PHYTOCHROME B CONTROLS SHOOT ARCHITECTURE BY REGULATING PHYTOCHROME INTERACTING FACTORS AND PHYTOHORMONES

A Dissertation

by

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ABSTRACT

Plant architectural responses to changes in the ratio of red light to far-red light (R: FR) are mediated by phytochromes (phy), especially phyB. phyB function is transduced through interactions with the PHYTOCHROME INTERACTING FACTORS (PIFs) family of transcription factors. This study assessed the roles of *Arabidopsis thaliana PIF4*, *PIF5* and *PIF7* in mediating shoot architectural responses to high and low R:FR. The genetic interactions between various PIFs and phyB were also examined. The results indicated that PIF4/PIF5 and PIF7 are required for suppression of branch outgrowth under low R:FR, or with the loss of functional phyB. Compared to wild-type, lower levels of axillary bud abscisic acid (ABA) were detected in the *pif7* and *pif4pif5* mutants under low R:FR. The loss of functional phyB elevated axillary bud sensitivity to exogenous ABA. It was also demonstrated that the abscisic acid biosynthetic enzyme NCED3 was essential for aspects of phyB mediated regulation of branching. The analysis of transcript abundances of a panel of auxin-responsive genes in *pif* and *phyB* mutants in the study suggested that PIF4/PIF5 may mediate branching responses by regulating the transcription of auxin-signaling genes. PIF7 mediated effects on bud outgrowth may involve regulation of both ABA abundances and sensitivity in buds. In summary, PIF4/PIF5 and PIF7 affect branching by regulating auxin-signaling in shoots, ABA biosynthesis and sensitivity in buds in response to the R:FR in coordination with phyB. Assessment of the kinetics of axillary bud outgrowth and ABA levels in buds revealed a significant change in bud ABA levels as early as 1 h after alteration of the

R:FR. This indicates that buds are able to rapidly respond to variations in the R:FR. Ethylene is known to mediate plant responses to variation in the R:FR. The assessment of plant architectural changes in the ethylene insensitive mutants *ein2-1* and *etr1-2* revealed a minor contribution of ethylene in mediating branch outgrowth responses to the R:FR. EIN2 and ETR1 were shown to regulate normal gravitropic responses in rosette branches.

DEDICATION

This dissertation is dedicated to my father, a farmer.

"*krisito nasti durbhiksham*"-farming prevents starvation

(Sanskrit source: unknown)

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CHAPTER I

INTRODUCTION

Plant growth and development is extremely plastic, undergoing morphological changes to adapt to fluctuating environmental conditions. Such plasticity is driven in part by the addition of new meristems or by the activation of existing dormant meristems to give rise to new plant organs (Bonser and Aarssen, 2001). Branches form new axes on the shoot and are an important component of shoot architecture. Branches develop from axillary meristems in the leaf axils. These meristems may give rise to a bud that may remain dormant or may grow out to form a branch. Various genetic factors and their interactions with environmental factors are known to affect the branching process (Finlayson et al., 2010; Kebrom et al., 2013). The location of branches on the stem and the patterns of their elongation contribute to the diversity in shoot architecture.

Branching (or tillering in monocots) has great relevance in modern agriculture. Branching has been a criteria for selection during the domestication of food crops. Strong evidence for selection at loci controlling branching in modern cultivars is apparent in various monocots (Doust, 2007) and dicot genomes (Martin-Trillo et al., 2011). Branching is an important trait for agricultural yield and biomass (Khush, 2001) and for ecological adaptation . Our understanding of the branching process in plants has increased in recent years. Several studies have provided insights into the molecular mechanisms and physiological events regulating branching. The role of the phytohormone auxin in controlling apical dominance is well known. A recent addition to

the list of hormones regulating branching includes strigolactone (SL) derivatives (Gomez-Roldan et al., 2008). The polar auxin transport stream and its interaction with other hormones such as cytokinins, abscisic acid (ABA) and SL are known to regulate various aspects of branch outgrowth (Chatfield et al., 2000; Crawford et al., 2010; Domagalska and Leyser 2011). Given the complexity of factors influencing the branching process, it seems likely that additional 'branching hormones' will be discovered in the near future.

Genetic studies on the model plant *Arabidopsis thaliana* have advanced our understanding of branching. Various studies have delineated the roles of hormones and their signaling components influencing axillary buds (reviewed by Beveridge and Kyozuka, 2010; Domagalska and Leyser, 2011). Bud localized transcription factors such as BRANCHED1 have been shown to integrate developmental and environmental cues to regulate bud fate (Aguilar-Martinez et al., 2007). Perhaps the outstanding challenge is the discovery of how various environmental inputs are translated to events that control bud fate. The confounding effects of both developmental programming and environmental regulation of bud outgrowth makes the process very complex.

Light is one of the environmental factors regulating axillary branch development. Changes in the light quality, particularly in the red (R) and far-red (FR) spectra, have been shown to control branching. The variation in the ratio of red light to far-red light (R:FR) perceived by phytochromes (phy), primarily by phyB, has been shown to control branching (Kebrom et al., 2006; Finlayson et al., 2010; Kebrom et al., 2010; Su et al., 2011; Reddy et al., 2013; Reddy and Finlayson, 2014). Low R:FR or loss of phyB

function was shown to suppress branch outgrowth in Arabidopsis (Finlayson et al., 2010). The low R:FR suppression of branching was shown to be due in part to higher ABA levels in the suppressed buds (Reddy et al., 2013). Furthermore, phyB was shown to suppress auxin signaling in the shoot to promote branching (Reddy and Finlayson, 2014).

Recently, a group of basic helix-loop-helix (bHLH) transcription factors termed PHYTOCHROME INTERACTING FACTORS (PIFs) that participate in transducing R:FR signals have been identified (Ni et al., 1999; Huq and Quail, 2002; Huq et al., 2004; Leivar et al., 2008a). Several studies have shown that specific PIFs (PIF1, PIF3-PIF7) physically interact with phyB in the nucleus to mediate various R:FR responses. Previous reports have also shown that PIFs interacts with G-Box elements in the promoters of specific light responsive genes (Hornitschek et al., 2012; Li et al., 2012). Several PIFs have also been shown to directly affect a wide range of phytohormone biosynthesis and signaling components to elicit phyB mediated low R:FR responses (Leivar and Quail, 2011). Notably, PIF4, PIF5, PIF7 have been shown to participate in the regulation of auxin signaling (Nozue et al., 2011; Hornitschek et al., 2012; Li et al., 2012). PIF7 has been shown to affect ABA signaling by repressing the expression of *DEHYDRATION RESPONSIVE ELEMENT BINDING-1C* (Kidokoro et al., 2009). These studies and several other reports suggest that PIFs are the major transcription factors regulating downstream events of phyB signaling. The role of the PIFs in regulating the inhibition of axillary branching by low R:FR is unknown.

Given the need for plants to cope with changing environmental conditions, it appears possible that multiple hormones and their cross-talk modulate many fundamental physiological processes. We now know that the R:FR regulates plant architecture and phyB is the major photoreceptor in the R:FR signaling pathway. However, the role of phyB and its signaling partners (PIFs) in modulating various branching parameters is poorly understood. The hypothesis is that PIFs modulate the transcription of auxin and ABA biosynthesis and signaling genes to regulate axillary branch outgrowth. To test this hypothesis the shoot architectural responses to high and low R:FR were assessed in loss of function PIF4/PIF5 and PIF7 mutants.

A recent investigation compared the transcriptome of axillary buds under low R:FR and providing plants grown under low R:FR with high R:FR (Reddy et al., 2013). The gene expression analysis indicated significant changes in the expression levels of genes involved in the biosynthesis and signaling of various phytohormones, auxin, cytokinins, ABA and ethylene. Furthermore, ABA levels in buds were shown to be important in R:FR regulation of bud outgrowth. It was also shown that ABA levels declined in lower axillary buds within 12 hours (h) of increasing the R:FR, while bud outgrowth was significantly promoted by 24 h when plants were moved from low R:FR to high R:FR at 3 days post-anthesis. As a part of this dissertation study, a more detailed analysis of the kinetics of the bud elongation and changes in the ABA levels were assessed at various time points earlier to 24 hours.

The findings of Reddy and coworkers (2013) also suggest the possible role of ethylene signaling components in regulating R:FR mediated bud outgrowth. Currently,

not much is known about the relevance of ethylene in the R:FR mediated branching regulation. To evaluate the influence of ethylene on axillary branching, ethylene insensitive mutants *ein2* and *etr1* were assessed.

The objectives of the dissertation research are:

1. To characterize the role of phyB and PIFs (PIF4, PIF5, PIF7) in the regulation of various shoot architectural parameters, in response to low R:FR (Chapter III).
2. To assess the kinetics of R:FR modulation of elongation and ABA abundance in the lower rosette buds of Arabidopsis (Chapter IV).
3. To assess the interaction between ethylene and the R:FR in the modulation of shoot architectural parameters such as axillary branching and branch angle (Chapter V).

The literature relevant to this dissertation is reviewed in chapter II. The details of the research conducted to pursue the above mentioned objectives of the dissertation are described in subsequent chapters.

CHAPTER II

REVIEW OF RELEVANT LITERATURE

Branching in dicots, or tillering in monocots, influences plant architecture. Axillary branch development can be separated into several events. The first is the formation of an axillary meristem in the leaf axil, the second is the formation of a bud from the axillary meristem and the third is the outgrowth of the bud. Buds may grow out to form branches or remain dormant or semi-dormant. In *Arabidopsis thaliana* under long days the initiation of axillary buds occurs after the transition to reproductive stage. The buds in the youngest leaf axils grow out first to form branches under long days (Grbic and Bleecker, 2000). Primary branches develop from buds in the cauline leaf axils on the inflorescence stem and in the rosette leaf axils. The buds on these primary branches have the potential to develop into a second order branch, and this pattern may reiterate to produce higher orders of branches. Axillary meristem initiation is influenced by various genetic factors, whereas bud outgrowth is profoundly influenced by the complex interactions arising from both developmental and environmental regulation (McSteen and Leyser, 2005; Leyser, 2009)

2.1 Genetics and initiation of axillary meristems in Arabidopsis

It is generally considered that axillary meristems are products of shoot apical meristems (SAM). However, this may not be the case of Arabidopsis axillary meristems. The reversion of a portion of differentiated cells to meristematic state in the leaf axils gives rise to axillary meristems (Long and Barton, 2000).

The impact of axillary meristem initiation on plant architecture can be grossly noticed in *REVOLUTA* (*REV*) loss of function mutants. The defect in *REV*, a class III HD-ZIP transcription factor, leads to failure in the initiation of axillary meristems resulting in plants that appear to be less branched (Talbert et al., 1995). Loss of *LATERAL SUPPRESSOR* (*LAS*) function also prevents axillary meristem initiation (Schumacher et al., 1999). A Myb domain protein, *REGULATORS OF AXILLARY MERISTEMS* (*RAX*) has also been reported to regulate axillary meristem initiation affecting both cauline branches and rosette branches (Muller et al., 2006). These transcription factors function not only to promote axillary meristem development, but also regulate other developmental processes.

A few genetic components regulating axillary meristem initiation in Arabidopsis and monocots have been identified to be relatively conserved. For instance, a nonfunctional *MONOCULM1* (*MOC1*) gene, orthologous to *LS*/*LAS*, results in the absence of tillers in rice (Li et al., 2003). Several genes required for tiller buds initiation in monocots have been characterized including *BARREN STALK1* (Gallavotti et al., 2004) and *LAX PANICLE* (Komatsu et al., 2001; Komatsu et al., 2003).

Hormonal inputs are known to influence axillary meristems in various ways. A highly proliferative branching habit results from the loss of the gene encoding a cytochrome P450 enzyme, *SUPERSHOOT* (*SPS*) (Tantikanjana et al., 2001). Lesion in *SPS* increased number of meristems in leaf axils and short branches, leading to a bushy phenotype. This is attributed to higher levels of cytokinin accumulation. Abnormalities in auxin transport and signaling affecting organ formation at the shoot apical meristem

are also known to influence axillary bud initiation (Reinhardt et al., 2000). It is likely that other hormones such as ethylene, gibberellins (GAs), and abscisic acid (ABA) may influence lateral bud development individually or in certain combinations. In summary, a complex interaction between hormone homeostasis and transcription factors in the development of axillary meristems may exist.

2.2. Regulation of axillary bud dormancy

2.2.1 Hormonal regulation of branching

After the initiation of axillary meristems, the meristems may develop into an axillary bud. The subsequent outgrowth of the bud is influenced by hormones. Various classes of hormones and their signaling components have been associated with the regulation of bud outgrowth. Auxin has been implicated as the master regulator of apical dominance. Auxin derived from the main shoot apex has been shown to regulate axillary bud outgrowth according to the classical theory of apical dominance. Decapitation of the shoot removes the major auxin source and stimulates lateral bud outgrowth, while the exogenous application of auxin (IAA) to the decapitated shoot reinstates the apical dominance. However, the precise mode of action of auxin remains unclear. Coherent auxin biosynthesis and signaling have been observed to be important for branching. For instance, *35S*:*YUCCA1* genotypes that are overproducers of auxin possess reduced branch numbers (Zhao et al., 2001), whereas the auxin signaling mutant *axr1-12* branches profusely (Stirnberg et al., 1999).

One hypothesis contends that auxin effects on bud outgrowth are mediated by the establishment of auxin export from buds into the polar auxin transport stream (PATS)

(Bennett et al., 2006; Ongaro et al., 2008). According to this hypothesis, export of bud-sourced auxin into the PATS of the shoot promotes bud growth. In intact shoots, the PATS capacity is saturated and decapitation- induced depletion of auxin permits auxin efflux from buds, thereby promoting bud outgrowth. Following the decapitation of the main shoot the bud localization of the auxin efflux transporter PIN1 was observed to become polarized. This polarization may facilitate auxin flow from the bud into the stem and supports the auxin transport canalization hypothesis (Balla et al., 2011). In this study, the authors observed auxin export out of the buds in the decapitated pea plants. Furthermore, polarization of PIN1 auxin efflux carriers facilitating auxin export into the stem was also reported. The study also reported suppressed expression of the dormancy markers *PsDRM1and PsAD1* during the first few hours (~1h) of decapitation. The expressions of *DRM1* and *DRM2* are increased in response to elevated ABA associated with environmental stress (Stafstrom et al., 1998). It would be interesting to examine the changes in ABA abundances in buds in response to decapitation in Arabidopsis at various time intervals.

Many studies have implicated an auxin-dependent second messenger in the regulation of axillary branch outgrowth. Although auxin is considered a master regulator, apically derived auxin does not enter the bud to inhibit bud outgrowth (Booker et al., 2003). Furthermore, application of auxin directly to the buds has no influence on bud outgrowth (Shimizu-Sato and Mori, 2001). Another evidence of an auxin-dependent signal was noticed in pea plants, wherein a time lag was apparent between decapitation and depletion of auxin in stem, while the bud had already initiated growth (Morris et al.,

2005). Furthermore, it was observed that some buds in pea plants were not responsive to the reduction of auxin levels in the stem induced by girdling or by application of auxin transport inhibitor naphthylphthalamic acid (NPA), whereas, these buds were responsive to decapitation (Ferguson and Beveridge, 2009). These investigations and other studies suggest that bud outgrowth is controlled by both an auxin- dependent and an auxin-independent decapitation- induced rapid signal (Cline, 1996; Beveridge et al., 2000). The identity of the auxin- independent signal is currently unknown.

Studies on members of the MORE AXILLARY GROWTH (MAX) family provided evidence for an additional signal controlling branching (Bennet et al., 2006). MAX (MAX1, MAX2, MAX3, and MAX4) loss of function mutants display hyper branching. *MAX1* encodes a cytochrome P450 enzyme, while *MAX3* and *MAX4* encode carotenoid cleavage dioxygenases. MAX1, MAX3 and MAX4 are proposed to be responsible for the biosynthesis of an unknown signal, with MAX1 acting downstream of MAX3 and MAX4 (Sorefan et al., 2003; Booker et al., 2004). MAX1 has been proposed to modify the mobile signal either to facilitate its transport or perception (Booker et al., 2005). MAX2 encodes an F-box LRR protein that appears to function in the perception of the unknown signal (Stirnberg et al., 2002). The MAX- dependent signal was hypothesized to be derived from the roots based on observations from grafting experiments (Bainbridge et al., 2005).

A recent breakthrough in the search for the unknown root- derived signal is the discovery that the MAX-dependent signal is a strigolactone (SL) or derivative (Gomez-Roldan et al., 2008). Strigolactones are derivatives of carotenoids and carotenoid

cleavage dioxygenases (CC7/MAX3, CCD8/MAX4) are enzymes involved in strigolactone biosynthesis. The *max3* and *max4* mutants produce reduced levels of strigolactones (Brewer et al., 2009; Hayward et al., 2009; Crawford et al., 2010). Application of the strigolactone analogue GR-24 suppressed bud outgrowth in the *axr1* and *tir1* auxin signaling mutants, known for prolific branching phenotypes, indicating that the MAX signal acts downstream of auxin (Brewer et al., 2009). In contrast, application of GR24 could not rescue the branching phenotype of *BRANCHED1* (*BRC1*) mutants, suggesting that *BRC1* functions downstream of strigolactones. These observations support the hypothesis that auxin- dependent signaling controls branching via a SL- derivative. The SL-derivative is a signaling molecule transported from root to shoot, that acts to directly or indirectly alter the activities of regulators of the branching process. Expression of a sorghum MAX2 homolog was associated with the R:FR and defoliation and was hypothesized to contribute to regulating axillary branching responses (Kebrom et al., 2010).

In addition to auxin, cytokinins are also known to affect branching process (Muller and Leyser, 2011). A highly proliferative branching habit caused by loss of function of a cytochrome P450 enzyme, SUPERSHOOT (SPS), was associated with higher levels of cytokinin accumulation (Tantikanjana et al., 2001). The lesion in SPS resulted in an increased number of axillary meristems in leaf axils, coupled with increased bud outgrowth, leading to a bushy phenotype. Plants that accumulate cytokinins due to loss of *ALTERED MERISTEM PROGRAM1 (AMP1*) gene also exhibit

hyper-branching (Helliwell et al., 2001). In the majority of cases, genetic studies exploring the role of cytokinins in branching suffer from pleiotropy.

Although ABA is known best as a hormone mediating responses to biotic and abiotic stress, a few reports have suggested a role for ABA in suppressing branching (Tucker, 1977a, 1977b, 1978). Application of ABA to axillary buds suppressed bud outgrowth, while application to the shoot apex induced branching (Tucker 1977b). Higher ABA levels were detected in suppressed buds in *Phaseolus vulgaris* and subsequent reductions in ABA levels due to decapitation were documented (Gocal et al., 1991). Several studies in broad beans and pea documented a strong association between ABA and apical dominance (Arney and Mitchell, 1969; Hartung and Steigerwald, 1977). Arney and Mitchell (1969) proposed that the ABA synthesized locally in the buds effectively inhibits bud outgrowth. This appears to be plausible, because the young buds might not have well-established xylem vascular connections (Sorokin and Thimann, 1964). Histological observation indicated that the establishment of vascular connection with the buds was evident 52-56 hours after decapitation in pea (Sorkin and Thimann, 1964). In another study, about 4-9 times higher ABA levels were documented in buds compared to the nodes and internodes in broad bean plants (Everat-Bourbouloux and Charnay, 1982). Arabidopsis bud anatomy, formation and the signals regulating outgrowth may be different from pea and beans. Further studies may be necessary to reveal the mechanisms of ABA-associated dormancy and its relation to the auxin mediated events in bud outgrowth in Arabidopsis.

Chatfield and coworkers suggested that ABA may affect polar auxin transport (Chatfield et al., 2000). The kinetics of Arabidopsis bud outgrowth were tested in a split-plate assay system, where excised cauline stem segments containing a bud were placed between two agar blocks supplemented with the hormone of interest. In an assay conducted with ABA insensitive mutants (*abi1, abi2*), there was no substantial difference in the bud outgrowth response to auxin in mutant genotypes compared to wild-type plants (Chatfield et al. 2000). Furthermore, application of ABA to the apical part of the segment was not effective in inhibiting bud growth. This suggests that ABA mediated suppression may not be acting from the shoot apex. However, there are possibilities that ABA may be affecting auxin transport in buds and stem. Nevertheless, the study was not comprehensive because the cauline buds on inflorescence stem may not be representative of lower buds in the rosettes. A recent study comparing branching between ABA biosynthesis mutants (*nced3-2* and *aba2-1*) and wild-type has revealed that ABA regulates the suppression of the lower rosettes branches in response to low R:FR (Reddy et al., 2013).

A recent report has shown that reduction is ABA biosynthesis also results in a concomitant decrease in strigolactones (López-Ráez et al., 2010). In tomato, defects in genes encoding an ABA biosynthetic enzyme, 9- CIS EPOXYCAROTENOID DIOXYGENASE (NCED), or application of abamineSG to block NCED enzyme activity were found to suppress strigolactone biosynthesis. It is well known that ABA levels are elevated when plants are stressed due to drought. It is possible that the strigolactone-derivative may be a messenger that conveys information about the below

ground environment. A favorable root environment (less ABA) could promote vigorous plant growth and enhanced branching (less strigolactone biosynthesis).

Ethylene is another important hormone regulating many physiological processes in plants. Currently, no mechanistic studies showing the direct effect of ethylene on apical dominance are available. A few reports have shown that auxin in association with ethylene could influence apical dominance (Prasad and Cline, 1985; Chatfield et al., 2000). Though no defect in axillary branching was observed in Arabidopsis ethylene insensitive mutants (*etr1-1*), the lateral bud elongation rates were reported to be slower compared to wild-type plants (Chatfield et al., 2000).

Another study indicated that ethylene is involved in the regulation of dormancy in perennials such as trees (Ruonala et al., 2006). The transgenic expression of a dominant mutant version of Arabidopsis *ETR1* in birch trees was shown to release apical dominance. Furthermore, compared to wild-type trees, the transgenic birch trees had lower levels of ABA in the buds and were also insensitive to exogenous application of ABA. In contrast, elevated sensitivity to ABA has been reported in Arabidopsis *ein2-1* and *etr1-1* mutants during germination (Beaudoin et al., 2000). These differences may be due to distinct mechanisms operating in the dormancy control of perennial trees compared to annual Arabidopsis.

Dormancy in the buds of perennials such as trees is strongly regulated by many environmental factors, with photoperiod exerting a strong effect. The phytochromes are known to be involved in transmitting the photoperiod signals (Franklin and Quail, 2010). Low R:FR or non-functional phyB have been shown to regulate ethylene production in

sorghum (Finlayson et al., 1998) and Arabidopsis (Vandenbussche et al., 2003). An intricate network involving ethylene, ABA and phytochromes in the control of lateral bud outgrowth may exist in annuals. Photoperiod induced bud dormancy might not be useful in annuals owing to their short life cycle.

Despite, extensive research on the mechanisms of branching control by auxin, cytokinins, strigolactones, and ABA, a comprehensive model integrating all of these components remains to be conceptualized.

2.2.2 Environmental factors affecting bud outgrowth

2.2.2.1 The R:FR regulates branching

Light is one of the environmental cues modulating plant development. The light mediated signaling system is comprised of a large network of components known to regulate a wide range of physiological processes (Quail et al., 1995). The red and far-red regions of the spectrum are very informative to plants. Reduced R:FR generated by neighboring plants has been shown to influence plant form. Plants respond to this competition signal in several ways. In many cases axillary branch outgrowth is suppressed, which may allow resource investment into vertical growth to outgrow competitors (Smith, 2000).

The changes in the R:FR are monitored by phytochromes (phyA-E in Arabidopsis). Among the Arabidopsis phy family members, phyB plays a key role in eliciting the shade avoidance response and to a lesser extent phyD and phyE also act in combination with phyB (Halliday and Whitelam, 2003). *phyB* displays a constitutive shade avoidance phenotype with elongated hypocotyls and petioles, tall stature and early

flowering, which are typical responses to low R:FR. Though the *phyE* phenotype is indistinguishable from wild- type, *phyBE* shows an exaggerated shade avoidance phenotype. The shade avoidance response was abolished in *phyBDE*, suggesting distinct yet overlapping functionality in the genetic network controlling shade avoidance (Franklin et al., 2003).

Plant responses to low R:FR and/or loss of phyB function, such as early flowering, elongation of stems and petioles, and hyponasty have been addressed extensively by several reports (Halliday et al., 1994; Franklin et al., 2003). Recent studies have focused on elucidating the role of phyB and the R:FR in modulating branching in dicots (Finlayson et al., 2010; Su et al., 2011; González-Grandío et al., 2013; Reddy et al., 2013; Reddy and Finlayson, 2014) and monocots (Kebrom et al., 2006; Kebrom et al., 2010). Branching mechanisms in monocots and dicots are fairly conserved. The TCP domain transcription factor TEOSINTE BRANCHED1 (TB1) in maize and its homologs *Sorghum bicolor TB1* and *Arabidopsis thaliana BRC1* (*BRANCHED1*) integrate the R:FR and phyB signals to negatively regulate tillers or branch outgrowth in diverse species (Doebley et al., 1995; Kebrom et al., 2006; Aguilar-Martinez et al., 2007; Finlayson, 2007). In Arabidopsis, suppression of rosette branching in response to low R:FR or loss of phyB has been tightly correlated with the accumulation of *BRC1* transcripts in the axillary buds (Finlayson et al., 2010). Low R:FR or phyB deficiency arrest axillary buds prior to outgrowth, while the early process of axillary meristems initiation remains unaffected (Kebrom et al., 2006; Aguilar-Martinez et al., 2007; Finlayson et al., 2010).

2.2.2.2 Phytochrome Interacting Factors (PIFs) -components of R:FR signaling

Phytochromes exist in two isoforms, a R absorbing form denoted P_r, and a FR absorbing form denoted P_{fr}. P_{fr} is the biologically active form. In the dark, phytochromes (P_r) are cytosolic, but upon exposure to the light the active form (P_{fr}) migrates to the nucleus and initiates a signaling cascade. The abundances of the inactive and active forms are influenced by the R:FR. Low R:FR, leading to relatively low concentrations of the active form (P_{fr}), triggers the shade avoidance response (Franklin and Quail, 2010). The current model holds that the Pfr form interacts with a set of transcription factors which in turn regulate a network of light responsive genes influencing plant growth (Nagy and Schafer, 2002; Bae and Choi, 2008).

Several recent studies on light signaling pathways have deepened our understanding of the components linking light and plant growth. The current models of light signaling have been integrated with bHLH transcription factors known as PHYTOCHROME INTERACTING FACTORS (PIFs) connecting phytochromes to downstream events affecting various processes (Leivar and Quail, 2011). Studies have identified functional roles of PIFs in translating R and FR stimuli into distinct phenotypic responses (Ni et al., 1999; Huq and Quail, 2002; Huq et al., 2004; Khanna et al., 2007; Leivar et al., 2008a; Li et al., 2012). Notably, PIF1 and PIF3 to PIF5 are known to repress photomorphogenesis in the dark, while exposure to light relieves the repression by phy mediated phosphorylation/degradation of the PIFs (Al-Sady et al., 2006; Shen et al., 2007). PIF7 is distinct from the other PIFs as the phosphorylation state is believed to determine its activity, with dephosphorylated PIF7 being inactive (Li et al.,

2012). In addition to seedling photomorphogenesis, studies have provided evidence for the pivotal roles of PIF4, PIF5 and PIF7 in regulating diverse attributes of plant growth (Huq and Quail, 2002; Li et al., 2012). PIF4 has been suggested to regulate flowering time in response to elevated temperature (Kumar et al., 2012). PIF4 and PIF5 have been shown to redundantly regulate leaf hyponasty, and petiole and hypocotyl elongation in response to shade (Lorrain et al., 2008; 2009) and temperature (Koini et al., 2009). Several reports comparing single or multiple *pif* mutants indicated that the PIFs act either separately or synergistically to regulate early light signaling events. Although the functions of PIFs are becoming increasingly apparent in the context of early light regulated growth, much remains to be known about their relevance in the context of more mature plant responses to low R:FR.

2.3 Light and hormone cross-talk influence plant architecture

2.3.1 The R:FR influences auxin biosynthesis, transport and signaling

Light regulates various morphological and physiological responses in plants. These responses include an array of cellular processes involving cell division, differentiation, and expansion primarily controlled by several classes of phytohormones (Woodward and Bartel, 2005). An interesting study in Arabidopsis has provided information on the contribution of auxin to shade avoidance responses. The existence of a light regulated pathway for auxin biosynthesis mediated by TRYPTOPHAN AMINOTRANSFERASE-1 (TAA1) was uncovered in an Arabidopsis mutant screen looking for short hypocotyl phenotypes that fail to elongate under reduced R:FR (Tao et al., 2008). A strong correlation between free IAA levels and the stem elongation rate was

also observed in pea, *Pisum sativum* (Sorce et al., 2008). Dark grown pea plants accumulated higher levels of free IAA than those grown under R, FR or white light indicating a light- mediated modulation of auxin levels. This evidence suggests a strong association between phytochromes and auxin biosynthesis in modulating responses to R and FR.

A model suggesting a link between shade avoidance and auxin was proposed by Moreli and Ruberti (2002). In high R:FR, auxin synthesized in the shoot apex moves basipetally through the central cylinder stream, whereas under low R:FR the movement occurs through peripheral outer cell layers forming a lateral stream (Morelli and Ruberti, 2002). The long distance transport of auxin from the site of synthesis to target cells is mediated by auxin influx and efflux carriers. The polarity of auxin transport is due to the basal localization of plasma membrane bound efflux carriers including the PIN proteins. R:FR induced changes in the polar auxin transport was demonstrated by monitoring the cellular localization of PIN-FORMED3 (PIN3) auxin efflux carriers (Keuskamp et al., 2010). The arrangement of PIN3-GFP markers in hypocotyls changed from basal to lateral orientation in endodermal cells in response to low R:FR. This evidence suggests that auxin transport changed from the central part of the stem to the lateral part. Furthermore, *pIAA19:GUS* staining was observed in lateral parts of hypocotyl when plants were exposed to low R:FR. The influence of AUX/IAA and PIN family members in the suppression of axillary branching by low R:FR remains unknown.

Recent studies on PIF4, PIF5 and PIF7 transcription factors have revealed direct regulation of auxin biosynthesis and signaling (Hornitschek et al., 2012; Li et al., 2012).

The PIF7 transcription factors were shown to bind to promoters of *YUCCA* genes involved in auxin biosynthesis (Li et al., 2012). Another study has shown that PIF4 promotes YUCCA- mediated auxin biosynthesis in response to elevated temperatures in Arabidopsis (Franklin et al., 2011). These studies suggest an interaction between light signaling and auxin biosynthesis that is modified by unfavorable environmental conditions.

Auxin signaling is also known to be affected by the R:FR. In *phyAphyB,* the transcript abundance of *IAA1* and *IAA3*/*SHY2* were up-regulated in shoots compared to wild type (Salisbury et al., 2007). Phytochrome B interaction with SHY2/IAA3, an Aux/IAA protein, was detected by a pull-down assay (Tian et al., 2003). However, mutations in C-terminal PAS domains of phyB do not eliminate this interaction and SHY2/ IAA3 turnover is not influenced by either dark or light treatment. *GH3*s are among the early auxin induced class of genes and play a role in negatively regulating auxin activity by facilitating amino acid conjugation to IAA. phyB dependent regulation of *WES1*, a *GH3-related* gene was reported (Park et al., 2007). Loss of phyB was shown to elevate *WES1/GH3.5* transcript abundances. A recent study has provided insights into the interaction between auxin signaling and phyB function in the branching process (Reddy and Finlayson, 2014). The apical dominance in loss of function phyB plants was attributed to elevated auxin signaling.

Genetic studies employing loss of function mutants in auxin signaling suffer from pleiotropic effects on various physiological processes. For instance, loss of PIN1 auxin transporters in Arabidopsis manifests in a defective inflorescence appearing as a

pin-head (Okada et al., 1991). Loss of function of one member of the Aux/IAA auxin repressor family, IAA16, promoted branch outgrowth, however the mutant plants had other pleotropic defects such as reduced fertility and dwarfness (Rinaldi et al., 2012). Assessing transcriptional changes of genes in the auxin signaling network may provide insights into light mediated regulation of auxin homeostasis.

2.3.2 The R:FR influences ABA biosynthesis and signaling

Although ABA is a well-known hormone, ABA biosynthesis and signaling have not been studied in relation to the R:FR. A small number of reports link ABA to R/FR light action. FR treatment as an end-of- day pulse was effective in suppressing lateral branches with a parallel enrichment of IAA and ABA in mature leaves and shoots in tomato plants (Tucker, 1978). A recent Arabidopsis axillary bud transcriptome study comparing wild-type and *brc1* mutants implied that ABA signaling components are important for stimulation of bud dormancy under FR enriched conditions (González-Grandío et al., 2013). Another study found that ABA levels were low in suppressed lower rosette buds of Arabidopsis plants grown under low R:FR (Reddy et al., 2013). Increasing the R:FR promoted bud elongation and this was accompanied by a significant reduction in bud ABA levels. This indicates that the R:FR regulates ABA levels in buds. The ABA deficient *aba2*-1 and *nced3*-2 mutants exhibited elevated branching under low R:FR compared to WT, indicating that ABA is necessary for proper branching responses to the R:FR.

Much remains to be understood in terms of the involvement of ABA in axillary bud outgrowth. Given the recognized roles of different phytohormones in regulating

branching, it is unclear how the various environmental cues modulate branching and the detailed mechanistic links are still poorly understood.

CHAPTER III

PHYTOCHROME INTERACTING FACTORS MEDIATE ARABIDOPSIS SHOOT ARCHITECTURAL RESPONSES TO THE RED:FAR-RED LIGHT

3.1 Introduction

Plant responses to changes in light quality are mediated by photoreceptors. In *Arabidopsis thaliana* phytochromes (phyA-phyE) regulate various aspects of plant growth and development in response to red light (R) and far-red light (FR). A change in the photoreceptor form and cellular location initiates the light signaling cascade. The red light triggers the conversion of phytochrome from the inactive (Pr) form to the biologically active (Pfr) form, whereas FR has an opposite effect. The R and FR mediated reversible interconversion between the two forms of phytochromes is the basis for the regulatory roles of these light wavelengths on plants (Casal et al., 2004; Bae and Choi, 2008). The current understanding of R:FR signal transduction is that the activated photoreceptor (Pfr) is translocated from the cytosol to the nucleus where it physically interacts with PHYTOCHROME INTERACTING FACTORS (PIFs) (Bauer et al., 2004; Al-Sady et al., 2006; Leivar and Quail, 2011). PIFs are basic helix-loop-helix (bHLH) domain transcription factors identified as negative regulators of photomorphogenesis in dark growing Arabidopsis (Ni et al., 1999; Huq and Quail, 2002; Huq et al., 2004; Khanna et al., 2007; Leivar et al., 2008a; Moon et al., 2008; Li et al., 2012). Molecular assays indicated that the active form of phyA or phyB interacts with PIFs (PIF1, PIF3 to PIF5) in the nucleus. The phys (phyA-phyE)

have been shown to modify PIF phosphorylation status, thereby activating proteasome mediated PIF degradation (Al-Sady et al., 2006; Shen et al., 2007; Lorrain et al., 2008). PIF7 is distinct from other PIFs, as it accumulates in the nucleus under high R:FR (Leivar et al., 2008a). The reversible phosphorylation and de-phosphorylation status of PIF7 in response to changes in R:FR, mediates downstream transcriptional responses of the light signaling cascade (Li et al., 2012). Similar to other PIF members, it is suggested that the PIF7 may interact with phyB (Leivar et al., 2008a). The stability of the PIFs is relevant to plant responses to the R:FR.

In addition to regulation of seedling photomorphogenesis, studies have implicated PIF4, and PIF5 in regulating diverse attributes of plant growth (Huq and Quail, 2002; Shen et al., 2007; Li et al., 2012). PIF4 has been shown to regulate flowering time in response to elevated temperature (Kumar et al., 2012). PIF4 and PIF5 have also been shown to regulate elongation of petiole and hypocotyl in response to the R:FR (Lorrain et al., 2008, 2009) and temperature (Koini et al., 2009). Several reports comparing lines with single or multiple pif-mutations indicated that the PIFs act either separately or synergistically to regulate R:FR modulated plant development (Leivar et al., 2008b). Although the functions of PIFs are becoming increasingly apparent in the context of early light regulated development, much remains to be known about their relevance in the context of mature plant architectural responses to far-red enrichment or due to foliar shade.

Light quality is one of the environmental factors influencing the development of plant shoot architecture, including axillary branching. Light quality, especially the R:FR

influences the branching habit of plants (Finlayson et al., 2010; Reddy et al., 2013). Plastic branching responses to the R:FR result from the influence of a variety of factors. Some of these factors are known to be bud specific while others reflect integrated effects of overall plant development. Previous reports have shown that the position of the bud in the plants is an important factor determining the potential to grow out. The elongation of lower buds in the Arabidopsis rosettes was shown to be tightly controlled by the R:FR. The outgrowth of lower buds was promoted when plants grown under low R:FR were moved to high R:FR (Reddy et al., 2013). These studies indicated that branching is tightly regulated by the R:FR.

Various hormones and their signaling pathways have been associated with axillary branching. The process of auxin mediated apical dominance has been studied extensively (reviewed in Domagalska and Leyser, 2011). Recently, a new class of hormone(s) derived from strigolactones (SL) were implicated in suppressing branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). The auxin signaling component AXR1 has also been demonstrated to be necessary for regulation of apical dominance (Hayward et al., 2009; Stirnberg et al., 1999). Loss of AXR1 function promotes prolific branch outgrowth. Besides auxin and SL, other phytohormones such as cytokinins and abscisic acid (ABA) have also been implicated in the control of bud outgrowth (Cline and Oh, 2006; Ferguson and Beveridge, 2009; reviewed by Muller and Leyser 2011). The involvement of various hormones in regulation of bud outgrowth suggests that multiple pathways control branching processes.

Several recent studies have focused on understanding the mechanistic link between hormones and environmental signals in branching. Light quality, especially the R:FR modifies plant architecture (Casal et al., 2004). Suppression of rosette branching due to low R:FR or non-functional phyB has been demonstrated previously (Finlayson et al., 2010). Recently, evidence for an interaction between the R:FR and ABA levels in the buds has been revealed (Reddy et al., 2013). Higher ABA abundances in the lower rosettes buds were associated with the suppressed state of these buds. In another report, elevated auxin signaling in the shoot was linked to enhanced branch suppression in *phyB* mutants (Reddy and Finlayson, 2014). These studies highlight that the R:FR mediated regulation of branching involves multiple mechanisms operating in the shoots as well as in buds. Furthermore, it suggests that these regulatory mechanisms may act synergistically or separately.

Plant architectural changes to the R:FR have been linked to marked alterations in the biosynthesis and signaling of various hormones (Stamm and Kumar, 2010). Recent studies have demonstrated that phyB-PIF signaling functions affect multiple events in the hormone metabolism and signaling (Khanna et al., 2007; Kidokoro et al., 2009; Franklin et al., 2011; Hornitschek et al., 2012). Light mediated perturbations in hormonal status are also known to influence various events of plant growth and development including axillary branch outgrowth. The current study was aimed at characterizing the roles of PIF4, PIF5 and PIF7 in the modification of shoot architecture in responses to the R:FR.

3.2 Results

3.2.1 PIFs modulate plant architectural responses to the R:FR

The effects of low R:FR or phyB loss of function on various attributes of plant architecture have been described in previous studies (Finlayson et al., 2010). To assess the contribution of PIFs in mediating architectural responses to the R:FR, the loss of function mutant genotypes *pif4pif5* and *pif7* were studied in detail. A comparative assessment of branching in monogenic *pif4*, *pif5* and the double mutant *pif4pif5* revealed an additive action of these two genes. To extend the studies on analyzing interactions between phyB and PIFs, the triple mutant *phyBpif4pif5* and the double mutant *phyBpif7* were also included in the study.

3.2.1.1 Plant height

Increased shoot elongation is a typical response to shade or low R:FR. The height of *pif4pif5* was promoted less by low R:FR compared to wild-type (Fig 1A). No such differences were observed in *pif7* mutants. However, loss of PIF4PIF5 or PIF7 resulted in a significant height reduction under high R:FR. In comparison to monogenic *phyB* mutants, there was a significant reduction of height in *phyBpif4pif5* plants, but no apparent difference in the height of *phyBpif7* was noted. Attenuation of responses to low R:FR in terms of hypocotyl elongation in *pif4 and pif4pif5* seedlings has been previously reported (Huq and Quail, 2002; Leivar et al., 2008b; Lorrain et al., 2009). The reduction in shoot height of *phyBpif4pif5* observed in this study suggests that PIF4/PIF5 negatively regulate phyB responses. Although loss of *pif7* reduced the height of plants under high

R:FR, the elongation responses to low R:FR were retained. This would suggest that shoot elongation responses to low R:FR are partly regulated by PIF4/PIF5 and PIF7.

3.2.1.2 Flowering time and rosette leaves

Plants flower early in response to low R:FR (Halliday et al., 1994; Kim et al., 2008). Although the transition from the vegetative to the reproductive stage involves both developmental and environmental cues, low R:FR induced early flowering is known to be mediated by phyB (Endo et al., 2013). In Arabidopsis, flowering time is generally inferred from the number of rosette leaves.

Under high R:FR, loss of PIF4/PIF5 function resulted in marginally reduced rosette leaf numbers compared to WT, whereas *pif7* flowered later than wild-type and accumulated more rosette leaves (Fig. 1B). There was no significant difference between wild-type and *pif4pif5* under low R:FR, in contrast, *pif7* accumulated more rosette leaves compared to the wild-type plants. Interestingly, loss of either PIF4/PIF5 or PIF7 in the *phyB-9* background delayed flowering and increased the number of rosette leaves compared to *phyB*. This could imply that PIF4/PIF5 and PIF7 actuated early flowering in *phyB*-9 mutants. In addition to phyB, phyE has also been implicated in regulating flowering time (Halliday et al., 1994). Besides R and FR signals, inputs influencing the floral transition are delivered by a huge network of genes controlled by developmental and photoperiodic cues (Andres and Coupland, 2012).

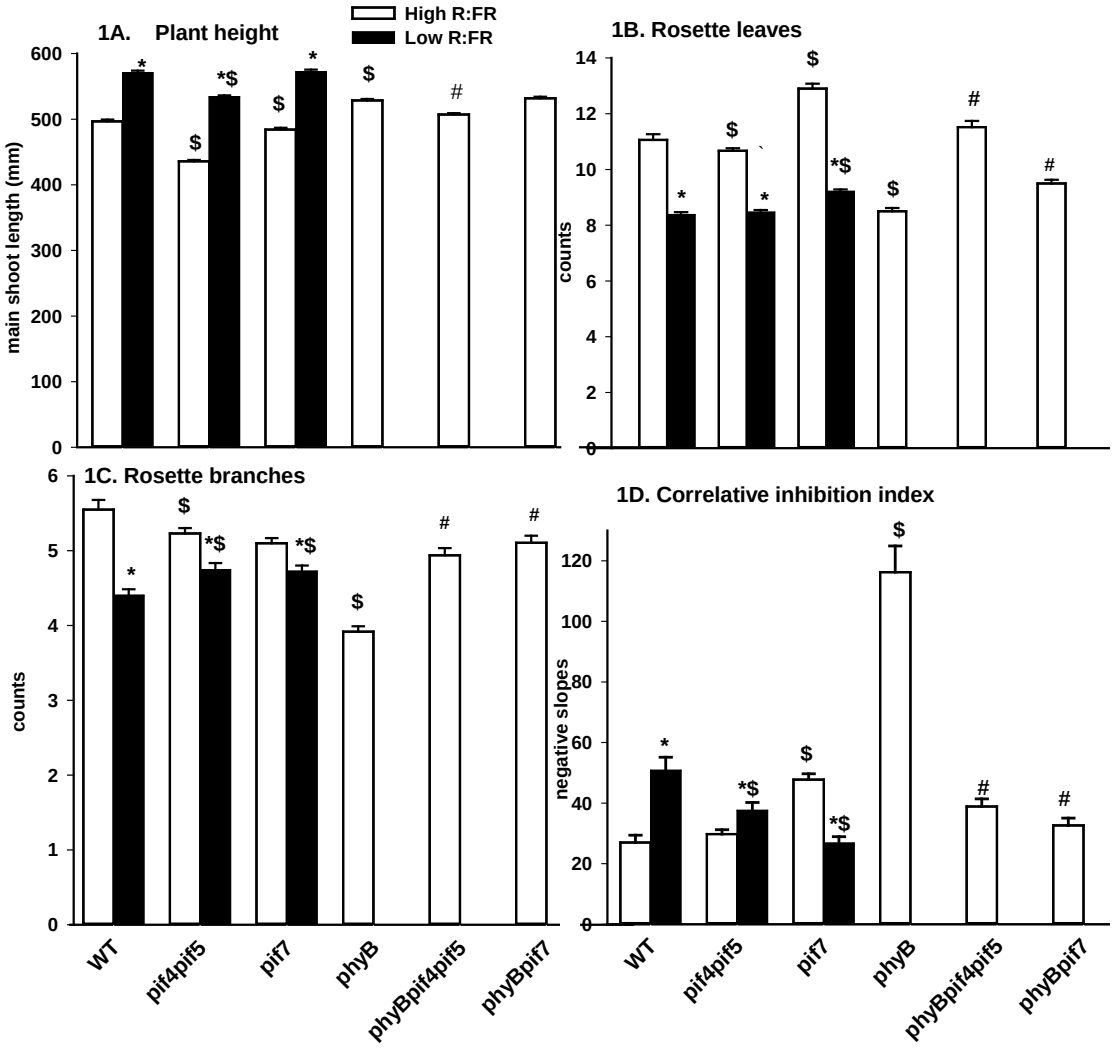

Figure 1. Shoot architectural parameters: A) plant height, B) number of rosette-leaves, C) number of rosette branches, D) correlative inhibition index of various genotypes at 10 days post-anthesis. Genotypes with functional phyB were grown under high R:FR and low R:FR and those without functional phyB were grown under high R:FR. Asterisks (*) and dollar signs ($) indicate a significant difference ($p<0.05$) between light treatments and within light treatment compared to wild-type respectively. Hash (#) indicate a significant difference ($p<0.05$) compared to *phyB*. Data represent means ± SE; n=36

3.2.1.3 Rosette branch numbers

Rosette branches arise from axillary buds in the rosette leaf axils and each of these buds has the potential to grow out to form a branch (Finlayson et al., 2010). Under long days, the bud in the youngest leaf axil elongates first to form a branch. In this study, the number of elongated rosette branches (>3mm) were recorded at 10 days post-anthesis. Under low R:FR, more branches were recorded in plants without functional PIF4/PIF5 and PIF7 compared to wild-type (Fig. 1C). Significant increases in the rosette branch numbers in *phyBpif4pif5* and *phyBpif7* compared to *phyB* were also observed. Strong apical dominance due to low R:FR or phyB deficiency has been reported earlier (Finlayson et al., 2010; Reddy et al., 2013, Reddy and Finlayson, 2014). This suggests that PIF4/PIF5 and PIF7 negatively regulate axillary bud outgrowth in response to low R:FR or loss of phyB function.

3.2.1.4 Correlative inhibition of axillary branches

Since both the number of branches and their relative elongation rates govern the shoot architecture, the lengths of individual rosette branches were measured. In Arabidopsis, the upper branches and the shoot apex are known to suppress the lower branches by a process termed correlative inhibition (Finlayson et al., 2010; Reddy and Finlayson, 2014).

In comparison to wild-type plants, the increase in correlative inhibition due to low R:FR was significantly attenuated in *pif4pif5* and *pif7* (Fig. 1D). Likewise, loss of PIF4/PIF5 and PIF7 function promoted branching in the *phyB* null background. However, loss of PIF7 function also resulted in increased correlative inhibition under

high R:FR. Elevated correlative inhibition due to deficiency in phyB or low R:FR has been previously reported (Finlayson et al., 2010; Su et al., 2011; Reddy et al., 2013). Loss of PIF4/PIF5 function under low R:FR or in the *phyB* null background moderately reduced the correlative inhibition. However, loss of PIF7 function completely abolished correlative inhibition due to low R:FR or phyB deficiency. These results indicate that different mechanisms control branching responses to low R:FR or loss of functional phyB. The visual phenotypes of various genotypes under are provided in Fig 2.

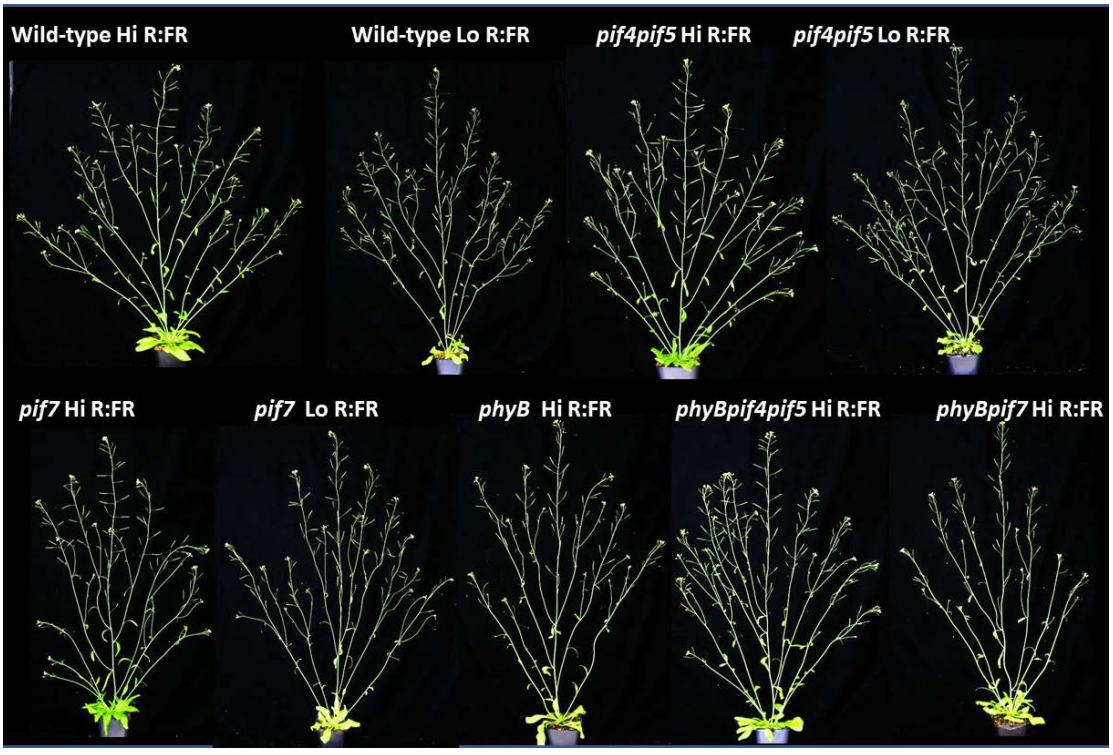

Figure 2. Visual phenotypes of various genotypes at 10 days post-anthesis. Plants were grown under high R:FR (Hi R:FR) and low R:FR (Lo R:FR).

3.2.2 ABA abundance in axillary buds

To further investigate the interactions between PIFs and hormones involved in branching process, the ABA abundance in axillary buds was quantified. Previously, the ABA abundance in buds ‘n’ and ‘n-2’ was correlated with bud outgrowth responses to the R:FR (Reddy et al., 2013). In the current study, largely in all the genotypes, under both high and low R:FR, the ABA levels in the ‘n-2’ buds were higher than outgrowing ‘n’ buds (Fig. 3).

Higher ABA levels were recorded in both ‘n’ and ‘n-2’ buds from *pif7* grown under high R:FR relative to wild-type. In contrast, lower ABA levels in *pif7* were detected under low R:FR light. Bud ABA levels were much lower in *pif4pif5* loss of function mutants compared to wild-type under identical R:FR. In the *phyB* null background, loss of PIF4/PIF5 function elevated bud ABA content, whereas reduced ABA abundance was observed in *phyBpif7*.

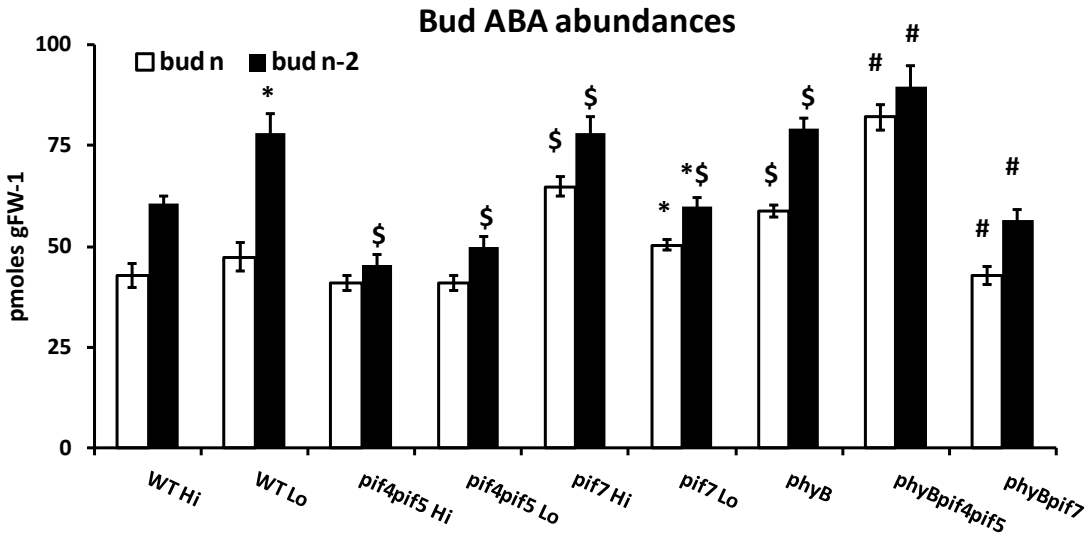

Figure 3. ABA abundances in bud 'n' and 'n-2' of various genotypes with functional phyB grown under high R:FR and low R:FR and without functional phyB grown under high R:FR. All comparisons are performed at identical bud positions. Asterisks (*) and dollar signs ($) indicate a significant difference ($p<0.05$) between light treatments and within light treatment compared to wild-type (WT) respectively. Hash (#) indicate a significant difference ($p<0.05$) compared to *phyB*. Data represent means ± SE; n=4. Hi- high R: FR; Lo- low R:FR.

3.2.3 Plant ethylene production

The R:FR is also known to influence ethylene biosynthesis. Loss of phyB function has been shown to elevate ethylene production in sorghum (Finlayson et al., 1998) and Arabidopsis (Vandenbussche et al., 2003). In the current study, ethylene production by the various genotypes under low R:FR and high R:FR was measured. Plants at 14 days post-sowing were used to measure ethylene because measurements from axillary buds were cumbersome.

Ethylene production was dramatically elevated in *phyB* mutants and *phyBpif4pif* mutants compared to other genotypes in the study (Fig. 4). However, there was a

substantial reduction in ethylene production in *phyBpif7* compared to *phyB*. Compared to wild-type, plants with non-functional PIF4/PIF5 had higher ethylene levels under high R:FR. *pif7* had reduced ethylene levels under low R:FR.compared to wild-type levels.

Earlier reports have suggested that low R:FR stimulates ethylene biosynthesis (Finlayson et al., 1998; Vandenbussche et al., 2003). In this study, ethylene levels were not significant different between low R:FR and high R:FR in wild-type plants. In comparison to wild-type, loss of PIF4/PIF5 elevated ethylene biosynthesis under high R:FR, whereas there was no apparent difference under low R:FR. Previously, ectopic expression of PIF5 has been reported to promote ethylene levels, whereas no apparent changes in ethylene levels were recorded in loss of function *PIF5* (Khanna et al., 2007). Currently, there are no reports on action of PIF4 on ethylene biosynthesis.

The results from this study revealed that loss of PIF7 negatively regulated ethylene production under low R:FR or in *phyB* mutants background. It may be concluded that PIF7 mediates ethylene production under low R:FR or in the case of non-functional phyB. Further investigations may be necessary to assess the mode of action of PIF7 on ethylene biosynthesis.

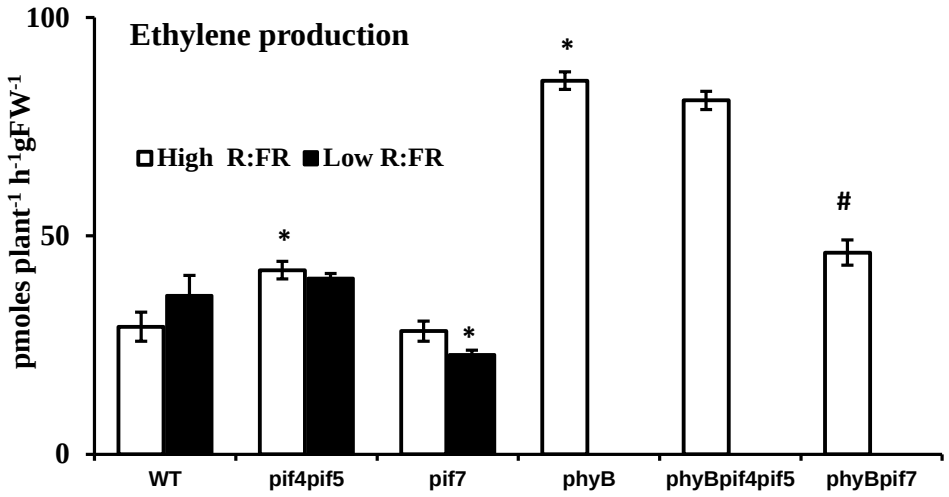

Figure 4. Ethylene production rate of various genotypes. Genotypes with functional phyB were grown under high R:FR and low R:FR and those without functional phyB were grown under high R:FR. Asterisks (*) indicate a significant difference ($p<0.05$) between light treatments or genotypes. Hash (#) indicate a significant difference ($p<0.05$) compared to *phyB*. Data represents means ± SE; n=4 plants

3.2.4 Effect of the R:FR and non-functional phyB and PIFs on sensitivity of buds to exogenous ABA

To test if the sensitivity to ABA is affected in due to R:FR or loss of functional phyB and PIFs, 100 picomoles of ABA was applied to the top five rosette buds at two days prior to the expected day of anthesis. At 8 days post-anthesis, branch lengths were measured and the ratio of the length of ABA treated buds compared to that of the control at their respective positions was computed.

The application of ABA suppressed the lower buds (n-1, n-2) more in comparison to 'n' bud in all the genotypes (Fig. 5). Among the loss of function genotypes assessed under high R:FR, *pif7* showed a reduced sensitivity to ABA. Under low R:FR, loss of PIF4/PIF5 and PIF7 enhanced sensitivity of buds to ABA in comparison to wild-type.

Loss of phyB elevated the sensitivity of buds to ABA in wild-type, *pif4pif5* and p*if7* genotypes. This suggests that phyB regulates bud outgrowth by modulating ABA sensitivity in buds. Furthermore, bud ABA abundances were higher in *phyB* than in the wild-type (Fig. 3). To assess an interaction between ABA biosynthesis gene NCED3 and phyB, the double mutant combination *phyB-9nced3*-2 were synthesized and branching parameters were recorded (Fig. 6).

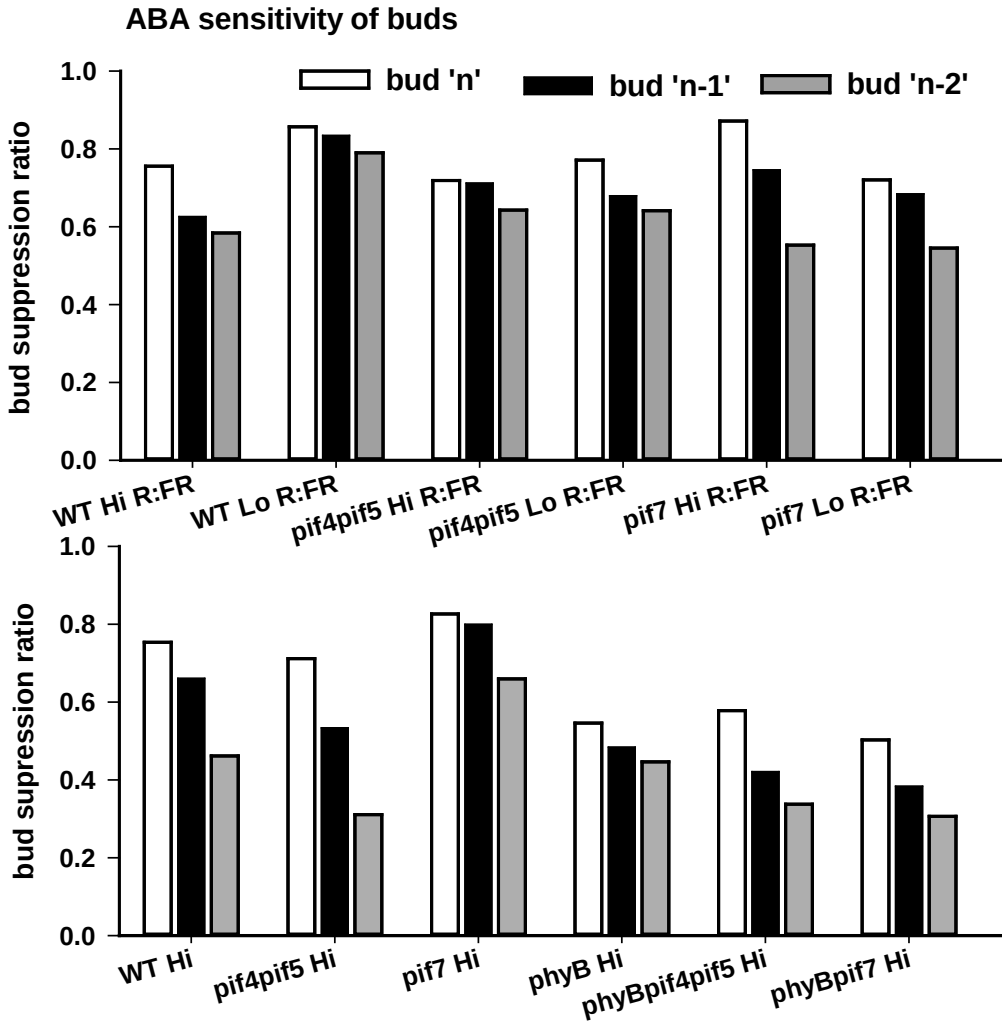

Figure 5. ABA sensitivity of rosette buds assayed by application of exogenous ABA to buds. 100 picomoles of ABA was applied to rosette buds at 2 days prior to expected anthesis. At 8 days post anthesis, the bud length at each position was measured. The ratios of ABA treated to control bud lengths were computed. Genotypes with functional phyB were grown under high R:FR (Hi) and low R:FR (Lo) and those without functional phyB were grown under high R:FR. There were 18 plants per treatment per genotype.

3.2.5 Genetic test to assess the interaction between ABA biosynthesis and phyB.

The elevated ABA levels in the *phyB* buds and plants grown under low R:FR correlated with the suppressed state of buds. The role of ABA in generating the branching phenotype of phyB deficient plants was investigated, by testing the genetic interaction between the ABA biosynthesis gene NCED3 and phyB. The double mutant genotype was developed by combining the *nced3*-2 and *phyB-9* mutations and the phenotype of *phyBnced3-2* was assessed (Fig. 6). There was no difference between monogenic *phyB* and double mutant *phyBnced3*-2 with respect to rosette leaf numbers. However, there was a significant increase in the number of elongating branches and a reduction in the correlative inhibition in *phyBnced3-2* in comparison to *phyB*. This shows that wild-type ABA biosynthesis is necessary for the typical reduced branching of *phyB*, and suggests that phyB may negatively regulate *NCED3* expression to promote branch outgrowth. The promotion of lower branches in *phyBnced3-2* plants indicates that ABA exerts a strong suppressive effect on these buds. These results are in agreement with a previous report showing promotion of lower branches due to loss of *NCED3* and *ABA2* in plants grown under low R:FR (Reddy et al., 2013).

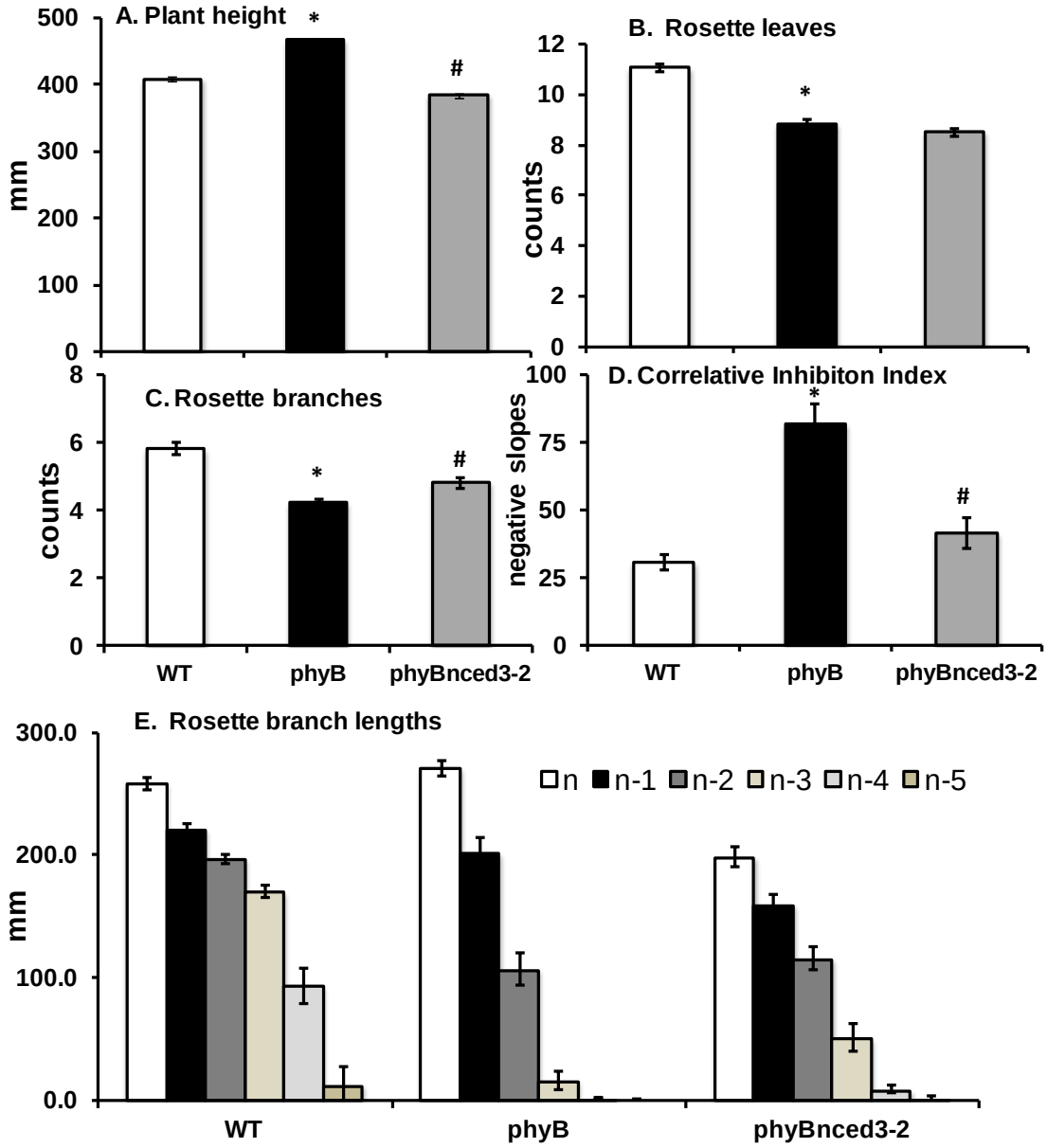

Figure 6. Branching parameters of wild-type, *phyB* and *phyBnced3*-2 genotypes. Architectural parameters include: A) plant height, B) number of rosette leaves, C) number of rosette branches, D) correlative inhibition index E) axis length of rosette branches of various genotypes at 10 days post anthesis. Plants were grown under high R:FR. Asterisks (*) indicate a significant difference ($p<0.05$) between wild-type (WT) and *phyB*. Hash (#) indicate a significant difference ($p<0.05$) compared to *phyB*. Data represent means ± SE; n=18.

3.2.6 Auxin-responsive gene expression

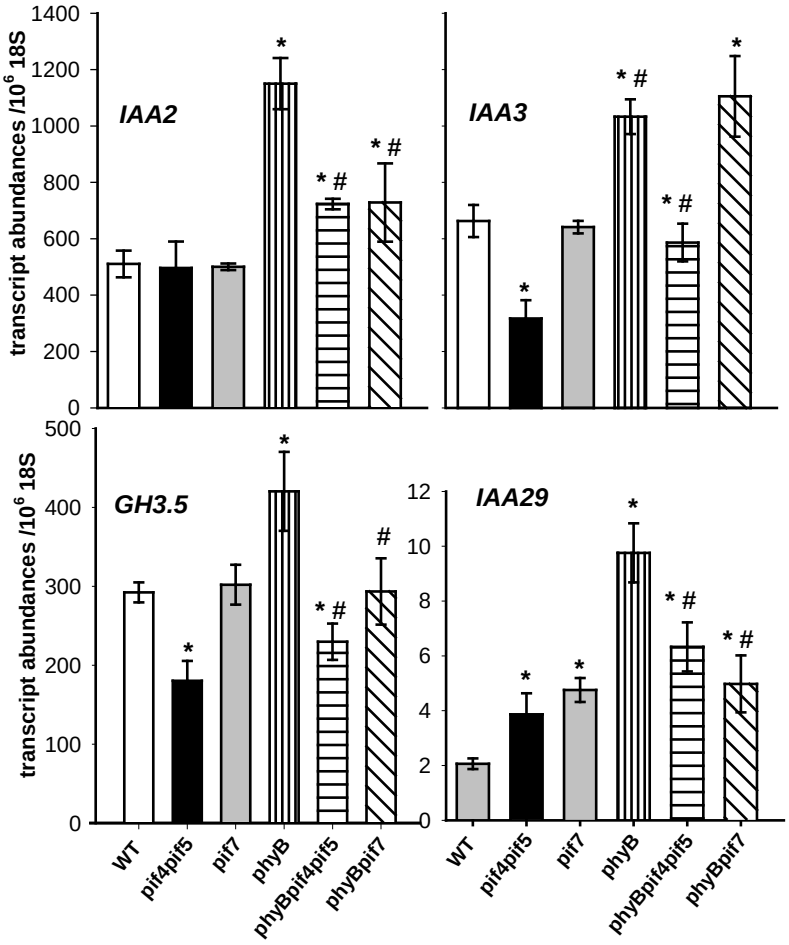

Figure 7. The transcript abundances of auxin-responsive genes in stem sections of various genotypes grown under high R:FR. Asterisks (*) indicate a significant difference ($p<0.05$) from WT. Hash (#) indicate a significant difference ($p<0.05$) compared to *phyB*. Data represent means ± SE; n=4.

Previously, elevated expression of a panel of auxin-response genes was correlated with the increased apical dominance phenotype of *phyB* (Reddy and Finlayson, 2014). The expression of similar auxin-responsive genes was investigated in stem segments in this study

In phyB mutants, the transcript abundances of *IAA2*, *IAA3*, *IAA29* and *GH3.5* were significantly elevated (Fig. 7). The loss of PIF4/PIF5 function in the *phyB* background conferred a significant reduction in the expression of the genes assayed. This suggests that PIF4/PIF5 function to increase auxin signaling in *phyB.* In wild-type background, non-functional PIF4/PIF5 enhanced the expression of *IAA29* whereas, suppressed the expression of *IAA3*, *GH3*.5. However, expression of *IAA2* was similar between *pif4pif5* and wild-type plants.

There were no differences in the expression of the auxin responsive genes *IAA2*, *IAA3* and *GH3*.5 between *pif7* and wild-type. However, *IAA29* expression was elevated in *pif7* compared to expression levels in the wild-type. Loss of PIF7 function in the *phyB* background revealed a differential response. In *phyBpif7*, the expression of the auxin-responsive genes *IAA2*, *IAA29* and *GH3*.5 were reduced whereas, *IAA3* was unaffected in comparison to phyB. This shows that IAA3 may not be regulated by PIF7.

3.3 Discussion

Architectural changes to low R:FR or loss of phyB function have been addressed in previous studies (Kebrom et al, 2006; Finlayson et al., 2010; Su et al., 2011, Reddy et al., 2013, Reddy and Finlayson, 2014). The bHLH transcription factors PIF4, PIF5 and PIF7 act in the signaling pathway downstream of phyB, and have been previously

characterized for their roles in a few aspects of photomorphogenesis and shade-avoidance responses (Leivar et al., 2008a; Lorrain et al., 2009; Li et al., 2012). The current investigation was carried out to assess the roles of PIF4/PIF5 and PIF7 in regulating architectural responses to the R:FR and phyB deficiency. The loss of PIF4/PIF5 and PIF7 partially suppressed various shoot architectures response to low R:FR (Fig. 1). Apical dominance associated with the loss of phyB function was also diminished in *phyBpif4pif5* and *phyBpif7*. PIF4/PIF5 and PIF7 function to promote shade responses by suppressing the number of elongated rosette branches and elevating the correlative inhibition of branches. The roles of PIF4 and PIF5 in mediating plant responses to low R:FR or the partial suppression of the *phyB* phenotype have been shown in previous studies (Lorrain et al., 2008). The suppression of branching (elevated correlative inhibition) due to the loss of PIF7 function under high R:FR was surprising and warrants further inquiry. Previous studies have indicated that unlike other PIFs, PIF7 protein is not degraded under high R:FR (Leivar et al., 2008a; Li et al., 2012). Furthermore, PIF7 has previously been shown to negatively regulate the expression of stress responsive DREB transcription factors in a circadian pattern (Kidokoro et al., 2009). It is possible that an elevated stress response in *pif7* may suppress the branching process.

ABA levels were quantified in axillary buds to determine if PIFs regulate its accumulation. Previously, elevated bud ABA levels were correlated with the suppressed state of lower rosette buds and the ABA biosynthetic enzymes NCED3 and ABA2 were shown to be necessary for low R:FR mediated bud dormancy (Reddy et al., 2013). The

results of the current study corroborate the earlier findings, as elevated ABA levels occurred in the ‘n-2’ buds in all the genotypes. The ABA levels also correlated well with the elongation potential of all of the buds except for *phyBpif4pif5*. The ABA levels in *phyBpif4pif5* were higher than monogenic *phyB* even though it generated more elongated branches than *phyB.* The unanticipated higher ABA levels in *phyBpif4pif5* could reflect differential ABA sensitivity in this line, or the elevated ABA might be overridden by stronger effects of auxin signaling that could exhibit a greater influence on bud fate. The observed promotion of branches in *phyBnced3-2* compared to *phyB* in this study would suggest that ABA biosynthesis/signaling is a component of phyB effects on branching.

The variation in the sensitivity of buds to ABA was tested by exogenous application of ABA to buds. The loss of functional *PIF4/PIF5 or PIF7* conferred enhanced ABA sensitivity to buds under low R:FR or in the *phyB* null background. The influence of R:FR on ABA sensitivity in buds is a not a well-known mechanism. The regulation of ABA sensitivity in buds may be mediated by phyB and PIF action. ABA perception is known to be regulated by various member belonging to PYR/PYL family and G-protein coupled receptors (Raghavendra et al., 2010). A previous investigation has shown that various members of PYL/PYR family were differentially expressed in loss of function *phyB*-5 (GonzÁLez et al., 2012). ABA sensitivity is also known to be regulated by various members of PROTEIN PHOSPHATASE 2C gene family such as *ABA INSENSITIVE 1* (*ABI1*), *ABI2* (Hirayama and Shinozaki, 2007). It is apparent that ABA biosynthesis, perception and signaling underlie co-ordination of huge network of cellular components. Regardless of the ABA perception mechanism, the results from the

present investigation (Fig. 5) demonstrated that loss of phyB or PIFs affect bud ABA sensitivity.

The loss of phyB has been shown to promote ethylene biosynthesis (Finlayson et al., 1998; Vandenbussche et al., 2003). To determine if PIF4/PIF5 and PIF7 mediate this response, ethylene production was quantified in various loss of function genotypes. Higher levels of ethylene biosynthesis were evident in *phyB* and *phyBpif4pif5*, whereas a strong reduction in ethylene production was noted in *phyBpif7*. Furthermore, under low R:FR *pif7* produced less ethylene compared to wild-type. This reveals that PIF7 promotes ethylene biosynthesis under low R:FR or with loss of functional phyB. Previously, it has been shown that ectopic expression of PIF5 enhanced ethylene levels (Khanna et al., 2007). Ethylene has previously been shown to be necessary for some shade-avoidance responses (Pierik et al., 2004), however there are no previous studies demonstrating the influence of ethylene on axillary branching. A parallel study has revealed that axillary branching was affected in the ethylene insensitive genotypes *ein2-1* and *etr1*-2 under low R:FR.

Auxin signaling in shoots is known to have a major impact on branching. According to the canalization hypothesis, the shoot may act as a sink for auxin exported from the buds (Li and Bangerth, 1999; Domagalska and Leyser, 2011). Auxin levels and auxin-signaling may influence PIN and other auxin efflux carriers, and thereby determine the auxin export rate from the buds into stems. The basis of the apical dominance in the *phyB* mutants has been linked to elevated auxin signaling (Reddy and Finlayson, 2014). A panel of auxin-responsive genes previously assayed was included in

this study to probe auxin signaling status. Loss of *pif4pif5* in *phyB* backgrounds suppressed the expressions of auxin-responsive genes assessed in this study. Previously perturbation of auxin-signaling due to loss of PIF4/PIF5 function has been reported (Hornitschek et al., 2012). In the present study, the loss of PIF7 function differentially affected the expressions of various auxin-responsive genes assessed. A previous study assessing *pif7* seedling responses to shade demonstrated that the transcript levels of auxin-signaling genes *IAA2, IAA3, IAA29* and the auxin metabolism gene *GH3.5* were not responsive to changes in the R:FR (Li et al., 2012). The evidences from various studies suggest that PIF4, PIF5 and PIF7 modulate the expression of several members of YUCCA auxin-biosynthetic genes (Hornitschek et al., 2012; Li et al., 2012). It may be necessary to quantify the auxin abundances in stems segments of various genotypes included in the present study. The transcriptional regulation of auxin-responsive and signaling components is influenced by multiple factors such as light, circadian rhythm, auxin transport and biosynthesis (Vanneste and Friml, 2009). Feedback regulation of AUX/IAA transcriptions are also documented at various instances (Dreher et al., 2006). A recent model of auxin perception hypothesizes that the AUX/IAA proteins partners with auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) (Calderón Villalobos et al., 2012). Functional characterization of the AUX/IAA proteins may be necessary to establish a causal link between branching and auxin-signaling. However, plants with loss of various auxin-signaling functions often display pleiotropic defects. A direct link between the auxin signaling and branching may be inferred from loss of

function *axr1-12* mutants (Leyser et al., 1993). *phyB* mediated branch suppression also required functional AXR1 (Finlayson et al., 2010).

In summary, the results suggest that PIF4, PIF5 and PIF7 promote apical dominance under low R:FR or due to loss of phyB function by modulating auxin signaling in the stems and ABA levels in the buds. Axillary branching is a complex process involving various hormones such as SL and cytokinins in addition to auxin and ABA. Furthermore, shade-avoidance responses are regulated by a huge network of genes. The bud specific regulator BRC1 has been shown to affect bud fate under low R:FR (Aguilar-Martinez et al., 2007; Finlayson et al., 2010). Further investigation on the transcript abundances of bud localized regulators in relation to PIF4/PIF5 and PIF7 may be useful.

3.4 Materials and Methods

3.4.1 Plant materials and growth conditions

Arabidopsis ecotype Col-0 (CS 60000) was used throughout the study. The *pif4-101* (Garlic_114_G06) and *pif5* (SALK-087012) mutants were kindly provided by Christian Fankhauser (University of Lausanne, Switzerland). *phyB-9* has been previously described (Reed et al., 1993). *pif7-1* and *phyB-9pif7*-1were kindly provided by Dr. Peter Quail. *nced3-2* has been described previously (Urano et al., 2009). The *phyB-9* mutation in *nced-2phyB-9* was confirmed by cloning and sequencing to identify the point mutation. Double and triple mutant combinations used in the study were generated in the laboratory and genotyped by following the standard PCR protocol to confirm T-DNA insertions and the homozygosity in mutant genotypes.

Seeds were stratified at 4°C for 3 days and then grown in trays with six-cell inserts (36 plants per tray) using LC-1 soilless potting mixture. Plants were fertilized weekly with 5 mL of Hoagland's nutrient solution at a week's interval. Plants were grown under 18 h/6 h light/dark photoperiods with 24°/18 ^{o}C day/night temperatures in a growth chamber with T5 fluorescent lamps as the light source. An overhead array of LEDS emitting FR (735 nm) was used to simulate competition signals. The light was set to 185 μmol m^{-2} s^{-1} PPFD. Plants were grown under a high R:FR of 4.0 for seven day at which time the FR LEDs were turned-on to provide low R:FR (0.075). One set of plants was maintained under high R:FR. A barrier in the middle of the growth chamber was used to prevent the light from one side of the chamber reaching the other. Architectural parameters were recorded at ten days post anthesis (DPA) as described in Finlayson *et al.* 2010. A bud more than 3 mm of length was considered a branch. Comparisons between means were made using a two-tailed t-test with $\alpha = 0.05$. The experiments were repeated twice and data were pooled.

3.4.2 Hormone abundance estimation

Phytohormones were quantified by GC-MS. Axillary rosette buds (n, n-2 positions) were harvested just prior to the elongation of bud 'n'. Buds were collected into a 1.7 mL microfuge tube chilled in liquid nitrogen and the fresh weights of the buds were noted. Samples were collected in four replicates per genotype per treatment. Each sampled replicate consisted of approximately 12-15 buds. Isotope dilution was used by including labeled $^{2}[H]_{6}$-ABA. The extraction method previously described by Reddy et al., (2013) was followed. The ion source was operated in the negative chemical

ionization for ABA. Two sets of ions were monitored for each hormone, and the larger fragment was used for quantification (ABA- 260, 266, 278, 284 m/z).

3.4.3 Gene expression analyses

Plants were grown as described above until anthesis. Fifteen millimeters basal inflorescence stem sections adjacent to the rosette leaves were collected into 1.7mL micro-centrifuge tubes chilled in liquid nitrogen. There were 8 stems sections per replicate harvested in four replicates per genotype. The stem tissues were ground in liquid nitrogen and processed for RNA isolation using Trizol following the manufacturers' recommendations. DNA contamination was removed by DNAse digestion and RNA was re-extracted with Trizol. Two micrograms of digested RNA was used to synthesize cDNA using the NEB proto-script reverse transcriptase kit. The cDNA was used as template in a reaction combined with 50 nM each of forward and reverse primers and ABI Sybr-green PCR master mix. The reaction was run on an ABI 7900 SDS instrument. Known quantities of gene fragments cloned into plasmid were amplified in parallel to develop a standard curve indicating the relationship between Ct cycles and copy numbers. The absolute transcript abundances derived from the standard curve was normalized with reference to *18S* rRNA. There were four biological replicates per genotype and each replicate was tested in three qPCR reaction replicates.

3.4.4 Ethylene measurements

Ethylene production was estimated in the various genotypes by harvesting plants into 3 mL syringes and incubating for 20 min for headspace analysis. Plants were sampled between 12 PM to 1 PM. Three identical syringes were filled and incubated for

the same duration to account for background ethylene rates. The sample was injected into a GC-PID (10SPlus gas chromatograph (Photovac, Ontario, Canada) to estimate the ethylene concentration. The fresh weights of the samples were measured after sampling. The GC was equipped with a 49 inch long Carbopak BHT column of 0.125 inch diameter and a pre-column of 17 inches in length. The gas chromatograph was calibrated with a known standard of 10 ppm ethylene gas before running samples. The peaks were integrated and converted to express ethylene production per g fresh weight of sample.

3.4.5 ABA application to axillary buds

Plants were grown under both high and low R:FR as described above. ABA was dissolved in ethanol to make 10 μM stock. From this stock, 100 picomoles of ABA along with 0.03% Silwet (Lehle seeds Inc) were applied to the top five rosette buds with a glass syringe fitted with a long needle. The control plants received the same solution without ABA. ABA application was started at least two days before the expected date of anthesis and continued until the final measurement at 8 days post-anthesis. The individual rosette branches were measured and ratio of ABA to control branch lengths was computed.

CHAPTER IV

KINETICS OF BUD OUTGROWTH IN RELATION TO ABSCISIC ACID LEVELS IN THE AXILLARY BUDS

4.1 Introduction

The phytohormone abscisic acid (ABA) is involved in various plant physiological processes. In addition to stress responses, ABA also regulates dormancy in seeds, buds, and root propagules such as rhizomes, bulbs, and tubers. Dormancy is an important process in agriculture. Dormancy in seeds is beneficial for grain storage of various crops, and for the perpetuation of crop production. The detrimental effects of defects in ABA mediated dormancy can be observed in pre-harvest sprouting of seeds of maize harboring mutations in the *viviparous1* (*VP1*) gene. *ZmVP1* has been identified as an ortholog of the Arabidopsis ABI3 transcription factor, a component of ABA signal transduction (Suzuki et al., 2003). It is evident that ABA biosynthesis and signaling are critical for plant growth and development.

In addition to seed dormancy, the temporary arrest of growth in meristems located at the shoot apex or in leaf axils has been hypothesized to be controlled by ABA (Horvath et al., 2003). Several studies have attempted to analyze the relationship between ABA and apical dominance in various species within diverse physiological contexts (Everat-Bourbouloux and Charnay, 1982; Gocal et al., 1991; Cline and Oh, 2006). Two recent studies on shade or light quality mediated axillary bud arrest in Arabidopsis have provided strong evidence for the involvement of ABA in axillary bud

dormancy (González-Grandío et al., 2013; Reddy et al., 2013). These studies used different approaches to analyze transcriptional changes in axillary buds and several categories of hormone biosynthesis and signaling genes responsive to changes in the R:FR were identified. Genetic analysis with the ABA deficient mutants *nced3-2* and *aba1-2* verified the role of ABA biosynthesis in the R:FR mediated control of bud dormancy in Arabidopsis (Reddy et al., 2013). However, the potential interactions between ABA and other hormonal signals co-regulated by light cannot be overlooked.

There is currently little information linking ABA biosynthesis and signaling to the R:FR. Some studies investigating the underlying gene expression changes in response to the R:FR during photomorphogenesis in plants have identified a few ABA signaling components regulated by phytochromes and light signaling proteins (Tepperman et al., 2001; Chen et al., 2008). Another study documented elevated ABA levels in mesocotyls of maize seedlings receiving end-of-day FR (Dubois et al., 2010). Loss of phyB was shown to reduce sensitivity of plants to elevated ABA levels in response to stress (GonzÁLez et al., 2012). Together these reports suggest that the R:FR influences ABA biosynthesis and signaling. Further experiments are required to gain insights into the molecular mechanisms of phytochrome regulation of ABA biosynthesis and signaling.

ABA levels decline in lower axillary buds within 12 h of increasing the R:FR, while bud outgrowth was promoted within 24 h (Reddy et al., 2013). A more detailed analysis of the kinetics of the response would increase our understanding of the role of ABA in the process. To identify if the dynamics of ABA levels correlate with the

promotion of bud outgrowth, the elongation rates of buds and the bud ABA content was determined at various time intervals over a 12 h span following the alteration of the R:FR. In the current investigation the ‘n-2’ rosette bud was studied because it was reported to be highly responsive to changes in the R:FR (Reddy et al., 2013). It was hypothesized that a decrease in bud ABA levels precedes the increase in bud elongation in response to high R:FR.

To test this hypothesis, plants were grown under low R:FR from 1 day after sowing and then moved to high R:FR at 3 days post-anthesis. The elongation of lower rosette buds at position ‘n-2’ is tightly regulated by the R:FR in plants grown in this way.

4.2 Results

4.2.1 Bud outgrowth kinetics and ABA abundances in buds

A significant difference in bud length was observed 24 h after providing the plants with high R:FR (Fig. 8A). A significant difference in the rate of bud elongation between the two light treatments was apparent at 6 h (Fig. 8B). Furthermore, the difference in the bud elongation rates at 24 h was larger in magnitude than at the other times.

Bud ABA levels were measured at 1 h, 3 h, 6 h, and 12 h after increasing the R:FR (Fig. 9). A significant decline in bud ABA abundance was noticed as early as 1 h in the plants provided with high R:FR compared to those retained in low R:FR. Furthermore, ABA levels were also found to be significantly lower at 3, 6 and 12 h in

the buds of plants given high R:FR compared to the buds from plants retained under low R:FR.

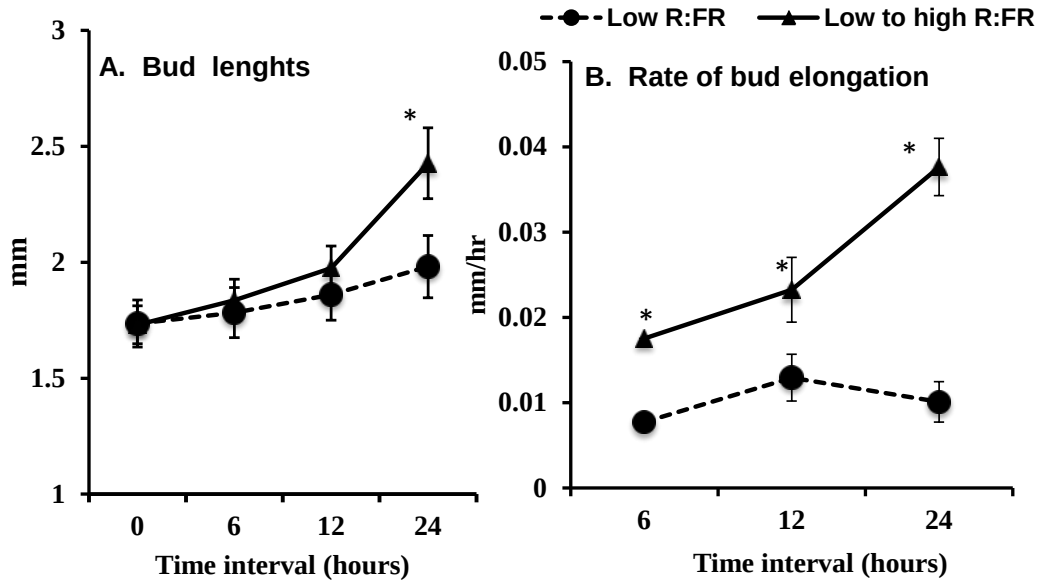

Figure 8. Bud outgrowth kinetics and elongation rates at various time points. A) bud lengths at various time intervals B) rate of bud elongation at various time points. Asterisks denote a significant difference between the two treatments ($p<0.05$); The data represent means ± SE. n=15.

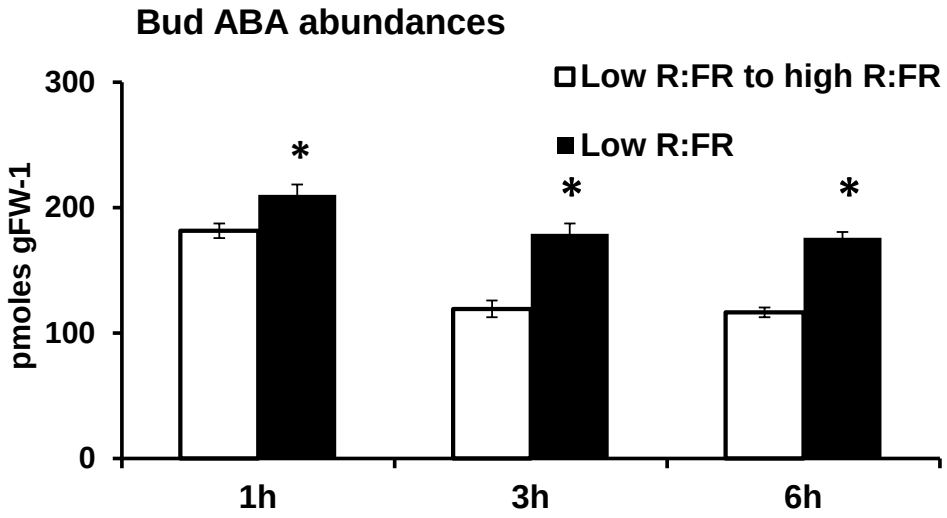

Figure 9. The 'n-2' rosette bud ABA levels at various times after providing plants grown under low R:FR with high R:FR. Asterisk denotes a significant difference between two light treatments ($p<0.05$); the data represent means ± SE. n=4**.**

4.3 Discussion

In this study the measurement of bud elongation kinetics accompanied the time-course analysis of ABA levels in 'n-2' buds. The measurement of bud lengths at various time points indicated significant changes in the bud elongation rate within 6 h of exposure to high R:FR compared to buds of plants under low R:FR. However, it was difficult to assess very small increments in bud elongation at the early time points because of the minute size of the buds. Another problem is that the growing tips of the buds were often covered with small leaves, rendering the detection of the growing tip difficult.

The significant reduction in bud ABA levels as early as one hour after providing plants grown under low R:FR with high R:FR indicates the existence of a mechanism that enables rapid response to variations in the R:FR. The reduction in ABA content in the buds in response to high R:FR could be due to a decline in ABA biosynthesis or an increase in catabolism in the buds. Previously, two genotypes with defective ABA biosynthesis, *nced3-2* and *aba2-1*, were shown to possess a higher frequency of rosette branches and had less correlative inhibition compared to wild-type plants under both high and low R:FR (Reddy et al., 2013). The results of the current study support the contention that ABA levels in the buds are regulated by the R:FR and are a critical component of the light regulated bud elongation program.

In this study, ABA levels were found to decrease within an hour of increasing the R:FR. An explanation for the rapid response of ABA levels to changes in the R:FR may be attributed to phyB-PIF dynamics. Studies have shown that active phyB in the nucleus interacts with PIFs bound to promoters of various light responsive genes (Martínez-García et al., 2000). When the R:FR is high, biologically active phyB translocates to nucleus to modify PIFs and negate the transcription of various classes of light responsive genes (Al-Sady et al., 2006). Several studies have shown that PIFs modulate the transcription of various phytohormone biosynthesis and signaling genes (Khanna et al., 2007; Franklin et al., 2011; Hornitschek et al., 2012). At this time there is no evidence showing PIF regulation of ABA biosynthesis genes. However, cis-element bound PIF7 was shown to negatively regulate the transcription of *DREB1* genes of the ABA signaling pathway (Kidokoro et al., 2009).

4.4 Materials and methods

4.4.1 Plant materials and growth conditions

The wild-type accession Col-0 was used in the study. Plant growth and light conditions were as described in Reddy et al., (2013). Briefly, wild-type Arabidopsis was grown under low R: FR (0.05) from 1 day after sowing. Low R:FR was provided by FR emitting LEDs in combination with T12 fluorescent lamps. The growth chamber was divided by a light barrier into two sides, with one set of plants per side. At 3 days after anthesis, the FR LEDs were turned off in one side to increase the R:FR. The ‘n-2’ buds from plants continually grown under low R:FR and those provided with high R:FR were harvested at 1 h, 3 h, 6 h and 12 h for ABA estimations. Buds were harvested in four replicates with at least 30 buds per replicate. The buds were harvested in tubes placed in liquid nitrogen. The fresh weights of buds were taken after sampling. The buds were stored at -80C until processing for ABA estimation by GC-MS as described in Chapter 3.

4.4.2 Growth measurements

For growth measurements, 15 uniform plants ith respect to number of rosette leaves, height growth and nearly equal bud length from each light treatment were identified and numbered. The plants from both the light treatments were imaged at 0, 6, 12 and 24 h after the initiation of high R:FR using a digital camera fitted onto a tripod stand and equipped with a macro-lens was used. The ‘n-2’ buds were imaged with a reference scale. ImageJ software was used to process the images to determine the bud lengths.

CHAPTER V

ETHYLENE REGULATION OF PLANT ARCHITECTURE

5.1 Introduction

Ethylene is a major phytohormone, controlling many diverse physiological processes in plants. Ethylene is critical for many aspects of plant life. Some important processes regulated by ethylene include senescence, abscission, epinasty and fruit ripening. It is also a component of plant responses to abiotic and biotic stress. Ethylene has also been shown to be involved in mediating plant responses to canopy shade and water submergence (Pierik et al., 2003, 2004). Many studies indicate that ethylene biosynthesis is strongly regulated by light quality. Low R:FR or loss of phyB function increased ethylene production in sorghum (Finlayson et al., 1998) and Arabidopsis (Vandenbussche et al., 2003). A recent investigation on PHYTOCHROME INTERACTING FACTOR5 (PIF5) revealed a strong link between light signaling and ethylene biosynthesis in Arabidopsis (Khanna et al., 2007). It was shown that the ectopic expression of PIF5 elevated ethylene biosynthesis.

Ethylene is synthesized from methionine. A small gene family encodes ACC synthase (ACS) enzymes that convert S-adenosyl L-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC). The multiplicity of ACSs allows diverse inputs to regulate the biosynthesis of ethylene (Chang and Shockey, 1999). Various events such as biotic and abiotic stress, light, the circadian clock and other hormones are all known to regulate the transcription of various *ACS*s. Ethylene perception is mediated

by five receptors in Arabidopsis (ETR1, ETR2, ERS1, ERS2, and EIN4). Inactivation of these receptors upon binding ethylene negatively regulates the activity of CTR1 (CONSTITUTIVE TRIPLE RESPONSE 1). Inactive CTR1 relieves the repression of EIN2 (ETHYLENE INSENSITIVE 2) which then activates EIN3. The EIN3 transcription factor promotes the expression of several ethylene responsive genes. Loss of functional EIN2 and ETR1 has been shown to strongly suppress plant responses to ethylene (Chang et al., 1993; Dugardeyn and Van Der Straeten, 2008).

Various attributes of plant growth are regulated by the concerted action of ethylene and other hormones (Pierik et al., 2004; Buer et al., 2006; Stepanova and Alonso, 2009). For instance, crosstalk between ethylene and GA signaling has been shown in some light mediated processes. The dependency of ethylene on GA to maximize shoot elongation in response to shade signals has been previously demonstrated (Pierik et al., 2004).

Studies on the interaction between ethylene and auxin have led to speculation on the possible role of ethylene in mediating apical dominance (Prasad and Cline, 1985, 1987; Chatfield et al., 2000). The absence of lateral bud dormancy under short days in birch trees expressing a mutant version of *AtEIN2* has been reported (Ruonala et al., 2006). A role for ethylene has been suggested in the environment induced latency of bud outgrowth in perennials (Horvath et al., 2003; Horvath, 2009). An axillary bud transcriptome study reported that a few members of the ethylene signaling pathway were responsive to changes in the R:FR (Reddy et al., 2013). These reports suggest that ethylene may play a role in the regulation of branching.

Apart from branch numbers and length of branches, the branch angle is also an important feature of shoot architecture. Branches emerging on the vertical stem are acted upon by gravitational pull to bend it downwards. The angle at which the lateral organs are subtended on the vertical stem may be described in terms of the Gravitropic Set - point Angle (GSA) (Digby and Firn, 1995). The GSA of '0' represents vertically downward suspension of an organ, whereas, an angle of 90 degrees indicates that the plant organ is parallel to the horizontal surface. Plant intrinsic signals and various environmental cues or their interactions could be contributing to the GSA of an organ or a branch (Digby and Firn, 1995). However, the mechanisms underlying this phenomenon are not well understood.

The significance of gravity in plant development can be inferred from various events in the plant's life. After seed germination, shoot orientation is negatively gravitropic, while the root displays positive gravitropism. This indicates an interaction between plant development and gravity. R sensing by phytochromes was shown to be involved in the abnormal downward growth of tomato shoots in plants harboring a mutation in *lazy-2* (Gaiser and Lomax, 1993). The *lazy-2* displayed normal growth similar to wild-type in the dark, whereas exposure to red light induced defects in upward growth of the shoots. This indicates that R and gravity perception or signaling interact to influence the gravitropism of plant organs.

A recent study has revealed the role of auxin in regulation of the GSA (Roychoudhry et al., 2013). Arabidopsis plants with higher abundances of auxins displayed more vertical growth of cauline branches (increased GSA) compared to wild-

type. Auxin signaling was also an important component, where elevated auxin response promoted a more vertical orientation of cauline branches. Deficiency in auxin biosynthesis and also reduction in auxin response led to a decrease in the GSA compared to wild type. Perturbed auxin homeostasis due to decapitation of the shoot apex was capable of altering the GSA. These results show a strong influence of auxin in regulation of the GSA.

Environmental signals such as light, gravity and other influences on hormonal homeostasis are integrated to shape the shoot architecture (Prasad and Cline, 1987; Finlayson et al., 2010). The current study assessed the influence of ethylene and the R:FR on Arabidopsis shoot architectural traits. Ethylene insensitive genotypes resulting from lesions in genes encoding EIN2 and ETR1 were used in this study. The interactions between the R:FR and rosette branch GSA in ethylene sensitive mutants were also evaluated in the study.

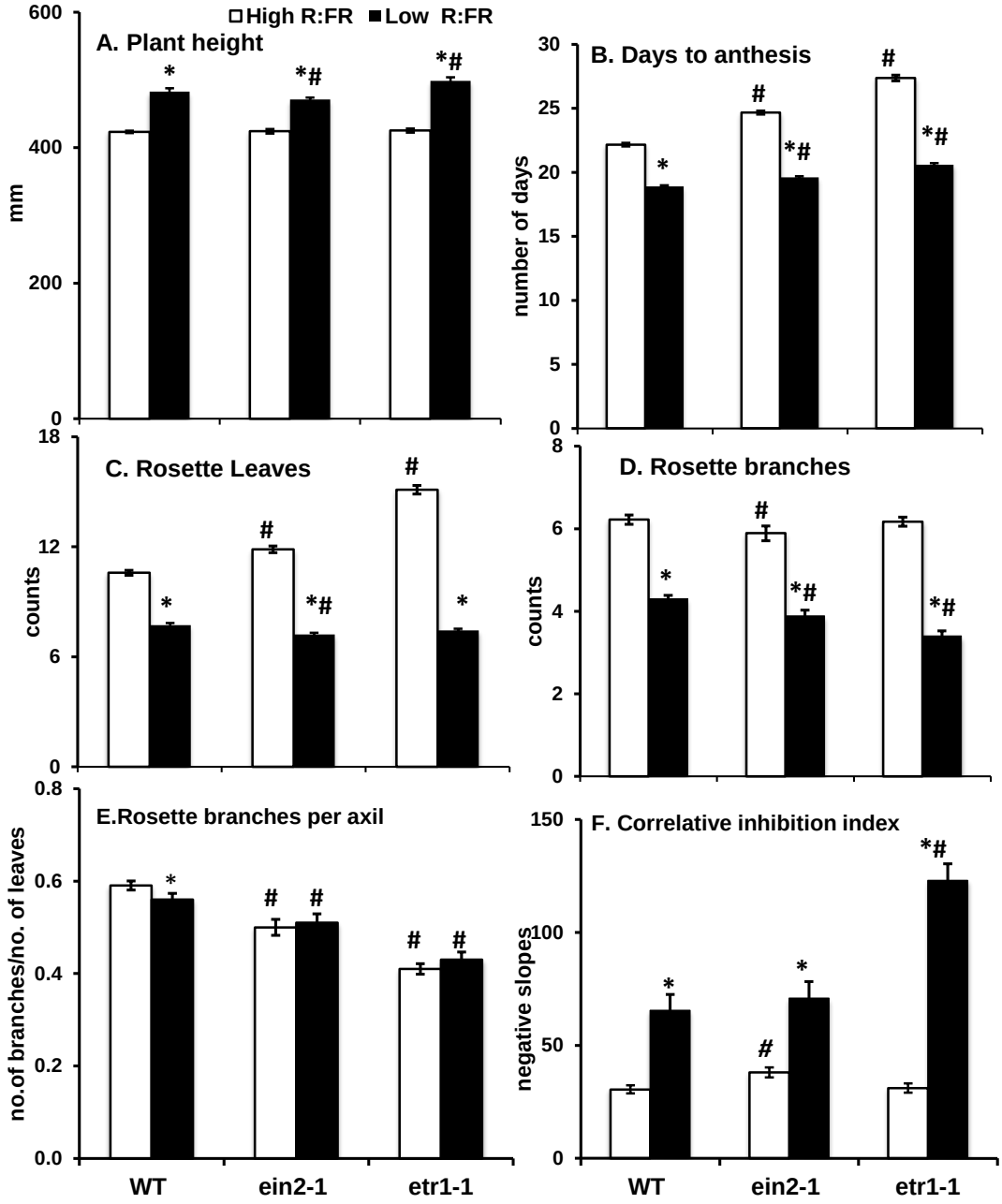

Figure 10. Assessment of wild-type and ethylene insensitive mutant genotypes under high and low R:FR. Data on shoot architectural parameters were collected at 10 days post anthesis. Asterisks indicate a significant difference ($p<0.05$) between R:FR treatments and hash (#) indicates a significant difference within a R:FR treatment compared to wild-type ($p<0.05$). Data are means ± SE. n=36 per treatment per genotype.

5.2 Results

5.2.1 Influence of the R:FR on shoot architecture of ethylene insensitive genotypes

To test the interaction between ethylene perception and the R:FR, the loss of function *ein2-1* mutant, the gain of function *etr1-1* mutant and wild type were grown under both high and low R:FR. Ethylene insensitive mutants showed a slight delay in germination. During the initial phase of growth, post germination establishment of *ein2-1* and *etr1-1* was slightly retarded as they showed defects in penetration of roots into the potting earth indicating deficiency in the gravitropic response. At post-establishment, the early stages of ethylene insensitive mutants had smaller leaf size compared to wild-type plants. However, at late stages of plant development, a robust expansion of rosette leaf area was observed in *etr1* and *ein2*. From the visual observations at 10 days-post anthesis, it was evident that ethylene insensitive mutants had larger leaf areas compared to wild-type.

Low R:FR significantly promoted the height of the wild-type shoots. *ein2-1* and *etr1-1* also displayed a similar response to low R:FR. However, minor differences in shoot height between wild-type and the two ethylene insensitive mutants genotypes were noted under low R:FR (Fig. 10).

Early flowering in response to low R:FR was retained in *ein2-1* and *etr1-1*. There was a delay in the flowering time in the ethylene insensitive mutants compared to wild-type plants in both the light treatments, however, the differences were greater under high R:FR. Under high R:FR, the *etr1-1* plants flowered late and accumulated more rosette leaves compared to *ein2-1* and wild-type plants.

There were significant differences in the number of rosette branches between *ein2-1* and wild-type under both high and low R:FR. However, *etr1-1* displayed a dissimilar response to low R:FR and high R:FR. *etr1-1* had significantly more rosette branches compared to wild-type under low R:FR, whereas there was no apparent difference under low R:FR.

A significant difference in the parameter accounting the ratio of branches to leaves was noted between ethylene insensitive mutants and wild-type plants. Reduction in branch to leaves ratio was noted in *ein2* and *etr1* under both high and low R:FR in comparison to wild-type. Though comparison of branch counts *per se* between *etr*-1 and wild-type revealed no statistical difference under high R:FR, a reduction in the number of elongating branches was evident from the computation of ratio of branches to total rosette leaves.

Low R:FR enhanced the correlative inhibition of branches in all the genotypes. There were minor differences in correlative inhibition between *ein2-1* and wild-type under high or low R:FR. However, a differential response to the R:FR was observed in *etr1-1* plants. The correlative inhibition index of *etr1-1* was significantly greater than that of wild-type plants under low R:FR, but not under high R:FR.

5.2.2 Gravitropic set-point angle of rosette branches

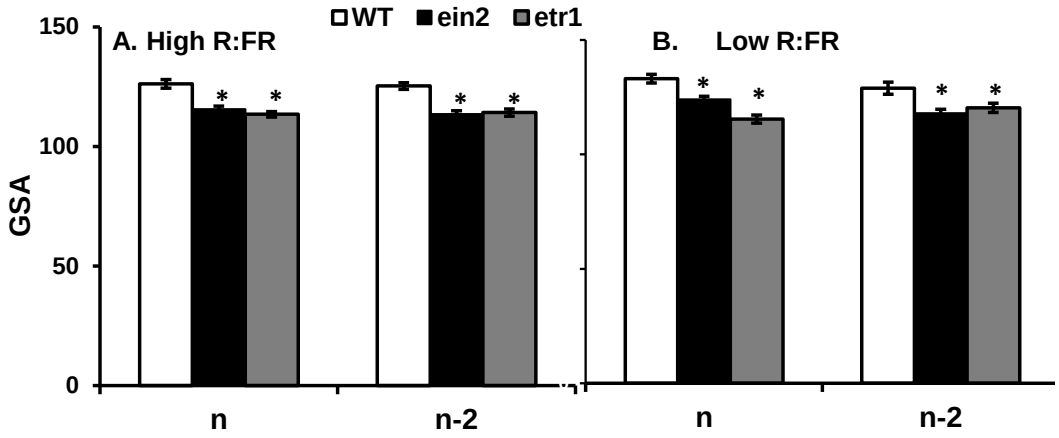

Figure 11. Gravitropic set-point angle of branch 'n' and 'n-2' of wild-type, *ein2* and *etr1* plants under high R:FR (A) and low R:FR (B). Asterisks (*) indicate significant difference ($p<0.05$) between genotypes comparing respective bud positions. Data represents mean ± SE. n=16 per genotype per treatment.

The GSA of *ein2-1* and *etr1-1* 'n' and 'n-2' rosette branches was lower than that of wild-type plants under both high and low R:FR (Fig.11). The lower GSA values reflect the more horizontal outgrowth of these branches. The GSA of all the genotypes was similar under both high and low R:FR, indicating that the R:FR does not control branch angle. The GSA in the ethylene insensitive mutants was affected by the developmental stage, and an elevated GSA was observed through the early elongation stage of the rosette branches.

5.3 Discussion

5.3.1 Ethylene influences plant architectural responses to R:FR

Identification of ethylene receptors and signaling components has resulted in a better understanding of ethylene mediated processes. Ethylene has been observed to mediate several aspects of plant development including shade avoidance, submergence responses (Voesenek and Blom, 1989; Pierik et al., 2009) and root gravitropic responses (Buer et al., 2006). However, there have been no reports describing the role of ethylene in Arabidopsis shoot architecture responses to the R:FR. In the present study, the shoot architecture of ethylene insensitive mutants *ein2-1* and *etr1-1* was assessed under both low and high R:FR.

Loss of ethylene sensitivity had no significant effect on shoot height under high R:FR, indicating the possible role of other factors controlling the elongation response. Low R:FR promotion of shoot elongation has been attributed to the concerted action of multiple hormones such as auxin, ethylene and gibberellins (Pierik et al., 2009). The deficiency in ethylene responsiveness due to non-functional *ein2-1* or *etr1-1* might have been compensated by the action of other hormones.

The R:FR sensing phyB is known to regulate the photoperiod mediated flowering response (Halliday et al., 1994). The accelerated flowering response to low R:FR was retained in *ein2-1* and *etr1-1* genotypes, however, there was a delay in anthesis in ethylene insensitive genotypes grown under both low and high R:FR. Similar observations on *ein2-1* and *etr1-1* have been reported by other studies (Ogawara et al., 2003). Ethylene is one of the multiple factors regulating the transition of the vegetation

meristem to the reproductive meristem (Horvath, 2009). Furthermore, the transition from the vegetative to the reproductive stage is a complex process mediated by both developmental and environmental factors. For instance, ethylene interaction with GA signaling has been shown to regulate the floral transition in short days (Achard et al., 2007).

In the current study, EIN2 loss of function influenced correlative inhibition under high R:FR. In contrast, loss of *etr1-1* affected the correlative inhibition under low R:FR. The observed elevation in the correlative inhibition in the *etr1-1* plants grown under low R:FR may be due to their delay in germination and seedling establishment. The delay in germination of *etr1-1* seeds results in an early exposure to low R:FR during experimentation, because low R:FR treatment was provided at 7 days after sowing irrespective of the germination speed of genotypes included in the study. The correlative inhibition of rosettes branches is sensitive to developmental stage, where exposure to low R:FR during earlier stages of seedling growth was found to elevate branch inhibition (Reddy et al., 2013).

5.3.2 Ethylene insensitivity reduces the GSA of rosette branches

In this study, lesions in *EIN2* and *ETR1* significantly reduced the GSA of both ‘n’ and ‘n-2’ buds compared to wild-type. The R:FR did not alter the GSA of rosette branches of any of the genotypes. The reduction in the GSA of ethylene insensitive mutants could be due to a variety of factors. One possibility is that ethylene insensitivity induces a perturbation in auxin transport or signaling. Previous studies have

demonstrated the role of auxin in mediating gravitropic responses of cauline branches (Roychoudhry et al., 2013).

Auxin gradients in plant organs stimulate a bend or curvature according to the Cholodny-Went theory. In organs such as branches, the upward curvature may be due to higher auxin in the lower flank and reduced auxin in the upper flank. In the current study, estimation of auxin abundances in upper and lower flanks of rosette buds may be necessary. Furthermore, the influence of ethylene insensitivity on auxin signaling may need to be assayed.

5.4 Materials and Methods

5.4.1 Plant materials and growth conditions

Arabidopsis ecotype Col-0 (CS 60000), *etr1-1* and *ein2-1* mutants were used in the study. The *etr1-1* and *ein2-1* seeds were stratified at 4°C for 7 days due to dormancy in seed germination, and wild type seeds were stratified for 3 days before sowing. For assessment of architectural parameters plants were grown in trays with six-cell inserts (36 plants per tray) using LC-1 soilless potting mixture. Plants were grown in individual pots for the purpose of assessment of the GSA. Plants were fertilized weekly with 5 mL of diluted (1:5) Hoagland's nutrient solution at a week's interval. Plants were grown under 18 h/6 h light/dark photoperiods with 24°/18 ^{o}C day/night temperatures in a growth chamber with T5 fluorescent lamps as the light source. The light was set to 184 μmol m^{-2} s^{-1} PPFD. Plants were grown under high R:FR (4.0) for seven days and thereafter a set of plants was provided with low R:FR (0.075). Architectural parameters were recorded at ten days post anthesis (DPA) as described in Finlayson *et al.* 2010. A

bud more than 3 mm of length was considered a branch. Comparisons between means were made using a two-tailed t-test with $\alpha = 0.05$.

5.4.2 GSA measurements on rosette branches

Wild-type (Col-0), *etr1-2* and *ein2-1* mutants were grown in individual pots. Images of individual plants were made using a digital camera. Image J software was used to measure the GSA of 'n' and 'n-2' rosette branches. The angle was measured when the branch lengths were 3-4 cm long. The GSA was measured as the angle between the horizontal plane and the growing tip of the branches. There were 16 replicates of each genotype under the two R:FR conditions. Comparisons between means were made using a two-tailed t-test with $\alpha = 0.05$.

CHAPTER VI

SUMMARY

Plant responses to changes in the R:FR have been attributed to phytochrome mediated R:FR perception and various downstream signaling events. In *Arabidopsis thaliana*, phyB is known to play a major role in R:FR signaling. phyB mediates several aspects of plant architecture including the branching habit of plants (Finlayson et al., 2010). Recent studies have identified a group of bHLH transcription factors interacting with phyA/phyB designated as Phytochrome Interacting Factors (PIFs). Available evidence suggests that PIF4, PIF5 and PIF7 predominantly regulate transcription of various genes participating in plant responses to the R:FR (Leivar and Quail, 2011). This investigation assessed the roles of PIF4, PIF5 and PIF7 in the regulation of the R:FR mediated control of branching in Arabidopsis. The kinetics of the R:FR regulation of bud outgrowth and bud ABA accumulation was studied to determine the potential role of ABA in the early response. The effect of ethylene insensitivity and its interaction with the R:FR on branching and branch angle was also investigated.

The approaches used in this study involved integrated genetic, physiological and molecular assays. Plant architectural parameters were assessed by growing the loss of function genotypes *pif4pif5* and *pif7* under both high and low R:FR. To investigate the interaction between phyB and various PIFs, *phyB*, *phyBpif4pif5* and *phyBpif7* plants were also included in the study. Compared to wild-type, the correlative inhibition of rosette branching was attenuated in *pif4pif5* and *pif7* under low R:FR. Introgression of

pif4pif5 and *pif7* mutations into the phyB-null background suppressed the apical dominance of *phyB*. This suggests that PIF4/PIF5 and PIF7 are necessary for the correlative inhibition of branches under low R:FR. Lower levels of bud ABA were observed in *pif7* under low R:FR and in *phyBpif7* compared to wild-type and *phyB* respectively, indicating that PIF7 may exert control over bud elongation through modulation of ABA abundance. Elevated levels of ABA in the *phyBpif4pif5* do not correspond to the reduced correlative inhibition compared to phyB loss of function. This may suggest that PIF4/PIF5 effects on branching are predominantly mediated through auxin. The loss of phyB function enhanced the sensitivity of buds to exogenous ABA. Furthermore, the analysis of branching in *phyB-9nced3-2* mutants revealed that the ABA biosynthetic gene *NCED3* is an essential component of phyB mediated repression of branching. The analysis of transcript abundances of a selection of auxin-responsive genes in the various mutants in the study suggested that PIF4/PIF5 mediate branching responses by regulating the transcription of auxin-signaling genes. Taken together, PIF4/PIF5 and PIF7 regulation of branching involve multiple hormones and their interactions with phyB.

ABA has been identified as a key regulator of bud outgrowth in response to the R:FR (Reddy et al., 2013). The elongation rate of axillary bud (n-2) showed a significant increase by 6 h after exposing plants to high R:FR following growth under low R:FR. The time-course analysis of the ABA levels in buds revealed significant reductions in bud ABA levels as early as one hour from initiating the change from low to high R:FR.

This suggests the existence of a mechanism in buds that enables them to rapidly respond to variations in the R:FR.

Ethylene biosynthesis is known to be responsive to variations in the R:FR. To assess the influence of ethylene on the regulation of plant architecture, the ethylene insensitive mutants *ein2-1* and *etr1-2* were grown under both high and low R:FR. In comparison to wild-type, the lesion in *EIN2* elevated the correlative inhibition under high R:FR. In contrast, loss of *ETR1* resulted in stronger branch inhibition under low R:FR compared to the wild-type. The growth of ethylene insensitive plants was defective in other developmental aspects such as delayed germination, late flowering, and small size of leaves during early stages and robust expansion of leaves at late stages of development compared to wild-type. Comparison of the rosette branch gravitropic-set point angle between wild-type and *ein2-1* and *etr1-1* revealed that ethylene perception affected the branch angle. A lower gravitropic-set point angle was observed in *ein2-1* and *etr1-2* regardless of the R:FR. Auxin has been suggested to control gravitropic responses in various studies. It is possible that ethylene insensitivity may affect auxin transport or signaling. However, the perception of gravity may vary with the plant organ and changing stages of development of an organ. Further investigation into the interaction between auxin-signaling components and ETR1/EIN2 may reveal the underlying mechanism(s) by which ethylene affects the gravitropic response of rosette branches.

In conclusion, this study showed that branching responses to the R:FR are mediated by the interaction between phyB and its interacting factors. It is possible that

PIF4/PIF5 and PIF7 regulates ABA biosynthesis in buds and auxin- signaling in shoots in response to the R:FR. The bud elongation kinetics and time-course analysis of bud ABA levels revealed the existence of a rapid mechanism in buds to respond to changes in the R:FR. The assessment of ethylene insensitive mutants indicated that ethylene mediates shoot architectural aspects of branch development and branch angle.

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