

**MOLECULAR STUDIES OF ENHANCED APICAL DOMINANCE
OF PHYTOCHROME B MUTANT SORGHUM**

A Dissertation

by

TESFAMICHAEL HINTSA KEBROM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Molecular and Environmental Plant Sciences

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Approved by:

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ABSTRACT

Molecular Studies of Enhanced Apical Dominance of Phytochrome B Mutant Sorghum.

(December 2006)

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Light is one of the environmental signals that regulate axillary shoot development. However, little is known about molecular and physiological mechanisms regulating the development of the axillary shoot in response to light signals. Molecular events associated with the enhanced apical dominance of phytochrome B mutant sorghum (*Sorghum bicolor*) were analyzed to reveal processes mediating axillary shoot development in response to light. The enhanced apical dominance of *phyB-1* mutant sorghum is due to inhibition of bud outgrowth and is accompanied by upregulation of the dormancy-associated gene (*SbDRM1*) in the buds. Increased expression of the *Teosinte Branched1* (*SbTBI*) gene (encoding a putative transcription factor that represses bud outgrowth) suggests that the inhibition of bud outgrowth in *phyB-1* sorghum is due to the absence of active phyB to repress *SbTBI*. The results were confirmed by growing wild type seedlings at high plant density or with supplemental far-red (FR) light that induces enhanced apical dominance. However, the *SbTBI* gene is not involved in the inhibition of bud outgrowth induced by defoliation in wild type

seedlings. The results indicate variations in molecular mechanisms among different signals inhibiting branching. Increased expression of *SbMAX2* (which encodes an F-box protein that represses bud outgrowth) in buds repressed by light and defoliation suggests common mechanisms at the downstream end of pathways inhibiting branching.

The expression levels of several cell cycle-related genes including *SbPCNA*, *SbHis4*, *SbCycD2*, *SbCycB* and *SbCDKB* were down-regulated in the repressed buds of FR-treated and defoliated seedlings indicating the suspension of cell division in those buds. However, these cell cycle-related genes were continuously expressed in the repressed buds of *phyB-1*, suggesting that inhibition of bud outgrowth in *phyB-1* is not associated with down-regulation of cell cycle-related gene expression. The down-regulation of cell cycle-related genes in the buds of FR-treated wild type seedlings indicates that other sensors, in addition to phyB, regulate bud outgrowth in response to FR enrichment. The approaches used and results achieved will provide direction for future research on this important topic.

DEDICATION

To my mother, Mizan Zeratsion

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CHAPTER I

INTRODUCTION

Yield improvement of major cereal crops in the past was mainly achieved through indirect modification of plant architecture to maximize nitrogen use efficiency. Dwarf wheat and other crops with reduced stature allow the use of higher rates of nitrogen fertilization with little problem of lodging. Further improvement in yield is expected to be achieved by developing varieties with plant architecture that maximizes efficient utilization of other resources such as light and space, and by modifying partitioning of dry matter to reduce the allocation to non-productive tillers and branches. This goal may be achieved in part through a better understanding of mechanisms of axillary shoot development in plants.

Understanding the mechanisms regulating the development of axillary shoots will contribute to the improvement of other aspects of plant production such as regrowth of forage crops, ratooning of sugar cane and rice, propagation of plants by vegetative organs such as tubers, stems and roots, storage of tuber crops such as potato where sprouting during storage is a problem, and control of vegetatively propagated perennial weeds. Furthermore, in the future, there could be a need for a shift from annual to

This dissertation follows the style and format of Plant Physiology.

perennial crop production systems due to concerns about the use of agrochemicals and their effects on the environment, soil erosion, as well as the need to reduce the cost of annual sowing and cultivation (Moffat, 1996). Development of perennial cereal crops will mainly depend on creating cultivars with adventitious or axillary buds that can initiate dormancy during unfavorable periods and regrow during favorable periods from dormant buds. Therefore, a complete understanding of axillary shoot development will contribute to the modification of plant architecture to improve crop yield and many other aspects of crop production at present and in future agricultural systems.

Axillary shoot development involves initiation of meristems in the axil of leaves to form buds and the subsequent outgrowth of these buds (Shimizu and Mori, 2001). In species that do not normally produce shoot branches, or in which branching is limited, removal of the apex of the main shoot induces branching. The phenomenon of inhibition of branching by the shoot apex in those species is known as apical dominance or correlative inhibition or paradormancy (reviewed in Cline, 1997). In most species, application of the plant hormone auxin to the stump soon after decapitation inhibits branching indicating apical dominance is due to the inhibition of bud outgrowth by auxin synthesized in the shoot apex (Thimann and Skoog, 1933; Cline, 1996). The mechanism of action of auxin in apical dominance was first suggested to be due to the direct inhibition of bud outgrowth by apically derived auxin reaching the buds (Skoog and Thimann, 1934). Since it was later found that apically derived auxin does not enter the buds to inhibit bud outgrowth (Hall and Hillman, 1975; Morris, 1977), the mechanism of

action of auxin in apical dominance has been suggested to be through the regulation of other plant growth promoters or inhibitors such as cytokinins (CKs) or abscisic acid (ABA) (Stafstrom, 2000).

It is well documented that the level of ABA in dormant buds is reduced after decapitation (Knox and Wareing, 1984; Gocal et al., 1991). Application of auxin to the apical end of excised stem sections of ABA-insensitive mutants of *Arabidopsis* (*Arabidopsis thaliana*) inhibited bud outgrowth, suggesting that ABA may not be a second messenger for the inhibition of bud outgrowth by apically derived auxin (Chatfield et al., 2000). However, there is evidence for CKs as second messengers for the inhibitory action of auxin on bud outgrowth. Direct application of CK to buds promotes their outgrowth (Turnbull et al., 1997). Decapitation significantly increases the level of CK in the xylem exudate and buds, whereas application of auxin to the decapitated shoot prevents an increase in the level of CK (Bangerth, 1994; Turnbull et al., 1997). These results suggest direct or indirect involvement of CK in regulating shoot branching (Napoli et al., 1999). Recent work indicated that decapitation of pea (*Pisum sativum*) upregulates the expression of a gene encoding adenosine phosphate-isopentenyltransferase (IPT), and the level of CK, in the stem (Tanaka et al., 2006). IPT is a key enzyme in the biosynthesis of CK. Application of auxin to the stump down regulated the expression of the *IPT* gene and prevented an increase in the level of CK (Tanaka et al., 2006). The authors concluded that apically derived auxin inhibits bud outgrowth by repressing the biosynthesis of CK in the stem. However, other

experimental approaches such as branching mutant analysis and gene expression studies indicate that other signals are also involved in mediating the regulation of branching by auxin.

Several mutants defective in branching have been identified in different species (Napoli et al., 1999; Ward and Leyser, 2004). These mutants are defective either in forming axillary meristems or in controlling bud outgrowth. Using these mutants, several genes specifically involved in the initiation of the axillary meristem or the outgrowth of axillary buds have been identified and cloned. The *LATERAL SUPPRESSOR* genes of tomato (*LS*; *Lycopersicon esculentum*) and Arabidopsis (*LAS*) and the *MONOCULMI* gene of rice (*MOC1*; *Oryza sativa*), which is orthologous to *LATERAL SUPPRESSOR*, are among the key genes that are required for axillary meristem initiation (Schumacher et al., 1999; Greb et al., 2003; Li et al., 2003). The *MORE AXILLARY GROWTH (MAX)* genes of Arabidopsis (Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004; Booker et al., 2005), and the *TEOSINTE BRANCHED1 (TBI)* gene of maize (Doebley et al., 1997) are involved in regulating bud outgrowth. The *MAX* and *TBI* genes are inhibitors of bud outgrowth and loss of function in these genes leads to an increased branching phenotype.

Four *MAX* genes has been identified (named *MAX1* to *MAX4*) that are involved in the branching inhibiting *MAX*-related signal. *MAX1*, *MAX3* and *MAX4* encode proteins likely involved in the synthesis of a novel carotenoid-derived hormone-like signal and

the product of *MAX2* is likely involved in the perception of the signal (Booker et al., 2005). The putative MAX-related hormone has not been identified. In addition to CK, the putative MAX-related hormone has been a candidate as a second messenger for the action of auxin in apical dominance (Leysner, 2005). Recent reports suggest that the MAX pathway inhibits branching by controlling auxin transport (Lazar and Goodman, 2006; Bennett et al., 2006).

Orthologs of several of the MAX genes have been identified in other species including the *RAMOSUS1* (*RMS1*) genes in pea (Foo et al., 2005), the *DECREASED APICAL DOMINANCE1* (*DADI*) gene in petunia (*Petunia hybrida*) (Snowden et al., 2005), and the *DWARF3* (*D3*) and *HIGH-TILLERING DWARF1* (*HTD1*) in rice (Ishikawa et al., 2005; Zou et al., 2005). The *TBI* gene encodes a putative transcription factor that is involved in suppressing bud outgrowth (Doebley et al., 1997; Hubbard et al., 2002). *TBI* orthologs have also been identified in rice (*OsTBI*) and several other monocots (Lukens and Doebley, 2001; Takeda et al., 2003). These genes function in a similar manner suggesting the presence of conserved mechanisms regulating branching in diverse species. However, there are also differences in the way some of these genes are regulated in different species. For example, the *RMS1* of pea and *DADI* of petunia are orthologous to *MAX4* of Arabidopsis. The expression of both *RMS1* and *DADI* but not *MAX4* is subject to feedback regulation (Foo et al., 2005; Snowden et al., 2005; Bainbridge et al., 2005).

To understand molecular mechanisms regulating axillary shoot development, gene expression changes associated with bud dormancy and outgrowth have been investigated (Stafstrom, 2000). Genes upregulated in dormant buds and reduced by decapitation include the dormancy-associated genes, *PsDRM1*, *PsDRM2*, *PsAD1*, and *PsAD2*, identified in pea (Stafstrom et al., 1998b; Madoka and Mori, 2000). However, the function of the products of these genes is not known.

Since inhibition of bud outgrowth is due to suppression of cell division (Anderson et al., 2001), patterns of expression of cell cycle-related genes have been investigated in order to identify how inhibitory signals may act on cell division to inhibit bud outgrowth. The expression of several cell cycle-related genes including *PCNA*, *Histones*, *CyclinB* and *CyclinD* were found to be low in dormant buds and upregulated when buds are released from dormancy by decapitation (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998).

Although hormonal signals and molecular mechanisms regulating axillary shoot development in plants have been investigated for the past several decades, a complete understanding that enables modification of the branching habit of plants and improvements in their productivity is still lacking. Furthermore, in addition to regulation by hormonal signals, shoot branching is also regulated by light signals (Cline, 1991). Plants may integrate light signals with hormonal signals to regulate branching. However, molecular mechanisms associated with the regulation of branching by light

signals have not been investigated. The objective of this project is to investigate molecular events associated with the regulation of branching by light signals.

The analysis of light signaling mutants defective in branching is one approach that can be used to study molecular mechanisms involved in the regulation of branching by light. Plants use several families of photoreceptors including phytochromes which absorb the red (R) and far-red (FR) light, and cryptochromes and phototropins which absorb UV-A or blue light, to monitor their environment and synchronize their growth and development to the prevailing environmental conditions (Fankhauser, 2001; Gyula et al., 2003).

The phytochrome family of photoreceptors regulates several developmental processes including seed germination, de-etiolation, vegetative development and flowering time (Fankhauser, 2001; Sawers et al., 2005). Phytochrome (phy) is composed of a *PHY* gene-encoded apoprotein covalently attached to a linear tetrapyrrole chromophore molecule (Gyula et al., 2003). The phytochrome apoprotein is encoded by five genes (*PHYA-PHYE*) in *Arabidopsis* and three genes (*PHYA-PHYC*) in cereals (Gyula et al., 2003; Sawers et al., 2005). The phytochromes exist in the active (Pfr) and inactive (Pr) forms that absorb the FR and R light, respectively. Phytochromes are synthesized in the inactive Pr form and upon perceiving red light are converted into the active Pfr form. Conversely, FR light converts the Pfr form back into the red absorbing, inactive Pr form.

However, a biologically active signal is generated when the Pfr form of phyA absorbs FR light demonstrating that not all phytochromes are inactivated by FR light (Shinomura et al., 2000; Wang and Deng, 2003).

Under daylight conditions where the R:FR ratio is around 1.15 (Franklin and Whitlam, 2005), the proportion of Pfr:Pr is high and induces molecular and biochemical changes to coordinate normal developmental processes. However, FR light reflected by neighboring plants lowers the proportion of Pfr:Pr and triggers the shade avoidance response including increased plant height, enhanced apical dominance and early flowering (Smith, 1982; Reed et al., 1993). It is well established that the shade avoidance response is mediated mainly by phyB (Franklin and Whitlam, 2005). PhyB mutants with enhanced apical dominance have been identified in several species including sorghum and Arabidopsis (Childs et al., 1992; Childs et al., 1997; Reed et al., 1993). Studying the molecular changes associated with the enhanced apical dominance of phyB mutants may give clues about mechanisms and signaling networks regulating branching in response to light and the involvement of hormonal signals in mediating the response.

The *phyB-1* sorghum fails to produce branches during the vegetative stage, and is taller and earlier maturing than the wild type (Fig. 1.1). A frame shift mutation in the gene coding for the phyB apoprotein introduces a premature stop codon in the 3' region of the *PHYB* gene that leads to lack of phyB in the *phyB-1* mutant sorghum (Childs et al.,

1997). It may be hypothesized that the failure of the *phyB-1* sorghum to produce branching is due to lack of active phyB to initiate a signal to up-regulate the expression of branching promoting genes or to down regulate branching inhibiting genes locally within the bud or from a distance.



Figure 1.1. 40-d-old phytochrome B null mutant (*phyB-1*, left) and wild type (right) sorghum plants.

The molecular events associated with the enhanced apical dominance of phyB mutants has been investigated using the phytochrome B mutant (*phyB-1*, cv. 58M) and wild-type (cv. 100M) sorghum as a model system. The strong apical dominance of *phyB-1*, early formation and ease of excising axillary buds are some of the advantages of using the *phyB-1* and wild type sorghum system to study the regulation of branching by light signals. In addition, a large number of sorghum seedlings can be grown in a small area and the dormancy and outgrowth of the bud in the axil of the first leaf (enabling comparison of buds at exactly the same position) can be studied within a short period (ten days after planting). Furthermore, the availability of expressed sequence tags (ESTs) in the data base and high resolution genome map (Mullet et al., 2001; Pratt et al., 2005) that can facilitate the identification and cloning of genes makes sorghum an ideal plant for studying the regulation of shoot branching by light in monocots. Recent work indicates there are conserved molecular mechanisms regulating branching in diverse species, including monocots and dicots (Li et al., 2003; Ishikawa et al., 2005). Conversely, although it is well known that application of auxin to the stump of decapitated plants restores apical dominance, greenhouse grown decapitated *Arabidopsis* plants did not respond to auxin (Cline, 1996). Since there is great variation among plants in development, morphology, physiology and adaptation to the environment (Mullet et al., 2001), an in depth understanding of axillary shoot development and other plant developmental processes could be achieved using both monocots and dicots and comparative approaches.

The expression of several branching and cell cycle-related genes were examined in the *phyB-1* and wild type sorghum axillary buds. In chapter II, the regulation of two important branching related genes, the *Teosinte Branched1 (TBI)* and *Dormancy associated genes (DRMI)*, by light signals perceived by phyB and their association with bud dormancy and outgrowth is reported.

The mechanism of action of auxin in apical dominance has been suggested to be through the recently identified *MAX*-related signal (Lazar and Goodman, 2006; Bennett et al., 2006). Genes involved in the biosynthesis of the putative *MAX*-related hormone or the receptor/transduction of the signal could also be involved in the regulation of branching by light. Therefore, expression changes of *MAX* gene homologs were analyzed in the *phyB-1* and FR light-treated wild type seedlings. In the past, axillary shoot development has been investigated using dicots such as pea and Arabidopsis, because of the ease of decapitation and grafting. Since the shoot apical meristem of grasses such as sorghum during the vegetative stage is located at the base of the plant and is enclosed by several leaves, it is difficult to decapitate and compare gene expression changes associated with decapitation-induced and light-induced branching in the sorghum system. However, defoliation of wild type sorghum seedlings at early stages of growth inhibits bud outgrowth. Branching-related gene expression changes were studied in the axillary buds of defoliated seedlings to see if inhibition of bud outgrowth in response to light and defoliation are mediated by similar molecular events. The results are reported in chapter III.

Apically derived auxin inhibits bud outgrowth by down regulating the expression of several cell cycle-related genes to inhibit cell division (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998). Expression changes of cell cycle-related genes were investigated in the repressed buds of *phyB-1*; FR-treated and defoliated sorghum seedlings. The results are presented in chapter IV. Finally, the results of expression analyses of branching and cell cycle-related genes associated with the regulation of bud outgrowth by light signals and defoliation is summarized in chapter V.

CHAPTER II

**PHYTOCHROME B REPRESSES *Teosinte Branched1* EXPRESSION
AND INDUCES SORGHUM AXILLARY BUD OUTGROWTH IN
RESPONSE TO LIGHT SIGNALS***

INTRODUCTION

The highly ordered arrangement of leaves and branches and the final shoot architecture are associated with a plant's developmental strategy to ensure its survival and productivity under continuously changing growing conditions. A complex developmental program that integrates genetic mechanisms, physiological processes, and environmental signals controls the overall form of the plant. The shoot architecture of crop plants has been modified during their domestication and improvement. One of the great achievements in the history of crop improvement, "The Green Revolution," was due to dwarfing of wheat (*Triticum aestivum*) and other crops to increase nitrogen-use efficiency and reduce lodging. A more complete understanding of the developmental programs that control shoot architecture will help to further improve resource-use efficiency and yield of crop plants.

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Shoot architecture is largely determined by the number of axillary shoots produced. Axillary shoot development begins with the initiation of an axillary meristem in the axil of a leaf to form a bud (for review, see Ward and Leyser, 2004; McSteen and Leyser, 2005). Then, depending on internal and environmental signals, the bud may continue growing to form an axillary shoot or enter into dormancy. Different approaches have been used to study the regulation of axillary shoot development. Early decapitation studies and application of plant hormones showed that auxin produced in the shoot apex inhibits the outgrowth of axillary buds (Thimann and Skoog, 1933). Further research indicated that other plant hormones, such as cytokinins and abscisic acid, are also involved in regulating branching (for review, see Stafstrom, 2000; Shimizu and Mori, 2001).

Molecular and genetic approaches have been used to study the mechanisms of action of plant hormones and to identify genes involved in regulating branching. Genes that control axillary meristem initiation have been identified in various species (Schumacher et al., 1999; Greb et al., 2003; Li et al., 2003). Studies using branching mutants of *Arabidopsis* (*Arabidopsis thaliana*), pea (*Pisum sativum*), and petunia (*Petunia hybrida*) are revealing the mechanisms of action of complex signaling networks of plant hormones that regulate shoot branching (Leyser et al., 1993; Stirnberg et al., 1999; Booker et al., 2005; Foo et al., 2005; Snowden et al., 2005). Investigations using these mutants suggest the presence of a novel, as yet unidentified, plant hormone-like signal that integrates hormonal action during branching.

A genetic analysis of the morphological differences between maize (*Zea mays* subsp. *mays*) and its wild ancestor teosinte (*Zea mays* subsp. *parviglumis*) led to the cloning of the *Teosinte Branched1* (*TBI*) gene (Doebley et al., 1997). The *TBI* gene encodes a putative basic helix-loop-helix transcription factor that is involved in suppressing bud outgrowth (Doebley et al., 1997; Hubbard et al., 2002). An orthologous gene to maize *TBI* was cloned from rice (*Oryza sativa*; *OsTBI*) and functions in a manner similar to the maize *TBI* (Takeda et al., 2003). In both maize/teosinte and rice, *TBI* is expressed predominantly in the young axillary bud. Further studies of the functional properties of *TBI* will provide insights regarding the regulation of shoot branching at the molecular level.

Several genes that are specifically up-regulated or down-regulated during branching have been identified. The dormancy-associated genes of pea, *PsDRM1* (Stafstrom et al., 1998b) and *PsADI* (Madoka and Mori, 2000), are among the genes identified in cDNA libraries from dormant axillary buds. The mRNA abundance of *PsDRM1* and *PsADI* in axillary buds declines after decapitation. However, while the expression of these genes correlates strongly with bud dormancy, their functions are not known.

It is well established that light quality (red:far red [R:FR]) is one of the environmental signals that regulate shoot branching (Casal et al., 1986; Wan and Sosebee, 1998). However, little information exists regarding the molecular mechanisms regulating shoot branching in response to light. Light signals may interact with plant hormones to

regulate plant growth and development (Halliday and Fankhauser, 2003). Recent findings indicate that light and auxin interact in the regulation of adventitious root formation in *Arabidopsis* (Sorin et al., 2005). To fully understand the regulation of axillary shoot development, the interaction of light and hormonal and other environmental signals should be investigated.

Plants use light signals perceived by photoreceptors to coordinate all stages of growth and development from germination to flowering. The phytochrome family of photoreceptors is involved in deetiolation, vegetative development, and flowering time in both dicots and monocots (Mathews and Sharrock, 1996; Sawers et al., 2005). Mutation in one of the family members, *PHYTOCHROME B* (*PHYB*), affects several developmental processes, including branching in sorghum (*Sorghum bicolor*) and *Arabidopsis* (Childs et al., 1992, 1997; Reed et al., 1993).

We used the *phyB* null mutant sorghum (*phyB-1*) as a model for studying the regulation of branching by light. The strong apical dominance of *phyB-1*, and the early formation and ease of excising axillary buds make sorghum useful for studying the role of light in axillary shoot development. We asked whether light signals perceived by *phyB* control shoot branching by regulating the expression of branching genes previously identified in other species. We characterized the enhanced apical dominance in *phyB-1* sorghum and investigated the expression of branching-related genes, including the sorghum homologs of the *TBI* (*SbTBI*) and dormancy-associated (*SbDRM1*) genes in *phyB-1* and wild-type

axillary buds. In this article, we report the regulation of expression of the sorghum *SbTB1* and *SbDRM1* genes by light signals perceived by phyB, and phyB's association with dormancy and outgrowth of axillary buds.

RESULTS

Enhanced Apical Dominance in *phyB-1* Mutant Sorghum

The *phyB-1* mutant sorghum fails to produce branches during vegetative development, whereas the near-isogenic wild-type plants branch profusely. The branching deficiency in *phyB-1* could theoretically arise from either a defect in axillary meristem initiation or bud outgrowth. We found that the defect occurs in bud outgrowth since equivalent buds are formed early in the development in both *phyB-1* and wild-type sorghum (Fig. 2.1). The buds in the axil of the first leaf of both *phyB-1* and the wild type grow at the same rate until 7 d after planting (DAP). Then they begin to show different developmental fates (Fig. 2.2A). While the buds of the wild type continue elongation, bud outgrowth is inhibited in *phyB-1*. Axillary buds are formed at all nodes of the main shoot of *phyB-1* (data not shown). These buds remain dormant and branching is observed only when the main shoot begins flowering. The *phyB-1* seedlings were taller than the wild type, showing enhanced growth of the main shoot as a result of constitutive shade avoidance (Fig. 2.2B).

Expression of *SbTB1* in Different Organs of Sorghum Seedlings

SbTB1 (accession no. AF322132) exists as a single copy in the sorghum genome with 93.9% nucleotide identity with the maize *TB1* gene (Lukens and Doebley, 2001). The maize *TB1* mRNA is 1.5 kb in size and accumulates in husks, axillary inflorescence primordia, and axillary meristems (Doebley et al., 1997; Hubbard et al., 2002). *OsTB1* is expressed in the basal part of the shoot apical meristem, in vascular tissue in the pith, and in the entire axillary bud (Takeda et al., 2003). We investigated the abundance of *SbTB1* in different parts of sorghum seedlings. The *SbTB1* mRNA of about 1.7 kb in size was detected only in the buds (Fig. 2.3).

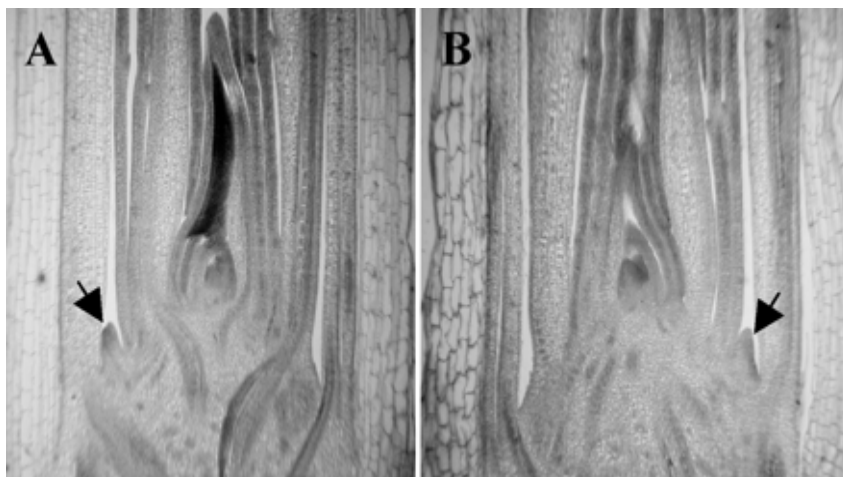


Figure 2.1. Median longitudinal sections of 4-d-old sorghum shoots. Arrows indicate buds in the axil of the first leaves of *phyB-1* (A) and wild-type (B) sorghum seedlings.

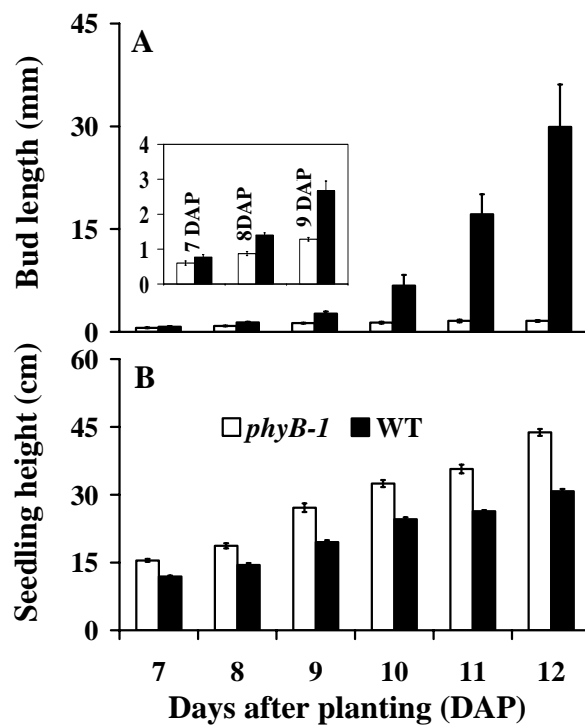


Figure 2.2. Bud length (A) and seedling height (B) of *phyB-1* and wild-type sorghum. Inset shows bud lengths at 7 DAP, 8 DAP, and 9 DAP (not visible in the main figure). Data are mean \pm SE; $n = 10$.

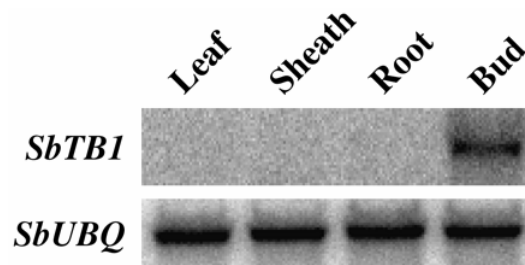


Figure 2.3. *SbTB1* mRNA abundance in leaf, sheath, root, and bud of sorghum seedlings at 9 DAP. The sorghum ubiquitin gene (*SbUBQ*) was used as a loading control.

Expression of *SbDRM1* in Different Organs of Sorghum Seedlings

PsDRM1 is expressed in dormant axillary buds, and its expression is suppressed by decapitation and auxin (Stafstrom et al., 1998b; Stafstrom, 2000). *PsDRM1* is also expressed in mature stems and roots. The Arabidopsis gene orthologous to *PsDRM1*, *AtDRM1*, has similar expression patterns to *PsDRM1* (Stafstrom et al., 1998a; Tatematsu et al., 2005). A BLAST search (TBLASTN; Altschul et al., 1991) identified a sorghum expressed sequence tag, (accession no. CN135114, hereafter called *SbDRM1*) encoding the entire *SbDRM1* open reading frame with a deduced amino acid sequence 59% and 53% identical to the *PsDRM1* (accession no. AAB84193.1) and *AtDRM1* (accession no.

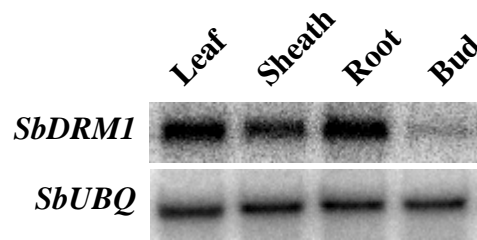


Figure 2.5. *SbDRM1* mRNA abundance in leaf, sheath, root, and bud of sorghum seedlings at 9 DAP. The sorghum ubiquitin gene (*SbUBQ*) was used as a loading control.

Abundance of *SbTB1* and *SbDRM1* mRNA in *phyB-1* and Wild-Type Axillary Buds

The expression of the *SbTB1* and *SbDRM1* genes in sorghum axillary buds was correlated with the dormant state of the buds. At 7 DAP and 9 DAP, *SbTB1* mRNA was detected in the axillary buds of both *phyB-1* and wild-type seedlings (Fig. 2.6A). However, *SbTB1* abundance was more than 2-fold higher in the *phyB-1* buds compared to the level in the wild type (Fig. 2.6B). The expression pattern of *SbDRM1* was different from that of *SbTB1* mRNA. At 7 DAP, when their size was comparable (Fig. 2.2), *SbDRM1* message is hardly detected in the buds of *phyB-1* and the wild type (Fig. 2.6A). At 9 DAP, when the buds of the wild type were rapidly elongating while those of *phyB-1* were suppressed, the mRNA level of *SbDRM1* was more than 5-fold higher in the buds of *phyB-1* than in the wild type (Fig. 2.6C).

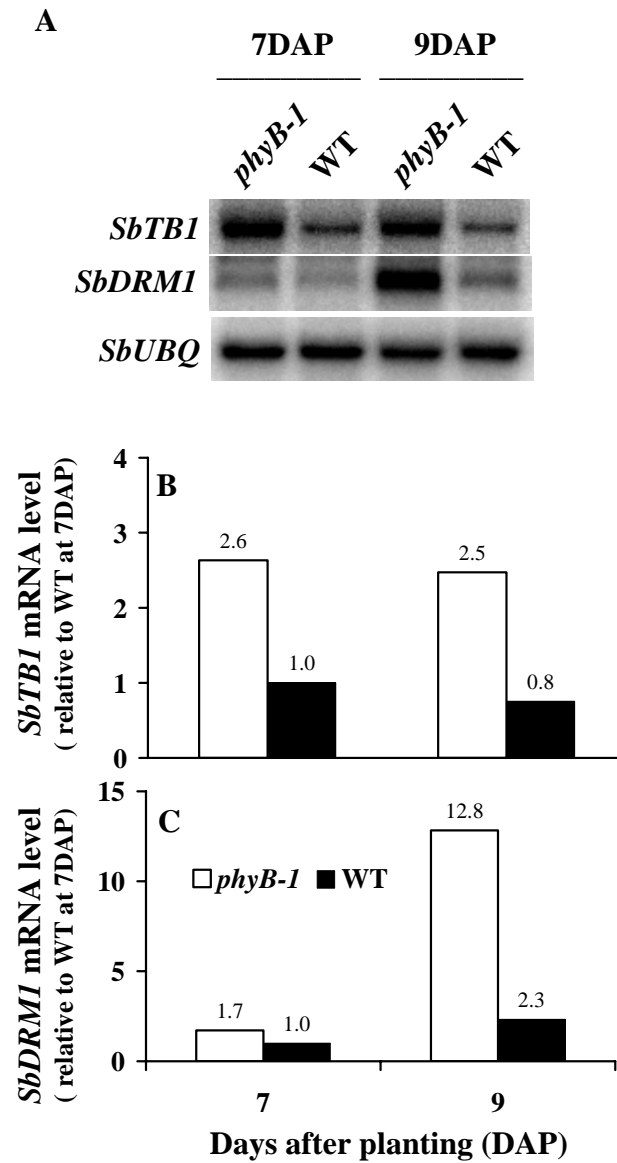


Figure 2.6. A, *SbTB1* and *SbDRM1* mRNA abundance in the buds from the first leaf axils of *phyB-1* and wild-type sorghum seedlings. B and C, Relative quantitation of *SbTB1* and *SbDRM1* mRNA levels, respectively. The sorghum ubiquitin gene (*SbUBQ*) was used as a loading control.

***SbTB1* and *SbDRM1* mRNA Abundance in Axillary Buds of Wild-Type Seedlings Grown at High and Low Densities**

The enhanced apical dominance of *phyB-1* sorghum is consistent with a constitutive shade avoidance response. Shade avoidance responses are also observed in the natural environment when wild-type plants are grown at high density that lowers the R:FR. In an attempt to simulate the constitutive shade avoidance response of *phyB-1* sorghum in the wild type, wild-type seedlings were grown at high (3,000 seedlings m⁻²) and low (300 seedling m⁻²) plant densities. In those seedlings grown at a high density, axillary bud outgrowth was suppressed at about 9 DAP (Fig. 2.7A). While high density affected axillary bud elongation, the height of seedlings at both planting densities was the same during the measurement period (Fig. 2.7B).

SbTB1 mRNA accumulation in the buds of wild-type seedlings grown at both plant densities decreased over time (Fig. 2.8). The abundance of *SbTB1* mRNA was higher in the axillary buds from high plant density compared to low plant density at both 7 DAP and 9 DAP (Fig. 2.8). At 7 DAP, *SbDRM1* mRNA abundance was only 2.2-fold higher in high density compared to low density (Fig. 2.9). However, relative to wild type at 7 DAP, the level of *SbDRM1* mRNA at 9 DAP was increased to 18.6- and 3.7-fold at high and low plant densities, respectively. At 9 DAP, *SbDRM1* abundance was therefore 5-fold higher in buds from high compared to low planting density.

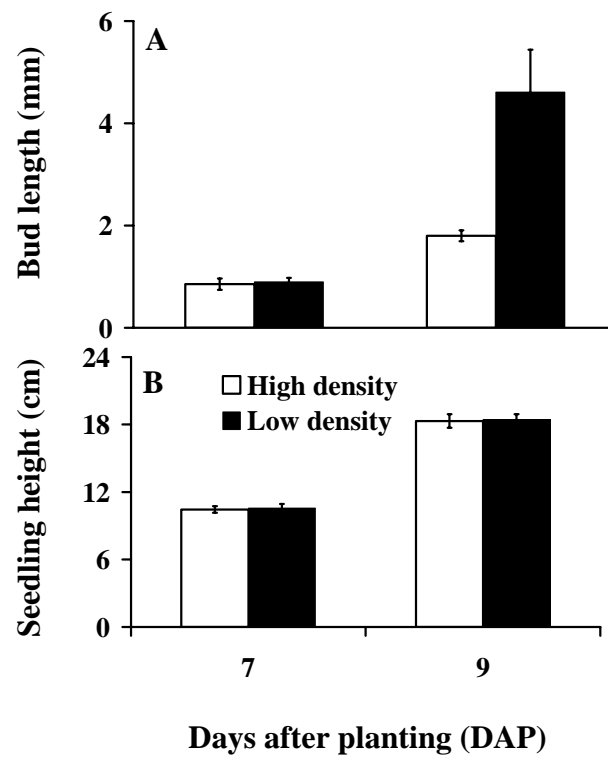


Figure 2.7. Bud length (A) and seedling height (B) of wild-type sorghum seedlings grown at high and low plant densities. Data are mean \pm SE; $n = 9$ or 10 .

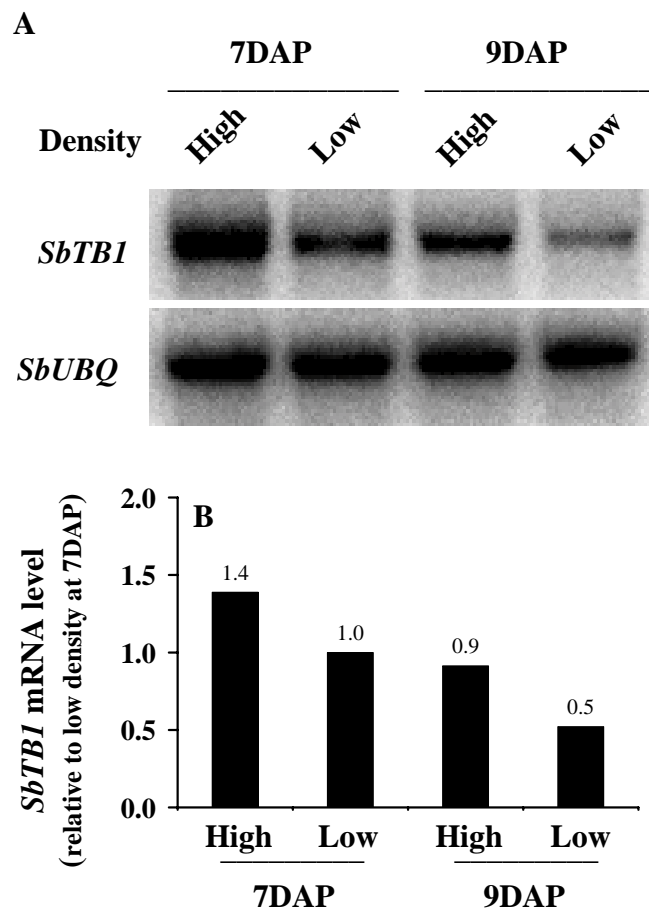


Figure 2.8. A, Abundance of *SbTBI* mRNA in axillary buds in the axil of the first leaves of wild-type seedlings grown at high and low densities at 7 DAP and 9 DAP. B, *SbTBI* mRNA quantitation relative to buds of seedlings grown at low density at 7 DAP. The sorghum ubiquitin gene (*SbUBQ*) was used as a loading control.

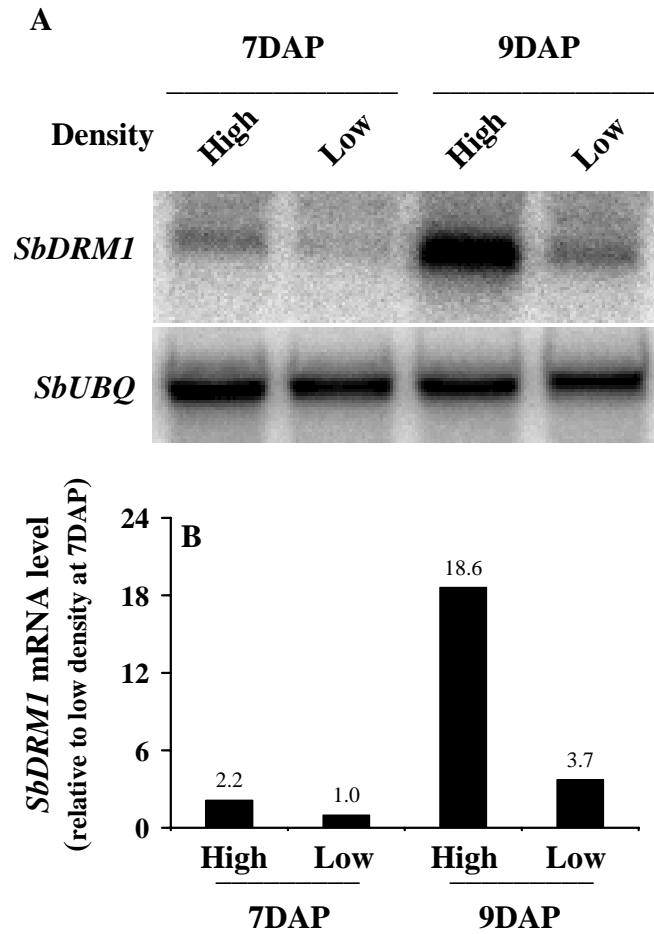


Figure 2.9. A, Abundance of *SbDRM1* mRNA in axillary buds in the axil of the first leaves of wild-type seedlings grown at high and low densities at 7 DAP and 9 DAP. B, *SbDRM1* mRNA quantitation relative to buds of seedlings grown at low density at 7 DAP. The sorghum ubiquitin gene (*SbUBQ*) was used as a loading control.

***SbTB1* and *SbDRM1* mRNA Abundance in Axillary Buds of Wild Type Grown with and without Supplemental FR**

Low R:FR reduces the proportion of the active form of phyB (Pfr), thereby triggering the shade avoidance response. Wild-type seedlings were grown until 7 DAP, when bud length in the axil of the first leaf and seedling height were measured (control, 7 DAP). Then, starting at 7 DAP and continuing for the next 2 d, some of the seedlings were irradiated with FR light from the sides (FR, 9 DAP), while others continued growth without supplemental FR light (control, 9 DAP). The supplemental FR light treatment suppressed the outgrowth of buds in the axil of the first leaf (Fig. 2.10A) and increased seedling height (Fig. 2.10B).

The abundance of *SbTB1* and *SbDRM1* mRNAs reflects the enhanced apical dominance induced by low R:FR (Fig. 2.11A). Compared to the abundance in the control at 7 DAP, the *SbTB1* mRNA abundance at 9 DAP was slightly reduced in the control, whereas it was increased in the FR-treated seedlings. The mRNA abundance of *SbTB1* was 2.8-fold higher in the FR-treated seedlings compared to the control at 9 DAP (Fig. 2.11B). The *SbDRM1* mRNA level was almost 18-fold higher in axillary buds of seedlings treated with FR compared to control (Fig. 2.11B).

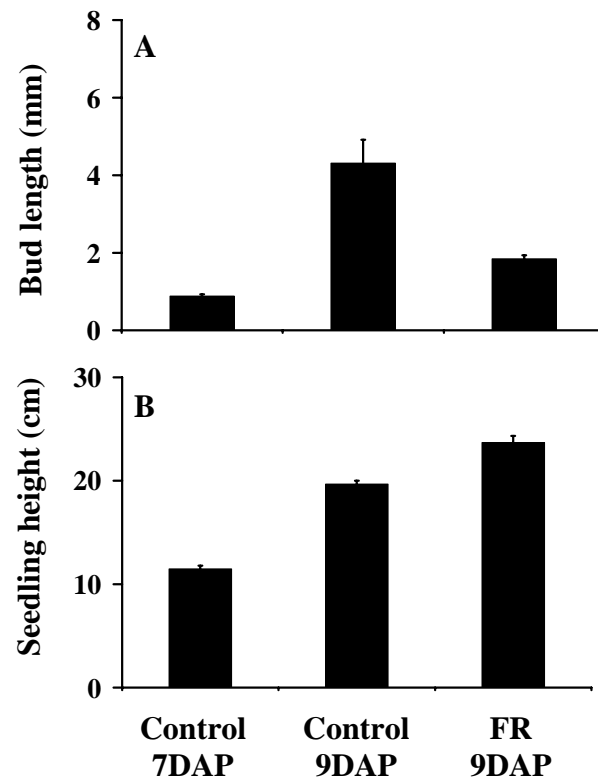


Figure 2.10. Effect of supplemental FR light on bud outgrowth (A) and seedling height (B) of wild-type sorghum. Data are mean \pm SE; n = 7.

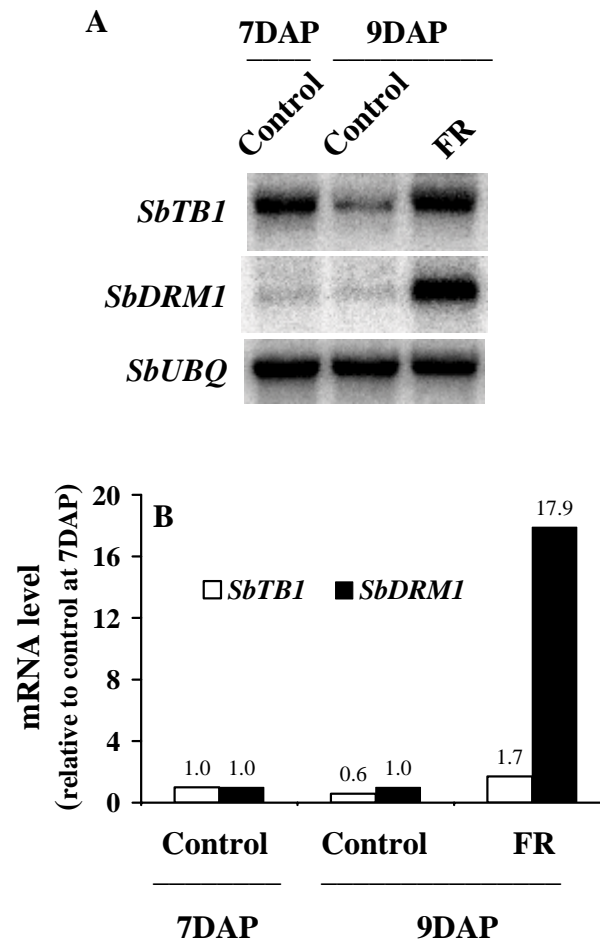


Figure 2.11. A, Abundance of *SbTB1* and *SbDRM1* mRNA in axillary buds of wild-type seedlings grown with or without supplemental FR light. B, Relative quantitation of *SbTB1* and *SbDRM1* mRNA levels. The sorghum ubiquitin gene (*SbUBQ*) was used as a loading control.

DISCUSSION

We investigated the regulation of branching by light signals perceived by phyB in the branching-deficient *phyB-1* null mutant sorghum. Mutants used previously to study the molecular mechanisms of branching are defective either in initiation of axillary meristems or outgrowth of buds (Napoli et al., 1999; Ward and Leyser, 2004). Our results show that the initiation of axillary meristems and formation of buds occur normally in *phyB-1*; however, the outgrowth of those buds was inhibited, indicating that the role of light signals in branching is limited to bud dormancy and outgrowth. Bud outgrowth was also inhibited in wild-type plants grown at high plant density or with supplemental FR light, demonstrating the regulation of axillary buds by environmental signals soon after their formation.

Supplemental FR light treatment of wild-type seedlings, started at 7 DAP, inhibited the outgrowth of buds in the first leaf axil of all treated seedlings. However, when FR treatment was started at 9 DAP, the buds in the first leaf axil of some seedlings were arrested, while in others they escaped the inhibitory signal and elongated (data not shown). It is noteworthy that all the buds in the axil of the second leaves of those plants treated with FR at 9 DAP were arrested (data not shown). The inconsistency in the response of buds in the axil of the first leaf to delayed FR light treatment was observed in repeated experiments. The "escape" phenomenon may be similar to the regulation of bud dormancy and outgrowth by auxin (for review, see Cline, 1997), in which

decapitation-induced bud outgrowth can be suppressed by exogenous auxin early after decapitation but not later. These results indicate the presence of a developmental window at which FR light and auxin can inhibit bud outgrowth. The data raise the question of whether the effect of light on bud dormancy and outgrowth is through auxin, or whether both light and auxin act on a common target that regulates the process.

Previous research has indicated that *DRMI* expression correlates with bud dormancy, and we have used it here as an indicator of a bud's physiological status. The inhibition of bud outgrowth in *phyB-1* sorghum is reflected in the high level of *SbDRMI* mRNA in these axillary buds (Fig. 2.6). The results are confirmed by the high level of *SbDRMI* mRNA in the arrested buds of wild type grown at high plant density or with supplemental FR (Figs. 2.9 and 2.11). There was a small increase in *SbDRMI* mRNA at 9 DAP compared to 7 DAP in wild type grown under standard conditions (Fig. 2.6) and in wild type grown at low densities (Fig. 2.9). These results may seem contradictory to the typical correlation of *DRMI* expression with dormancy but may indicate an increase in the proportion of nondividing cells in the rapidly elongating buds of the wild type.

The pattern of *SbDRMI* accumulation agrees with that observed in pea and Arabidopsis, implying that conserved mechanisms of dormancy and outgrowth may operate in both monocots and dicots—an observation also supported by the orthologous function of *MAX*-related genes in rice (Ishikawa et al., 2005). Since auxin represses *PsDRMI* in pea (Stafstrom, 2000), the suppression of *SbDRMI* mRNA accumulation by light suggests

that similar dormancy mechanisms are induced by both auxin and light. Phytochrome regulation of gene expression has been shown to be both auxin dependent and auxin independent (Tanaka et al., 2002). Whether the suppression of *SbDRM1* mRNA by light is auxin dependent or auxin independent needs to be investigated. It is not known whether decapitation-induced and non-decapitation-induced bud outgrowth (i.e. via light) are regulated through the same pathway (Napoli et al., 1999). Further studies on the regulation of expression of *SbDRM1* mRNA and related genes by light signals will help resolve such questions.

The *TBI* gene was identified as one of five quantitative trait loci that distinguish the morphology of maize from its wild ancestor, teosinte (Doebley and Stec, 1991). Compared to teosinte, maize exhibits enhanced apical dominance. Sequence comparison of the maize and teosinte *TBI* alleles indicated no difference in the predicted coding region of the two alleles (Doebley et al., 1997); however, the expression of the maize *TBI* was twice that of teosinte, suggesting differences in the regulation of expression of the gene (Doebley et al., 1997). Increased expression of the *TBI* gene in axillary organs was correlated with suppression of growth of those organs (Doebley et al., 1997; Hubbard et al., 2002). Increased expression of *OsTBI* was also found to suppress axillary bud outgrowth but not their formation (Takeda et al., 2003). The elevated *SbTBI* mRNA abundance in *phyB-1* axillary buds compared to wild type and in wild type grown with low R:FR suggests that *SbTBI* gene expression is regulated by light

signals perceived by phyB. Therefore, phyB may exert its influence on branching in part through modulation of *SbTBI* mRNA levels.

Branching in maize is relatively insensitive to planting density; whereas branching in teosinte is reduced at high plant density and increases at low plant density. Doebley et al. (1995) suggested that *TBI* expression in maize is decoupled from regulation by environmental signals and is constitutively expressed, while teosinte *TBI* is expressed under unfavorable growing conditions and repressed under favorable growing conditions. Our results suggest that the regulation of *TBI* expression in wild-type sorghum more closely resembles that proposed for teosinte, while the expression in *phyB-1* sorghum more closely resembles that proposed for maize.

It should be noted that the response of wild-type sorghum seedlings to high planting density was not the same as their response to low FR light treatment during the experimental period. Although bud elongation was inhibited in both treatments, only FR induced an increase in seedling height, which is one of the typical shade avoidance responses. The pattern of accumulation of *SbDRMI* mRNA at 9 DAP at high planting density and in the FR-treated seedlings was similar in each case, indicating both treatments inhibited bud outgrowth through similar downstream mechanisms. However, the level of *SbTBI* mRNA at 9 DAP at high planting density was lower than at 7 DAP at both high and low planting densities (Fig. 2.8), while FR light treatment increased the level of *SbTBI* mRNA at 9 DAP compared to the level at 7 DAP (Fig. 2.11). Takeda et

al. (2003) reported that the branching habit of the *fine culm 1* mutant rice, which contains a loss-of-function mutation of *OsTBI*, responds to planting density. They suggested that the regulation of branching in rice in response to density is independent of *OsTBI* but dependent on other factors. Since high density did not affect plant height and the effect on *SbTBI* expression is relatively small, it is unlikely that suppression of bud outgrowth in our experiment is mainly the result of a phyB-mediated shade avoidance response. In addition to changes in light quality, other undetermined density-derived cues may also inhibit bud outgrowth at high planting density.

SbTBI mRNA is highest in the younger wild-type buds (7 DAP) and decreases with time, suggesting that early in development these buds acquire a signal that inhibits their outgrowth. The fate of these buds, whether to continue growth or enter into dormancy, is determined at a later stage depending on the perception of light and possibly other signals required for their elongation. The results imply that the absence of active phyB to suppress *SbTBI* accumulation in the *phyB-1* axillary buds leads to dormancy, while the suppression of *SbTBI* accumulation in the wild-type axillary buds by active phyB leads to bud outgrowth. Transgenic potato (*Solanum tuberosum*) plants overexpressing phyB produced more branches at high plant density (Boccalandro et al., 2003). These results are consistent with the hypothesis that active phyB suppresses accumulation of the *TBI* mRNA, thereby promoting bud outgrowth. Recently, a potato gene (*sttcp1*) with a function similar to that of the maize *TBI* gene was cloned (Faivre-Rampant et al.,

2004). It would be interesting to study the level of expression of the *sttcp1* gene in the phyB-overexpressing and wild-type potato.

Devlin et al. (2003) studied genome-wide gene expression changes in *phyB* mutant, *phyA phyB* double mutant, and wild-type *Arabidopsis* in response to shade and identified 301 genes that demonstrated altered expression. Some of these genes encode proteins that are known to function in light and hormone signaling, transcription regulation, and protein degradation. In some developmental programs, phytochrome action involves a direct interaction with transcription factors to regulate the expression of light-responsive genes. Phytochromes may also act through posttranslational regulation of the level of transcription factors by directed proteolysis to indirectly regulate gene expression (Quail, 2002). The mechanism by which phyB regulates *SbTBI* abundance remains to be discovered.

The *TBI* gene belongs to the TCP family of transcription factors with a noncanonical basic helix-loop-helix domain that is predicted to function in DNA-binding and protein-protein interactions (Cubas et al., 1999). Previous studies indicated that the TCP proteins stimulate or repress the growth of an organ in which they are expressed by regulating the expression of several cell cycle-related genes (Kosugi and Ohashi, 1997, 2002; Gaudin et al., 2000; Tremousaygue et al., 2003; Li et al., 2005). Since TB1 represses the growth of organs in which it is expressed, it may directly or indirectly repress growth by interfering with the progression of cell division. We are investigating the expression of several cell

cycle genes, such as *PCNA*, ribosomal protein genes, *Cyclins*, and *CDKs*, in the *phyB-1* and wild-type sorghum axillary buds to elucidate the regulation of bud outgrowth by TB1 in response to light.

It is not yet understood whether hormonal signals interact with TB1 to suppress bud outgrowth (McSteen and Leyser, 2005). We have identified several sorghum expressed sequence tags with homology to key genes involved in regulating hormonal signals during axillary shoot development, including *MAX* related (Booker et al., 2005) and *AXRI* (Leyser et al., 1993; Stirnberg et al., 1999), and we are investigating their patterns of expression in the sorghum system to understand the complex signaling networks involved in regulating axillary shoot development.

Tatematsu et al. (2005) identified cis-elements that regulate the expression of genes involved in axillary bud outgrowth in *Arabidopsis* by analyzing the transcriptome of axillary shoots collected from all positions. Since axillary shoots at different phyllotactic positions are at different developmental stages, it is difficult to delineate the role of those elements at the different stages. Using both the monocots such as sorghum where working with buds comparable in size and at similar positions is easier and dicots such as pea and *Arabidopsis* where decapitation, grafting, and genetic manipulation are easier, a more complete understanding of the regulation of plant architecture may be achieved.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Wild-type and *phyB-1* mutant sorghum (*Sorghum bicolor*) seedlings were grown in a growth chamber with incandescent and fluorescent lamps, maintained at 31°C/22°C day/night temperatures with a 12-h photoperiod. The photosynthetically active radiation was approximately $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the R:FR 3.0. Seeds were sown at a rate of 300 m^{-2} on trays containing 7-cm-deep cells filled with growth medium prepared as described by Beall et al. (1991). To study the effect of plant density, seeds were sown at a rate of 300 and 3,000 m^{-2} for the low and the high plant density, respectively. Seedlings near the perimeter of the trays were avoided during sampling. To study the effect of shade signals (R:FR) on bud outgrowth, seedlings were grown in 50-mL tubes, one seedling per tube, and the tubes were supported on a rack arranged at a seedling density of 900 m^{-2} . At this rate, shading due to density was not observed as detected by measurement of bud elongation until 10 DAP (data not shown). At 7 DAP, uniform seedlings were selected, divided into two groups, arranged on a rack at a seedling density of 300 seedlings m^{-2} , and then one group was treated with supplemental FR (FR, 9 DAP) while the other group was grown as control (control, 9 DAP). Supplemental FR was applied using FR light-emitting diode arrays directed from the sides starting at 7 DAP. FR-treated and control seedlings were grown in the same growth chamber under the

same conditions except that the FR-treated seedlings had additional FR light from the sides.

Histological Studies

Young elongating shoots of *phyB-1* and the wild type were collected about 5 mm from the base of seedlings and fixed in FAA (70% ethanol, 37% formaldehyde-acetic acid, 18:1:1) for at least 24 h, and then stored in 70% ethanol. The young stems were dehydrated in a tertiary butyl alcohol series, embedded in Tissueprep (Fisher Scientific), sectioned at 15 μm with a rotary microtome, and placed on microscope slides that were kept at 40°C to 50°C for at least 24 h. The slides were then stained in Safranin-O fast-green series using an HMS series programmable slide stainer (Carl Zeiss). Two drops of Permount mounting medium (Fisher Scientific) were placed on each slide and covered with a cover glass. The slides were then observed with a bright-field Zeiss microscope.

Bud Length and Seedling Height Measurement

Subtending leaves were removed, and buds in the axil of the first leaf were excised and their lengths measured under a dissecting microscope using a micrometer. A ruler was used to measure buds longer than 3 mm. Seedling height was also measured as the height from the base of the shoot to the tip of the tallest leaf.

RNA Isolation and Northern Analysis

For gene expression studies, seedlings were harvested, roots were washed, and buds in the axil of the first leaves were excised under a dissecting microscope. The buds were immersed in lysis/binding solution (Ambion) on ice and were stored at -80°C until RNA extraction. The roots of seedlings were kept moist during sampling. Sampling was done between 11 AM to 2 PM during the day. Total RNA was extracted using the Trizol method (Life Technologies), and then separated on a denaturing glyoxal agarose gel and transferred to a Hybond membrane (Amersham Biosciences). Membranes were probed with *SbTB1*, *SbDRM1*, and *SbUBQ* (*S. bicolor ubiquitin*) genes. Probes were prepared by PCR amplification from a cDNA prepared from sorghum axillary buds using primers for *SbTB1*, forward 5'-GGTGGTGGTTCAAATGGTTC-3' and reverse 5'-TACAATGGCTCCTCAACACG-3'; for *SbDRM1*, forward 5'-TGGTGGCTTTGTGAGTGAAG-3' and reverse 5'-TTATCAGCAACAGCGACAGC-3'; and for *SbUBQ*, forward 5'-GGAAACATAGGGACGCTTCA-3' and reverse AAGGAGTCCACCCTTCACCT-3'. Northern hybridization was done at 65°C and washed with 0.1x SSPE or SSC and 0.5x SDS. Membranes were exposed to an imaging plate and analyzed using a phosphor imager. After subtracting background, the target mRNA photostimulated luminescence of each sample was divided by the corresponding ubiquitin mRNA photostimulated luminescence. The level of the control wild-type target mRNA at 7 DAP was adjusted to one, and comparable samples were adjusted by the same factor to provide relative abundance. Northern analyses were done at least

twice from buds collected from a minimum of 25 seedlings grown at different times, and representative results are presented.

CHAPTER III

EXPRESSION OF *Teosinte Branched1*, *MAX* AND AUXIN TRANSPORTER GENES IN SORGHUM AXILLARY BUDS REPRESSED BY LIGHT SIGNALS AND DEFOLIATION

INTRODUCTION

Light regulates axillary shoot development by controlling bud outgrowth. Previous studies showed that the inhibition of bud outgrowth in the *phyB-1* mutant sorghum is associated with increased expression of the *Teosinte Branched1* (*SbTBI*) and dormancy associated (*SbDRM1*) genes (Kebrom et al., 2006, chapter II). The *TBI* gene is a repressor of bud outgrowth (Hubbard et al., 2002; Takeda et al., 2003). The results suggested that light signals regulate bud outgrowth by controlling the expression of the *TBI* gene (Kebrom et al., 2006, chapter II). However, axillary bud outgrowth is regulated by a complex signaling network that integrates environmental and endogenous signals and genetic mechanisms. For a better understanding of the process regulating bud outgrowth by light, the interaction of light and other dormancy signals should be investigated. This can be done by studying the known branching related genes and their possible roles in mediating light and other inhibitory signals.

The analysis of branching mutants defective in controlling bud outgrowth has been one approach used to study the signaling networks involved (Napoli et al., 1999; Ward and

Leyser 2004). Analyses of the *Arabidopsis more axillary growth (max)* mutants has provided evidence for the presence of a novel hormone-like signal that inhibits bud outgrowth (Booker et al., 2005).

The putative hormone regulating bud outgrowth has not been identified. However, three genes (*MAX1*, *MAX3* and *MAX4*) encoding proteins likely required for the synthesis of the signal and another gene, *MAX2*, encoding a likely signal transduction component, have been cloned (Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004; Booker et al., 2005). *MAX1* encodes a protein that belongs to the cytochrome P450 family; whereas, *MAX3* and *MAX4* encode different carotenoid-cleaving dioxygenases. Reciprocal grafting of scions and rootstocks of *max1*, *max3* and *max4* mutants indicated that *MAX1* acts downstream of *MAX3* and *MAX4*. Based on the homology of *MAX3* and *MAX4*, it seems likely that the signal is derived from a carotenoid precursor (Sorefan et al., 2003; Booker et al., 2005). The *MAX2* gene encodes an F-box protein that may function in the ubiquitin-proteasome pathway (Stirnberg et al., 2002). Other F-box proteins have been demonstrated to participate in hormone signal transduction. For example, *TIR1* is now known to act as an auxin receptor (Dharmasiri et al., 2005; Kepinski and Leyser, 2005).

Mutations in any one of the four *MAX* genes causes increased bud outgrowth, and buds of *max* mutants are resistant to auxin indicating that the *MAX*-related hormone is necessary for the inhibitory action of auxin (Leyser, 2005). The increased branching

phenotype of *max1*, *max3* and *max4* shoots but not the *max2* shoot can be reverted to wild type branching by grafting to wild type rootstock (Booker et al., 2005). These results led to the conclusion that the carotenoid-derived signal is mobile, whereas signal transduction involving MAX2 acts in the shoot (Booker et al., 2005). Increased branching mutants identified in pea, petunia and rice have also been found to have mutations in genes orthologous to the MAX genes of Arabidopsis, providing evidence that the MAX-related signal is common in higher plants (Sorefan et al., 2003; Snowden et al., 2005; Ishikawa et al., 2005; Zou et al., 2005). However, expression of the MAX4 orthologs of pea (*RMS1*) and petunia (*DADI*), but not the MAX4 of Arabidopsis, are under feedback control suggesting differences among species the way the MAX-related signal may be regulated (Bainbridge et al., 2005; Foo et al., 2005; Snowden et al., 2005).

Recent results indicate that the MAX-related signal inhibits branching by regulating auxin transport (Lazar and Goodman, 2006; Bennett et al., 2006). Several auxin influx (*AUX1*) and efflux (*PIN*) carrier proteins facilitate the polar transport of auxin (Woodward and Bartel, 2005) which induces vascular tissue development (Berleth et al., 2000). Release of axillary shoots from correlative inhibition by decapitation of the main shoot is accompanied by rapid cambial development and vascular tissue differentiation; whereas, application of auxin to the stump inhibits cambial development (Morris, 1977). Therefore, the export of auxin from the bud may induce the formation of vascular tissue connections between the bud and the stem enabling bud outgrowth (Bennett et al., 2006). Expression of the *AUX1* and *PIN* genes is higher in the bud and stem of *max* mutants

compared to wild type suggesting that the *MAX*-related signal controls auxin transport, and therefore, bud outgrowth, by directly or indirectly regulating the expression of the auxin transporter genes (Lazar and Goodman 2006; Bennett et al., 2006). The *MAX*-related signal may integrate different signals inhibiting bud outgrowth. Therefore, expression analysis of the *MAX* and auxin transporter genes in the *phyB-1* and wild type sorghum axillary buds will provide clues regarding the role of *MAX*-related signal in regulating axillary shoot development in response to light.

Since the shoot apical meristem of sorghum is located at the base of the plant and is enclosed by leaves, it is not possible to conduct decapitation experiments in sorghum. This restricts our ability to compare molecular mechanisms regulating bud outgrowth in response to light and hormonal signals. However, defoliation of sorghum seedlings at early stages of development inhibits bud outgrowth.

In this study, the expression of the *TBI*, *MAX* and auxin transporter genes were investigated in sorghum axillary buds repressed by light signals and defoliation. The results suggest that the *TBI* gene functions in the regulation of bud outgrowth by light signals; whereas, the *MAX2* gene is involved in regulating bud outgrowth by both light and defoliation.

RESULTS

Response of Wild Type Seedlings to Defoliation

Bud outgrowth in the axil of the first leaf of the *phyB-1* mutant and the wild type sorghum seedlings treated with supplemental FR light starting at 7 DAP are inhibited at about 9 DAP (Kebrom et al., 2006, chapter II). Defoliation of wild type seedlings starting at 7 DAP also inhibited the outgrowth of axillary buds in the axil of the first leaves very rapidly (Fig. 3.1).

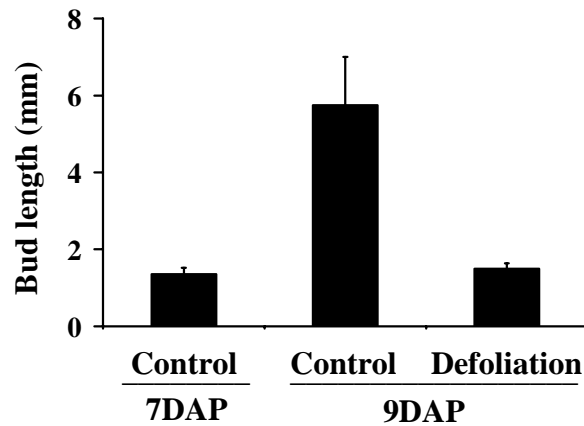


Figure 3.1. The effect of defoliation of wild type sorghum seedlings, starting at 7 DAP, on the outgrowth of buds in the axil of the first leaves. Data are mean \pm SE; n=10.

Identification of Sorghum ESTs Homologous to the Arabidopsis *MAX1* and *MAX2*

A BLAST (TBLASTN; Altschul et al., 1991) search for sorghum ESTs homologous to the Arabidopsis MAX1 protein identified a 460 nucleotide long sorghum EST, accession no. BG049021. The *MAX1* sorghum EST homolog was translated in all possible reading frames using a translation tool available at <http://ca.expasy.tools/dna.html>. Several open reading frames were identified. The longest open reading frame (ORF) had no stop codons. However, no protein in the data base is similar to this ORF. The next longest ORF was 92 amino acids long and blasting this sequence identified cytochrome P450 monooxygenases CYP711A (*MAX1* like proteins) of several species. The amino acid sequence identity between the sorghum ORF with Arabidopsis MAX1 and *Medicago trunculata* CYP711A is 63%. The 460 nucleotide sorghum EST includes about 276 nucleotides (92 amino acids) of coding region followed by a stop codon and an untranslated region. The translated region aligns to the 3' end of the Arabidopsis MAX1 (accession no. NP_565617) and the homologous protein in *Medicago* (accession no. ABC59098) (Fig. 3.2).

A similar search for a sorghum EST homologous to the *MAX2* gene of Arabidopsis identified accession no. CD207478. Translation of this EST resulted in one ORF of about 160 amino acids (which is a *MAX2* like ORF). BLASTP of the *MAX2*-like amino acid sequence identified the rice MAX2 homolog (accession no. BAD69289), the pea RAMOSUS4 (RMS4, accession no. ABD67495) and the Arabidopsis MAX2 (accession

no. NP_565979) proteins with 80%, 66% and 65% identity, respectively. The MAX2 protein of Arabidopsis is 58% and 47% identical to the pea and rice MAX2 homologs, respectively. The sorghum MAX2-like translated region of the EST aligns to the 3' region of the known MAX2 proteins (Figure 3.3). The sorghum *MAX1*-like and *MAX2*-like ESTs are referred to as *SbMAX1* and *SbMAX2*, respectively.

```

Medicago      1  EMKQRHPYAFIPFGIGPRACIGQKFSMQEIKLSLTHLYKKYLFRHSADME
Arabidopsis    1  EEKHRHPYAFIPFGIGPRACVQRFALQEIKLTLHLYRNYIFRHSLEME
Sorghum        1  EQRGRHPCAHIPFGIGPRACVQRFALQELKLSMVHLYQRFLFRRSPOME
consensus      1  * . ***.*.*****.***.*..**.*..***.....**.* **

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```

Medicago      51  SPLELEYGIVLNFKHGVKFSVIKR
Arabidopsis    51  IPLQLDYGIILSFKNGVKLRTIKR
Sorghum        51  SPAELQFGIVLNFKNGVKLVAVER
consensus      51  .....*.....**.*

```

Figure 3.2. Alignment of translated partial *SbMAX1* cDNA sequence with the Arabidopsis and *Medicago* MAX1 amino acid sequences.

```

Pea          1  EGDCRGQPKP-AVSEFGLSILTCYPQLSKMQLD CGDTRGYVYTAPSGQMD
Arabidopsis  1  EGDCRGKRRP-AEPEFGLSCLALYPKLSKMQLD CGDTIGFALTAPPQMD
Sorghum     1  EGDCRARP - - -GPF FGLRYLAGSPVLAKMKLDLSEAVGYALTAPAGQMD
Rice       1  EGDCRTCPRPAPRTIFGLSDLAGFPVLAKMKLDLSEAVGYALTAPTQMD
consensus  1  *****.....*.....*.....*.....*.....*.....*.....*

Pea          50  LSLWERFFLNGIGSL-SLNELHYWPPQDEDVNQRSLSLPAAGLLQECYTL
Arabidopsis  50  LSLWERFFLTGIGSL-SLSELDYWPPQDRDVNQRSLSLPGAGLLQECYTL
Sorghum     47  LSLWERFVLOGIDSLMTLYELDYWPPQDKEVNCQSLTLPVAVGLLQGCVGL
Rice       51  LSLWERFV LHGIESLQTL YELDYWPPQDKDVHHRSLTLPVAVGLLQRCVGL
consensus  51  *****.....*.....*.....*.....*.....*.....*.....*

Pea          99  RKLFIHGTTHEHF MNFFLKIPNL RDVQLRE DYYPAPEND-MSTEMRVGSC
Arabidopsis  99  RKLFIHGTAHEHF MNFLLRIPNL RDVQLRADYYPAPEND-MSTEMRVGSC
Sorghum     97  RKLFIHGTTHEHF LTFFLKVPNL RDMLRE DYYPAPESDMMNTTEMRAESW
Rice      101  RKLFIHGTTHEHF MTFFLSIPNL RDMLRE DYYPAPENDLMFTEMRAESW
consensus  101 *****.....*.....*.....*.....*.....*.....*.....*

Pea          148  SRFEDALNRRITCD
Arabidopsis  148  SRFEDQLNSRNIID
Sorghum     147  LRFENQLNIRLIED
Rice       151  LRFEVQLNSRQIDD
consensus  151  ***.....*.....*.....*.....*.....*.....*.....*

```

Figure 3.3. Alignment of translated partial *SbMAX2* cDNA sequence with the Arabidopsis, pea and rice MAX2 amino acid sequences.

Identification of Auxin Transport Related Sorghum ESTs

A 750 nucleotide long sorghum EST, accession no. CX622221, is homologous to the maize auxin influx carrier gene (*AUX1*, accession no. AJ011794). The nucleotide identity between the sorghum EST and the *AUX1* gene of maize is 86% and the amino acid identity between translated partial cDNA of the sorghum EST and the maize *AUX1* protein accession no. CAB65535 is 85% (Fig. 3.4). The sorghum EST, accession no. CX622221, is referred to as *SbAUX1*.

There are eight auxin efflux carrier genes (*PIN1-PIN8*) in Arabidopsis (Paponov et al., 2005). There are also several sorghum ESTs highly similar to *PIN* genes in Arabidopsis and other species. The 595 nucleotide long sorghum EST, accession no. CX620904, is homologous to the *PIN1* gene in several species. A translated partial cDNA of the sorghum EST is 96% and 83% identical to the *PIN1* amino acid sequences of rice (accession no. Q5SMQ9) and Arabidopsis (accession no. Q9C6B8), respectively (Fig. 3.5). The sorghum EST, accession no. CX620904, is referred to as *SbPIN1*.

```

Sorghum      1  WHGGSVYDAWFSCASNQVAQVLLTLPYSFSQLGMA SGVVFQLFYGLMGSW
Maize       1  WHGGSAYDAWFSCASNQVAQVLLTLPYSFAQLGML SGVLFQLFYGLMGSW
consensus   1  *****

```

```

Sorghum     51  TAYLISVLYVEYRTRKERD-KVDFRNHVIQWFEVLDGLLGKHWNRVGLFF
Maize      51  TAYLISILYLEYRTRREREREKAA DFRNHVIQWFEVLDGLLGRHWNRAGLAF
consensus  51  *****

```

```

Sorghum    100  NCTFLLFGSVIQLIACASNIYYINDKYDKRTWTYVFGACCATTVFIPSFH
Maize     101  NCTFLLFGSVIQLIGCASNIYYVNDRLDKRTWTYVFGACCATTVFIPSFH
consensus 101  *****

```

```

Sorghum    150  NYRIWSFLGLLMTTYTAGYLTIA
Maize     151  NYRVWSFLGLVMTTYTAWYMAVA
consensus 151  *****

```

Figure 3.4. Alignment of translated partial *SbAUX1* cDNA sequence with the maize AUX1 amino acid sequence.


```

Sorghum      1  MITATDFYHVMTAVVPLYVAMILAYGSVRWWRIFSPDQCSGINRFVALFA
Rice         1  MITAADFYHVMTAMVPLYVAMILAYGSVKWWRIFTPDQCSGINRFVALFA
Arabidopsis  1  MITAADFYHVMTAMVPLYVAMILAYGSVKWWRIFTPDQCSGINRFVALFA
consensus   1  ****.*****.*****.***.***.*****

Sorghum      51  VPLLSFHFISTNNPYTMNLRFAADTLQKLMVLAMLTAWSHLSRRGSLEW
Rice         51  VPLLSFHFISTNNPYTMNLRFAADTLQKLMVLAMLTAWSHLSRRGSLEW
Arabidopsis  51  VPLLSFHFIAANNPYAMNLRFAADSLQKVIIVLSLFLWCKLSRNGSLDW
consensus   51  *****.*****.*****.*****.*****.*****.*****

Sorghum      101  TITLFSLSLTPNTLVMGIP
Rice         101  TITLFSLSLTPNTLVMGIP
Arabidopsis  101  TITLFSLSLTPNTLVMGIP
consensus   101  *****

```

Figure 3.5. Alignment of translated partial *SbPIN1* cDNA sequence with the rice and Arabidopsis PIN1 amino acid sequences.

***SbDRM1* and *SbTB1* mRNA Abundance in *phyB-1*, FR Light-Treated and Defoliated Seedlings of Sorghum**

The level of expression of the *SbTB1* and *SbDRM1* genes in the axillary buds of *phyB-1* and wild type, and wild type grown with supplemental FR light were previously investigated by Northern analysis (Kebrom et al., 2006, chapter II). The levels of these genes were also investigated by qPCR here (Fig. 3.6 and Fig. 3.7). Consistent with the results of the Northern analysis, the levels of these genes were higher in dormant buds compared to growing buds. However the fold changes as determined by qPCR were higher than those determined by Northern blot analysis. The mRNA abundance of *SbDRM1* in the defoliated buds was higher than the control. The fold change of *SbDRM1* at 9 DAP in the *phyB-1* vs. wild type, FR vs. control and defoliated vs. control was 15.3, 22.0, and 32.8, respectively. Compared to growing buds of wild type or control, the *SbTB1* mRNA abundance in the repressed buds of *phyB-1* and FR light-treated at 9 DAP was 6.0 and 2.9 fold higher, respectively. However, the dormant state of the buds in the defoliated seedlings was not associated with an increase in the *SbTB1* gene (Fig. 3.7). In fact, the level of *SbTB1* in the buds from defoliated seedlings was repressed in a manner similar to the reduction of the level of expression in the buds of the control seedlings at 9 DAP.

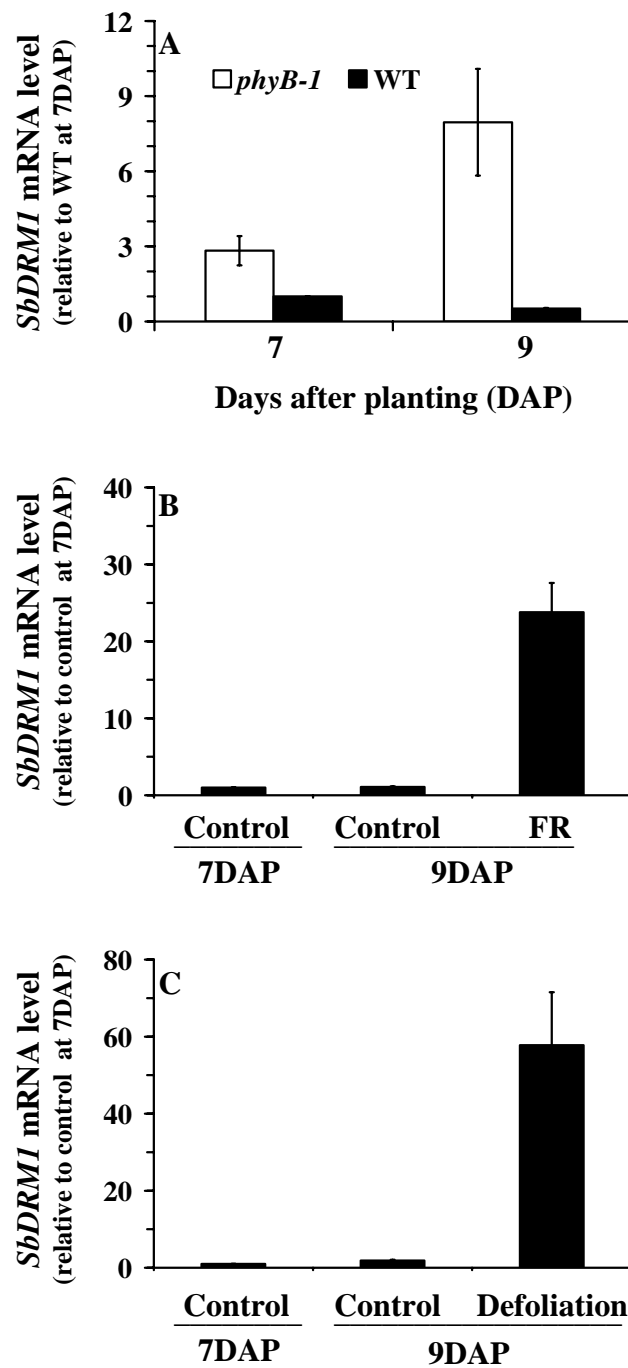


Figure 3.6. Relative expression level of *SbDRMI* in the buds from the first leaf axil of *phyB-1* and wild type (A), FR-treated and control wild type (B) and defoliated and control wild type (C) sorghum seedlings. Bars are means \pm SE of the mean, n=3 biological replicates.

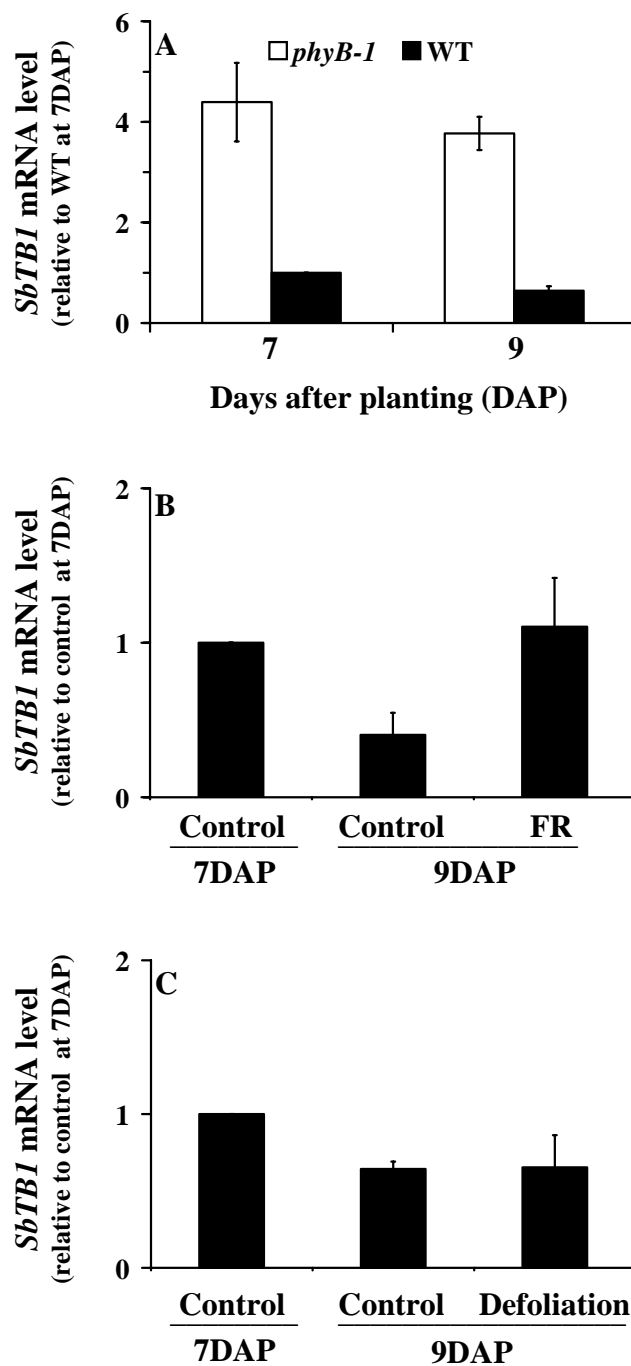


Figure 3.7. Relative expression level of *SbTBI* in the buds from the first leaf axil of *phyB-1* and wild type (A), FR-treated and control wild type (B) and defoliated and control wild type (C) sorghum seedlings. Bars are means \pm SE of the mean, n=3 biological replicates.

***SbMAX1*, *SbMAX2*, and Auxin Transporter mRNA Abundance in *phyB-1*, FR Ligh-Treated and Defoliated Seedlings of Sorghum**

The level of expression of *SbMAX1* in the repressed buds of *phyB-1* compared to the wild type was the same at 7 DAP but more than two fold higher at 9 DAP (Fig. 3.8). However, there was no difference in the level of *SbMAX1* mRNA in the buds of the FR light-treated and defoliated seedlings compared to the control (Figs. 3.9 and 3.10). *SbMAX2* expression was consistently more than two fold higher in the *phyB-1* buds compared to that of the wild type at 9 DAP (Fig. 3.8). The *SbMAX2* expression was also more than two fold higher in the repressed buds of wild type seedlings treated with FR light and defoliation compared to that of the control (Figs. 3.9 and 3.10).

The patterns of expression of the auxin transporter genes, *SbAUX1* and *SbPIN1*, were similar. The level of expression of both genes was higher in the *phyB-1* buds compared to the wild type at both 7 DAP and 9 DAP (Fig. 3.8). However, there was little difference in the level of expression of these genes in the buds of FR light-treated and defoliated wild type seedlings compared to the control (Figs 3.9 and 3.10).

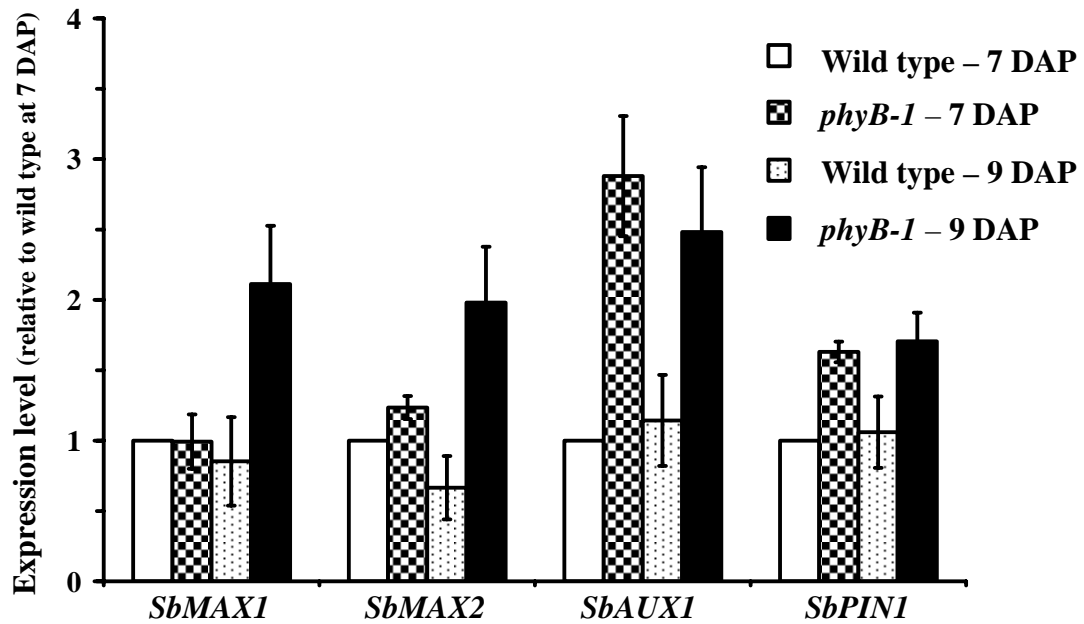


Figure 3.8. Relative expression level of *MAX* and auxin transporter genes in the buds from the first leaf axil of *phyB-1* and wild type sorghum seedlings. Bars are mean \pm SE of the mean, n= 3 biological replicates.

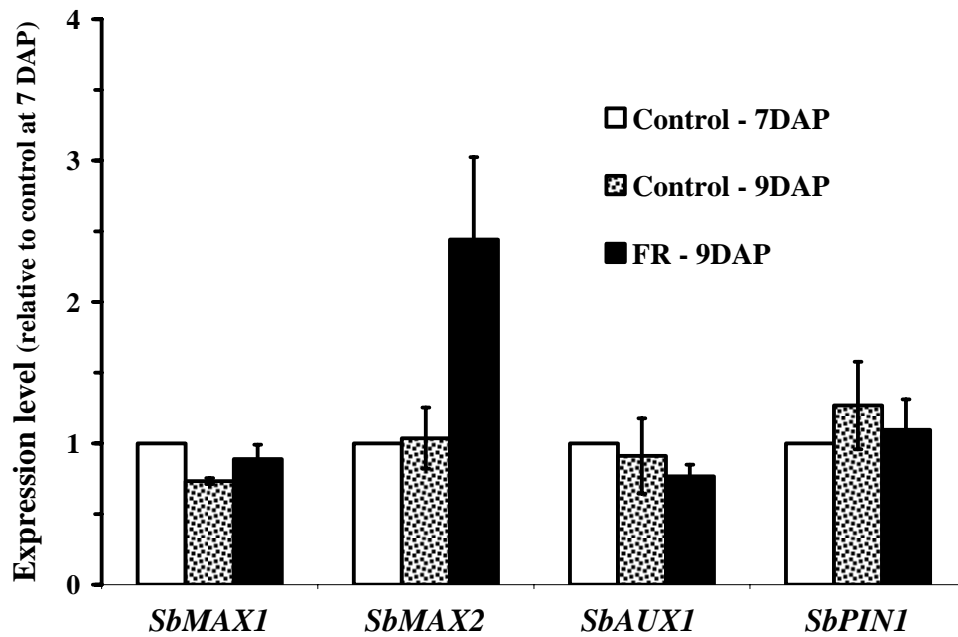


Figure 3.9. Relative expression level of *MAX* and auxin transporter genes in the buds from the first leaf axil of FR-treated and control wild type sorghum seedlings. Bars are mean \pm SE of the mean, n= 3 biological replicates.

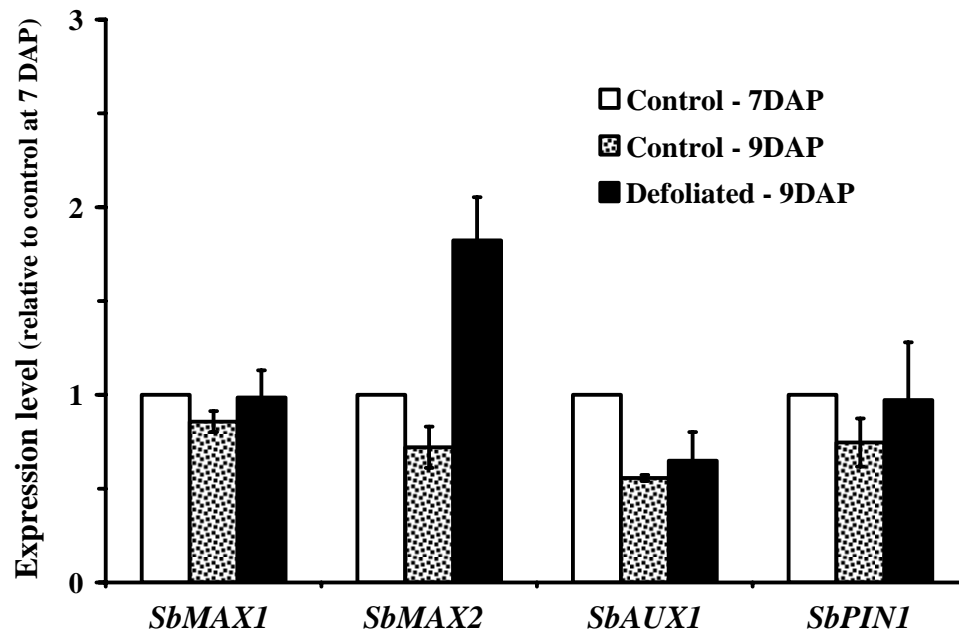


Figure 3.10. Relative expression level of *MAX* and auxin transporter genes in the buds from the first leaf axil of defoliated and control wild type sorghum seedlings. Bars are mean \pm SE of the mean, $n=3$ biological replicates.

DISCUSSION

Expression analysis of branching related genes in the *phyB-1* sorghum axillary buds and buds inhibited by signals other than light can help identify genes as well as mechanisms specifically involved in regulating branching in response to light. Previous studies showed that tillering of grasses can be stimulated or suppressed by defoliation (Murphy and Briske, 1992). The responses to defoliation may depend on several factors including the developmental stage of the plant and the extent of defoliation (Murphy and Briske, 1992; Zhang and Romo, 1995). The effect of defoliation of wild type sorghum seedlings on bud outgrowth has been studied. The results show that continuous defoliation starting at 7 DAP inhibits outgrowth of buds in the axil of the first leaves. FR light treatment started at 7 DAP inhibits the outgrowth of buds in the axil of the first leaves and buds at the same position in the *phyB-1* sorghum seedlings also stop growing after 7 DAP. Therefore, the *phyB-1*, FR light-treated and defoliated seedlings were used as a model system to analyze and compare molecular mechanisms regulating bud outgrowth in response to light and defoliation.

Previous studies by Northern blot analysis showed that the inhibition of bud outgrowth in the *phyB-1* and FR light-treated seedlings is associated with increased expression of the *SbTB1* and *SbDRM1* genes (Kebrom et al., 2006; chapter II). Analysis of these genes by qPCR also showed similar results. However the fold change was higher when assessed by qPCR compared to by Northern blotting. The variation in the fold change

could be due to differences in the sensitivity of the methods used. However, both Northern and qPCR analysis showed that the level of these genes was consistently higher in the repressed buds of *phyB-1* and FR light-treated seedlings.

The expression of the *SbDRM1* gene was upregulated in the buds of defoliated seedlings indicating the induction of dormancy in those buds by defoliation. The *DRM1* gene is expressed in dormant buds of several dicot species such as pea and its expression is down regulated by decapitation (Stafstrom et al., 1998b; 2000). The results from pea and sorghum suggest that the *DRM1* gene is upregulated by inhibitory endogenous and environmental light signals and it is probably at the downstream end of pathways inhibitory to bud outgrowth.

The level of expression of the *SbTB1* gene in the buds of FR light-treated seedlings was 2.9 fold higher than the level in the control at 9 DAP. However, the level was not much higher than in the control at 7 DAP. These results support the hypothesis that for continued outgrowth of buds after 7 DAP, the *SbTB1* gene expression has to be repressed by phytochrome B. However, the expression of the *SbTB1* gene was repressed in the buds of defoliated seedling suggesting that inhibition of bud outgrowth by defoliation does not require *SbTB1* (Fig. 3.7C).

The *MAX1* gene suppresses branching in Arabidopsis (Stirnberg et al., 2002; Booker et al., 2005). The *SbMAX1* gene is upregulated in the *phyB-1* buds at 9 DAP. The

association of the increased expression of the *SbMAX1* gene with the entry into dormant state of the *phyB-1* buds at 9 DAP is consistent with the function of the *MAX1* gene, which is bud outgrowth inhibition. However, the expression of the *SbMAX1* gene was not increased in repressed buds of FR light-treated and defoliated seedlings. The *MAX1* gene has been shown to act in the bud and stem to regulate bud outgrowth (Lazar and Goodman, 2006). Therefore, more studies in different tissues are required to establish the role of *MAX1* in the inhibition of bud outgrowth by light.

Recent studies indicate that apically-derived auxin inhibits bud outgrowth by indirectly limiting the capacity of the buds to export auxin (Lazar and Goodman, 2006; Bennett et al., 2006). The *MAX*-related signal regulates the transport of auxin in the stem and buds of *Arabidopsis* (Lazar and Goodman, 2006; Bennett et al., 2006). It has been hypothesized that the *MAX1*-related signal positively regulates the expression of the flavonoid genes in the bud and stem, and that these flavonoids interfere with the transport of auxin from the buds into the stem (Lazar and Goodman, 2006). In fact, the level of expression of flavonoid genes is higher in the repressed buds of wild type *Arabidopsis* compared to the outgrowing buds of the *max1* mutant; whereas, the level of expression of auxin transporter genes is higher in the outgrowing *max1* mutant bud compared to the wild type (Lazar and Goodman, 2006). In this study, there was little difference in the level of expression of *SbAUX1* and *SbPIN1* in the repressed buds of FR light-treated and defoliated seedlings compared to control, suggesting that the inhibition of outgrowth of these buds was not associated with changes in the level of expression of

the auxin transporter genes (Figs. 3.9 and 3.10). However, the expression of the *SbAUX1* and *SbPIN1* genes was upregulated in the dormant buds of *phyB-1*. The level of the auxin transporter genes in *phyB-1* is not consistent with the status of the buds. In addition, with the increased expression level of the *SbMAX1* gene at 9 DAP, the level of the auxin transporter genes in *phyB-1* should have been downregulated (Fig. 3.8). The higher level of expression of the *AUX1* and *PIN1* genes in the repressed *phyB-1* buds compared to rapidly elongating wild type buds could be due to the presence of a feedback regulatory mechanism. For example, several branching mutants (*ramosus* (*rms*)) characterized by an increased branching phenotype have been identified in pea (Beveridge, 2000). The *RMS1* gene was cloned and is orthologous to the *MAX4* gene of Arabidopsis encoding a carotenoid cleavage dioxygenase (Sorefan et al., 2003). The expression of the *RMS1* gene is increased in several *rms* mutants (Foo et al., 2005). *RMS1* inhibits bud outgrowth and the increased expression of the *RMS1* gene in the *rms* mutants was proposed to be due to feedback upregulation in response to unregulated bud outgrowth in the *rms* mutants (Foo et al., 2005). Branching is regulated by several factors. Therefore, the unanticipated increase in the level of the auxin transporter genes in the dormant *phyB-1* buds may be related to internal mechanisms within the *phyB-1* plant that attempt to maintain bud outgrowth in response to other growth promoting signals.

The expression of the *SbMAX2* gene was more than two fold higher in the repressed buds of *phyB-1*, FR light treated and defoliated seedlings, consistent with its function as

a repressor of bud outgrowth. Its increased expression by those different inhibitory mechanisms supports the hypothesis that it functions at the downstream end of the bud outgrowth inhibitory pathway. In fact, Arabidopsis MAX2 acts in the shoot and likely plays a role in the perception or signal transduction of the *MAX*-related signal (Booker et al., 2005). The *MAX2* gene encodes an F-box protein probably involved in ubiquitinating proteins for degradation by the 26S proteasome (Stirnberg et al., 2002). However, the target protein of MAX2 has not yet been identified. Since the *max2* mutants are characterized by increased bud outgrowth, MAX2 may target a protein or proteins required for promoting bud outgrowth (Stirnberg et al., 2002).

In conclusion, analyses of *SbTBI* and *SbMAX* genes suggest that the *SbTBI* gene is involved in repressing bud outgrowth in response to inhibitory light signals, whereas the *SbMAX2* gene is involved in both light and defoliation. The *MAX2* gene could be involved in suppressing bud outgrowth in response to several growth inhibiting signals. The involvement of the *MAX*-related signal in the regulation of branching by light and defoliation needs further investigation. Detailed knowledge of the function of the *TBI* and *MAX2* genes as well as the *MAX*-related signal may aid in developing crops with branching habits that maximize yield.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Wild type and *phyB-1* mutant sorghum (*Sorghum bicolor*) seedlings were grown in a growth chamber, in 50-mL tubes filled with a growth medium prepared as described by Beall et al. (1991). One seedling was grown per tube and the tubes were supported on a rack arranged at a density of 300 m⁻². Seedlings were grown until 9 DAP and bud sampling were done at 7 DAP and 9 DAP. For FR light treatment, wild type seedlings were treated with supplemental FR light directed from the sides using FR light-emitting diodes beginning 7 DAP as described in Kebrom et al. (2006). The light sources, temperature, photoperiod, photosynthetically active radiation and R:FR conditions of the growth chamber are also described in Kebrom et al. (2006) (chapter II).

For defoliation experiments, wild type seedlings were grown in 50-mL tubes as given above. At 7 DAP, some of the seedlings were harvested, buds in the axil of the first leaves were excised, and their length measured (Control 7 DAP). Then, also at 7 DAP, uniformly growing seedlings were selected; all the leaf blades of half of the uniformly growing seedlings were removed continuously for the next two days (Defoliation 9 DAP) and the other half continued growth as a control (Control 9 DAP). At 9 DAP, buds in the axil of the first leaves of the defoliated and control seedlings were excised and their lengths were measured. Bud length measurements were conducted under a

dissecting microscope using a micrometer. Buds longer than 3 mm were measured using a ruler.

RNA Isolation and Gene Expression Analysis

For gene expression analysis, buds in the axil of the first leaves of different samples of sorghum seedlings were excised under a dissecting microscope, immersed in lysis/binding solution (Ambion) on ice and stored at -80°C until RNA extraction. Total RNA extraction was done using the Trizol method (Invitrogen). Total RNAs were treated with TURBO DNase (Ambion) or RQ1 RNase-free DNase (Promega) to remove DNA before first strand cDNA synthesis. First strand cDNA synthesis was performed using SuperScript III reverse transcriptase kit (Invitrogen) and random hexamer primers from about 1.5 to 2.5 μg DNase treated total RNA in a 20 μl reaction (+RT). A similar reaction was also prepared for every sample without adding SuperScript III reverse transcriptase as a negative control for DNA contamination (-RT). The samples were treated with RNase H and the synthesized first strand cDNA (+RT) and -RT was diluted to 80 μl with sterile water. Gene expression analysis was accomplished by quantitative PCR (qPCR). In every 10 μl qPCR reaction, 0.5 μl of the 80 μl RT (+/-) product was used.

qPCR was run on an ABI prism 7900HT Sequence Detection System (Applied Biosystems). qPCR reactions were prepared using the SYBR Green JumpStart Taq

ReadyMix kit (Sigma-Aldrich). About 250-300 μ M of each gene specific primer pair was used per reaction (Table 3.1). Both +RT and –RT were run at the same time. The –RT was run to check for any genomic DNA contamination due to incomplete digestion of DNA from total RNA prior to cDNA synthesis. In most of the –RT reactions, amplification products were not detected indicating little genomic DNA contamination. The dissociation curves were also analyzed to make sure that there was no non-specific amplification product or primer-dimer formation. The 18S ribosomal RNA (rRNA) was used as an internal control.

The relative level of expression of each gene in different samples was determined from an average of three biological replicates. The threshold cycle value (CT) from each biological sample was an average of two qPCR reactions run at the same time. The difference between the two qPCR CT values is an indicator of pipetting error and a difference of more than 0.5 log warranted a repeated measurement. The expression levels reported are relative to the level of the wild type at 7 DAP in the *phyB-1*/wild type or relative to the level of control at 7 DAP in the FR light or defoliation treatments. The relative level of expression in a biological sample was calculated and then the average of three biological replicates was determined. The relative expression level or fold change of a target gene in a biological sample was calculated using the following equation:

$$2^{-(\text{CT target gene} - \text{CT 18S}) \text{ of treatment}} - ((\text{CT target gene} - \text{CT 18S}) \text{ of control})$$

Table 3.1. Branching and auxin transporter gene-specific primers used in gene expression analysis by qPCR

Gene	Primer Pairs (Forward/Reverse, 5'...3')
<i>SbDRM1</i>	CATGGGTGCCAACCTCTTC/ATCGATCTAACGGTGGTTGC
<i>SbTB1</i>	TGATGAGCACCTCTCCCTCT/ GAGCTCGAACCATTTGAACC
<i>SbMAX1</i>	GCTGCCATGTCGTAGTATCG/CGAGAGATCAGTTTCCACGA
<i>SbMAX2</i>	ATGGACGAAGAGCTTCCTGA/ATTATTGGCCTCCCCAAGAC
<i>SbAUX1</i>	CCTACCTCATCAGCGTCCTC/ CAAACCACTGGATGACATGG
<i>SbPIN4</i>	CCTTGTTTCTCGCTCAGTCC/ GCGCGCTACAACACTACTCC
<i>Sb18S</i>	ATTCTATGGGTGGTGGTGCAT/TCAAACCTCGCGGCCTAAA

CHAPTER IV

**CELL CYCLE-RELATED GENE EXPRESSION CHANGES IN
SORGHUM AXILLARY BUDS REPRESSED BY LIGHT SIGNALS
AND DEFOLIATION**

INTRODUCTION

Following their formation, axillary buds may continue growth to form an axillary shoot or their growth may be arrested leading to dormancy. Growth arrest of buds is typically due to blockage in the cell division cycle (Anderson et al., 2001). Cell division is a complex process that involves a sequence of cell cycle phases of DNA duplication (S phase) and mitotic division (M phase) of a cell into two daughter cells. The transition of a new daughter cell following M phase to S phase is separated by a gap (G1) phase during which a cell increases its size and synthesizes materials required for DNA replication. It is also during G1 phase that a cell makes the decision to enter or exit the cell cycle depending on environmental and developmental signals (Shen, 2001). The S phase is separated from the M phase by the G2 phase during which a cell checks for successful completion of the S phase.

The progression of the cell cycle is controlled by the level and activity of cyclins and cyclin-dependent kinases (CDKs) as well as CDK activating and inhibiting proteins

(Reviewed in Dewitte and Murray, 2003). However, entry into, or exit out of, the cell cycle is determined by environmental and hormonal signals that act on the cell cycle machinery by regulating the transcription and post-transcriptional modifications of cell-cycle related genes and proteins (Jacobs, 1995; Stals and Inze, 2001; Inze, 2005).

Previous studies showed that most cells in dormant buds are arrested at the G1 phase of the cell cycle (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998). The mRNA levels of several cell cycle-related genes, such as *PCNA*, *Histone H4*, *CyclinD*, *CyclinB* and *CDK*, are very low in dormant buds of pea (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998). Decapitation leads to upregulation of the cell cycle-related genes and induction of bud outgrowth. The results indicate that apical dominance observed in intact pea plants results from the direct or indirect action of apically derived auxin on mechanisms regulating the expression of cell cycle-related genes in the buds.

Axillary buds in the *phyB-1* sorghum stop growing soon after their formation (chapter II). Plant growth is due to both cell division and cell expansion (Hemerly et al., 1999; Ingram and Waites, 2006), but it is the production of cells by the process of cell division that maintains the growth of a plant (Mizukami, 2001). Therefore, it may be hypothesized that suspension of growth of buds in the *phyB-1* is due to inhibition of cell production. Inhibition of bud outgrowth in the FR light-treated and defoliated wild type seedlings could also be due to inhibition of cell division. However, the inhibition of bud outgrowth by light involves elevated expression of the *TBI* gene, while the inhibition by

defoliation is not associated with similar changes (chapter III). Therefore, the downstream targets or components of the cell cycle machinery regulated during inhibition of bud outgrowth by light and other signals could be different. To gain clues about the downstream targets of light signals regulating bud outgrowth, expression of the cell cycle-related genes was analyzed in the repressed buds of *phyB-1*, FR light-treated and defoliated sorghum seedlings. The results show little difference in the mRNA level of cell cycle-related genes in the repressed buds of *phyB-1* compared to outgrowing buds of wild type sorghum seedlings. However, the expression of the cell cycle-related genes was dramatically reduced in the repressed buds of FR-treated and defoliated sorghum seedlings.

RESULTS

Identification of Cell Cycle-Related Genes in Sorghum

Several cell cycle-related ESTs of sorghum were identified by Blast searches. There is high homology between the sorghum ESTs and maize or rice cell cycle related-genes. Since nucleotide identity is very high, only the accession no., nucleotide identity and E-values are shown in Table 4.1. The Proliferating Cell Nuclear Antigen (*PCNA*) and Histone H4 (*His4*) are required for DNA replication and modification during the S phase, respectively. Since it is only expressed during the S phase, *His4* is a marker for the S phase (Devitt and Stafstrom, 1995). Cyclin D2 (*CycD2*) is a G1 cyclin and its

expression in maize is stimulated by sugar and cytokinin (Gutierrez et al., 2005). Cyclin B (*CycB*) is a mitotic cyclin and it is expressed during the G2 and M phase of the cell cycle (Dewitte and Murray, 2003). Sorghum ESTs representing A and B type Cyclin Dependent Kinases (*CDKs*) were also identified. The mRNA level of *CDKA* has been shown to be almost the same during the different phases of the cell cycle; however, *CDKB* is only expressed during the S, G2 and M phases (Dewitte and Murray, 2003).

Table 4.1. Accession no., length, % identity and E-value of sorghum cell cycle-related genes

Gene	Sorghum EST accession no.	length (nt)	Homology (Species, accession no.)	% identity, E-value
<i>SbHis4</i>	CF771582	487	Maize, M13370	97%, 1e-121
<i>SbPCNA</i>	CN131224	805	Maize, X79065	95%, 0.0
<i>SbCycD2</i>	BI140861	565	Maize, AF351189	95%, 7e-133
<i>SbCycB</i>	CN128140	785	Maize, U10079	89%, 5e-150
<i>SbCDKB</i>	BG487760	562	Rice, AB239918	92%, 2e-108

The five cell cycle-related genes were chosen for analyses by qPCR because they represent the different phases. In addition to indicating changes in their expression level in dormant and growing tissues, they may also show if dormant buds are blocked at a specific stage during the cell cycle by different treatments. The genes described are also among those studied in growing and dormant buds of pea (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998). This allows a comparison of the changes in the level of expression of these genes in growing and dormant buds of sorghum and pea.

Expression Level of Cell Cycle-Related Genes in the Repressed Buds of *phyB-1*, FR Light-Treated and Defoliated Seedlings of Sorghum

Buds in the axil of the first leaves of *phyB-1* and the wild type sorghum seedlings grew at the same rate until 7 DAP. At 9 DAP, the buds in *phyB-1* became dormant, while the buds in the wild type continue growing (chapter II). At both 7 DAP and 9 DAP, there was little difference in the expression level of several cell cycle-related genes in the buds of *phyB-1* and the wild type (Fig. 4.1). At 7 DAP, the levels of most of the cell cycle-related genes were slightly upregulated in the buds of *phyB-1* compared to the level in the buds of the wild type seedlings. The level of expression of the *CycD2* gene was upregulated (2.3 fold higher) in the buds of the wild type seedlings at 9 DAP compared to the level at 7 DAP. However, in *phyB-1*, the level of *CycD2* at 9 DAP was only slightly higher compared to the level at 7 DAP. In fact, when comparing the level of the cell cycle-related genes at 9 DAP between the repressed buds of *phyB-1* and growing

buds of the wild type, only *CycD2* showed an appreciable difference. There was little difference in the level of expression of the other four genes in the buds.

Buds in the axil of the first leaves of the wild type seedlings treated with FR light or that were defoliated became dormant at about 9 DAP (chapters II and III). The level of expression of the cell cycle-related genes was dramatically reduced in repressed buds of FR light-treated and defoliated seedlings (Fig. 4.2 and Fig. 4.3). There was a greater reduction in the level of cell cycle-related mRNAs in the buds of defoliated seedlings compared to those treated with FR light.

Gene expression changes over a longer period of growth were monitored in *phyB-1* to determine if trends in expression were persistent. The buds in the first leaf axil of *phyB-1* seedlings became dormant after 7 DAP (chapter II). At 9 DAP, the level of expression of *SbDRM1* was upregulated indicating the dormant status of the buds. The expression level of the *SbDRM1* gene remained high even after 9 DAP (Fig. 4.4A). However, the levels of expression of most of the cell cycle-related genes were not reduced greatly even at 15 DAP (Fig. 4.4B).

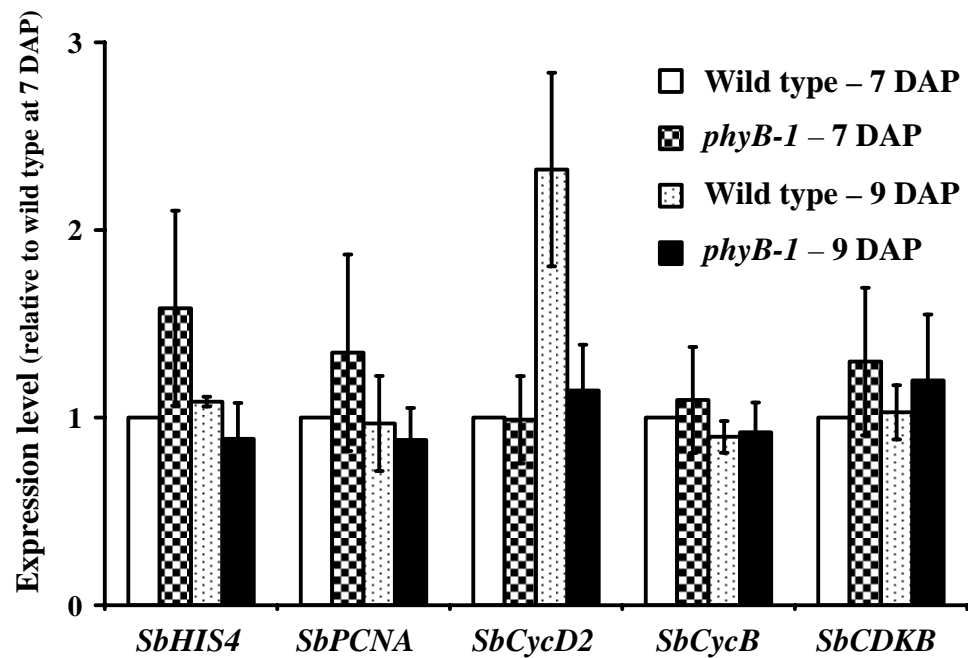


Figure 4.1. Relative expression level of cell cycle-related genes in the buds from the first leaf axil of *phyB-1* and wild type sorghum seedlings. Bars are mean \pm SE of the mean, n= 3 biological replicates.

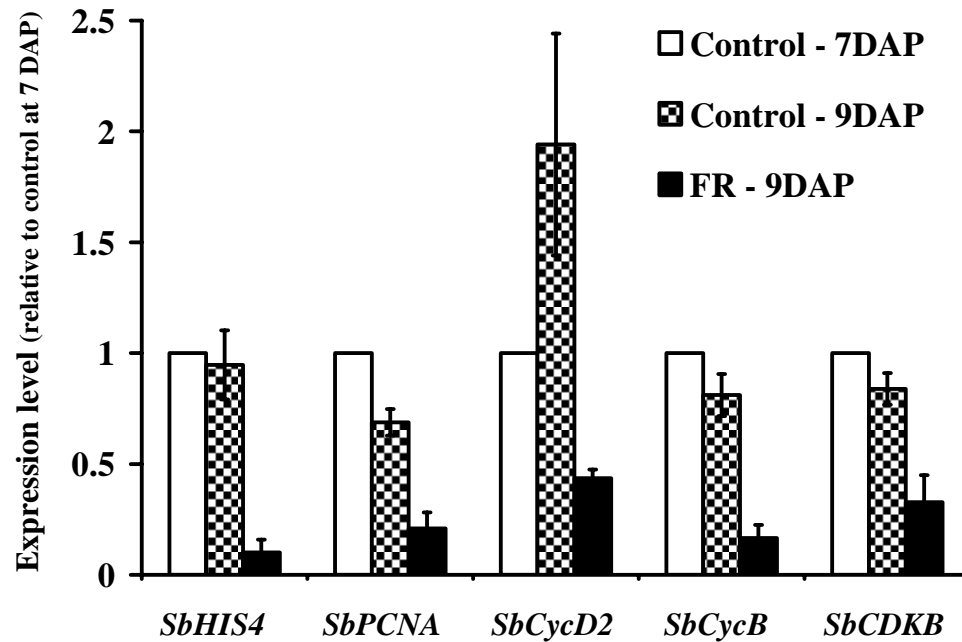


Figure 4.2. Relative expression level of cell cycle-related genes in the buds from the first leaf axil of FR light-treated and control sorghum seedlings. Bars are mean \pm SE of the mean, n= 3 biological replicates.

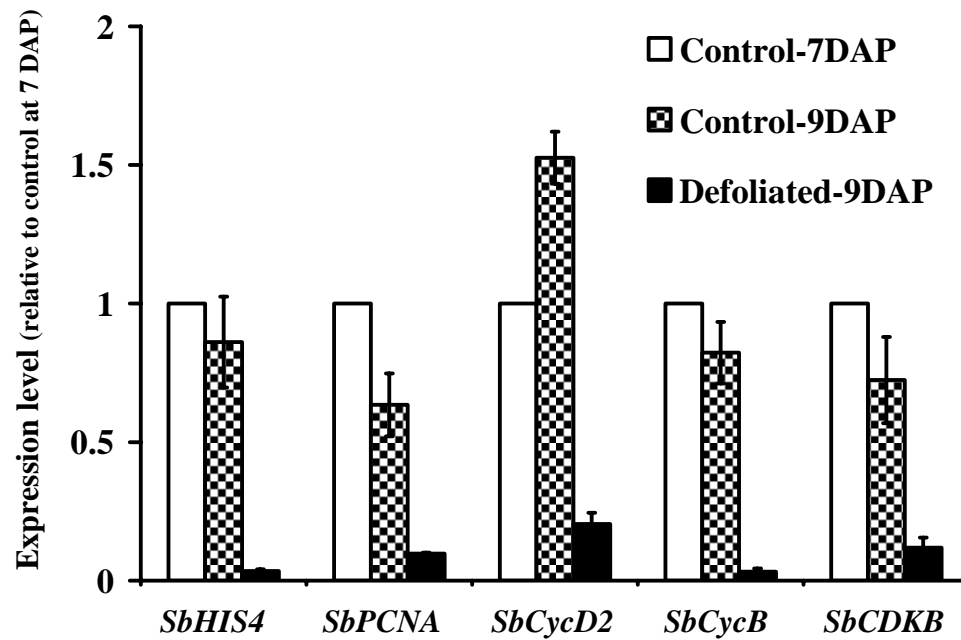


Figure 4.3. Relative expression level of cell cycle-related genes in the buds from the first leaf axil of defoliated and control sorghum seedlings. Bars are mean \pm SE of the mean, n= 3 biological replicates.

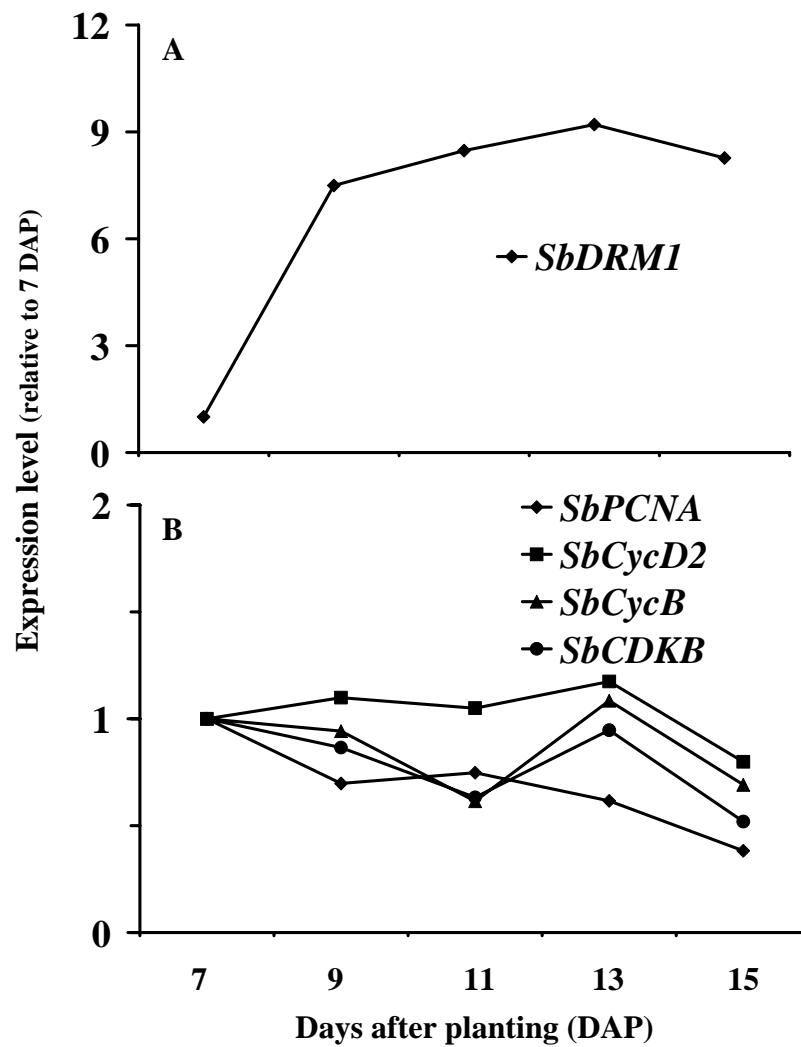


Figure 4.4. Relative expression level of the sorghum dormancy associated (*DRM1*) (A) and cell cycle-related (B) genes in the buds from the first leaf axil of *phyB-1* seedlings. Results are from one biological sample.

DISCUSSION

Bud outgrowth is inhibited in the *phyB-1* mutant as well as in the FR light-treated and defoliated wild type seedlings of sorghum. Suspension of growth in these buds is reflected by the high level of expression of the *SbDRM1* gene, which is a maker for dormancy (chapters II and III). Growth suspension or dormancy is due to inhibition of cell division (Anderson et al., 2001). Signals inhibiting bud outgrowth may act on the components of the cell division machinery to stop cell division and hence prevent bud outgrowth.

Expression analyses of several key cell division-related genes indicate that there are differences in the mechanisms by which bud outgrowth is inhibited by different signals. Although the buds of *phyB-1* stop growing at about 9 DAP (chapter II), the level of expression of the cell cycle genes was not reduced (Fig. 4.1). Conversely, inhibition of bud outgrowth induced by FR light and defoliation treatments was associated with dramatic reductions in the expression level of genes associated with the cell cycle (Figs. 4.2 and 4.3). Such down-regulation is similar to the results observed with decapitation experiments in pea (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998). They found that the level of expression of cell cycle-related genes is very low in the dormant buds of intact pea plants, and is upregulated when buds are induced to grow by decapitation. To confirm that the cell cycle-related genes are continuously expressed in the *phyB-1* buds, their expression was analyzed in the *phyB-1* buds until 15 DAP (Fig. 4.4). These results

demonstrate that the level of expression of the cell cycle-related genes, even at 15 DAP, changed only slightly from 7 DAP - a time before the buds became dormant. The expression level of the *DRM1* gene was high from 9 DAP and beyond, indicating that the dormant status of the buds during analyses period. These results suggest that inhibition of bud outgrowth in the *phyB-1* buds is not associated with suppression of the expression of cell cycle-related genes. The slight upregulation of most cell cycle-related genes in the *phyB-1* buds at 7 DAP may be related to the rate of growth of the buds. Prior to 7 DAP, the growth rate of *phyB-1* buds is slightly higher than that of the wild type (data not shown).

Expression analyses of branching-related genes showed that the suppression of bud outgrowth in the *phyB-1* mutant sorghum is associated with high level expression of the *TB1* gene in those buds (chapters II and III). *TB1* has been documented as a repressor of growth (Doebley et al., 1997; Hubbard et al., 2002; Takeda et al., 2003). *TB1* belongs to the TCP family of proteins identified in several plant species (Cubas et al., 1999; Cubas, 2002). There are two classes of TCP proteins (Class I and Class II). The Class I proteins stimulate growth; whereas, those of Class II repress growth (Li et al., 2005). Several TCP proteins have been shown to function as transcription factors and act as enhancers or repressors of gene expression (Kosugi and Ohashi, 1997; Gaudin et al., 2000; Kosugi and Ohashi, 2002; Li et al., 2005). Several target genes of the TCP proteins have been identified and most are growth or cell cycle-related genes (Kosugi and Ohashi, 1997; Gaudin et al., 2000; Li et al., 2005).

Lukens and Doebley (1999) hypothesized that *TBI* may inhibit the growth of an organ by suppressing cell division. They analyzed the expression of the *CyclinB* gene in the ear primordia of maize plants containing *TBI* alleles of teosinte (*TBI*-teosinte) or maize (*TBI*-maize). The *TBI*-maize allele is expressed two fold higher than the *TBI*-teosinte allele. Maize plants with the *TBI*-maize allele have ear primordia with only pistillate florets; whereas, those with the *TBI*-teosinte allele possess both staminate and pistillate florets (Doebley et al., 1997; Lukens and Doebley, 1999). Higher expression of the *TBI* gene in ear primordia suppresses the growth of stamens and lodicules (Doebley et al., 1997). However, there was no significant difference in the level of expression of *CyclinB* between the ear primordia of maize plants with *TBI*-maize or *TBI*-teosinte allele (Lukens and Doebley, 1999). The authors suggested the need for more studies to determine whether *TBI* acts on cell division or some other mechanism that inhibits the growth of axillary organs. Findings from this study indicate that the repression of bud outgrowth in the *phyB-1* is associated with the failure to repress *TBI* gene expression in the buds (chapters II and III). Analyses of the expression of cell cycle-related genes, including the maize *CyclinB* homolog studied by Lukens and Doebley (1999), showed little difference in the repressed buds of *phyB-1* and growing buds of the wild type seedlings (Fig. 4.1). Therefore, the results reported here are consistent with those described in the *TBI* studied in maize. The lack of correlation between *TBI* expression and repression of cell cycle-related genes suggest that *TBI* may repress bud outgrowth by mechanisms unrelated to the regulation of these genes. This hypothesis is further supported by the findings from the defoliation experiments. Repression of bud

outgrowth by defoliation is not associated with changes in the expression of the *TBI* gene (chapter III). Inhibition of bud outgrowth by defoliation is associated with down-regulation of the cell cycle-related genes. These results suggest that molecular mechanisms regulating bud development by phyB may be mediated by *TBI* and that bud dormancy is initiated or occurs with little change in the expression level of cell cycle-related genes. However, the mechanisms of repression of bud outgrowth in the *phyB-1* and *TBI* mutants need further analyses to determine the downstream targets.

Although the buds of *phyB-1* become dormant at 9 DAP, when their length is about 1.5 mm, they grow to a length of 5-10 mm by about one month after planting indicating that the buds of *phyB-1* do not stop growing completely (data not shown). In fact, by definition, dormancy is the absence of visible growth of an organ containing a meristem over a short period of time, but buds may continue growth at a very slow rate and visible changes in growth may be observed over a long period of time (Lang, 1987). This increased length of the buds of *phyB-1* observed over longer durations demonstrates that part of the bud is continuing cell division or elongation. Therefore, continued expression of the cell cycle-related genes is not inconsistent with the developmental program of this structure.

It is well established that the rate of cell division varies in the different zones of a shoot apical meristem (Reddy et al., 2004). Cells in the peripheral zones of the apical meristem divide at a more rapid rate than those in the central zone (Reddy et al., 2004).

Therefore, the inhibition of bud outgrowth in *phyB-1* could be due to inhibition of cell division in specific regions of the bud probably the peripheral zone or the zone from which primordia arise. Transgenic tobacco plants expressing the *CycD2* gene of *Arabidopsis* showed an increased rate of leaf initiation (Cockcroft et al., 2000). The expression level of *SbCycD2* in the wild type bud was enhanced at 9 DAP when the buds were rapidly elongating. However, the level was not enhanced in the repressed *phyB-1* buds. Thus, the suspension of growth in *phyB-1* buds may be associated with the expression level of *SbCycD2* in specific regions of the buds. This could be analyzed by using *in situ* hybridization to investigate where *SbTB1*, *SbDRM1*, *SbCycD2* as well as other genes are specifically expressed in the buds.

It is interesting to observe that the expression level of the cell cycle-related genes in *phyB-1*, FR treated and defoliated seedlings at 9 DAP is correlated with the relative level of expression of the *SbDRM1* gene (Table 4.2). The level of *SbDRM1* expression is highest in the buds of defoliated seedlings followed by FR treatment and lowest in *phyB-1*. The level of reduction of the cell cycle-related genes is also highest in the buds of defoliated seedlings followed by FR treatment and little changed in *phyB-1*. The *DRM1* gene is a marker for dormancy and the high level of expression in the defoliated seedlings, along with dramatic reductions in the level of expression of cell cycle-related genes may indicate a stronger arrest or that more cells or regions of the bud are arrested. On the other hand, light signals may inhibit growth in specific regions of the bud or in specific cells.

Table 4.2. Expression fold-changes of *DRM1* and cell cycle-related genes in the buds of *phyB-1*, FR-treated and defoliated seedlings relative to wild type or control at 9 DAP.

Gene	<i>phyB-1</i> /wild-type	FR/Control	Defoliation/Control
<i>SbDRM1</i>	15.3	22.0	32.8
<i>SbPCNA</i>	-1.6	-4.1	-6.5
<i>SbHis4</i>	-1.4	-14.1	-26.8
<i>SbCycD2</i>	-2.0	-4.5	-8.3
<i>SbCycB</i>	1.1	-6.8	-43.8
<i>SbCDKB</i>	1.2	-3.9	-7.2

Inhibition of bud outgrowth in the wild type seedlings by FR treatment is associated with the reduction in the level of expression of the cell cycle-related genes. These results are different from the results in the *phyB-1* mutants. The shade avoidance response is primarily mediated by phyB (Franklin and Whitelam, 2005). However, the presence of a shade avoidance response in the *phyB* mutant of Arabidopsis at low R:FR indicates that other phytochromes are involved in the process (Sullivan and Deng, 2003; Franklin and Whitelam, 2005). In fact, Arabidopsis *phyA phyB* double mutants are more elongated than the single *phyB* mutant (Johnson et al., 1994). Gene expression changes in the buds of FR light-treated sorghum seedlings suggest that photoreceptors other than phyB are also involved in mediating the response of bud outgrowth to supplemental FR light.

Continuous supplemental FR light induces a high irradiance response (HIR) which is mediated by phytochrome A (phyA) and affects many processes including altering the mRNA abundance of several genes, anthocyanin content, stem growth and floral development in different species (Casal, et al., 1998; Yanovsky et al., 1998). Bean (*Phaseolus vulgaris*) plants grown in white light supplemented with FR light showed enhanced elongation of internodes due to both increased cell division and cell elongation (Beall et al., 1996). Increased stem elongation is expected to be associated with increased sink strength that will divert resources into the growing stem at the expense of other structures, for example buds. However, Beall et al. (1996) did not address the effect of supplemental FR light on bud outgrowth or branching. The response of wild type seedlings to supplemental FR light includes increased seedling height which could

be due to both shade avoidance responses mediated by phyB and HIR mediated by phyA. The expression patterns of several branching and cell cycle-related genes have shown that the inhibition of bud outgrowth by FR light similar to both the inhibition in *phyB-1* and defoliation. Repression of bud outgrowth by FR is similar to that observed in *phyB-1* because in both cases *TB1* gene expression is maintained at high level. FR-mediated inhibition of bud outgrowth also is similar with defoliation-induced bud outgrowth inhibition because of the reduction in the level of the cell cycle-related genes. Buds released from inhibitory signals may not continue growth due to resource limitations (McIntyre, 2001). Growth inhibition following defoliation could be due to a drastic reduction in photosynthesis (Gautier et al., 1999; Schnyder and de Visser, 1999) and the inhibition of bud outgrowth by defoliation in the wild type sorghum seedlings is probably due to limitations in carbohydrate supply. Increased growth of the main shoot in the FR-treated seedlings could also enhance the sink strength of the main shoot depleting carbohydrates which could have been used for bud outgrowth. Therefore, the suppression of bud outgrowth by FR treatment may be due to the combined effects of inactivation of phyB, and associated growth suppression by TB1, and carbohydrate supply limitations due to enhanced growth of the main shoot.

In conclusion, analyses of cell cycle-related gene expression changes in the repressed buds of *phyB-1*, FR-treated and defoliated sorghum seedlings revealed variations in molecular mechanisms regulating bud development in response to different signals. Variations in cell cycle-related gene expression in the *phyB-1* and FR-treated buds

demonstrated the involvement of photoreceptors, other than phyB, in regulating branching in response to light signals and shows that the mechanisms of regulation vary depending on the photoreceptors. Differences in the level of *DRMI* and cell cycle-related genes in the buds of *phyB-1*, FR-treated and defoliated seedlings also suggest variations in the targets of those different signals within the bud.

MATERIALS AND METHODS

The plant material, growing conditions, sampling and methods of analysis of gene expression by qPCR are describe in the materials and methods sections of chapters II and III. The levels of expression of the cell cycle-related genes were analyzed in the buds of *phyB-1* and FR light-treated and defoliated wild type seedlings. In addition, the levels of expression of the cell cycle-related genes were analyzed in the buds in the axil of the first leaves of *phyB-1* seedlings starting at 7 DAP and every other day until 15 DAP. The *phyB-1* seedlings were grown in trays containing 7-cm-deep cells, and the growing medium and growth conditions were as described in the materials and methods section of chapter II. The gene-specific primers of cell cycle-related genes used in qPCR analyses are shown in Table 4.3. 18S ribosomal RNA was used as an internal control.

Table 4.3. Cell cycle-related gene-specific primers used in gene expression analyses by qPCR

Gene	Primer Pairs (Forward/Reverse, 5'...3')
<i>SbHIS4</i>	GACAACATCCAGGGCATCA/ GCGGGTCTCCTCGTAGATG
<i>SbPCNA</i>	TCCACCTCTGGAGAAATTGG/AACCGGCTCTTGCATCTCTA
<i>SbCycD2</i>	TTACAATTTTCGCACCGTTGA/GCTGAGTCATCCAAGCTCTGT
<i>SbCycB</i>	TCAAGCATCACACAGGCTTC/AACCCTCAGCTTGCTCTCAG
<i>SbCDKB</i>	GGTGGCGCTGAAGAAGAC/ATGGAGTGGGAGAGGAGGTT
<i>Sb18S</i>	ATTCTATGGGTGGTGGTGCAT/TCAAACCTTCGCGGCCTAAA

CHAPTER V

SUMMARY AND CONCLUSIONS

Shoot branching is an important agronomic trait that determines resource use efficiency and yield of crop plants. In-depth knowledge of the development of branches will help in modifying the shoot architecture of crops to maximize yield. The development of branches has been investigated systematically for more than 70 years. It involves the initiation of an axillary meristem and the outgrowth of buds and is regulated by complex interactions among several environmental and hormonal signals, and genetic mechanisms. Although environmental signals are key determinants of the development of an axillary shoot, apart from their effects on the number of branches produced by a plant, little has been done to understand how these signals regulate branching at the molecular level. The efforts of past studies have concentrated on molecular mechanisms regulating branching in response to hormonal signals and regulation by genetic mechanisms. Without a complete understanding of the regulation of branching by environmental, hormonal, and genetic factors and their interactions, yield improvement by manipulating the branching habit of plants may not be achieved easily. One of the most important environmental factors that regulate branching is light. The objective of this project was to investigate molecular mechanisms associated with the regulation of branching by light signals.

Mutant analysis has been one of the approaches used to study molecular mechanisms regulating plant developmental processes. The enhanced apical dominance of phytochrome B null mutant sorghum (*phyB-1*) as a model system was used for this study. Analysis of the *phyB-1* mutant sorghum indicated that buds are formed normally and that the enhanced apical dominance was due to inhibition of bud outgrowth. Therefore, the *phyB-1* mutant sorghum is useful for analyzing the regulation of bud outgrowth by light signals.

It is well established that high planting density or supplemental FR light treatments inhibit bud outgrowth. While buds in the *phyB-1* sorghum remain dormant during the vegetative stage, buds in the wild type outgrow and produce branches. The enhanced apical dominance of *phyB-1* was simulated in the wild type by growing wild type seedlings at high planting density or with supplemental FR light to compare with the inhibition of bud outgrowth in *phyB-1*.

One of the approaches used to study branching in dicots was decapitation; however, it is not possible to decapitate sorghum seedlings. Therefore, defoliation of wild type sorghum seedlings was employed to compare molecular changes associated with the regulation of branching by light with other signals. Defoliation of wild type seedlings inhibited bud outgrowth. However, the mechanism by which defoliation affects bud outgrowth is not known. Therefore, in this study, *phyB-1*, high planting density,

supplemental FR light, and defoliation were used to investigate molecular mechanisms regulating bud outgrowth in response to light signals.

Characterization of enhanced apical dominance of *phyB-1* showed that the buds in the first leaf axil of the *phyB-1* mutant sorghum grow until 7 DAP and are comparable in size with the wild type buds at the same position. However, buds in *phyB-1* become dormant by 9 DAP; whereas, buds in the wild type continue growth. By treating wild type seedlings with FR light or defoliation at 7 DAP, it was possible to inhibit bud outgrowth at 9 DAP. A plant density of 3000 seedlings m² inhibited bud outgrowth at about 9 DAP, thus establishing a time point for studying bud outgrowth in *phyB-1* mutant and the wild type seedlings grown at high planting density, treated with FR light and defoliation.

Direct assessment of dormant vs. growing buds requires measuring bud elongation over relatively long period of time. However, it was found that expression of the sorghum *DRM1* gene correlated with growth status and it was therefore possible to determine if the buds were growing or dormant at a single time point by molecular rather than morphological means. Therefore, one of the achievements of this project was the establishment of a model system and sampling times that will help study buds just before and after their fate is determined in response to inhibitory signals. Additionally, the identification of the *DRM1* gene in sorghum and establishing it as a molecular marker

for determining the growing/dormant status of buds will serve as a tool useful for studying the process.

Using this established sorghum system as a model, the expression of several branching and cell cycle-related genes was investigated. One of the most important discoveries was the association of the branching inhibiting gene, *TBI*, with the enhanced apical dominance of the *phyB-1* mutant and its specific involvement in the inhibition of bud outgrowth by light signals. Based on the results of the expression analyses of the *TBI* gene in the different systems, a working hypothesis that “phytochrome B represses *TBI* gene expression to induce bud outgrowth” was developed, providing direction for future research. *TBI* is one of the genes that contributed to the domestication of maize from its wild ancestor teosinte (Doebley et al., 1995). A detailed understanding of the regulation of *TBI* gene expression could help in developing cultivars with an architecture that maximizes yield.

Recent studies indicate that the MAX-related signal is required for the inhibition of bud outgrowth by auxin (Leyser, 2005). It is not known if the MAX-related signal also mediates other signals which inhibit branching. Among the *MAX* genes, *MAX2* encodes an F-box protein that may function in the perception or signal transduction of the MAX-related signal (Stirnberg et al., 2002). Expression analyses in the sorghum system showed that suppression of bud outgrowth by light or defoliation is associated with increased expression of the *MAX2* gene. The results suggest that the *MAX2* gene acts

downstream of several signals inhibiting branching. Additional studies are needed to identify targets of MAX2 as well as the role of the putative MAX-related hormone or signal in the regulation of bud outgrowth by light and other factors.

Expression analyses of cell cycle-related genes revealed interesting results that will contribute to studies in phytochrome regulation of growth in general and bud outgrowth in particular. The suppression of bud outgrowth in the *phyB-1* sorghum is not associated with changes in the expression level of several cell cycle-related genes. However, the upregulation of the *CycD2* gene in the growing buds of the wild type seedlings, but not in the dormant buds of *phyB-1*, indicate that the *CycD2* gene could be a target for the regulation of bud outgrowth by light signals perceived by phyB.

The high level of expression of the *DRM1* gene accompanied by dramatic reduction in the expression level of the cell cycle-related genes in the buds of defoliated seedlings compared to *phyB-1* indicate variations in the type of inhibition of those buds. These results raise the question of whether there is variation in the regions within the buds targeted by different inhibitory signals. For example, leaf primordia vs. meristem or peripheral vs. central zones of a meristem. Further studies may reveal interesting biological processes associated with the suppression of bud outgrowth by light and other signals and help in identifying downstream targets for manipulating the branching habits of crops.

Another interesting finding concerns the molecular changes associated with the inhibition of bud outgrowth by FR treatment and high planting density. Although past studies have indicated that the inhibition of branching at high planting density or by FR light enrichment to be due to the shade avoidance response, similar to the constitutive shade avoidance phenotype of the *phyB-1* mutant, results of this study suggest the involvement of additional mechanisms. For example, the expression level of cell cycle-related genes is down-regulated in FR-treated but not in *phyB-1* buds and the expression level of *TBI* is higher in *phyB-1* buds compared with the buds of the wild type seedlings grown at high plant density or with supplemental FR light. Further study may help identify other density-derived and FR light-induced mechanisms that inhibit bud outgrowth.

Although the objective of the defoliation treatment was to compare the molecular changes associated with the repression of bud outgrowth by light and signals other than light, it opened an opportunity for future research. Recovery from defoliation is one of the important aspects in grazing or range land management and many other aspects of crop production. The effect of defoliation on tiller or branch growth has been investigated only at the whole plant level. Findings from this study demonstrate that it is worthwhile to explore signal transduction and response-regulation associated with the inhibition of bud outgrowth by defoliation. Such investigations could help in developing management strategies and/or new cultivars (using molecular tools) that will respond to defoliation as desired.

This study is the first to investigate the molecular changes associated with repression of bud outgrowth by light signals. The study benefited enormously from the availability of a well studied and characterized *phyB* mutant sorghum with enhanced apical dominance (Pao and Morgan, 1986; Childs et al., 1991, 1997; Foster et al., 1994; Finlayson et al., 1999) and sorghum ESTs available in the data base (Mullet et al., 2001; Pratt et al., 2005). The findings and approaches used will contribute significantly toward the complete understanding of the regulation of development of plant architecture by light and other signals.

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