence and Dr. Yanhai Yin. I am thankful to them for their contribution and advices to this work.

I thank to both present and past Becraft Lab members, Antony Chettoor, Joonbae Seo, Gibum Yi, and Yong-Sun Moon for their friendship and help. I was lucky to have such a good group to study with.

I am especially grateful to my parents and family for their support and encouragement. They were the main source of my motivation.