REGULATION OF BRANCHING BY PHYTOCHROME B AND PPFD IN *Arabidopsis thaliana*

A Thesis

by

NAN-YEN CHOU

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

MASTER OF SCIENCE

August 2008

Major Subject: Agronomy

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Approved by:

Chair of Committee	Scott Finlayson
Committee Members	Dirk Hays
	Hisashi Koiwa
Head of Department	David Baltensperger

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ABSTRACT

Regulation of Branching by Phytochrome B and PPFD in *Arabidopsis thaliana*.

(August 2008)

Nan-Yen Chou, B.A., National Taiwan University

Chair of Advisory Committee: Dr. Scott Finlayson

The branching or tillering of crops is an important agronomic trait with a major impact on yield. Maintaining an appropriate number of branches allows the plant to use limited light resources and to produce biomass or yield more effectively. The branching process includes the initiation of the axillary meristem leading to bud formation and the further outgrowth of the axillary buds. Phytohormones, including cytokinins and auxin, are known to play major roles in regulating axillary bud outgrowth.

Light signals, including light quantity and light quality, are among the most important factors regulating plant growth and are perceived by the action of specialized photoreceptors, including phytochromes. Phytochromes sense red (R) and far-red (FR) light and allow some plants to perceive and respond to competing neighbors by evoking the shade avoidance syndrome (SAS). One component of the SAS is inhibition of branching. Phytochrome B (phyB) is especially important in sensing shade signals and loss of phyB function results in a constitutive shade avoidance phenotype, including reduced branching. While it has been anecdotally reported that phyB-deficient *Arabidopsis* branches less than wild type, a detailed study of the defects in the process is lacking. In this research, the interactions between light signals, phytochromes and

phytohormones in the regulation of branching were assessed using an integrated physiological, molecular and genetic approach.

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1. INTRODUCTION

The branching or tillering of crops is an important agronomic trait with a major impact on yield. Maintaining an appropriate number of branches allows the plant to use limited light resources and to produce biomass or yield more effectively. A more thorough knowledge of branching mechanisms may help in further research to modify plants for higher biomass or yield. The branching process includes the initiation of the axillary meristem leading to bud formation and the further outgrowth of the axillary buds. Once formed, an axillary bud can either grow out to give rise to an individual branch or remain dormant. Both axillary meristem initiation and bud outgrowth can potentially determine the final number of the branches on a plant.

Although two axillary meristems usually form in each axil, it is typical for only one meristem of these to give rise to a bud with the potential to form a branch (Grbic and Bleecker, 2000). Therefore, the number of rosette leaves can be considered as the primary rosette branching potential of the plant. Generally, only the upper 4-5 rosette buds of WT give rise to branches by the 10^{th} day after anthesis under long days with moderate PPFD and R:FR. Under long day conditions bud outgrowth and elongation usually occurs after anthesis, starting from the topmost bud, and progresses sequentially to the lower buds (Grbic and Bleecker, 2000). The first axillary meristem forms in the youngest leaf axil, which is in the topmost position.

A network of environmental signals, complex hormone actions, and genetic mechanism allows plants to determine the final branching pattern. It was mentioned by Kull and Tulva (2002) that there are many environmental factors that can influence the branching ability of plants, such as temperature, nutrient availability, amount of water, light quality, and light intensity.

Light signals are among the most important factors regulating plant growth and are perceived by the action of several specialized photoreceptors, which include phytochromes, cryptochromes, and phototropins (Quail 2002a). Phytochromes can

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This thesis follows the style of Plant Physiology.

absorb the entire visible spectrum, however they are best known as red (R) and far-red (FR) light receptors. There are five species in the phytochrome family in *Arabidopsis*, phytochrome A-E, playing different, but some what overlapping roles in the regulation of plant development. Phytochrome A (phyA) and B (phyB) are generally considered the two main phytochromes among the various species. Light is often a limiting factor for plant growth; therefore plants may compete with the adjacent vegetation for more light. When plants are growing in a crowded community, they sense the neighboring competitors through lowered R:FR (as low as 0.05 in closed canopies) perceived by phytochromes (Vandenbussche et al., 2005). To overcome the shading stress, they have to either tolerate shade or avoid shade. Plants having the shade avoidance responses exhibit a suite of syndromes, such as elongated stems, early flowering, and increased apical dominance resulting in decreased branching in dicot plants and tillering reduction in monocot plants. These morphological changes in response to shade are collectively termed the "shade avoidance syndrome" (SAS) which serves as a strategy for plants to avoid shade, enhance light capture, and increase the chances of reproductive success. It has been well documented that the *phyB* mutants of various species possess constitutive shade avoidance responses (Yanovsky et al., 1995; Franklin et al., 2003).

The *Arabidopsis hy3* mutant was found to possess a constitutive shade avoidance response in the aspects of cotyledon expansion, anthocyanin synthesis, hypocotyl elongation, petiole elongation, and stem elongation that was due to loss of phytochrome B function (Somers et al., 1991). Considered together with the overexpression of phytochrome B, which results in an extremely short hypocotyl phenotype (Wagner et al., 1991), it was suggested that phytochrome B plays the major role in transducing R:FR signals. Mutants deficient in phyB have now been reported in several other species, including cucumber (López-Juez et al., 1992), *Brassica rapa* (Devlin et al., 1992), *Arabidopsis* (Reed et al., 1993), tomato (Van et al., 1995b) sorghum (Childs et al., 1997), *Nicotiana plumbaginifolia* (Hudson et al., 1997), rice (Takano et al., 2005), and maize (Sheehan et al., 2007).

In addition to reduced R:FR, a reduction in blue light and total light intensity (PPFD: photosynthetic photon flux density) can also contribute to a shade avoidance phenotype (Vandenbussche et al., 2003b). It has been suggested that *Arabidopsis* responses to

quantity shading may share a similar auxin-dependent mechanism with those of quality shading (Vandenbussche et al., 2003b; Vandenbussche et al., 2005).

The phyB deficient sorghum mutant, *phyB-1*, was used to study the role of phyB-perceived light signals in the regulation of branching and its relationship with other branching-related genes. Several branching-related genes have been identified to be up- or down-regulated during branching. The *TEOSINTE BRANCHED1* (*TB1*) gene was first identified in maize and its expression was negatively correlated with the bud outgrowth (Doebley et al., 1995). The expression level of the *TB1* ortholog in sorghum was also found to be negatively correlated with the growth of axillary buds repressed by light signals (Kebrom et al., 2006). The *Arabidopsis TBL1 (BRC1)* gene (*TEOSINTE BRANCHED1-LIKE1*, *BRANCHED1*) was found to be a homolog of the *TB1* gene, and has been identified as a branching-inhibiting gene in *Arabidopsis* (Aguilar-Martinez et al., 2007, Finlayson, 2007). Another branching-related gene, Dormancy-associated gene (*DRM1*), was proposed to be an early marker of bud dormancy since its expression was found to be very responsive to decapitation, auxin treatment, and other phenomena that regulate bud outgrowth (Stafstrom et al., 1998; Tatematsu et al., 2005; Kebrom et al., 2006; Aguilar-Martinez et al., 2007, Finlayson et al., 2007). The expression of *DRM1* was also found to be regulated by light signals and phyB in sorghum, and its expression correlated with that of *TB1* (Kebrom et al., 2006).

A thorough understanding of branching processes in *Arabidopsis* is still unclear. Moreover, the roles of phytochromes and PPFD have not been investigated in detail. Additionally, the relationship and the dominance of phytochromes and other branching related genes are also unknown. The main objective of this research is to determine the roles of phytochromes A and B under high and low PPFD in the regulation of branching in *Arabidopsis thaliana*. Wild type Columbia (Col-0), the *phyB* null mutant, the *phyA* null mutant, and the *phyAphyB* double mutant will be used to quantify branching responses under varying light intensities (PPFD). The main hypothesis is that the *phyB* mutant will produce fewer branches compared to WT; however the effect is predicted to be suppressed by high PPFD as a consequence of higher energy input. The branching patterns of the various genotypes under high and low light are proposed to coincide with up- or down-regulation of several branching-related genes regulated by phytochromes.

To thoroughly understand the mechanism of branching through the interaction of light, hormones and several branching-related genes, the expression of the auxin responsive gene *DRM1*, CK responsive gene *ARR5*, and the branching genes *TBL1*, and *BRC2* in response to light signals will also be investigated.

2. LITERATURE REVIEW

2.1 Arabidopsis thaliana

Arabidopsis thaliana is a member in *Brassicaceae* or mustard family. *Arabidopsis* itself is not an economically important plant. However, it is closely related to other *Brassicaceae* or mustard members, such as canola, cabbage, broccoli, cauliflower, and turnip. Moreover, *Arabidopsis* has become a common tool in physiological, biochemical, genetic, and biological research over the past 40 years due to several advantages. *Arabidopsis* develops, reproduces, and responds to stimuli in a manner similar to many crop plants. It is a rapidly growing plant with a 28-day life cycle and it produces many seeds that are easy and cheap to grow. It is relatively small in size and requires little space, which allows researchers to grow them in greenhouses or in growth chambers instead of the field. Moreover, *Arabidopsis* has a small genome of 125 Mbp that has only 5 chromosomes in a haploid genome. It is also the first plant for which the genome was completely sequenced, which makes it an excellent model system for basic research. The genetic information from *Arabidopsis* helps us to understand genetic and physiological processes in other crop species.

Leaves of *Arabidopsis* can be divided into two groups: cauline and rosette leaves. Both cauline and rosette leaves can have two meristems formed in the axils, though in WT Ler this occurs rarely (Grbic and Bleeker, 2000). A previous study of branch development in the Landsberg *erecta* (Ler) ecotype indicated that cotyledonary axils do not give rise to axillary buds (Grbic and Bleeker, 2000). Cauline leaves are generally smaller than rosette leaves and are associated with longer internodes. *Arabidopsis* can complete its entire life cycle in six weeks. Under long days, it typically transitions from vegetative growth to reproductive growth and start producing flowers about three weeks after planting. Once it has gone through the floral transition, it stops producing more vegetative leaves. The numbers of cauline and rosette leaves are fixed at this stage.

2.2 Axillary meristem development

An axillary meristem is a small mound of cells in the axil of a leaf primordium that is isolated from the shoot apical meristem (SAM). Cell division is essential for the development of axillary meristems. Initially the cells produced by cell division in the

meristem continue to be meristem cells, so the meristem itself becomes larger. Once established, the axillary meristem will begin producing its own leaf primordia, and following leaf and stem formation, the axillary meristem becomes an axillary bud. In some species the leaf primordia of axillary buds develop into bud scales that envelop and protect the axillary bud. In *Arabidopsis* grown under long days axillary meristem formation initiates in a basipetal wave following the floral transition (Hempel and Feldman, 1994, Grbic and Bleecker, 2000). The buds that subsequently form may then remain arrested, or grow out to form branches, again beginning with the topmost position and progressing downwards through the rosette. In plants grown under conditions promting extended vegetative growth, or in late flowering mutants, axillary meristem initiation can occur in an acropetal wave at lower positions during the vegetative phase, and then in the typical basipetal wave in the upper positions following the floral transition (Grbic and Bleecker, 2000).

There are three stages of axillary meristem development that have been proposed: dormancy, transition, and sustained growth (Stafstrom and Sussex, 1992; Devitt and Stafstrom, 1995; Cline, 1997; Napoli et al., 1999; Shimizu-Sato and Mori, 2001; Morris et al., 2005; Beveridge, 2006). Bud dormancy describes a stage of a metabolically active axillary bud that grows with an extremely low or negligible rate (Dun et al., 2006). Each axillary bud has the potential to break dormancy and then give rise to an individual shoot. The determination of the axillary bud to stay dormant or start elongation is a key step in regulating plant architecture, which involves interactions among genotypes, environmental cues, and endogenous hormones. Once the identity of the axillary bud is determined, short and long range signals control whether or not the axillary bud can reach its potential to form a mature branch or whether it will stay dormant (Beveridge et al., 2003).

In many plants, the SAM plays a role in inhibiting the outgrowth of axillary buds after bud initiation. This phenomenon, whereby the leading shoot dominates the growth of the axillary buds below it, is referred to as apical dominance. Decapitation often leads to the release of apical dominance and further rapid outgrowth of axillary meristems, as in peas and *Arabidopsis* (Beveridge et al., 2000; Cline, 1996). This allows plants to maintain vigorous growth under competitive growing conditions (Beveridge et al., 2003),

however, this mechanism needs to be tightly regulated to prevent self-shading or diversion of resources away from reproductive organs. The removal of the shoot tip, which is the source of auxin, induces the axillary buds to enter a transition state between dormancy and growth. It has been proposed that the fate of the axillary buds in the transition stage (whether to revert back to dormancy or grow out) is partly regulated by communication among axillary buds and between the shoot top and axillary buds on the plant (Stafstrom et al., 1998; Shimizu-Sato and Mori, 2001). Auxin is the most well-known branching regulator and is transported from the shoot top and young leaves to influence bud outgrowth at more basal positions. Auxin may indirectly regulate the outgrowth of axillary buds by influencing the supply of CK to the axillary buds (Cline, 1994). It has been suggested that axillary buds at different locations exhibit varying responses to CK/auxin treatments. King and Van Staden (1988) found buds in pea plants at nodes 1 and 2 elongate in response to CK application, whereas those at nodes 3 and 4 cannot respond to CK unless the plant was decapitated.

Exogenous auxin can repress the outgrowth of axillary buds (Thimann and Skoog, 1933). It was further found that exogenous auxin applied to decapitated peas can significantly restore the apical dominance. However, decapitated *Arabidopsis* show relatively weak responses to exogenous auxin (Cline, 1996; Beveridge et al., 2000; Cline et al., 2001). These results strongly imply that auxin deficiency alone does not trigger initial bud outgrowth in *Arabidopsis*.

The discovery of genes expressed in the meristem and in early organ primordia is a useful method to study the regulation of meristem development. The *SHOOT MERISTEMLESS* (*STM*) gene is required for meristem initiation and maintenance (Barton and Poethig, 1993; Clark et al., 2006; Felix et al., 1996; Endrizzi et al., 1996). The *STM* gene encodes a homeodomain-containing protein of the *KNOTTED* class and is expressed in the meristem founder cells in the embryo (Long et al., 1996; Long and Barton, 1998). The *STM* transcript remains exclusively expressed in the meristem throughout the life span of the plant and is found in all types of meristems including primary, axillary, and floral. Thus, *STM* can be used as a marker of meristem fate determination, even early in the development of the meristems.

2.3 Shoot branching

In dicot plants, the elongation of the axillary bud is known as branching, whereas in monocot plants, branches are known as tillers. In the monocot system, the axillary meristem in the first few nodes can give rise to tillers.

A major contributor to the architecture of plants is the degree of shoot branching. The SAM of *Arabidopsis* remains active throughout the life span of plants and leads to life long apical dominance which serves as one of the major determinants of the degree of shoot branching. The pattern of shoot branching depends not only on the initiation of the axillary meristems, but also the formation and outgrowth of the axillary buds (Schmitz and Theres, 2005).

The process of branch development has been divided into five stages according to different molecular events and axillary meristem or bud sizes (Schmitz and Theres, 2005). The first stage is the establishment of axil identity (Aida et al., 1999; Takada et al., 2001; Vroeman et al., 2003; Shuai et al., 2002; Greb et al., 2003). The second stage is axillary meristem initiation, and the third is the organization of the meristem. The fourth stage is the formation of the axillary bud and the last (fifth) stage is the outgrowth of the bud. Auxin , the uncharacterized MAX-related hormone (discussed below) and TB1 (or homologs of TB1) are major regulators of branching that affect this last stage (Lincoln et al., 1990; Arumingtyas et al., 1992; Rameau et al., 2002; Stirnberg et al., 2002; Snowden et al., 2005; Takeda et al., 2003).

The axillary bud often remains dormant after formation. One or more cues are required for breaking bud dormancy, which depend on environmental, developmental, and genetic conditions. The environmental stimuli, hormone networks, and branching-related genes will be discussed later in this thesis.

2.4 Hormones involved in branching regulation

Apical dominance contributes abundantly to the activity of the axillary bud, which is regulated by a network of interacting hormones. Among them, auxin is the most well-established hormone in the regulation of branching. Basipetally transported auxin may prevent branching by reducing the synthesis and/or import of cytokinins (CK) into the bud (Sachs and Thimann, 1967; Li et al., 1995; Chatfield et al., 2000; Leyser, 2003;

Nordstrom et al., 2004; Tanaka et al., 2006). It has been proposed that the ratio of CK to auxin strongly determines branch development (Sachs and Thimann 1967; Bangerth, 1994; Li et al., 1995; Chatfield et al., 2000). This is supported by phenotypic observations in many *Arabidopsis* mutants impaired in different aspects of auxin and/or cytokinin physiology (Hobbie and Estelle, 1994; Catterou et al., 2002). The *AUXIN-RESISTANT1* (*AXR1*) protein of *Arabidopsis* mediates many auxin responses by inducing destabilization of the Aux/IAA transcriptional repressor proteins in response to auxin (Gray et al., 2001). The *axr1-12* mutant in *Arabidopsis* possesses impaired auxin signaling and thus loses many auxin-related responses, such as apical dominance, which leads to a hyperbranching phenotype (Lincoln et al., 1990; Stirnberg et al., 1999). This genetic evidence, along with classical physiological experiments, has been taken as support for a role of auxin in the regulation of shoot branching.

CK is an essential plant hormone that is involved in the regulation of many aspects of plant development, such as seed germination, meristem formation, apical dominance, and stem growth and differentiation (Heyl and Schulling, 2003; Mok and Mok, 2001). The roots are traditionally considered to be the major site for CK synthesis in plant (Chen et al. 1985). However, it was later found that the synthesis of CK can also occur in the aerial part of plants, especially in the young developing leaves with active cell division (Nordstrom et al., 2004).

Biosynthesis is not the only process regulating CK responses in meristems. CK signaling is also a target of the regulatory network controlling meristem activity. CK affects the expression of a variety of genes, among which are a family of *Arabidopsis Response Regulators* (*ARR*), which serve as transcriptional regulators in the phosphorelay-mediated CK signal transduction network in *Arabidopsis*. These genes can be divided into two types: type A and type B. Type A ARR proteins includes ARR3-9,15-17, and Type B includes ARR1-2,10-14,18-21. Expression of the genes in the type A family can be induced exclusively in response to exogenous CK, while type B ARRs do not respond transcriptionally to CK treatment (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). Among the type A ARRs, *ARR4* and *ARR5* were found to be able to respond transcriptionally to exogenous CK within 10 min (Brandstatter and Kieber, 1998; D'Agostino et al., 2000).

Several CK related mutants have been reported with altered branching phenotypes. *Arabidopsis hoc* (*high organogenic capacity*) was identified as a CK over-producing mutant, which is capable of generating shoots without exogenous growth regulators (Catterou et al., 2002). *hoc* possess a bushy phenotype with 2 fold higher CK level in the shoots compared to WT. It was found that *hoc* displays a de-etiolation phenotype in darkness, which can be mimicked by exogenous application of CK to WT in darkness (Catterou et al., 2002).

A relationship between CK and auxin was first proposed by Sachs and Thimann (1967), who suggested that endogenous auxin inhibits cytokinin production in the buds. It was recently found that auxin negatively regulates the level of CK in pea, both at nodes and in the roots, through the repression of the CK biosynthesis enzyme, adenosine-phosphate-isopentenyl transferase (IPT) (Tanaka et al., 2006). Previous research found that decapitation correlated with the export of CK from the roots and accumulation in the buds of chickpeas (Mader et al., 2003). It is also the similar case in pea (Balla et al., 2002).

The influence of a novel branching-related, carotenoid-derived hormone has recently been studied. Genes involved in this carotenoid-derived hormone pathway have been identified and described in *Arabidopsis* (*MORE AXILLARY GROWTH1-4* [*MAX1-MAX4*]), pea (*RAMOSUS1-5* [*RMS1-RMS5*]), petunia (*DECREASED APICAL DOMINANCE1-3* [*DAD1-DAD3*]), and rice (*DWARF3, DWARF10* and *HIGH TILLERING DWARF1*). *Arabidopsis* with loss of function mutations in these genes show multi-branching phenotypes with axillary buds resistant to the inhibitory effects of apically applied auxin (Stirnberg et al., 2002; Bainbridge et al., 2005, McSteen and Leyser, 2005, and Bennett et al., 2006). This suggests a branching-repressor role for these genes and the existence of an interaction with auxin to inhibit branching. The branching phenotype of *max1*, *max3*, and *max4* in *Arabidopsis*; *rms1*, *rms2*, and *rms5* in pea; *dad1* in petunia can be restored by grafting WT rootstock to mutant scion, suggesting that these mutants are deficient in a long range upwardly graft transmissible signal that inhibits branching (Turnbull et al., 2002; Sorefan et al., 2003; Beveridge et al., 1994; Foo et al., 2001; Morris et al., 2001; Napoli, 1996).

MAX3 and *MAX4* in *Arabidopsis* have been shown to encode divergent members of the carotenoid cleavage dioxygenase (CCD) family that can act on multiple linear and cyclic carotenoid substrates and generate a mobile signal (Booker et al., 2004; Sorefan et al., 2003). *MAX3* encodes for CCD7 (Booker et al., 2004; Schwartz et al., 2004), and *MAX4* encodes for CCD8 (Sorefan et al., 2003). Individual *MAX3* and *MAX4* over-expression transgenic plant have been developed to examine their effects on branching, however, no significant phenotypic differences were observed from WT (Booker et al., 2004; Bainbridge et al., 2005). This suggests that co-expression of *MAX3* and *MAX4* may be required for the production of the signal (Bainbridge et al., 2005). The MAX-dependent signal generated by MAX3 and MAX4 requires further modification by MAX1, which is a cytochrome p450 family member, to synthesize the MAX-dependent hormone (Booker et al., 2005).

MAX2 has been identified as an F-box LRR containing member of the SCF family of ubiquitin ligases that functions in protein degradation (Stirnberg et al., 2002). The MAX-dependent hormone requires MAX2 in the shoot for its perception and/or transduction (Stirnberg et al., 2002; Booker et al., 2005). It was suggested that the SCF^{MAX2} complex might act in the degradation of proteins that activate axillary bud outgrowth (Stirnberg et al., 2002). The role of MAX2 as a component of an SCF ubiquitin ligase was later confirmed by Stirnberg et al. (2007). It was found that over-expression of *MAX2* is able to rescue the phenotype of *max2* mutants with a functional F-box domain, while over-expression of *MAX2* is not effective in the *max2* mutant without the F-box domain. They were able to confirm that axillary bud outgrowth is controlled by the SCF^{MAX2} complex (Stirnberg et al., 2007). The active site of *MAX2* was recently found to be locally at the nodes or in the center of the axillary buds in response to the MAX-dependent hormone, and it is required at each node to suppress the associated axillary bud outgrowth (Stirnberg et al., 2007).

The interactions between auxin and the putative *Arabidopsis* MAX-dependent hormone have been studied. *max* mutant buds are resistant to the inhibitory effects of apically supplied auxin (Sorefan et la., 2003; Bennett et al., 2006). However, the *AXR1*-mediated auxin signaling pathway was not found to be directly involved in the MAX-dependent regulation of branching, while PIN1, an auxin efflux facilitator, was

(Bennett et al., 2006). Upon perceiving the MAX-dependent hormone signal, MAX2 appears to facilitate a decrease the accumulation of PIN1 and thus reduces auxin transport capacity in the main stem. *max* mutants were found to have increased auxin transport capacity resulting from increased abundance of PIN1 (Bennett et al., 2006). The accumulation of PIN1 in the stem may allow increased export of auxin from the axillary buds into the stem and consequently decrease the negative effects of auxin on cytokinin accumulation in the buds by increasing the synthesis of CK or the import of CK from the synthesis sites to the buds (Tanaka et al., 2006 Mader et al., 2003). This may cause a reduction in the apical dominance and lead to the hyperbranching phenotype of *max* mutants (Bennett et al., 2006).

Other studies have provided evidence that auxin application can enhance the expression of *MAX4*. The up-regulation of *MAX4* was detected in the root, especially the root tip (Sorefan et al., 2003), suggesting that *MAX4* is required for the negative action of auxin on bud outgrowth.

In summary, this MAX-dependent hormone can be considered as a second messenger of auxin in negatively regulating bud outgrowth into branches through the PIN1-dependent pathway.

Studies in a variety of species have identified genes orthologous to the MAX genes of *Arabidopsis* (Stirnberg et al., 2002; Bainbridge et al., 2005; McSteen and Leyser, 2005; Bennett et al., 2006). The gene products appear to possess properties similar to their *Arabidopsis* counterparts, with some variations. The abundance of pea *RMS1* and petunia *DAD1* are modulated by feedback regulation, while this effect on *MAX4* is less apparent (Bainbridge et al., 2005; Snowden et al., 2005; Simons et al., 2007). Additionally, auxin is able to increase the expression of *RMS1* in pea to a greater extent than observed with the *Arabidopsis* ortholog *MAX4*. The up-regulation can be detected in the nodes and has been proposed to be sufficiently rapid to inhibit bud growth in response to auxin application (Sorefan et al., 2003). Additionally, the relationship between CK and the SMS has been studied more extensively in pea. The RMS pathway was suggested to be able to regulate CK levels and *RMS1* and *RMS5* transcript abundances through a feedback signal that can move from the shoot to the roots (Foo et al., 2007). The expression of *RMS1* and *RMS5* are elevated in *rms* mutants, except for

rms2, where levels of *RMS1* and *RMS5* are reduced. This suggests a key role of *RMS2* in the feedback regulation of the RMS pathway (Foo et al., 2005; Johnson et al., 2006).

Various studies have shown that there is a correlation between the inhibition of bud outgrowth and the Abscisic acid (ABA) content of the bud. The amount of ABA is somewhat higher in the dormant buds compared to growing buds of *Phaseolus* and *Elytrigia* (Gocal et al., 1991; Pearce et al., 1995), which suggests a role for ABA in inhibiting bud growth. This negative role of ABA in the regulation of bud outgrowth was later supported by the finding that the ABA content of the axillary buds is under the control of auxin moving down from the shoot apex (Grossmann and Hansen, 2001).

Several lines of evidence have inferred an interaction between auxin and ABA in regulating axillary bud outgrowth. In *Arabidopsis*, ABA was found to enhance apical dominance caused by auxin when applied basally, while apically applied ABA was found to reduce the inhibition by auxin (Chatfield et al., 2000). Decapitation of many plants, which releases the apical dominance, is also accompanied by a reduction of ABA content in the axillary buds (Tamas, 1995; Geuns et al., 2001). Moreover, dormant buds are observed to have higher ABA content than non-dormant buds, while application of ABA to the shoot apex can release axillary bud inhibition (Chatfield et al., 2000). These data suggest a secondary role for ABA downstream of auxin in the regulation of bud outgrowth. However, using ABA-insensitive *Arabidopsis* mutants, *abi1-1* and *abi2-1,* evidence has been produced that suggests that auxin can inhibit axillary bud outgrowth independently of ABA activity. These results together suggest that ABA is not required for auxin to suppress the axillary bud outgrowth, but may be a contributing factor. There may be multiple bud outgrowth regulation pathways mediated by auxin.

Early research has found that application of GA can increase apical dominance by increasing auxin content (Holmes et al., 1970). Several molecular lines of evidence, using mutants deficient in *GIBBERELIC ACID INSENSITIVE* (*GAI*) and *REPRESSOR OF GAI-3* (*RGA*), suggest that GA may influence the formation of axillary meristems (Schmitz and Theres, 1999). *GAI* and *RGA* encode negative regulators of GA responses that appear to have partially redundant or overlapping functions (Dill and Sun, 2001). *gai* and *rga* loss of function mutants exhibit reduced GA responsiveness, but do not exhibit a mutant phenotype unless GA levels are abnormally low. The hyperbranching

phenotype of *ga1-3*, a GA deficient mutant, is partially rescued by loss of RGA function (Dill and Sun, 2001), and while *gai* alone does not have significant effects on axillary branching, double mutations in *gai* and *rga* can restore the *ga1-3* hyperbranching phenotype to WT levels (Dill and Sun, 2001). These findings also indicate that GAI and RGA regulate axillary branching through bud outgrowth instead of meristem formation.

The interaction between GA and auxin in the regulation of branching was also studied in tobacco and pea (Wolbang and Ross, 2001; Ross et al., 2001). Both GA_{20} and GA_1 levels were decreased in a decapitated tobacco plant due to the reduced conversion of GA_{19} to GA_{20} and GA_{20} to GA_1 and the promotion of GA deactivation pathways (Wolbang and Ross, 2001). Exogenous IAA applied to the decapitated plant counteracted these effects and allowed the enhancement of GA_{20} and GA_1. These results suggest GA plays a negative role in regulating branching and that auxin is required for GA biosynthesis in tobacco (Wolbang and Ross, 2001). It was proposed that auxin may serve in the role of messenger linking apical dominance with the biosynthesis of bioactive GA in tobacco.

2.5 Environmental factors influencing shoot branching

Shoot branching is genetically controlled, yet environmental variations, including parameters such as light intensity and R:FR also contribute to the branching pattern. Bud dormancy induced by environmental factors is termed ecodormancy (Horvath et al., 2003). In addition to environmental signals, stresses like cold or drought may lead to the prevention of bud outgrowth by inducing ABA synthesis in the plant (Gilmour et al., 1991).

2.5.1 Red: Far red ratio

The red: far red ratio (R:FR) is typically given as the photon irradiance between 655 and 665 nm divided by the photon irradiance between 725 and 735 nm. An optimized R:FR ratio is required for plants to time germination and for subsequent growth and development (Franklin et al., 2005). Phytochromes are the pigments known to be responsible for sensing R:FR. Among the various family members (phyA-phyE in

Arabidopsis), phytochrome B (phyB) plays the major role in sensing R:FR (Franklin et al. 2003; Chen et al. 2004).

The photosynthetic pigment in leaves, chlorophyll, absorbs light over most of the visible spectrum. FR is not absorbed by chlorophyll and is thus transmitted through the leaf, or reflected by the leaf, resulting in a reduction in the R:FR ratio near the plant. Therefore, the R:FR ratio decreases as the plant density increases and increases as the plant density decreases. Light is often a limiting factor for plant growth which leads to competition between adjacent vegetation for light. Plants use the R:FR as an indicator of potential nearby competitors and while some species tolerate shade, many others alter their development to try to outcompete their neighbors. To respond to, and avoid shade, many plants increase the elongation rate of stems and petioles with a corresponding reduction of leaf area (leaf shape), thickness (leaf structure) and chlorophyll content. Plants sensing low R:FR also elevate the leaf angle, increase the allocation of energy to shoot growth at the expense of root growth, and flower earlier. In addition to these modifications, apical dominance is increased resulting in reduced branching in dicot plants and tillering reduction in monocot. These morphological changes in response to shade are collectively termed the "shade avoidance syndromes" (SAS) which serves as a strategy for plants to avoid shade and enhance light capture in rapidly growing populations (Smith and Whitelam, 1997; Ballare, 1999).

Deregibus et al. (1983) showed that high R:FR could stimulate *Lolium multiflorum* to develop more tillers. Casal et al. (1986) showed that the tillering activity of *Lolium multiflorum* was regulated by the R:FR. Irradiation with R was shown to reverse the reduced tillering of plants grown under low R:FR. Moreover, irradiation of the base of *Lolium* with intermediate-low R:FR reduced tillering (Casal et al., 1987). It was concluded that phytochrome was involved in the process. This conclusion was further supported by work by Wan and Sosebee (1998) demonstrating that high R:FR was able to stimulate both basal and aerial tiller production in *Eragrostis curvula*.

2.5.2 Light quantity

Low R:FR is a well-characterized factor that contributes to the shade avoidance syndrome (Smith, 1982; Ballare, 1999). However, studies have shown that

approximately half of the total shade-avoiding responses can also be observed under neutral shading with constant R:FR and thus likely involve transduction pathways that are not phyB dependent (Stuefer and Huber, 1998). Induction of the shade avoidance syndrome as a result of low light intensity was reported for hypocotyl elongation in cucumber (Ballare et al., 1991) and stem elongation in tobacco (Casal and Sanchez, 1994). Two well characterized shade avoidance phenotypes in *Arabidopsis*, elongated petioles and unexpanded leaf blades, were also reported to occur under low light intensity (Tsukaya et al., 2002).

The shading responses caused by changes in light quality were examined using an ethylene and auxin insensitive *Arabidopsis* mutant, *ACC-related long hypocotyl 1* (*alh1*; Vandenbussche et al., 2003a). *alh1* has reduced responses to low PPFD and displays a phenotype similar to *phyB* (Vandenbussche et al., 2003b). From these data, it is logical to propose that light quantity may have effects similar to light quality on branching and may act through the regulation of auxin-dependent mechanisms. Presumably, these auxin-dependent mechanisms could influence R:FR responsive branching as well.

Arabidopsis grown under low PPFD (15 μmol m^{-2} s^{-1}) exhibits increased production of ethylene and expression of several auxin-induced genes (Vandenbussche et al., 2003b). One of the auxin-induced genes that is induced by low PPFD is *IAA3/SHY2*, which is required for the elongation growth of the *phyB* mutant (Tian et al., 1999). Therefore, it was concluded that phenotypic adaptations upon quantity shading are mediated by auxin. However, some of these findings are not consistent with the conclusion drawn by Finlayson et al. (2007) that only low R:FR can increase ethylene production in WT sorghum, while PPFD has no significant effects. The differences in ethylene production may be due to the depletion of CO_2 in the *Arabidopsis* system that caused inhibition of ethylene production rather than light signals *per se*. Ethylene production has been demonstrated to be negatively regulated by light quantity through photosynthetic depletion of CO_2 (Bassi and Spencer, 1982), an activator of the ACC oxidase enzyme (Smith and John, 1993). Therefore, light quantity may have similar effects on branching as light quality, acting via the regulation of auxin rather than ethylene. It is possible that enhanced auxin levels, transport or sensitivity may lead to typical shading responses, including increased apical dominance.

Blue light (B) acting through cryptochromes has been proposed to play a substantial role in regulating plant development including the shade avoidance syndrome, (Ballare et al., 1991; Kozuka et al., 2005). It was suggested by Pierik et al., (2004) that some of the shade avoidance responses, such as hyponasty and stem elongation in *Arabidopsis* can be caused by the reduced blue light intensity via an ethylene-dependent pathway. Sucrose availability was also reported to have different effects on the growth promotion of leaf blades and petioles in *Arabidopsis* (Kozuka et al., 2005). It was found that the sugar-insensitive mutants are unable to expand the leaf blades under blue light and expand normally under R, suggesting the blue light perceived by cryptochromes can regulate leaf blade expansion through a sugar-dependent pathway (Kozuka et al., 2005).

2.6 Phytochromes

Phytochromes are a family of plant photoreceptors that mediate many aspects of plant development in response to R: FR changes, such as seed germination, de-etiolation, chlorophyll accumulation, leaf development, stem elongation, floral induction, light regulated gene expression, modulation of the circadian clock, and anthocyanin accumulation. Phytochrome was first purified by Butler et al. in 1959 using etiolated oat seedlings which have relatively abundant phytochrome. Phytochrome is a soluble chromoprotein that consists of a chromophore (phytochromobilin in higher plants) and an apoprotein of about 125kDa. The functional holoprotein is a dimer with each of the polypeptides folding into two main domains, the N-terminal and C-terminal domains. The N-terminal domain is covalently attached to the phytochromobilin chromophore through a cystein residue while the C-terminal domain mediates dimerization. In *Arabidopsis*, phytochromes are encoded by five discrete genes, *PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE* (Sharrock and Quail, 1989; Clack et al., 1994).

Phytochromes are synthesized in the inactive R absorbing form ($P_{r,}$~ maximum absorbance 660 nm), and will turn into the FR absorbing form ($P_{fr,}$~ maximum absorbance 730 nm) upon exposure to R. The P_r form possesses the *Cis* isomer form of phytochromobilin, while the P_{fr} form possesses the *Trans* isomer form of phytochromobilin. The transformation between these two forms is reversible. Hence P_{fr} will undergo a conformational change into P_r upon absorbing FR. The P_{fr} is the

biologically active state, which can spontaneously transform into P_r in the dark, a process termed dark reversion. The ratio of the P_{fr} to P_r depends on the relative proportion of R and FR in the ambient light, the forward and reverse rates of photo-conversions between two forms, and the rates of thermal inter-conversion (Rockwell et al., 2006). Generally, the R:FR of the ambient light plays the major role in determining the ratio of P_{fr} to P_r, and thus the amount of active phytochrome

After photoactivation, phyA and phyB P_{fr} move from the cytosol to the nucleus and induce target gene expression by interacting with specific transcription factors. PIF3 (PHYTOCHROME INTERACTING FACTOR 3) has been demonstrated to be a transcription factor mediating transcription signaled by phytochromes (Monte et al., 2004; Kim et al., 2003; Martinez-Garcia et al., 2000). PIF3 is a member of the basic helix-loop-helix (bHLH) transcription factor family, is nuclear localized, and has surfaces to interact with both P_{fr}A and P_{fr}B in a light-dependent manner (Ni et al., 1998; Khanna et al., 2004; Al-sady et al., 2006). *Arabidopsis* PIF3-deficient mutants showed reduced sensitivity to both R and FR, but with a stronger reduction in sensitivity to R. This suggests that PIF3 is involved in mediating phyA and phyB signaling responses, but with a major role in the phyB signaling transduction pathway (Ni et al., 1998). Moreover, it was later found that the affinity of PIF3 to phyB was 10-fold higher than it is to phyA, which is consistent with a major role of PIF3 in mediating phyB signaling and a minor role in phyA signaling pathways (Zhu et al., 2000). PIF3 is involved in negatively regulating phyB-mediated hypocotyl elongation, which is independent of phyA. PIF3 was also found to negatively regulate phyA- and phyB-mediated cotyledon expansion and opening (Kim et al., 2003). PIF3 acts early in regulating the rapid (within 1 h) phytochrome-induced changes in gene expression triggered by the initial exposure of dark-grown seedlings to light (Monte et al., 2004). A number of bHLH proteins with similar functionality to PIF3 have been found to be involved in phytochrome signaling pathways and are known as PHYTOCHROME INTERACTING FACTORs (PIFs) or PHYTOCHROME INTERACTING FACTOR-LIKE (PILs) (Huq and Quail, 2002; Salter et al., 2003; Oh et al., 2004; Leivar et al., 2008).

Early studies (Mohr, 1962; Blaauw et al., 1968; Mandoli et al., 1981) have divided phytochrome-mediated responses into three classes according to their energy

requirements: low-fluence responses (LFRs), very-low-fluence responses (VLFRs), and high irradiance responses (HIRs). LFRs such as *Arabidopsis* seed germination, some de-etiolation responses and orientation of the chloroplasts require irradiances in the range of 10^{-1} to 10^{2} μmol m^{-2} of R, with a relatively short exposure time. VLFRs require much lower photon-fluence in the range of 10^{-6} to 10^{-3} μmol m^{-2} of R for a short time period. Because of the overlapping absorption spectra of the P_r and P_{fr}, even small a amount of FR is sufficient to generate $P_{fr}A$ (Shinomura et al., 1996). HIRs, which are both fluence and exposure time dependent, require high, continuous irradiation of FR or blue light. LFRs and VLFRs are usually R and FR photoreversible, but some of the VLFRs require that less than 1% of the phytochrome needs to be converted to P_{fr} to saturate the response, and therefore are not reversible.

Phytochromes have been classified into two groups, type I and type II, according to light stability (Furuya et al., 1989; Vince-Prue, 1991). Phytochrome type I is the gene product of *PHYA*, which is unstable in light and accumulates in dark-grown seedlings. The gene products of *PHYB-PHYE* are type II phytochromes (Vierstra et al., 1993; Chory et al., 1997) and are light stable. Type I phytochrome (phyA) is responsible for VLFRs and HIRs. Type II phytochromes are responsible for LFRs. Phytochromes are also classified into three groups according to their amino acid sequences and the similarities of the encoded proteins, phyA and phyC; phyB and phyD; and phyE (Clack et al., 1994; Mathews et al., 1997). phyD and phyB share 80% sequence similarity in the apoproteins, while phyA, B, C, and E share only 46% to 53% homology.

Studies with distinct phytochrome mutants alone, and various combinations, have indicated that each of them have distinct but somewhat overlapping roles. Among them, phyB is believed to be the major R:FR receptor mediating plant development. So far, mutants deficient in phyB have been reported in several species, including cucumber (López-Juez et al., 1992), *Brassica rapa* (Devlin et al., 1992), *Arabidopsis* (Reed et al., 1993), tomato (Van et al., 1995a) sorghum (Childs et al., 1997), *Nicotiana plumbaginifolia* (Hudson et al., 1997), and rice (Takano et al., 2005). Mutants deficient in phyA have been characterized in *Arabidopsis* (Dehesh et al., 1993; Nagatani et al., 1993; Parks and Quail, 1993., Whitelam et al., 1993), potato (Yanovsky et al., 2000), and rice (Takano et al., 2005). The *hy1* and *hy2* mutants in *Arabidopsis* (Parks and Quail,

1991), *pew* mutant in *Nicotiana plumbaginifolia* (Kraepiel et al., 1994), and elongated mesocotyl1 (em1) mutant in maize (Sawers et al., 2002) were characterized as defective in phytochrome chromophore biosynthesis. Since all phytochromes share the same chromophore, these mutants are deficient in all phytochromes.

The *Arabidopsis phyB* null mutant was found to be able to detect and respond to end-of-day (EOD) FR, which suggests the involvement of additional phytochromes in mediating development in response to R:FR (Whitelam and Smith, 1991; Robson et al., 1993). Evidence suggests the involvement of phyD and phyE (Devlin et al., 1996; Devlin et al., 1998; Devlin et al., 1999). Further investigations have revealed that phyD and phyE have redundant roles similar to phyB in response to R:FR, mediating leaf morphology and flowering time in *Arabidopsis* (Farnklin et al., 2003). phyD is the most closely related member to phyB. It appears to have similar photosensory and regulatory activity to phyB, but is a relatively minor contributor (Aukerman et al., 1997).

phyC is involved in photomorphogenesis throughout the plant life cycle, and is believed to play a significant role in blue light sensing (Franklin et al., 2003). However, the phenotype of the *phyC* mutant is dependent on phyB (Monte et al., 2003). Overexpression of phyC in *Arabidopsis* confers a phenotype with moderately enhanced sensitivity to R as regards hypocotyl growth inhibition with no detectable effects on sensitivity to FR (Qin et al., 1997). This suggests that phyC acts similar to phyB in photosensory responses, but distinct from phyA. It has been suggested that phyC, in response to R:FR changes, may act as the transcriptional suppressor of a *ATHB-2* (*HAT4*) homeobox gene (Franklin et al., 2003), encoding for a homeodomain-Leu zipper protein involved in cotyledon expansion and lateral root formation (Steindler et al., 1999).

After emergence of seedlings, phyA initially dominates the de-etiolation processes in the FR-enriched environment (Yanovsky et al., 1995). However, phyA rapidly declines to very low levels due to its light-lability characteristic. phyB then dominates in the fully-de-etiolated plants under a R-enriched environment (Smith and Whitlam, 1997; Whitelam and Devlin, 1997).

2.6.1 Phytochrome A

phyA is the only type I phytochrome, and has distinct functions compared with all the other phytochrome species. The active P_{fr} form was found to inhibit the transcription of its own gene by feedback regulation (Lissemore and Quail., 1988). The phyA protein is degraded rapidly in light, and accumulates only in the dark-grown seedlings (Quail, 1991). There is evidence inferring that phyA protein may be tagged and degraded by the ubiquitin system and 26S proteasome (Vierstra, 1994). The P_rA from has been demonstrated to have a half life of about 1 week, whereas the half life of $P_{fr}A$ is about 1-2 h (Clough and Vierstra, 1997). The abundance of *phyA* transcripts is dependent largely upon light signals perceived and transduced by the phyA signal transduction pathway that leads to a rapid decrease of *phyA* transcription. In addition, *phyA* mRNA is unstable in the light, leading to a significant reduction of *phyA* mRNA levels in *Arabidopsis* and tomato under continuous white light (Sharock and Quail, 1989; Somers et al., 1991; Quail, 1994).

phyA is involved in the regulation of development under continuous FR (Whitelam et al., 1993). The relative abundance of phyA can affect FR-mediated responses, such as hypocotyl elongation, seed germination (along with phyE), and de-etiolation. These responses together are called the FR high-irradiance response (FR-HIR) (Hennig *et al.* 2002; Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). *Arabidopsis phyA* mutants do not exhibit de-etiolation under FR (Whitelam et al., 1993; Dehesh et al., 1993). The *Arabidopsis* phyA deficient mutation at locus *fhy2* or *hy8* possesses elongated hypocotyls under FR, but not under white light. *Arabidopsis phyA* mutants grown under white light were initially considered to have a phenotype indistinguishable from WT (Whitelam et al., 1993). However, more recent investigations have inferred that phyA plays the major role, along with phyB and phyE playing minor roles, in inhibiting leaf and internode elongation growth in *Arabidopsis* under white light (Franklin et al., 2003).

2.6.2 Phytochrome B

PhyB, along with other type II phytochrome members, is expressed at low levels in both dark and light conditions. The products of type II phytochrome genes are

light-stable. PhyB transcripts in potato, rice and *Arabidopsis* are stable regardless of light treatments (Sharock and Quail, 1989; Somers et al., 1991; Dehesh et al., 1991; Heyer and Gatz., 1992; Clack et al., 1994).

Mutants deficient in phyB have been identified and investigated in many species. The *Arabidopsis phyB* mutant (*hy3*) displays a constitutive shade avoidance phenotype, which is pale, spindly, with long petioles, early flowering, and increased apical dominance (Reed et al., 1993). phyB deficient mutants of *Brassica rapa* (*ein*) also showed shade avoidance syndromes in the aspects of cotyledon expansion, anthocyanin synthesis, hypocotyl elongation, petiole elongation, and stem elongation. The *long hypocotyl* (*lh*) mutant of cucumber also exhibited a phenotype similar to *hy3* in *Arabidopsis*, displaying several shade avoidance phenotypes (López-Juez et al., 1992). Mutants deficient in phyB were also characterized in sorghum (Childs et al., 1997), rice (Takano et al., 2005), and maize (Sheehan et al., 2007). These mutants display many characteristics of a constitutive SAS.

Several studies investigating the interactions between phyA and phyB have been conducted. Red light pulses, perceived mainly by phyB, have an impact on hypocotyl elongation in *Arabidopsis phyA* mutants but not in the WT. This indicates a suppression of phyB function by the presence of phyA (Hennig et al., 1999). A study of the mutual signaling regulation of phyA and phyB in *Arabidopsis* found that phyA has a negative effect on phyB-mediated responses, such as LFRs (Cerdan et al., 1999). phyA does not affect the levels of phyB. It was also determined that phyA acts antagonistically to phyB signaling in VLFRs, whereas phyA acts synergistically with phyB signaling in HIRs (Cerdan et al., 1999). The signaling pathways of phyA and phyB have further been studied and both appear to have shared and independent early signaling pathways, with the downstream pathways possibly integrating to regulate further responses (Quail, 2002a; Quail, 2002b). Therefore, while some interactions between phyB and phyA have been documented, the full extent of these interactions remains unknown.

phyB plays a major role in sensing the R:FR, and mediating shade avoidance responses (Smith, 2000; Quail, 2002a; Quail, 2002b). Furthermore, the role of phyB in the regulation of branching has been studied in sorghum (Kebrom et al., 2006), and at

least reported anecdotally in *Arabidopsis* (Reed et al., 1993). However, little research has been conducted to study the role of any of the other phytochromes in branching.

2.7 Branching-related genes

An investigation into the genes whose expression is associated with dormancy or subsequent growth could provide insights into growth regulation of axillary buds. Genes involved in branching regulation can be classified into two categories according to the phenotypes of the mutants; those that regulate axillary meristem initiation, and those that regulate bud outgrowth. The *REVOLUTA* (*REV*), *LATER SUPPRESOR* (*LAS*), *PINHEAD*, and *REGULATORS OF AXILLARY MERISTEMS* (*RAX*) genes in *Arabidopsis* (Otsuga et al., 2001; Greb et al., 2003; McConnell and Barton, 1995; Talbert et al., 1995; Muller et al., 2006); the *BLIND* (*Bl*) and *LATERAL SUPPRESSO*R (*Ls*) in tomato (Mapelli and Kinet, 1992; Schmitz et al., 2002; Schumacher et al., 1999); the *BARREN STALK1* gene in maize (Gallavotti et al., 2004); the *MONOCULM1* and *LAX PANICLE* genes in rice (Li et al., 2003; Komatsu et al., 2003) have been shown to be involved in the initiation of the axillary bud formation, which is the early step of the lateral branching process. These mutants possess impaired axillary bud formation. *RAX* in *Arabidopsis* has been identified to be a homolog of *BLIND* (*Bl*) in tomato (Muller et al., 2006). *Arabidopsis LAS* and rice *MONOCULM1* have been shown to be homologs of *Ls* in tomato (Greb et al., 2003). *REV* homologous genes have been further studies in other species including maize (Juarez et al., 2004) and bamboo (Peng et al., 2007).

Genes affecting the outgrowth of axillary buds, the latter step of the lateral branching process, include *MAX1-4* in *Arabidopsis* (*RMS1-6* in pea; *DAD1-3* in petunia) and *TB*1 (or homologs of *TB1*) and are the major regulators at this stage (Arumingtyas et al., 1992; Rameau et al., 2002; Stirnberg et al., 2002; Snowden et al., 2005; Takeda et al., 2003).

The discovery of the *TB1* gene resulted from QTL analysis of morphological differences between maize and teosinte (Doebley et al., 1995). *tb1* mutants in teosinte and maize were later analyzed to determine the function of *TB1* in regulating branching (Hubbard et al., 2002). It was demonstrated that *TB1* is involved not only in regulating the number of branches, but the length as well. Branches from the basal nodes were

elongated the most in maize *tb1* mutants. It was also demonstrated that the hyper-branching phenotype of the *tb1* mutant was due to the presence of more than one axillary bud per leaf axil and the outgrowth of normally dormant buds, rather than an increase in the node number. The expression of *TB1* in maize was also found to occur mainly in axillary meristems, rather than in the shoot apical meristem or in ground tissue. It was determined that *TB1* expression is negatively correlated with axillary bud outgrowth in both maize and teosinte (Doebley et al., 1995). The TB1 protein is a putative transcription factor that locally suppresses bud outgrowth s in monocots.

Orthologs of *TB1* have been characterized in some other species. The *TB1* ortholog in rice (*OsTB1*) was identified based on its position and sequence similarity to the *TB1* gene in maize (Takeda et al., 2003). The function of *OsTB1* in rice was determined using over-expression transgenic plants and loss-of-function mutants. The *OsTB1* over-expression transgenic plants possessed a significantly reduced tillering phenotype. The *fine culm1* (*fc1*) mutant in rice contains the loss-of-function mutation of *OsTB1* gene (*fc1*) exhibiting a thin seedling, and hyper-tillering phenotype. The number of axillary buds did not show any significant differences. The function of *OsTB1* was thus determined to be that of a negative lateral branching regulator acting locally in the axillary buds after the buds were initiated. It was proposed that the *OsTB1* eliminates the promoting effects of CK or enhances the inhibiting action of auxin on the meristematic activity of axillary buds (Takeda et al., 2003). The regulatory circuit that modulates tillering associated with planting density was also studied using *fc1*. Mutant *fc1* planted under high density were found to have several dormant buds compared to *fc1* planted in normal conditions. This suggests the presence of other factors in rice that may control tillering along with *OsTB1* (Takeda et al., 2003).

TB1 is a member of the TCP domain family, named after its first characterized members, TB1 in maize (*Zea maize*), CYCLOIDEA (CYC) in snapdragon (*Anthirrinum majus*), and the PCFs in rice (Cubas et al., 1999), which appear to be transcription factors involved in regulating the cell cycle at the G_1/S transition phase (Boer and Murray, 2000). Members of the TCP domain family share a highly conserved domain, a non-canonical basic-helix-loop-helix (bHLH) structure in the N-terminal region that is involved in DNA-binding and dimerization. Some members of the TCP domain family

are known to act as transcription factors involved in various developmental control pathways and have been identified only in angiosperms so far. TCP domain family members are divided into two subfamilies, TCP-C and TCP-P, according to their primary sequence similarities of the DNA binding domain and length (Cubas et al., 1999; Cubas et al., 2002). The ones that are more closely related to CYC and TB1 belong to TCP-C subfamily, such as CYC, TB1, and TCP1-TCP5. The ones that are more closely related to PCFs fall into the TCP-P subfamily, such as PCF1-2, and TCP6-9. The TCP-C subfamily has been shown to be involved in development of flower, leaf shapes, and shoot branching (Cubas, 2004; Doebley et al., 1995). The TCP-C subfamily contains up to three phosphorylation sites within the bHLH domain sequences that code for nuclear localized signal proteins, while TCP-P contains only a portion of it (Hunter et al., 1992). The bHLH structure is required for both DNA binding and dimerization (Kosugi and Ohashi, 1997). Phosphorylation has been shown to affect nuclear localization, DNA binding, and transcriptional activation of regulatory proteins (Hunter and Karin, 1992). Members of the TCP-P subfamily are known to be involved in determining the organ border and the influencing cell growth and proliferation (Weir et al., 2004). However, the functions of members of the TCP-P subfamily are less well studied. The similarity of the phosphorylation sites within the bHLH domain between TCP-C and TCP-P subfamilies suggests that a similar regulatory mechanism may act on the activity of the different TCP proteins (Cubas et al., 1999). In *Arabidopsis*, the TCP domain family constitutes a small gene family of 24 members located on all five chromosomes (Cubas, 2002).

Other members of the TCP domain family also play important roles in plant development. CYC is involved in the floral meristem and primordia development. It inhibits stamen development at dorsal positions through the repression of the expression of the *CYCLIN D3* gene, one of the key factors of the G_1 phase of the cell division cycle (Gaudin et al., 2000). PCF1 and PCF2 are DNA-binding proteins that bind specifically to the *cis*-element of the promoter of *PCNA* (proliferating cell nuclear antigen) gene in rice. *PCNA* is expressed only in the meristem tissues and is involved in cell cycle control at the G1/S phase boundary and DNA replication (Kosugi and Ohashi, 1997; Kosugi, 1995).

PCFs are transcriptional activators, which are possibly involved in axillary bud outgrowth regulation of *Arabidopsis* (Tatematsu et al.,2005).

A direct ortholog of *TB1* in *Arabidopsis* or in other dicot plant genomes is absent (Lukens et al., 1999; Ward and Leysor, 2004). However, three homologs of TB1 have been identified in *Arabidopsis* and named BRANCHED1, BRANCHED2 and TCP1 (BRC1 and BRC2, Aguilar-Martinez et al., 2007) or TEOSINTE BRANCHED1-LIKE1 and TEOSINTE BRANCHED-LIKE2 (TBL1 and TBL2, Finlayson, 2007). Based on the phylogenies, it was hypothesized that two of the TCP family members, TCP18 (BRC1, TBL1) and TCP12 (BRC2, TBL2), might have functions similar to that of TB1 in maize. The *tbl1* mutant was found to exhibit a non-pleiotropic hyperbranching phenotype in which about 80 percent of primary buds grew into branches. The enhancement of branching ability was almost doubled compared to the branching of WT, which branched from about 47 percent of potential sites. The increased branching appears to be due mainly to the release of buds in lower positions that are normally repressed in WT. Moreover, the cotyledon axils never give rise to individual branches in WT, but those of *tbl1* sometimes do (Finlayson, 2007). The function of TCP1 remains obscure.

TBL1 expression was found to be abundant in unelongated axillary buds in the last rosette axil (Finlayson, 2007). In contrast, the expression of *TBL1* was not detectable or extremely low in buds that had already elongated and any other organs such as leaves, roots, or stems. The level of *TBL1* expression in the lower rosette buds of WT was about 4-fold higher than in the upper rosette buds, which is consistent with the observation that the upper buds usually successfully give rise to branches, while the lower ones usually do not. Together, it suggests that *TBL1* is required for plants to arrest the outgrowth of axillary buds (Finlayson, 2007).

The correlation of the expression of *BRC1* to environmental stimuli that regulate bud dormancy, such as growing density, was also studied. *BRC1* expression was doubled in the high density plants compared to the low density plants to arrest the bud outgrowth. It was concluded that BRC1 is required for bud dormancy in responses to environmental signals (Aguilar-Martínez et al., 2007).

TBL1 appears to act downstream of auxin and the MAX-dependent hormone, and the abundance of *TBL1* in buds is negatively correlated with the outgrowth of the primary and secondary axillary buds (Finlayson, 2007). This was further supported by the expression of *BRC1* that was found to be largely reduced in the buds of *max* mutants, in accordance with their hyperbranching phenotypes (Aguilar-Martínez et al., 2007). However, the involvement of the AXR1-dependent pathway in the regulation of *TBL1* (*BRC1*) through auxin is not determined yet. *BRC1* was suggested to be independent of the AXR1-dependent pathway as the expression was not significantly altered in *ycc1*, *axr1*, and *amp1* mutants. In contrast, other data indicated that the expression of *TBL1* appeared to be at least partly regulated by auxin through an AXR1-dependent pathway (Finlayson, 2007). It has been proposed that the MAX-dependent hormone acts to limit auxin transport in the main stem, remote from the axillary bud and to affect bud outgrowth, at least partially, by an *AXR1*-independent pathway (Bennett et al., 2006). If this is the case, then there should be at least one other compound connecting the MAX-dependent hormone pathway and the expression of *BRC1* (Aguilar-Martínez et al., 2007).

The role of the *TB1* gene (*SbTB1*) and its relationship to phyB in sorghum has been studied. The expression level of *SbTB1* mRNA was found to be higher in the buds of *phyB-1* mutants compared to WT (Kebrom et al., 2006). It was suggested that phyB mediates axillary bud outgrowth in response to light signals by suppressing the expression of the *SbTB1* gene. This result is consistent with the phenotype of *phyB-1*, which branches less than WT. WT was grown under low R:FR to reduce the proportion of the active $P_{fr}B$ and thereby enhance the apical dominance that leads to a reduced branching phenotype (Kebrom et al., 2006). The expression of *SbTB1* was found to be higher in the buds of low R:FR treated WT than those maintained under high R:FR. This reflects the increase apical dominance induced by the decrease of R:FR.

PsDRM1 and *PsDRM2* were first isolated from a dormant bud cDNA library of pea (Stafstrom et al., 1998). Both *PsDRM1* and *PsDRM2* mRNA abundances are relatively high in dormant buds. However, *PsDRM1* is a better marker of dormancy than *PsDRM2*. The expression level of *PsDRM1* decreases about 20-fold within 6 h of decapitation, and becomes undetectable after 12 h. In addition, *PsDRM1* expression is relatively high in

other non-growing organs, matured state roots and shoots, and low in the growing organs. The function of pea *DRM1* and its orthologs is not clear yet. However, its expression was found to be very responsive to auxin treatment or decapitation. The *DRM1* gene and its orthologs have been characterized as markers of bud dormancy (Stafstrom et al., 1998; Tatematsu et al., 2005; Kebrom et al., 2006). The expression of *DRM1* in buds of *Arabidopsis max2* and *axr1-12* mutants has been found to be lower than in WT, which is consistent with the hyper-branching phenotypes (Finlayson, 2007). However, it was suggested that the decreased expression of *DRM1* is not required for bud outgrowth since there was almost equivalent expression of *DRM1* in the axillary buds of hyperbranching *tbl1* and WT (Finlayson, 2007). Additonally, WT and *35S:YUCCA* buds also showed similar levels of *DRM1* expression, suggesting that auxin may not be directly involved in its regulation. The correlative relationship between *DRM1* expression and dormancy and the changes in the expression of *DRM1* in response to auxin, though imperfect, may provide an indication of the role of auxin in the regulation of branching mediated by phytochrome-perceived light signals.

Type A Arabidopsis Response Regulators (*ARRs*) are CK signal transduction components that are transcriptionally responsive to CK signals. On perception of the CK, a signaling cascade through a phosphorelay process activates the type B ARRs (ARR1, 2, 10, etc.) which act as transcriptional activators essential for cell proliferation and shoot formation (Hwang and Sheen, 2001). The activation of type-B ARRs results in transcription of repressor-type ARRs (type-A ARRs: ARR4, 5, 6, 7, etc.), which play a negative feedback role in the CK signaling cascade (Hwang and Sheen, 2001). The complete functions of most of these proteins are still unclear, however changes in the expression of *ARRs* (*ARR5*) in response to CK provides a means to assess the involvement of CK in the regulation of branching mediated by phytochrome-perceived light signals.

3. MATERIALS AND METHODS

3.1 Plant materials and growth conditions

Arabidopsis thaliana was used as plant material. Wild type (Col-60000), *phyB*, *phyA*, and *phyAphyB* mutants were in the Columbia ecotype background. Seeds were stratified in distilled water for 3 days at 4°C and then planted on a commercial soilless growing medium (Metromix 200). Plants were grown in trays (30 x 60 cm) with 36 cells and 1 plant per cell in a growth chamber with 18 h photoperiod (long days) at 24°C during the day and 18°C during the night. Each cell was fertilized with 7 ml of 1X Hoagland's solution once a week until harvest. Plants were maintained under low light (160 μmol m^{-2} s^{-1}) or high light (310 μmol m^{-2} s^{-1}) with a constant R:FR of 12. Light was measured with a Licor Li-1800 spectroradiometer and the R:FR was calculated as the photon flux from 650 to 670 divided by the photon flux from 720 to 740.

3.2 Branching analysis

First, the roles of phyA and phyB in regulating branching in *Arabidopsis* were determined by growing WT, *phyB*, *phyA*, *phyAphyB* under both low (160 μmol m^{-2} s^{-1}) and high light (310 μmol m^{-2} s^{-1}) and then conducting an architectural analysis on the 10^{th} day after anthesis. Thirty six individual plants of each genotype were examined. The architectural analysis included measurements of the time to flowering, the length of the main inflorescence, the number of primary and secondary rosette and cauline leaves, numbers and lengths of primary rosette and cauline branches (shoots > 3mm), numbers of secondary branches (shoots > 3mm), and numbers of primary and secondary rosette and cauline buds or meristems (shoots < 3mm). The number of rosette leaves was taken as the branching potential of the plant. The value of primary branches/ rosette leaves was used to compare the differences in bud outgrowth taking variations in rosette leaf number into account. Similarly, the value of cauline branches/cauline leaves was calculated to compare the differences in inflorescence branching.

The architectural analyses described above were applied to the various genotypes to assess the potential roles of phyA and phyB in the regulation of branching. A reduction in the number of branches with the same number of rosette leaves and axillary buds as WT would suggest a positive role of a phytochrome in regulating the outgrowth

and elongation of the axillary buds rather than the initiation of axillary meristems. A reduction in the number of axillary buds with the same number of rosette leaves compared to WT would suggest a positive role in promoting the initiation of axillary buds. A reduction in the number of rosette leaves in *phyB* with the same value of ([branches + axillary buds]/rosette leaves) as WT would suggest a role in regulating branching by modifying the number of rosette leaves.

3.3 Branching rate analysis

The elongation rate of the main inflorescence and the three topmost primary rosette branches was investigated by measuring the branch lengths from the day of the start of elongation (buds > 3mm) of the topmost bud until the 10^{th} day after anthesis. This measurement would determine the effects of elongation rate and the effects of the timing of outgrowth on the final length of the topmost three branches. The rate of elongation over a specified time period was derived using the slopes of the trend lines of the length values plotted against time.

3.4 Analysis of the mRNA abundance of branching-related genes

The roles of phytochromes and light in branching were further assessed by measuring the expression of various branching-related genes (including *TBL1*, *BRC2*, *ARR5*, and *DRM1*) in the three topmost rosette axillary buds from WT, *phyB*, *phyA*, and *phyAphyB* grown under both low and high light by quantitative real-time PCR (QPCR). Axillary buds of various genotypes from the three topmost rosette leaf axils were collected by position before the onset of the outgrowth. Three biological replicates were collected, with approximately 12 buds in each replicate. Harvested buds were kept in 25 µl of lysis/biding solution (Ambion) and immediately frozen at -20°C.

Total RNA was extracted with TRIzol (Invitrogen). RNA concentration and purity were estimated by spectrophotometry. Gel electrophoresis was used to verify RNA quality and ensure similar concentrations among samples. Three and a half units of RQ1 DNAse was added to 5 µg of RNA from each sample to digest DNA according to the manufacture's protocol (Promega) followed by re-extraction of the RNA with TRIzol

(Invitrogen). The concentration of each RNA sample after re-extraction was measured by spectrophotometery and each RNA sample was suspended to the same concentration.

cDNA was synthesized from the RNA using the Superscript III kit according to the manufacture's protocol (Invitrogen). Controls (minus RT) were also prepared by substituting water for the reverse transcriptase. RNAseH was added afterwards to remove the remaining RNA. The cDNA was then diluted 1:5 for further use.

QPCR was performed using 2 replicates of plus RT sample and 1 of minus RT sample (used to verify that genomic DNA contamination did not substantially affect results). 10 µl QPCR reactions were run using the SYBR Green Jumpstart kit (Sigma) on an ABI 7900 HT SDS instrument (ABI), following the manufacturer's recommendations. *TBL1*, *BRC2*, *ARR5*, and *DRM1* primers were used at 50 nM each forward and reverse. A standard curve for each primer set was generated from a dilution series of known concentrations of amplicons. Cycle threshold values of the target genes were determined and converted to the actual transcript number per reaction using the *18S* ribosomal RNA as a control. as a control. The dissociation curve of each reaction was checked to verify primer specificity. For *TBL1*, the primer combination TCTAGAAGCTTATGAACAACAACATTTTCAG (forward) and CCCGGGAATTCGACTAAAATGACGAAAAAGCC (reverse) was used. For *BRC2*, the primer combination TCTAGAAGCTTATGTTTCCTTCTTTCATTAC (forward) and CCCGGGAATTCTCAATTAGGGTTTTTAGTTA (reverse) was used. For *DRM1*, the primer combination TTGGAGTTCCAGGGCTCACT (forward) and ATGTTGTGGCTGGACCTCA (reverse) was used. For *ARR5*, the primer combination TTGCGTCCCGAGATGTTAGAT (forward) and TGAGTAACCGCTCGATGAACTTC (reverse) was used. For *18S* rRNA, the primer combination AAACGGCTACCACATCCAAG (forward), ACTCGAAAGAGCCCGGTATT (reversed) was used.

The expression levels of the various genes were compared in buds from different positions of individual genotypes, as well as between buds from the same position of various genotypes.

3.5 Histological analysis

Histological analysis was used to assess the early timing of the formation of the topmost rosette axillary meristems/buds.

WT and *phyB* were harvested at various developmental stages and the rosette parts were collected with the rosette leaves eliminated. The specimens were fixed in FAA (formaldehyde: alcohol: acetic acid: water = 10%: 50%: 5%: 35%) for 24 hrs immediately after harvest, and then stored in 70% ethanol. The specimens were then dehydrated according to a TBA (tertiary-butanol) dehydration schedule and stored in 100% TBA. Twenty five mm Hg of vacuum was applied for 20 minutes to remove the air in the specimens. The TBA was replaced with liquid paraffin and kept in the oven at 60°C for 3 days with daily paraffin changes. Twenty mm Hg of vacuum was applied to allow the paraffin to fill in the specimens. Specimens were then embedded in paraffin blocks, and stored at 4°C.

The specimens were sectioned to 15 μM with a micotome. Sections were put onto slides and kept on the slide warmer at 50°C for at least 24 h, and then stained in a safranin-O and fast-green series using an HMS programmable slide stainer (Carl Zeiss). Stained slides were covered with a cover glass using Permount mounting medium (Fisher Scientific). Slides were then observed with a brightfield Zeiss microscope.

3.6 Statistics

Eighteen biological replicates of each genotype/light treatment were used for architectural analysis, and each experiment was performed twice. The data from the two experiments were combined giving a total of 36 observations per genotype/light treatment. Three biological replicates of each genotype/light treatment were collected for the gene expression analysis, with approximately 12 buds in each replicate. Each experiment was performed twice, and the most representative data was presented.

All statistics analyses were run using SPSS software and Analysis of Variance (ANOVA). A Duncan's test was used for *post hoc* comparisons with significance at $\alpha = 0.05$.

4. RESULTS AND DISCUSSION

Objective 1. Quantification of the architecture of WT, *phyA*, *phyB*, and *phyAphyB* under high and low PPFD.

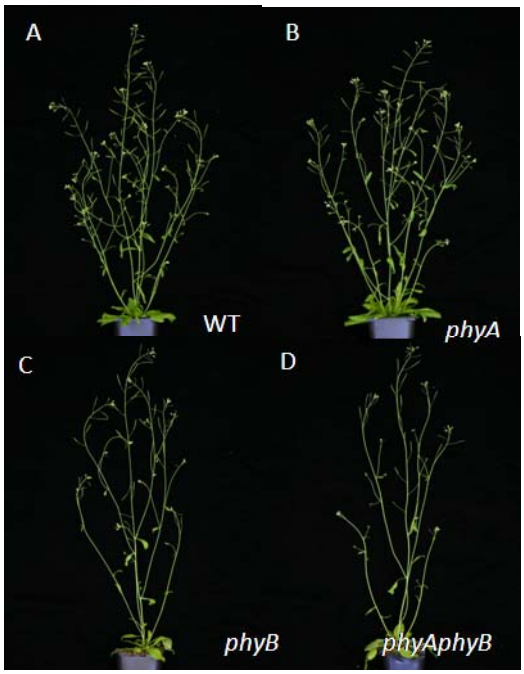

Figure 1. Phenotypes of WT (A), *phyA* (B), *phyB* (C), and *phyAphyB* (D) grown under low light.

Figure 1 shows that phenotypes of various genotypes grown under low light (PPFD: 160 µmol m^{-2} s^{-1}). Architectural analysis revealed that WT and phyA had similar numbers of rosette branches under low light. This result is consistent with earlier reports that indicated that the phenotypes of WT and *phyA* were similar when grown under white light (Whitelam et al., 1993 and Dehesh et al., 1993) and suggest that phyA does not have a significant role in regulating rosette branching. However, the number of rosette leaves in *phyA* was significantly higher than WT (Fig2. A), which contrasts with the previous reports of equivalent WT and *phyA* phenotypes (Whitelam et al., 1993 and Dehesh et al., 1993). The differences in the number of rosette leaves of *phyA* and WT further leads to a significantly higher number of axillary buds and a significantly lower value of branches/axil in *phyA* (Fig2. C, D). The branch numbers of

phyB and *phyAphyB* were similar to each other, but less than that observed in WT and *phyA* (Fig2. B). The numbers of rosette leaves, rosette branches, rosette axillary buds, and the value of rosette branches/axil of *phyB* and *phyAphyB* were significantly lower than WT and *phyA* (Fig2 A, B, C, D).

Taken together, these results suggest that under low light phyA has a negative role in determining the number of rosette leaves (branching potential), while phyB has a positive role in determining the number of rosette leaves. phyB has a greater effect on regulating the number of rosette leaves than phyA, resulting in a reduced number of rosette leaves in *phyAphyB* compared to WT, but not compared to *phyB* (Fig. 2B). The increased number of rosette leaves in *phyA* may be an indirect consequence of the negative influence of phyA on phyB-mediated responses (Cerdan et al., 1999). Loss of phyA function has been shown to result in the accumulation of $P_{fr}B$, which appears to play a positive role in the production of rosette leaves during the vegetative stage. Loss of phyA function did not have effects on phyB-mediated responses in phyB deficient mutants (Mazzella et al., 2001; Casal, 2002). These interactions may lead to the significantly higher number of rosette leaves in *phyA* compared to WT, whereas *phyB* and *phyAphyB* leaf numbers were reduced compared to WT, but similar to each other (Fig. 2A).

The significantly reduced branching potential of *phyB* and *phyAphyB* was one factor that contributed to their reduced branching phenotypes (Fig.2C). The value of rosette branches/axil was calculated to minimize the effects of branching potential and focus on the activity of the axillary buds. The significantly lower values of rosette branches/axil of *phyA*, *phyB*, and *phyAphyB* compared to WT suggest that both phyA and phyB play a positive role in determining the activity of the axillary buds and the subsequent regulation of bud outgrowth and elongation, with phyB having a greater effect than phyA (Fig. 2C).

All four of the genotypes had almost 100% bud initiation (rosette branches+axillary buds)/axil (Fig. 2D). It was previously suggested that he pattern of shoot branching depends on both of the initiation of the axillary meristems and the formation and outgrowth regulation of the axillary buds (Schmitz and Theres, 2005). Here in this research, we found that phyA and phyB do not have major effects on rosette axillary

meristem initiation. It is the outgrowth regulation of the axillary buds that determines the variations in the final architecture.

In summary, the similar branching phenotypes of WT and *phyA* and reduced branching phenotypes of *phyB* and *phyAphyB* under low light are due to both variations in number of rosette leaves that leads to variations in axillary meristem numbers (but not initiation frequency *per se*) and variations in axillary bud outgrowth and elongation.

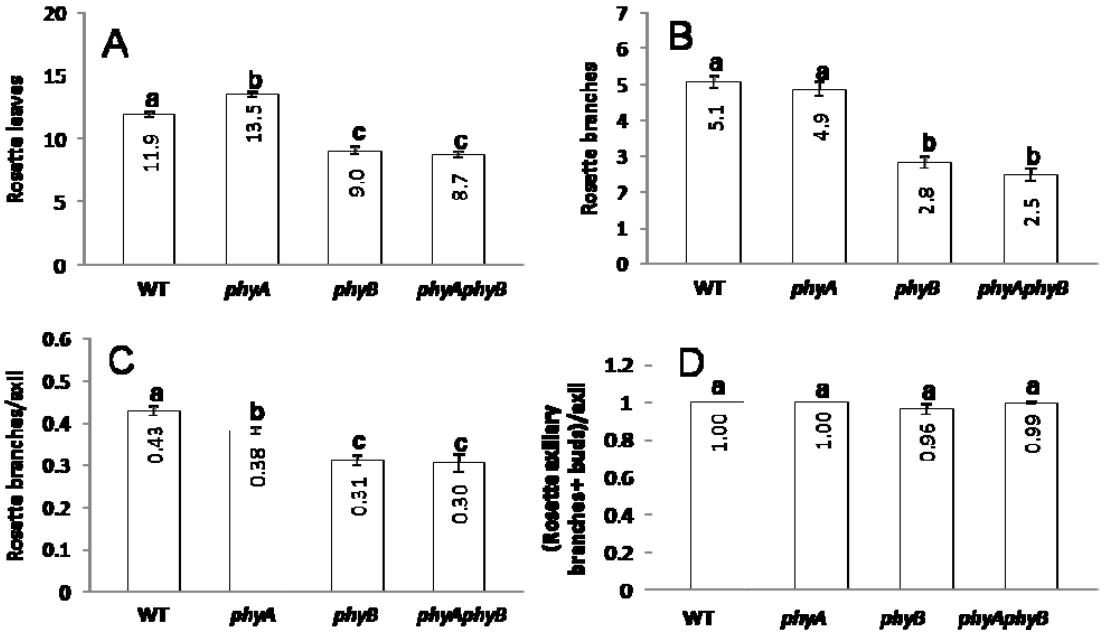

Figure 2. Numbers of rosette leaves (A), rosette branches (B), rosette branches/axil (C), and the value of (rosette branches + axillary buds)/axil (D) of WT, *phyA*, *phyB*, and *phyAphyB* under low light. Numbers within bars indicate the actual value. Bars with different letters are significantly different at $\alpha = 0.05$.

The numbers of primary cauline branches of the various genotypes were analyzed to determine the roles of phyA and phyB in the regulation of cauline branching (Fig. 3). *phyA* had significantly increased numbers of cauline branches, while *phyB* and *phyAphyB* did not show any significant differences in the number of cauline branches compared to WT (Fig. 3). All four of the genotypes had almost 100% bud initiation (cauline branches+axillary buds)/ axil (data not shown) and elongation. Therefore, phyA and phyB do not have effects on cauline axillary meristem initiation or outgrowth. The

greater numbers of cauline branches of *phyA* is due to an increase in the number of cauline leaves on the main inflorescence. This suggests that phyA has a negative role in determining the number of cauline leaves (branching potential), while phyB has no effect in determining the number of cauline leaves under low light.

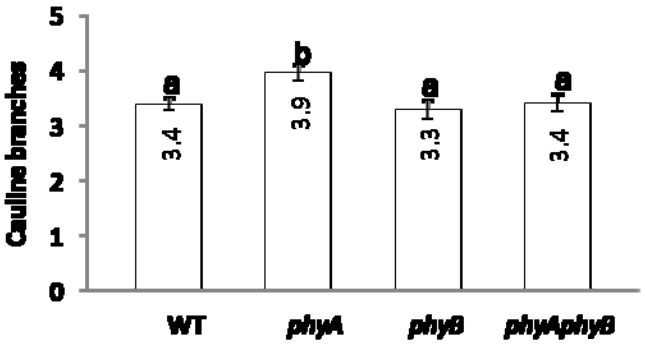

Figure 3. Number of primary cauline branches of WT, *phyA*, *phyB*, and *phyAphyB* under low light. Numbers within bars indicate the actual value. Bars with different letters are significantly different at $\alpha = 0.05$.

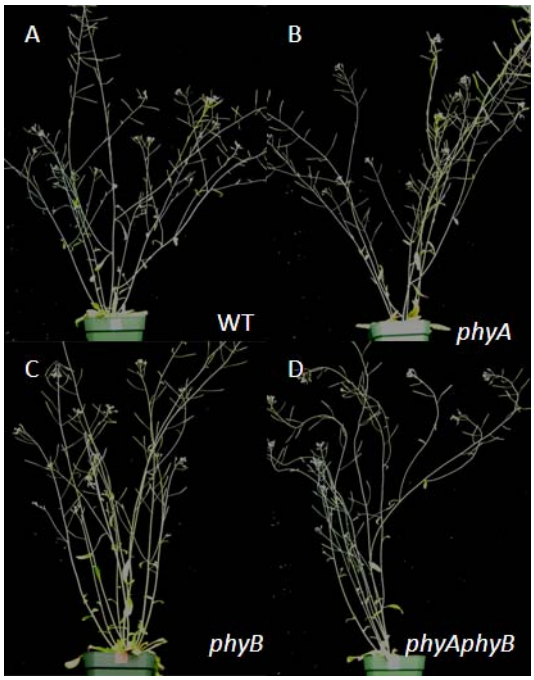

Figure 4. Phenotypes of WT (A), *phyA* (B), *phyB* (C), and *phyAphyB* (D) grown under high light.

Figure 4 shows the phenotypes of various genotypes grown under high light (PPFD: 310 μmol m^{-2} s^{-1}). Under high light, *phyA* did not show a significant difference in the number of rosette branches compared to WT (Fig. 5B). *phyB* and *phyAphyB* had significantly lower numbers of rosette branches than *phyA*, while no significant differences were observed between *phyB*, *phyAphyB* and WT (Fig. 5B). It should be noted that even the differences that were significant were relatively minor. Higher light intensity (PPFD) provides higher energy input to the plants, which appears to allow the plants to increase branching regardless of the genotype. However, by comparing the differences in the number of rosette branches under low and high light it was found that high light caused a 104% increase of rosette branches in *phyB*, 120% in *phyAphyB*; but only increased branching by 13% in WT, and 32.7% in *phyA* (Fig. 2B, 5B). This indicates that high light is able to overcome some shade avoidance phenotypes of phyB deficient mutants. Therefore, the effect of the lesion in phyB on rosette branch production was greatly reduced by high light intensity, causing a decrease in the differences of the rosette branch numbers between WT and *phyB*, *phyAphyB*.

phyB and *phyAphyB* had significantly fewer rosette leaves, with similar numbers of rosette branches compared to WT under high light (Fig. 5A, B). Higher energy input contributes more to the activity of the rosette axillary buds in phyB deficient mutants than other genotypes leading to similar rosette branches/axil in phyB deficient mutants and phyB sufficient genotypes (Fig. 5C). The equalization of rosette branches/axil results from increases in the branching potential and the activities of rosette axillary buds which are proportionally greater in the phyB-deficient genotypes. The effects of higher energy input on the regulation of the activity of the rosette axillary buds in WT and *phyA* are less obvious than in phyB deficient mutants.

phyA had a significantly higher number of rosette leaves (branching potential) than WT under high light (Fig. 5A) as well as under low light (Fig. 2A), indicating that phyA has a consistent negative role in determining the number of rosette leaves regardless of the light intensity.

Under high light all four genotypes initiated buds (rosette branches+buds) in every leaf axil (Fig. 5D). High light intensity did not have significant effects on the (rosette

branches+buds)/axil compared to low light (Fig. 2D, 5D). This suggests low light intensity provided sufficient energy for rosette meristems to form buds.

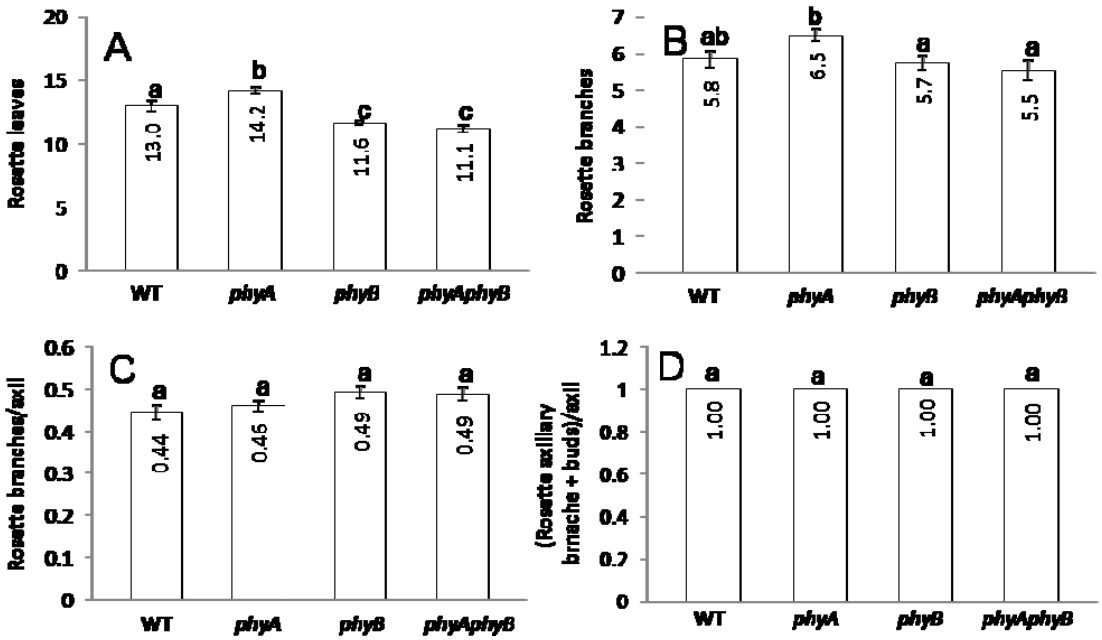

Figure 5. Number of rosette leaves (A), rosette branches (B), rosette branches/axil (C), and number of rosette axillary buds (D) of WT, *phyA*, *phyB*, and *phyAphyB* under high light. Numbers within bars indicate the actual value. Bars with different letters are significantly different at $\alpha = 0.05$.

Compared to WT, *phyA* had greater numbers of cauline branches, while *phyB* and *phyAphyB* numbers were the same as WT (Fig. 6). All four genotypes had almost 100% cauline bud initiation (cauline branches + axillary buds)/axil (data not shown) and outgrowth. Therefore, phyA and phyB do not have effects on cauline axillary meristem initiation or outgrowth. The elevated number of cauline branches in *phyA* was due to an increase in the number of cauline leaves on the main inflorescence. This again suggests that phyA has a negative role in determining the number of cauline leaves (branching potential) regardless of light intensity (Fig. 3, Fig. 6). phyB did not affect cauline leaf number under either high or low light.

The number of cauline branches in the various genotypes was elevated by higher light input. However, the effect was smaller compared to the effect of higher light input on the regulation of rosette branch number in the various genotypes (Figs. 2, 5). This suggests that the number of cauline branches is highly programmed and that the environmental influence is minor, whereas the environment can have greater effects on the rosette branching pattern.

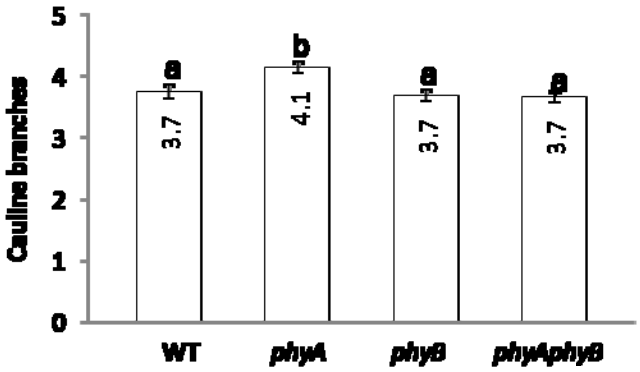

Figure 6. Number of primary cauline branches of WT, *phyA*, *phyB*, and *phyAphyB* under high light. Numbers within bars indicate the actual value. Bars with different letters are significantly different at $\alpha = 0.05$.

Figure 7 shows the relative ratios of secondary cauline branches/axil of the various genotypes grown under low light. It is clear that WT and *phyA* had similar secondary cauline branching patterns, suggesting that phyA does not influence this process (Fig. 7), while *phyB* has a pattern similar to *phyAphyB*. Therefore, phyA does not play a major role in regulating secondary cauline branching under these conditions, while phyB has a greater effect on the regulation of secondary cauline branches/axil.

The effects of the phyB lesion were greater in the more basal branches compared to WT (Fig. 7). These results demonstrate a positive role for phyB in regulating secondary cauline branching, with pronounced effects at nodes C(n) and C(n+1) (Fig. 7), but no effect at the upper nodes (Fig. 7).

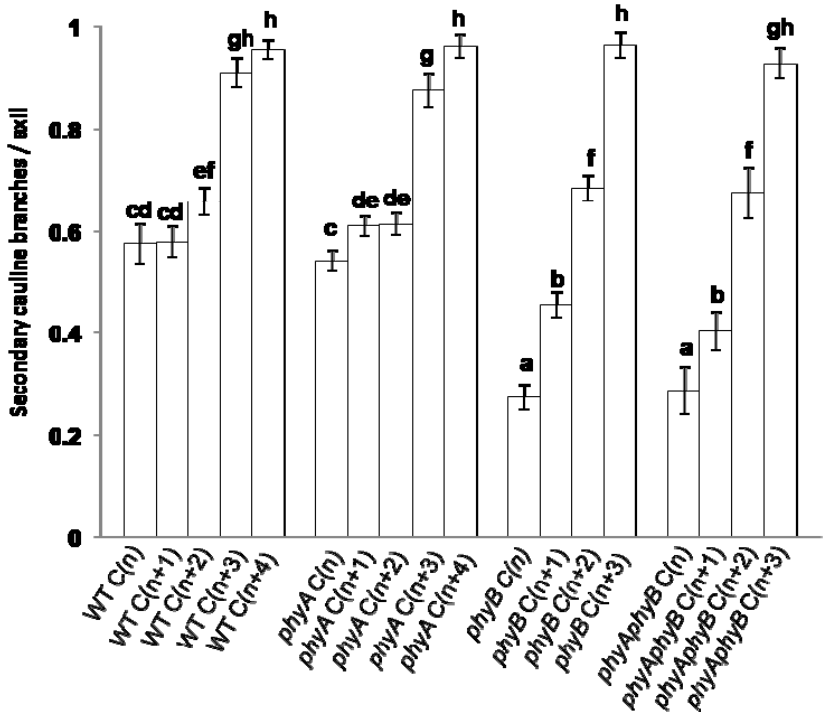

Figure 7. Relative ratios of secondary cauline branches/axil of WT, *phyA*, *phyB*, *phyAphyB* under low light. Bars with different letters are significantly different at $\alpha = 0.05$.

Figure 8 shows the relative ratios of secondary cauline branches/axil of the various genotypes grown under high light. Differences in secondary cauline branching were minor and somewhat inconsistent between genotypes, precluding a role for phyB in this process under high light (Fig. 8). Compared to low light, high light promoted secondary cauline branching in the two lower nodes in *phyB* and *phyAphyB*, but had little effect in WT and *phyA* (Fig. 8). This suggests that high light intensity is able to overcome the negative effects of loss of phyB function in the secondary cauline branching pattern. The effects of phyB deficiency are greatly reduced by high light, compared to low light, which results in a more similar secondary cauline branching pattern among the various genotypes (Figs. 7, 8).

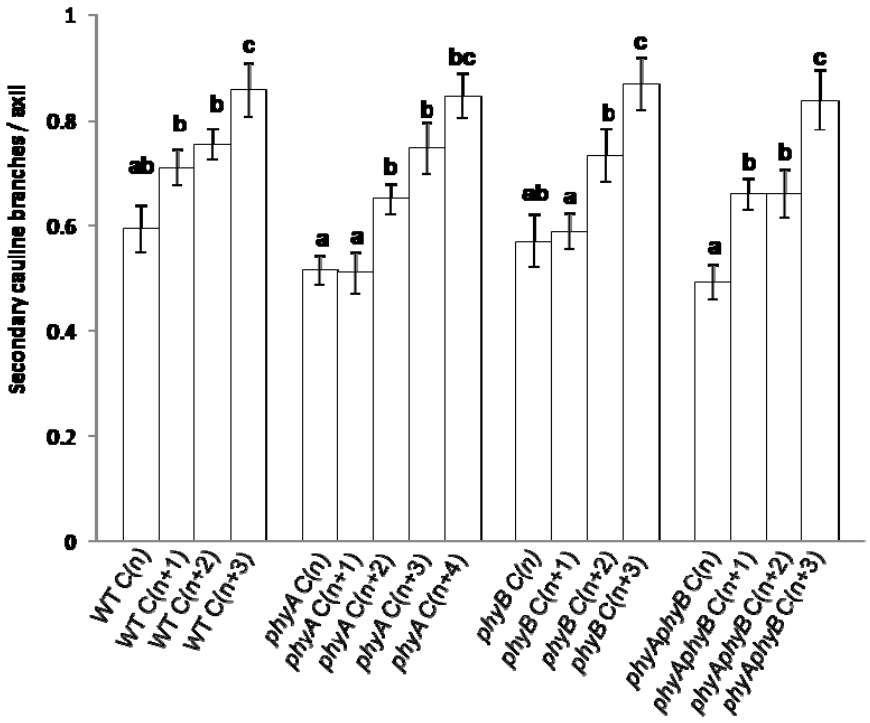

Figure 8. Relative ratios of secondary cauline branches/axil of WT, *phyA*, *phyB*, *phyAphyB* under high light. Bars with different letters are significantly different at $\alpha = 0.05$.

High light intensity was found to be able to restore the rosette branching pattern of phyB deficient mutants by an unknown mechanism. Sucrose, the product of photosynthesis, was found to be able to enhance the growth of leaf blades and promote leaf blade expansion in shaded conditions (Kozuka et al., 2005), and presumably extra energy could promote branch growth. On the other hand, high light intensity may not only play a role in offering more photosynthetic resources to the plants, but might also provide a role redundant to high light quality (R:FR) as a developmental signal. This hypothesis is consistent with the findings of Vandenbussche et al. (2003b) that suggest that *Arabidopsis* responses to quantity shading may share a similar mechanism with those of quality shading. Blue light was proposed to play the major role in contributing to the phenotypic changes of the shade-avoiding plants under different light intensity environments (Vandenbussche et al., 2005), and may be participating in the branching responses noted here. A growing body of evidence suggests that plants adjust growth to light intensity at a variety of specific wavelengths, including that of blue light

(Vandenbussche et al., 2003b; Millenaar et al., 2005; Pierik et al., 2004). Blue light has been suggested to play a role as a signal for the shade avoidance syndrome through the action of cryptochromes (Ballare et al., 1991; Kozuka et al., 2005). Taken together, it is possible that light intensity may have effects on plant development through producing different amount of photosynthate and/or by triggering a blue light dependent signaling pathway. The relatively greater changes in the branching patterns of *phyB* and *phyAphyB* under high light may indicate the functioning of two semi-independent signaling pathways. One pathway operates via sensing of R:FR by phyB and perhaps other phytochromes. The other pathway operates via light intensity effects and is dominant to the R:FR pathway. Therefore, if light quantity is low, R:FR signals become important, but when light quantity is high, R:FR plays a minor role in determining branching.

It should be noted that *Arabidopsis phyB* mutants can respond to both EOD FR and reduced R:FR and thus display some shade avoidance responsiveness (Whitelam and Smith, 1991; Robson et al., 1993; Devlin et al., 1996). These observations indicate that phyB is not the only mediator of the shade avoidance syndrome in *Arabidopsis*. There appear be additional phytochromes that participate in transducing shade signals in *Arabidopsis* (Whitelam and Smith, 1997), and cry1 and cry2 have been implicated as well (Kim et al. 2005). Moreover, it has been suggested that some photoreceptors serve not only as light quality detectors but also as photon counters (Ballare et al., 1999). PhyC was also found to have a significant role in blue light sensing (Franklin et al., 2003) and thus is a possible candidate for a blue light responsive light quantity sensor. In summary, high light quantity may play a role similar to high light quality and rescue the phyB deficient phenotype, possibly through the mediation of other phytochromes and/or cryptochromes.

It is known that every primary rosette leaf possesses two axillary buds, and at least one of them has a potential to grow out (Grbic and Bleeker, 2000). However, the data presented here indicate that the actual rosette branching is less than 50% of the branching potential under high light regardless of genotype. Our anecdotal observations suggest that phyB sufficient genotypes suffer stress when grown under PPFD greater than 310 μmol m^{-2} s^{-1} (data not shown), and show reduced numbers of rosette branches and rosette

branches/axil under these conditions. These findings indicate that light intensity of approximately 310 μmol m^{-2} s^{-1} is able to promote WT *Arabidopsis* to reach its maximum branching ability, which is about 50% of the actual branching potential. The inability of WT to branch beyond this level may be due to constitutive shading of the lower buds from the upper rosette leaves. To assess the branching ability of the lower buds in the future, light supplied directly to every individual axillary bud could be used to overcome the intrinsic shading factor.

Objective 2. To determine if the branching patterns of WT, *phyA*, *phyB*, and *phyAphyB* are regulated through the formation of the buds, the timing of the onset of bud elongation, and/or the elongation rates of the branches.

The timing of meristem initiation was investigated by examining thin sections of the leaf axils of WT and *phyB*. Under low light, axillary meristem formation in WT was found to occur at least as early as 14 days after planting (Fig 9A), and at least as early as 13 days after planting in *phyB* under low light (Fig 9B). Therefore, meristem formation occurred with similar timing regardless of phyB status.

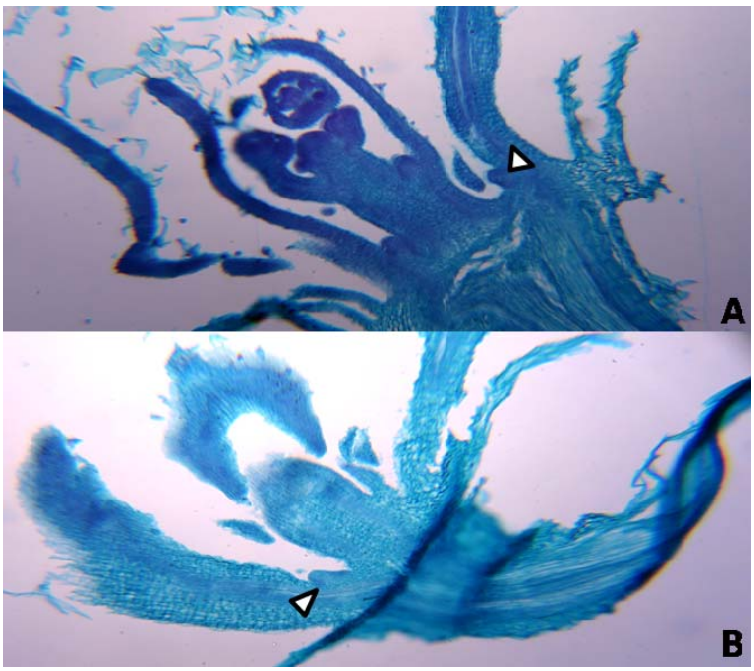

Figure 9. Median longitudinal sections of 14-d-old WT plant (A) and 13-d-old *phyB* plant (B) grown under low light. White arrows indicate the first initiated axillary meristems.

Figure 10 shows the interval in days between anthesis of the main inflorescence and the onset of elongation (buds > 3mm) of the three topmost rosette buds of WT, *phyA*, *phyB*, and *phyAphyB* under low light. The patterns of the delay in elongation were very similar in WT and *phyA*, but differed from *phyB* and *phyAphyB* which were also very similar to each other. The delay until the onset of elongation of the three topmost rosette buds in *phyB* and *phyAphyB* was consistently longer than in WT and *phyA* with the greatest difference at node R(n-2) and the smallest at node R(n) (Fig. 10). This suggests that the lesion in phyB is responsible for the delay of elongation in the phyB deficient mutants. Taken together, the data suggest that phyB plays a more dominant role in determining outgrowth of the axillary buds than meristem initiation timing.

In summary, phyA did not have a significant effect on the timing of elongation of the three topmost rosette buds, while phyB did. Thus it is apparent that phyB normally promotes rapid outgrowth of rosette axillary buds. The timing of the onset of elongation of the three topmost rosette buds contributes, in part, to the final architecture of the plants.

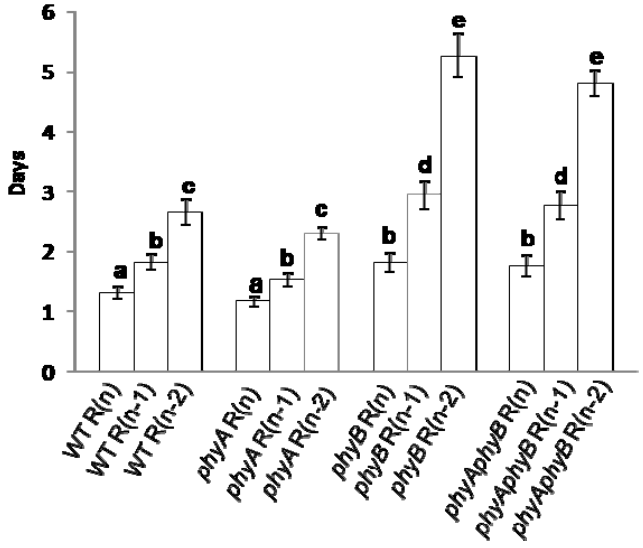

Figure 10. Interval between anthesis and the onset of elongation of the topmost three rosette buds of WT, *phyA*, *phyB*, and *phyAphyB* under low light. Bars with different letters within genotypes are significantly different at $\alpha = 0.05$.

Figure 11 shows the lengths of the main inflorescence and the three topmost rosette axillary buds each day from the onset of elongation of bud R(n) to the 10th day after anthesis under low light. *phyB* showed elongation patterns similar to those of *phyAphyB*. Branches R(n-1), and R(n-2) had slower elongation rates than branch R(n) throughout the measurement period (Fig. 11B, C, D). The finals lengths of branches R(n), R(n-1), and R(n-2) of phyB deficient mutants are shorter than those of phyB sufficient genotypes with significance. No significant differences were observed from the main inflorescence from various genotypes. This suggests that, under low light, phyB plays a positive role in the elongation of the branches, but not the main inflorescence. The elongation patterns of *phyA* were similar to WT under low light (Fig. 11A-D). Branches R(n-1) and R(n-2) of WT and *phyA* elongated at a lower rate than R(n) during the first six days, but after day six, the elongation rates of all three branches were similar (Fig. 11A-C). The similar phenotypes of *phyA* and WT seem to suggest that phyA does not play a role in regulating branch elongation.

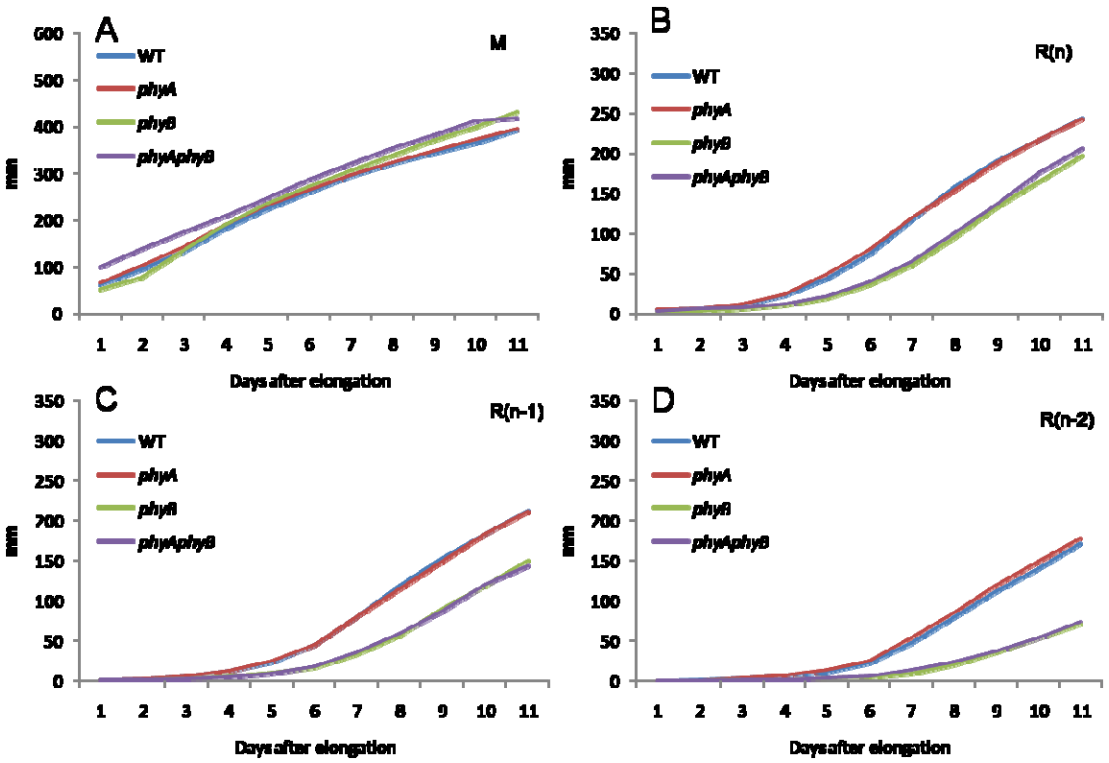

Figure 11. The lengths of the main inflorescence (M) (A), the topmost rosette branch R(n) (B), the next topmost rosette branch R(n-1) (C), and the third topmost rosette branch R(n-2) (D) of WT, *phyA*, *phyB*, and *phyAphyB* from the day of the onset of elongation of bud R(n) to the 10th day after anthesis under low light.

Linear regressions were fitted to the elongation data of the various genotypes, and the slope of each regression was taken as the overall elongation rate. Under low light, the elongation rate of the main inflorescence of the various genotypes was significantly higher than that of branch R(n), which was greater than branch R(n-1), which in turn was significantly higher than branch R(n-2) (Fig. 12A). Corresponding branches R(n), R(n-1), and R(n-2) of WT and *phyA* had similar elongation rates, while those of *phyB* and *phyAphyB* were dissimilar from WT and *phyA*, but similar to each other (Fig. 12A).

Thus, the branching phenotypes of the various genotypes are due in part to the elongation rates of the branches after outgrowth is initiated. The ranking of branch elongation is consistent with the proposed activity of the buds: R(n) > R(n-1) > R(n-2) (Fig. 12A).

The plots of branch length vs. time (Fig. 11B-D) indicated that elongation occurred in two phases- a slower early phase and a faster late phase, therefore, the calculation of elongation rates was separated into two intervals: from the 1st day - 3rd day (Fig. 12B), and from the 4th day - 10th day after anthesis for each branch position (Fig. 12C).

The elongation rates from the 1st day - 3rd day of elongation period were consistent with the trends observed from the 1st day of elongation to the 10th day after anthesis, while the elongation rates from the 4th day - 10th day after anthesis did not show significant differences, with one exception in *phyAphyB* and *phyB*. (Fig. 12).

The data also indicate that the final architecture of WT and *phyA* are mainly determined by early differences in branch elongation, while both early and late branch elongation contributes to the final architecture of *phyB* and *phyAphyB* under low light.

Overall, phyB plays a positive role in the onset of bud elongation timing and in determining the elongation rate, while phyA has no significant effects. This is perhaps not surprising since phyA is known to act only on the regulation of limited aspects of plant development after de-etiolation, such as hypocotyl growth and internode and leaf morphology (Whitelam et al., 1993; Franklin et al., 2003).

The previously reported synergistic relationship of phyA and phyB (Cerdan et al., 1999) is not observed in this research. If phyA acts synergistically with phyB signaling, we should expect a significantly lower elongation rate of *phyAphyB* compared to *phyB*. However, no significant differences were observed throughout the elongation measurement period. This suggests the synergistic relationship of phyA and phyB is limited and light dependent, which is consistent with the previous knowledge that phyA acts synergistically to phyB signaling in HIRs (Cerdan et al., 1999).

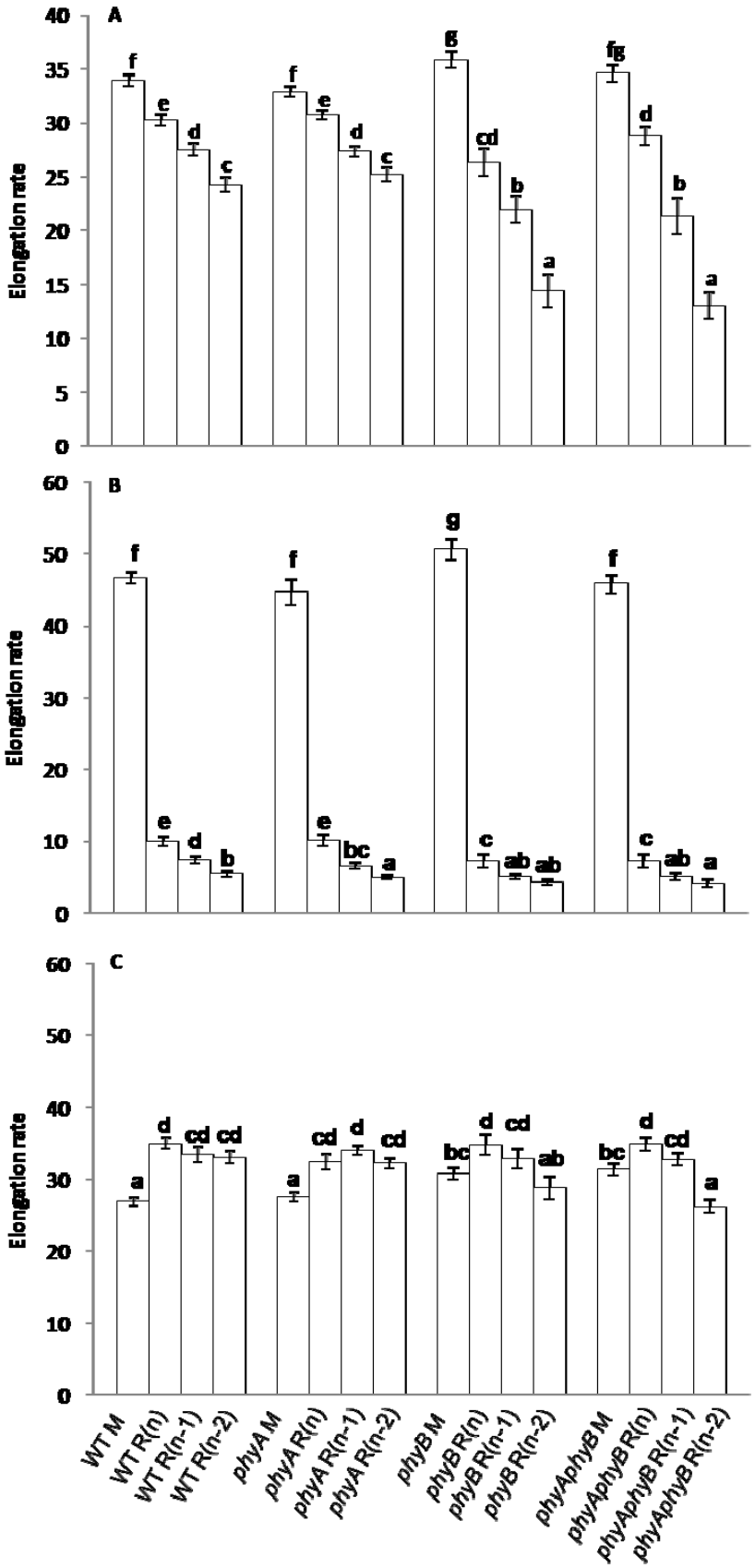

Figure 12. The elongation rates of the main inflorescence (M), the topmost rosette branch R(n), the next topmost rosette branch R(n-1), and the third topmost rosette branch R(n-2) of WT, *phyA*, *phyB*, and *phyAphyB* from the day of the onset of elongation to the 10th day after anthesis (A), from the day of the onset of elongation to the 3rd day (B), and from the 4th day after the onset of elongation to the 10th day after anthesis (C) under low light. Bars with different letters are significantly different at $\alpha = 0.05$.

Figure 13 shows the intervals between anthesis of the main inflorescence and the elongation of the topmost rosette buds of WT, *phyA*, *phyB*, and *phyAphyB* under high light. The differences in the delay until elongation of buds between the various genotypes at all positions were minor, and did not exceed 0.7 days (Fig. 13). The effect of phyB deficiency on elongation timing of the buds observed under low light was not apparent under high light.

Higher light input enables the upper buds from the various genotypes to start elongating earlier than under low light, and this promotion was relatively greater in the phyB deficient mutants than in WT and *phyA* (Figs 10, 13). Therefore, high light eliminated the inhibitory effects of the phyB lesion on the timing of the onset of elongation.

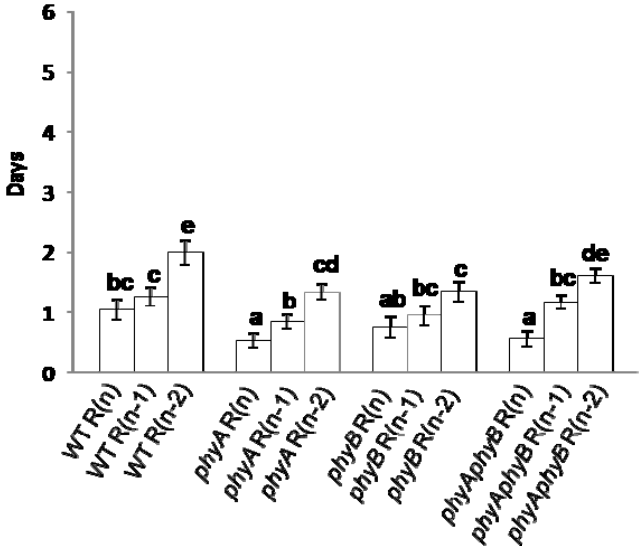

Figure 13. Interval between anthesis and the onset of elongation of the topmost three rosette buds of WT, *phyA*, *phyB*, and *phyAphyB* under high light. Bars with different letters within genotypes are significantly different at $\alpha = 0.05$.

In contrast to the elongation patterns of the various genotypes grown under low light, the lengths of the main inflorescence and the three topmost branches of *phyB* and *phyAphyB* were consistently greater than those of WT and *phyA* under high light (Fig. 14). This suggests a negative role for phyB in regulating elongation of both the main

inflorescence and the three topmost branches under high light (Fig. 14). The negative role of phyB in elongation of the main inflorescence is well established; however, phyB loss of function is typically believed to inhibit branching, as was observed under low light (Fig. 2, Fig. 11). Under high light this pattern was unexpectedly reversed. The branching phenotypes of phyB deficient mutants in many aspects were recued to almost the same level as phyB sufficient genotypes. This may be due to the compensation of high light intensity on the constitutive low light quality phenotypes of phyB deficient mutants, perhaps due to increased photoassimilation, and/or light quantity signaling described above.

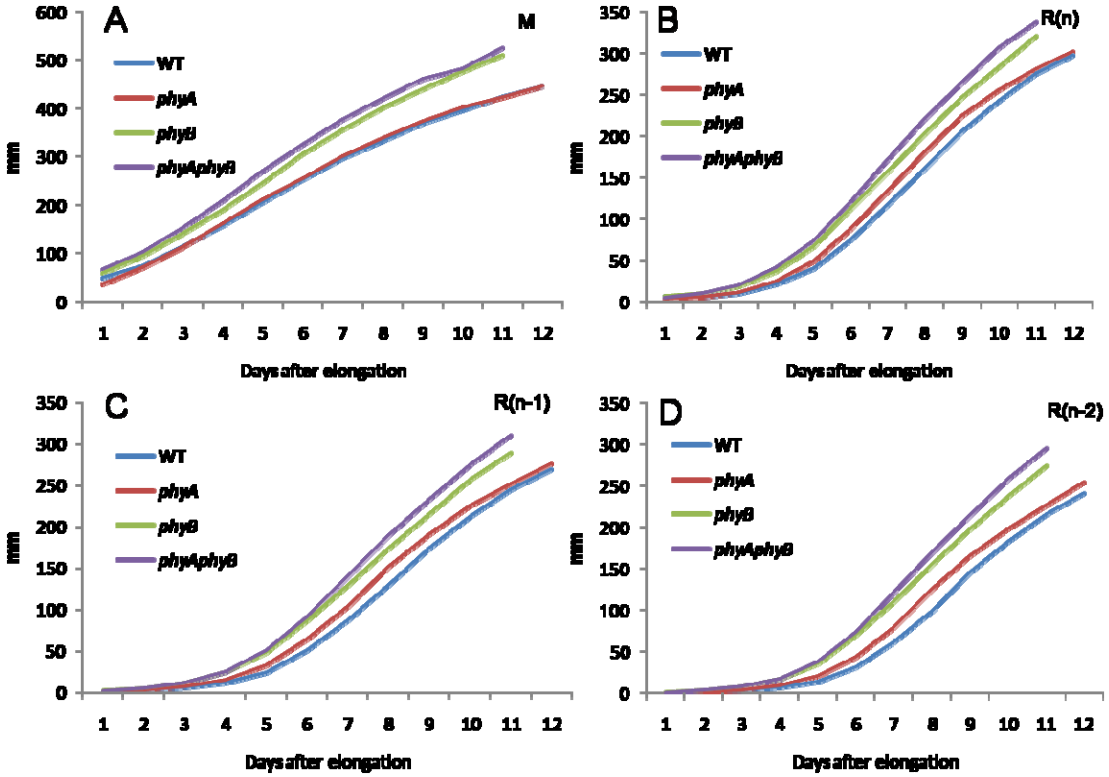

Figure 14. The lengths of the main inflorescence (M) (A), the topmost rosette branch R(n) (B), the next topmost rosette branch R(n-1) (C), and the third topmost rosette branch R(n-2) (D) of WT, *phyA*, *phyB*, and *phyAphyB* from the day of the onset of elongation of bud R(n) to the 10th day after anthesis under high light.

The ranking of the bud activity with respect to the timing of elongation onset was consistent among genotypes: R(n) > R(n-1) > R(n-2). However, the differences between the activities of the three topmost rosette axillary buds under high light were not as great as those under low light (Figs 10, 13).

Linear regressions were again fitted to the elongation data of the various genotypes, and the slope of each regression was taken as the overall elongation rate (Fig. 15A). Under high light, the main inflorescences of WT, *phyA*, *phyB*, and *phyAphyB* elongated at a significantly greater rate than branch R(n), which had an elongation rate significantly greater than R(n-1). No significant differences were observed between the elongation rates of R(n-1) and R(n-2) (Fig. 15A). Rosette axillary branches from *phyB* and *phyAphyB* elongated at a significantly higher rate than those from phyB sufficient genotypes (Fig 15A). While the trends of branch elongation rates within genotypes were still consistent- R(n) > R(n-1) > R(n-2), the differences in the branch elongation rates of phyB deficient and phyB sufficient genotypes were reversed in high compared to low light (Figs. 12A, 15A). This again suggests a role of light quantity in inducing shade avoidance responses in a manner similar to light quality as discussed above.

The calculation of elongation rates was again separated into two intervals: from the 1st day - 3rd day (Fig. 15B), and from the 4th day - 10th day after anthesis (Fig. 15C). The main inflorescence of the various genotypes under high light elongated significantly faster than R(n) from the 1st day to 3rd day of elongation. R(n) elongated significantly faster than R(n-1) and R(n-2). The elongation rates of R(n-1) were not significantly different from R(n-2) (Fig. 15B). The elongation rates of the three branches within genotypes were similar (Fig. 15C). Loss of phyB function resulted in an increase in the elongation rate of the top branches under high light. The elongation rates of the main inflorescence and the three topmost buds during first three days of elongation contributed more to the final architecture of the various genotypes. However, the reversal of the trends in elongation rates of the branches of phyB deficient and phyB sufficient genotypes under high light compared to those under low light was determined by both early and late phase elongation.

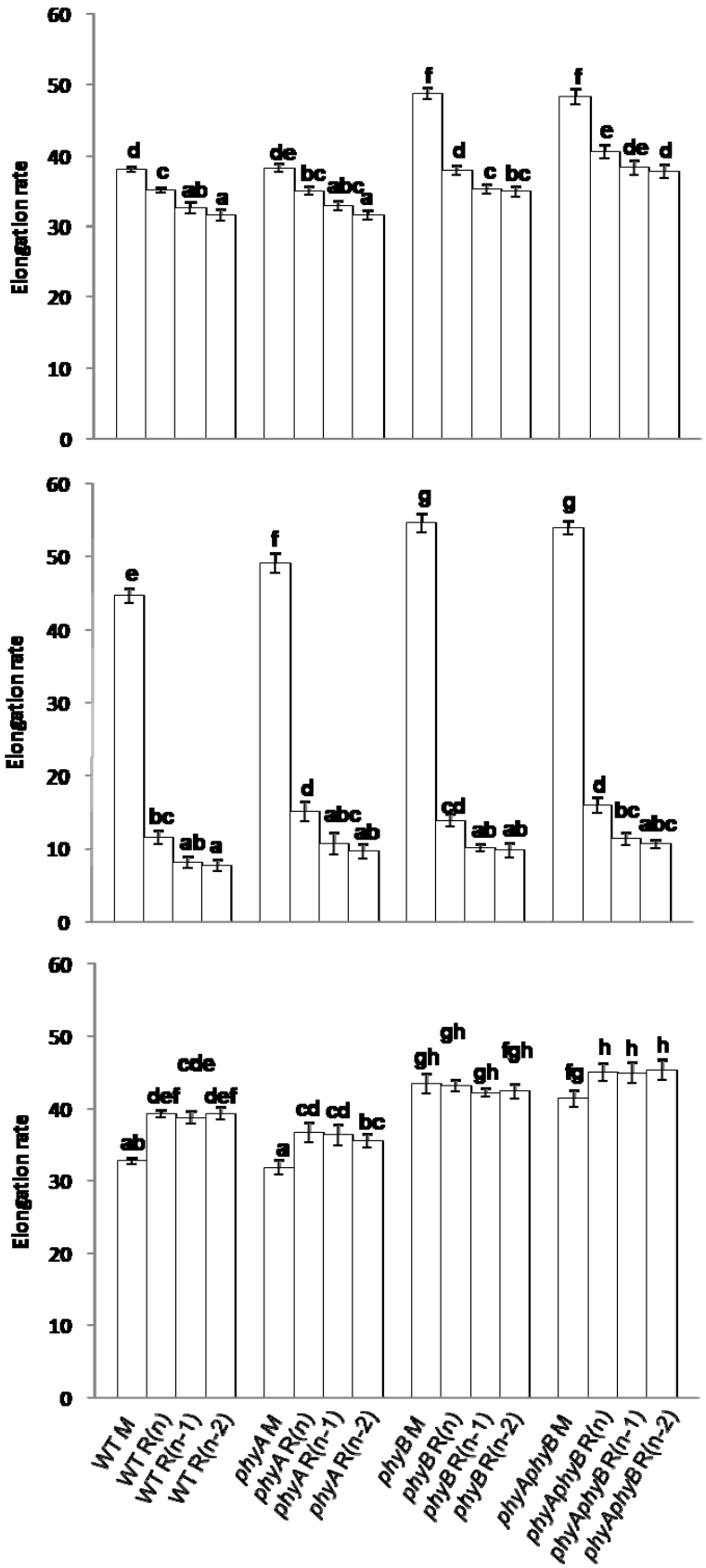

Figure 15. The elongation rates of the main inflorescence (M), the topmost rosette branch R(n), the next topmost rosette branch R(n-1), and the third topmost rosette branch R(n-2) of WT, *phyA*, *phyB*, and *phyAphyB* from the day of the onset of elongation to the 10th day after anthesis (A), from the day of the onset of elongation to the 3rd day (B), and from the 4th day after the onset of elongation to the 10th day after anthesis (C) under high light. Bars with different letters are significantly different at $\alpha = 0.05$.

Objective 3. Assessment of the changes in the expression of branching-related genes in buds of WT, *phyA*, *phyB*, and *phyAphyB* under high and low PPFD.

The expression of the branching-regulator *TBL1* has previously been demonstrated to be negatively correlated with the developmental stage of the axillary buds (Aguilar-Martinez et al., 2007, Finlayson, 2007). *TBL1* is expressed at modest levels in unelongated buds and decreases to very low levels once buds begin to grow out. Here, the changes in the expression of branching-related genes in buds of WT, *phyA*, *phyB*, and *phyAphyB* were assessed to determine how phyA and phyB regulate the molecular physiology of the bud, and also to confirm the proposed activity ranking of the axillary buds from the various genotypes.

The mRNA abundance of *TBL1* in the topmost three rosette axillary buds of the various genotypes under low light (Fig. 16) was generally in accordance with the hypothesized activity of the axillary buds [R(n) > R(n-1) > R(n-2)].

Loss of phyB function resulted in elevated expression of *TBL1*. This is consistent with the previous report that *TBL1* (*BRC1*) is required for plants to arrest the outgrowth of axillary buds (Finlayson, 2007; Aguilar-Martínez et al., 2007), and indicates that *TBL1* expression is regulated by phyB in a manner similar to *tb1* in sorghum (Kebrom et al., 2006). *TBL1* expression in the lower buds of *phyA* was reduced compared to WT, though no differences in branching were observed (Figs. 2B, 2C, 2D, 7, 10, 11). It is difficult to explain these results without considering the possibility of sampling errors. Additionally, the lack of statistically significant differences in some instances are likely due to the variation in the status of harvested buds. *TBL1* expression varies with the physiological status of the buds, which is difficult to determine visually. Errors in the timing of bud collection could have contributed noise to the analysis.

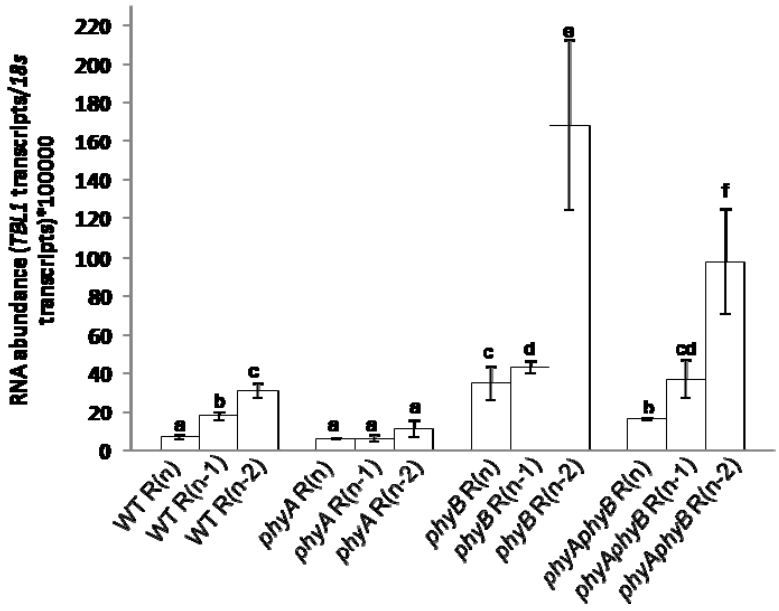

Figure 16. *TBL1* mRNA abundance in the topmost three rosette axillary buds from WT, *phyA*, *phyB*, and *phyAphyB* grown under low light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters are significantly different at $\alpha = 0.05$.

It is known that mutations in *TBL1* and *BRC2* result in hyper-branching phenotypes (Aguilar-Martı´nez et al., 2007; Finlayson, 2007). *BRC2* expression may not be as closely related to bud outgrowth as *TBL1* since loss of BRC2 function results in only a moderate hyper-branching phenotype (Aguilar-Martı´nez et al., 2007). Moreover, *tbl1/brc2* double mutants possess the phenotype of *tbl1* (Aguilar-Martı´nez et al., 2007; Finlayson, 2007). *BRC2* has been suggested to function in a manner similar to *TBL1* as an inhibitor of branching that is expressed in the axillary buds. The pattern of *BRC2* mRNA abundance was hypothesized to be similar to the pattern of *TBL1* mRNA abundance in the buds of the various genotypes. Therefore, the expression of *BRC2* in the topmost three rosette axillary buds was also assessed.

In general, bud R(n) had the least *BRC2* abundance, R(n-1) had an intermediate amount, and R(n-2) had the greatest. This trend was observed in WT, *phyB* and *phyAphyB*, though it was not always statistically significant in every case. *phyA* showed a slightly different pattern with lowest *BRC2* expression in bud R(n-1), however, this level was not statistically different from that of bud R(n) (Fig. 17). Differences in *BRC2*

expression between genotypes were minor.

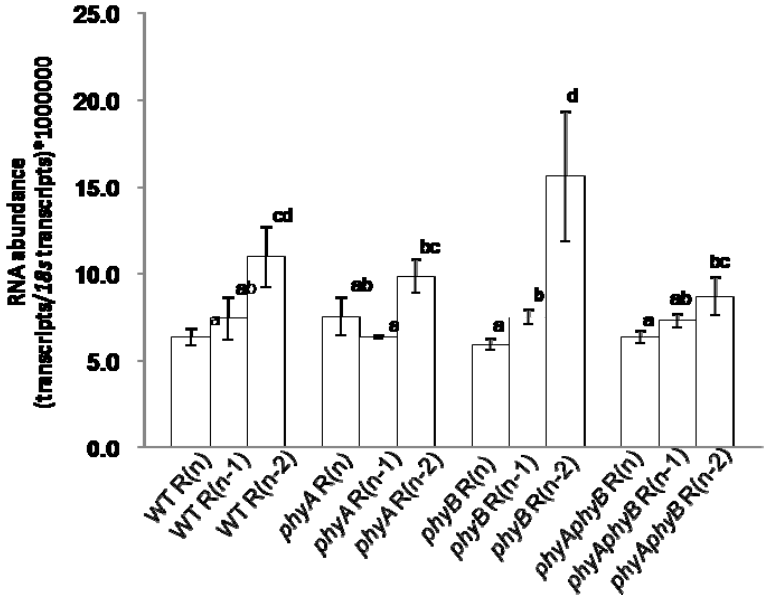

Figure 17. *BRC2* mRNA abundance in the topmost three rosette axillary buds from WT, *phyA*, *phyB*, and *phyAphyB* grown under low light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters are significantly different at $\alpha = 0.05$.

The ranking of the *TBL1* RNA abundance in the three topmost rosette axillary buds of WT, *phyA*, *phyB* and *phyAphyB* under high light was also generally in accordance with the outgrowth potential of the respective buds (Fig 18). In some cases the differences between buds at different positions within a genotype were not statistically significant, but the numerical trends consistently ranked the *TBL1* content lowest in bud R(n), intermediate in R(n-1) and highest in R(n-2). This pattern was similar to that observed under low light, but the *TBL1* mRNA abundance in the three topmost axillary buds of WT were reduced with the greatest reduction in R(n-1) (32%) under high compared to low light. The *TBL1* mRNA abundance in the three topmost axillary buds of *phyA* were reduced with the greatest reduction in R(n-2) (58%) under high compared to low light. The *TBL1* mRNA abundance in the three topmost axillary buds of *phyB* were reduced with the greatest reduction in R(n) (7%) under high compared to low light. The *TBL1*

mRNA abundance in the three topmost axillary buds of *phyAphyB* were reduced with the greatest reduction in R(n-2) (11%) under high compared to low light (Figs 16, 18). The differences in the expression of *BRC2* caused by high light are not as substantial as *TBL1*. However, it was found that most of the axillary buds of the various genotypes possess reduced expression of *BRC2* under high light compared to low light (Figs 17, 19).

The expression pattern of *TBL1* was in accordance with the hypothesis that higher light can increase the activity of rosette axillary buds. This suggests that the enhancement of elongation rate and the earlier onset of elongation that together contribute to the activities of axillary buds under high light was regulated by decreased expression of *TBL1* locally in the buds. The reduction of *TBL1* expression caused by high light is greater in the phyB deficient mutants than that in the phyB sufficient genotypes. This result is also in agreement with the branching analysis of the three topmost buds (branches) under low and high light. High light has more effect on the branching of phyB deficient mutants than phyB sufficient genotypes. Taken together, it suggests that phyB is not the only mediator of *TBL1* expression in *Arabidopsis*, since *TBL1* expression is still responsive to light quantity in the phyB deficient mutants. This is in accordance with our hypothesis that in addition to phyB, other photosensors and/or the plant's sugar status could regulate *TBL1* expression.

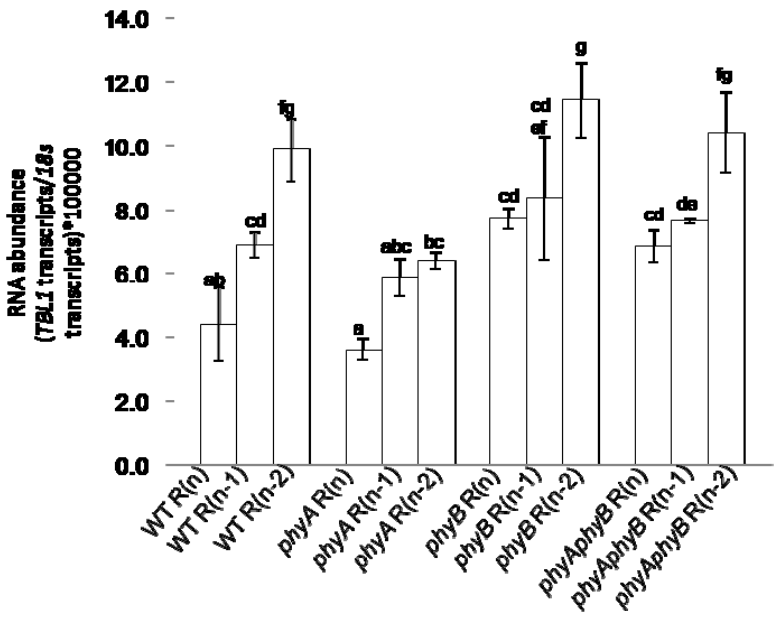

Figure 18. *TBL1* mRNA abundance in the topmost three rosette axillary buds of WT, *phyA*, *phyB*, and *phyAphyB* grown under high light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters are significantly different at $\alpha = 0.05$.

The *BRC2* mRNA abundance patterns within genotypes from plants grown under high light (Fig. 19) were dissimilar from those observed under low light (Fig. 17), with the exception of *phyAphyB*. *BRC2* levels were higher in WT than in *phyB*, which is consistent with the enhanced branching of *phyB* under high light (Figs 2, 5). *BRC2* expression levels in *phyB* and *phyAphyB* were reduced in high light compared to low light, with one exception in bud R(n) of *phyB*, suggesting a role for PPFD in regulating *BRC2* expression in addition to *TBL1* expression. It is possible that the somewhat anomalous *BRC2* expression patterns observed may be due in part to the very low expression level of this gene, which results in elevated noise.

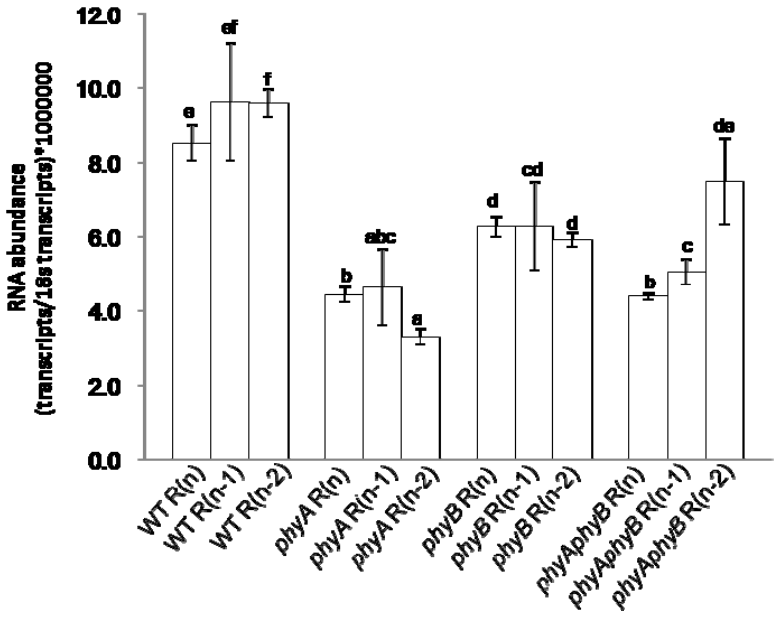

Figure 19. *BRC2* mRNA abundance in the topmost three rosette axillary buds from WT, *phyA*, *phyB*, and *phyAphyB* grown under high light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters are significantly different at $\alpha = 0.05$.

In summary, it may be concluded that the expression of *TBL1* in *Arabidopsis* is regulated by the light signals perceived by phyB, which is negatively correlated to bud outgrowth but not bud initiation. These are in accordance with the previous report that the expression *TBL1* (*BRC1*), negatively regulate axillary buds outgrowth (Aguilar-Martinez et al., 2007; Finlayson, 2007), and was proposed to be negatively regulated by phyB (Finlayson, 2007). These findings also agree with the previous research in other species, such as rice, which suggests that *OsTB1* expression suppress the axillary bud outgrowth instead of formation (Takeda et al., 2003). It was also consistent with the findings of Kebrom et al. (2006) that phyB regulates bud outgrowth through mediating the expression level of *SbTB1* in response to various light signals.

Objective 4. Assessment the association of hormones with the activity of buds of WT, *phyA*, *phyB*, and *phyAphyB* under high and low PPFD.

Previous research has related the *DRM1* expression level to the dormancy status of buds (Stafstrom et al., 1998; Tatematsu et al., 2005; Kebrom et al., 2006). The

expression of *DRM1* was also found to be regulated by light signals and phyB in sorghum, and its expression correlated with that of *TB1* (Kebrom et al., 2006). The *DRM1* expression level in the three topmost axillary buds of various genotypes was assessed as an indicator of the status of the buds and to probe the possible role of auxin in their development.

Under low light, the expression patterns of *DRM1* in the three topmost buds from WT, *phyA* and *phyB* and *phyAphyB* generally correlate with those of the *TBL1* expression levels (Fig 16, 20), with lowest levels in R(n) and highest levels in R(n-2). Between genotypes, the *DRM1* expression levels in the buds R(n) and R(n-1) from *phyB* and *phyAphyB* are significantly higher than the respective buds in WT, which is in accordance with the physiological analysis indicating that phyB has a positive role in determining bud activity. *DRM1* expression may provide an indication that phyB plays greater role in regulating the topmost two buds, as opposed to those at more basal positions since *DRM1* levels are nearly equivalent in bud R(n-2) of WT and *phy*B. The results could also suggest a possible role for auxin in triggering the outgrowth of axillary buds. However, *DRM1* expression is less representative of proposed bud activity, [R(n) > R(n-1) > R(n-2)] compared to *TBL1*, indicating that auxin is not the only regulator of bud outgrowth or that auxin is acting through mechanisms unrelated to *DRM1* expression. This may be consistent with the previous report that auxin deficiency alone does not trigger initial bud outgrowth in *Arabidopsis* (Cline, 1996; Beveridge et al., 2000; Cline et al., 2001).

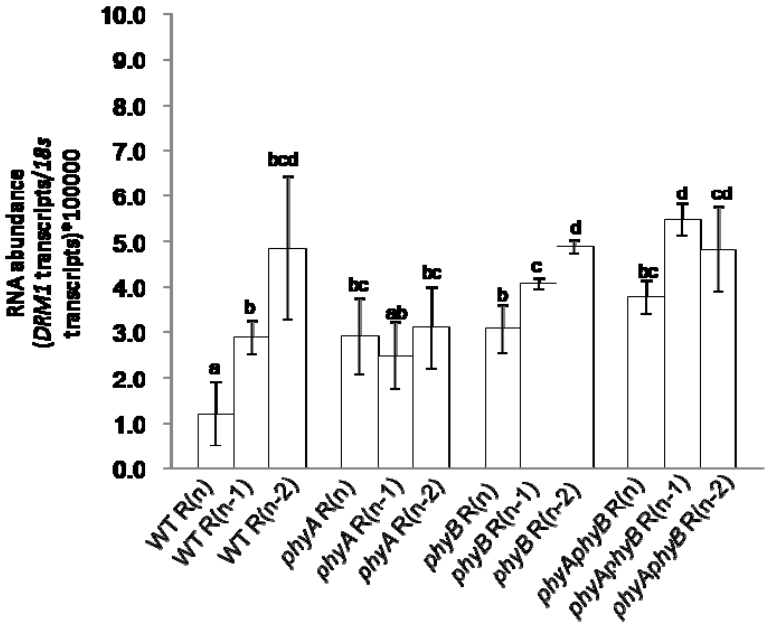

Figure 20. *DRM1* mRNA abundance in the topmost three rosette axillary buds from WT, *phyA*, *phyB*, and *phyAphyB* grown under low light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters are significantly different at $\alpha = 0.05$.

Under high light, *phyB* and *phyAphyB* show similar *DRM1* expression patterns as that of *TBL1* under high light. No differences in *DRM1* expression in the three topmost buds of WT were observed, and the pattern in *phyA* was opposite that predicted, though the differences were not large (Fig 21). However, the *DRM1* levels of *phyB* and *phyAphyB* were higher than WT and *phyA*, which is inconsistent with its proposed utility as an indicator of dormancy, an issue previously raised by Finlayson (2007). *TBL1* expression appears to be a better indicator of the developmental stages of axillary buds than *DRM1* expression.

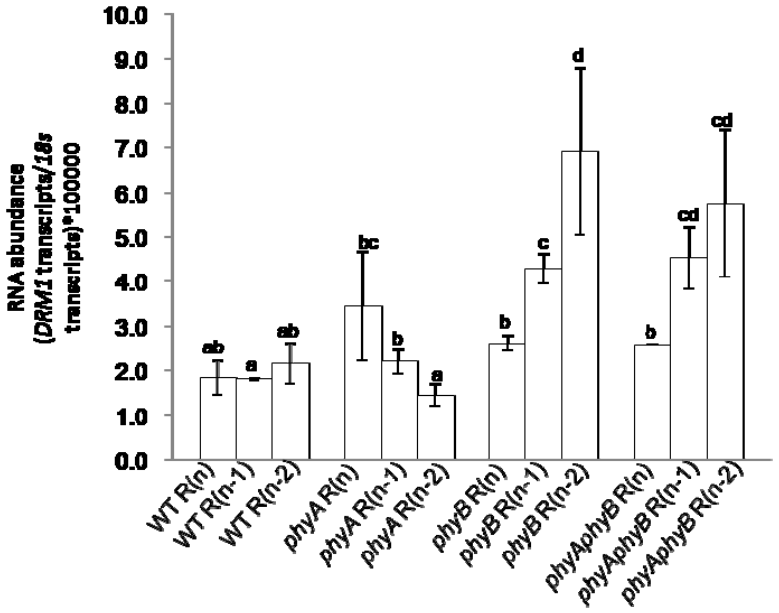

Figure 21. *DRM1* mRNA abundance in the topmost three rosette axillary buds from WT, *phyA*, *phyB*, and *phyAphyB* grown under high light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters are significantly different at $\alpha = 0.05$.

Previous research has indicated that *ARR5*, a type A *ARR*, responds exclusively to exogenous CK transcriptionally within 10 min (Brandstatter and Kieber, 1998; D'Agostino et al., 2000). *ARR5* expression was monitored to determine if there were differences in CK activity within the different buds.

Under low light, the expression patterns of *ARR5* of the three topmost buds from WT, *phyA* and *phyB* and *phyAphyB* generally correlated inversely with those of *TBL1* and *DRM1* expression levels (Fig 16, 22), with the lowest levels in R(n-2) and highest levels in R(n). The results could suggest a possible role for CK in triggering the outgrowth of axillary buds. This is also consistent with the role of auxin acting negatively on the amount of CK in axillary buds (Cline, 1994). However, *ARR5* expression is less representative of proposed bud activity, [R(n) > R(n-1) > R(n-2)] compared to *TBL1*.

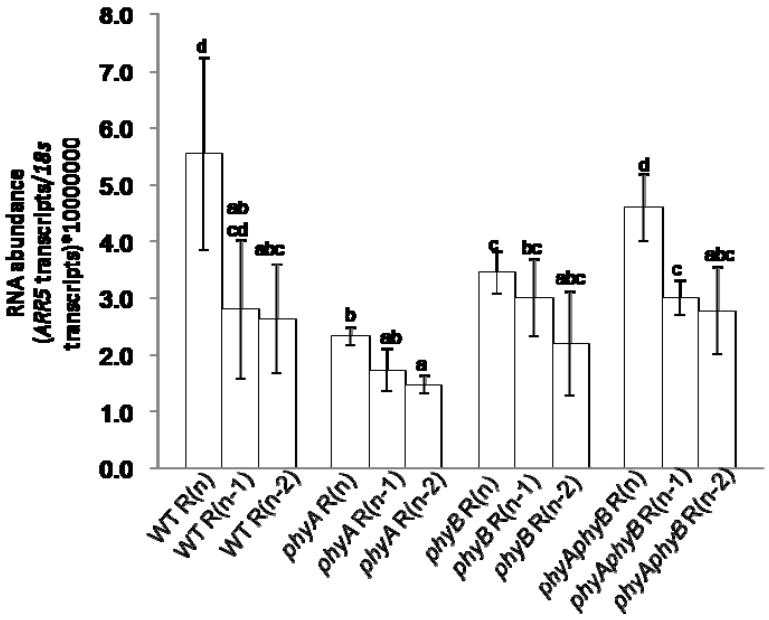

Figure 22. *ARR5* mRNA abundance in the topmost three rosette axillary buds from WT, *phyA*, *phyB*, and *phyAphyB* grown under low light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters are significantly different at $\alpha = 0.05$.

phyB and *phyAphyB* show *ARR5* expression patterns that are the inverse of *TBL1* under high light. The pattern in WT was as predicted highest in R(n), lowest in R(n-2), though no statistical differences between R(n-1) and R(n-2) were observed (Fig 23). These results are consistent with the role of CK, which has been previously suggested to be highly correlated with bud activity and negatively regulated by auxin (Sachs and Thimann, 1967; Li et al., 1995; Chatfield et al., 2000; Leyser, 2003; Nordstrom et al., 2004; Tanaka et al., 2006). No differences in *ARR5* expression in the three topmost buds of *phyA* were observed. However, the *ARR5* levels of *phyB* and *phyAphyB* were higher than WT and *phyA*, which is consistent with its proposed utility as an indicator of bud activity since these buds elongated earlier and at a higher rate than WT (Figs 13, 14, 15, 23).

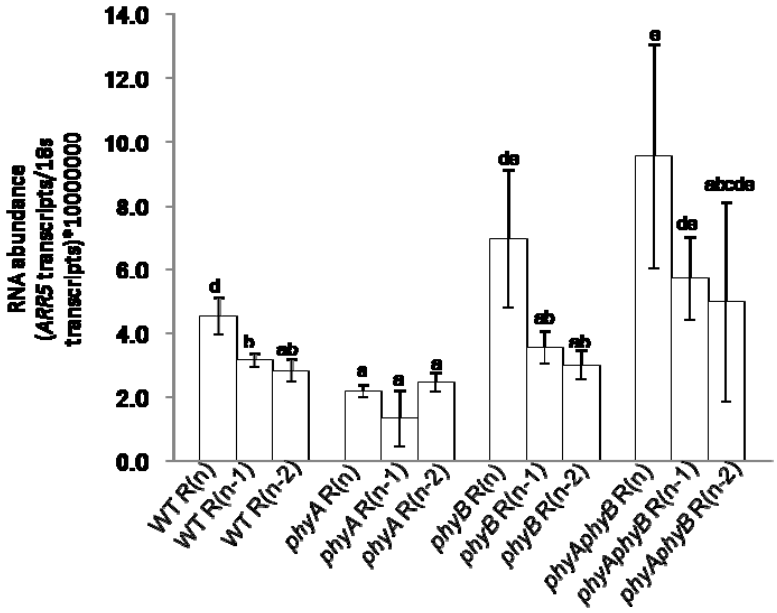

Figure 23. *ARR5* mRNA abundance in the topmost three rosette axillary buds from WT, *phyA*, *phyB*, and *phyAphyB* grown under high light. Results are the means of QPCR analysis of three biological replicates ± SEM. Bars with different letters within are significantly different at $\alpha = 0.05$.

5. SUMMARY AND CONCLUSIONS

Objective 1. Quantification of the architecture of WT, *phyA*, *phyB* and *phyAphyB* under high and low PPFD.

Branching is regulated by light signals perceived by phytochromes. While it has been anecdotally reported that *Arabidopsis phyB* mutants branch less than WT, no data defining the lesion in branching caused by loss of phyB function has been available. The hypothesis proposed herein was that phyB would have a large effect on the regulation of axillary bud outgrowth, while phyA would have little or no effect on branching. Therefore, it was predicted that *phyA* and WT would not have significant differences in branching patterns under low light intensity, while *phyB* and *phyAphyB* would have significantly reduced numbers of primary and secondary rosette branches, primary and secondary cauline branches, rosette leaves, and axillary buds. High light intensity (PPFD) was hypothesized to be able to rescue the reduction of branching and rosette leaves in *phyB* and *phyAphyB*. The blue wavelengths in white light may act as a signal of environmental quality and play a role similar to R:FR in determining shade avoidance. Such blue light responses could be evoked in phyB deficient mutants as well as plants with functional phyB, possibly through the action of cryptochromes. Light quantity might also affect branching by determining the amount of photosynthates available for branch growth.

WT and *phyA* were found to have similar branching patterns in the number of rosette branches, ratio of secondary cauline branches / axil. However, *phyA* was found to have more rosette leaves than WT under both low and high light conditions.

phyB and *phyAphyB* had similar branching patterns in the number of rosette branches and the ratio of secondary cauline branches/axil. Loss of phyB function led to reduced numbers of rosette leaves and branches, lowered rosette branches/axil and secondary cauline branches/axil, and delayed and slower elongation of the topmost three rosette branches.

The high light environment may have provided more photosynthate to allow WT, *phyB*, *phyA*, and *phyAphyB* to increase the number of rosette leaves and branches, the ratio of rosette branches/axil and secondary cauline branches/axil. The differences resulting from high light were greater in mutants deficient in phyB (*phyB* and *phyAphyB*)

than in WT and *phyA*. In fact, high light quantity partially rescued the phenotypes of *phyB* and *phyAphyB* resulting in branching patterns similar to WT and *phyA*. Along with increased photoassimilation, it is also possible that high light could provide a signal, perceived by other phytochromes and/or cryptochromes, that overrides the R:FR signal normally perceived by phyB.

Objective 2. To determine if the branching patterns of WT, *phyA*, *phyB*, and *phyAphyB* are regulated through the formation of the buds, the timing of the onset of bud elongation, and/or the elongation rates of the branches.

It was hypothesized that the branching patterns of WT, *phyA*, *phyB*, and *phyAphyB* are determined by both the formation of the buds and through elongation of the buds. Histological analysis indicated that phyB does not influence meristem initiation, but does play a positive role in promoting the onset of bud elongation and also the bud elongation rate.

phyA was hypothesized to possess a similar pattern of branching as WT. It was found that phyA does not play a role in regulating the onset of bud outgrowth or elongation.

The data also indicate that the final architecture of WT and *phyA* are mainly determined by early differences in branch elongation, while both early and late branch elongation contributes to the final architecture of *phyB* and *phyAphyB* under low light.

Loss of phyB function resulted in a delay in the onset of bud outgrowth under low light, but promoted it under high light. Obviously, the role of phyB in this, and other processes, is conditional upon the light quantity.

The early (day 1 to day 3) elongation of the topmost three rosette buds of WT and *phyA* contributed more to the final differences in length of the branches than the later (day 4 to day 10) elongation regardless of the variations in light quantity. While early elongation also contributed greater to the differences in final branch length in *phyB* and *phyAphyB*, later elongation was also a factor. The ranking of the elongation rate of the top most three branches was: R(n) > R(n-1) > R(n-2) under both low and high light intensity, in all genotypes assessed.

Objective 3. Assessment of the changes in the expression of branching-related genes in buds of WT, *phyA*, *phyB*, and *phyAphyB* under high and low PPFD.

Branching-related gene expression was investigated in the buds to determine if it was linked to light signals perceived by phytochromes. The expression levels of *TBL1* and *BRC2* in the topmost three axillary buds and their elongation rates in the various genotypes were proposed to be in accordance with the activity of the axillary buds. The ranking of *TBL1* and *BRC2* expression level in the topmost three axillary buds was therefore predicted to be: R(n) < R(n-1) < R(n-2) under both low and high light intensity. This hypothesis was based on the known roles of *TBL1* and *BRC2* as negative regulators of bud outgrowth. It was found that the trend of *TBL1* mRNA abundance in the topmost three buds was consistent within genotypes regardless of variations in the light. In accordance with the hypothesis, bud R(n) of various genotypes had the lowest expression levels, and buds R(n-2) had the highest expression levels. The differences in *TBL1* RNA abundance between buds were occasionally non-significant. Increasing the size of the sampling population and/or increasing the number of replicated measurements would likely help resolve this issue.

The trend of *BRC2* RNA abundance in the topmost three buds was similar to, but not as clear as, *TBL1* expression. This suggests that *BRC2* is a weaker negative regulator of axillary bud activity compared to *TBL1*. This is consistent with the more similar phenotypes of *brc2* and WT. The phenotype of the *tbl1*/*brc2* double mutant was indistinguishable from the *tbl1* single mutant (Aguilar-Martinez et al., 2007; Finlayson, 2007).

The axillary bud outgrowth was negatively regulated by *TBL1* (*BRC1*) expression (Aguilar-Martinez et al., 2007; Finlayson, 2007), which was proposed to be negatively regulated by phyB (Finlayson, 2007). The proposed hypothesis was that the level of *TBL1* mRNA in the axillary buds of *phyB* and *phyAphyB* would be higher than that in WT and *phyA*, which would suggest that it is through the expression of *TBL1* that phyB inhibits the growth of the axillary buds. The branching and *TBL1* expression patterns of *phyA* were predicted to not be significantly different from WT. Our results show that the *TBL1* expressions of the three top most buds from phyB deficient mutants are consistently higher than those in the respective buds from phyB sufficient genotypes,

which is in accordance with the hypothesized relationship of phyB and *TBL1* in branching regulation.

It was found that the expression of *TBL1* can be reduced in response to high light through a phyB-independent pathway. The reduction of *TBL1* expression in the axillary buds of various genotypes grown under high light is in accordance with the increased axillary bud activity caused by high light, with a higher reduction in the phyB deficient mutants. The data suggest that multiple pathways regulate *TBL1* expression in response to the light environment.

Objective 4. Assessment of the association of hormones with the activity of buds of WT, *phyA*, *phyB*, and *phyAphyB* under high and low PPFD.

The expression level of *DRM1*, an auxin responsive gene, was proposed to be negatively associated with the activity of axillary buds and positively associated with *TBL1* expression. It was hypothesized that the ranking of the *DRM1* expression level of the topmost three axillary buds would be: R(n) < R(n-1) < R(n-2) under both low and high light intensity.

Our results show that the expression level of *DRM1* in the axillary buds of *Arabidopsis* was not as highly correlated with the developmental status of the buds as *TBL1* expression was.

The expression level of *ARR5*, a CK responsive gene, was proposed to be positively associated with the activity of axillary buds and negatively associated with *TBL1* expression. It was hypothesized that the ranking of the *ARR5* expression level of the topmost three axillary buds would be: R(n) > R(n-1) > R(n-2) under both low and high light intensity. *ARR5* expression generally followed this trend, but was less well correlated with bud activity than *TBL1* expression was.

Figure 24 shows the proposed model of regulation of branching by light quality and quantity. The two pathways appear to converge to regulate *TBL1* expression in the buds. This also suggests that phyB is not the only regulator of *TBL1* expression in *Arabidopsis*.

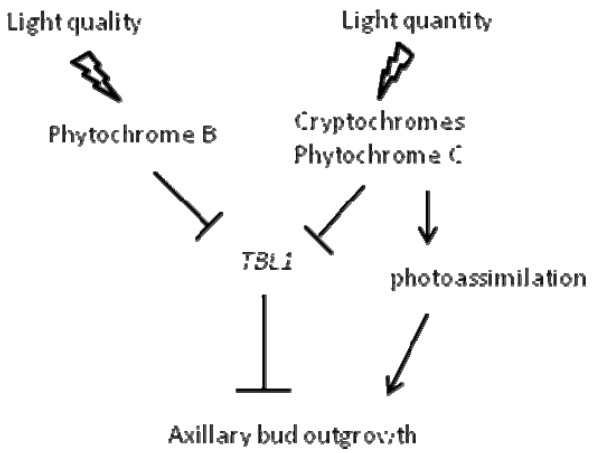

Figure 24. The proposed model of axillary bud outgrowth regulation by light quality and quantity perceived by photoreceptors mediating the expression level of *TBL1*.

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VITA

Name:	Nan-Yen Chou
Address:	Soil and Crop Sciences
	370 Olsen Blvd.
	College Station, TX 77843-2474
Email Address:	neuronneuronneuron@gmail.com
Education:	M.S., Agronomy, Texas A&M University, 2008
	B.A., Agronomy, National Taiwan University, 2005