ABSCISIC ACID REGULATION OF BRANCHING

A Thesis

by

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

MASTER OF SCIENCE

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December 2013

Major Subject: Agronomy

ABSTRACT

Axillary meristem production and activity contribute to the high plasticity of plant development which allows plants to respond to environmental changes. Branches arise from axillary buds that form in the leaf axil. These buds may remain dormant, or may grow out to form branches immediately. Alternatively, the axillary buds may also persist in a dormant state for an indefinite period of time until appropriate signals permit outgrowth to commence. Branching is regulated by phytohormones, including auxin acting via the polar auxin transport stream, and locally within the bud by the action of branching integrators like BRC1 and MAX2. Branching is also regulated by environmental factors such as competition signals (low Red light: Far-Red light [R: FR]) that inhibit bud outgrowth. Our recent studies indicate that ABA acts within the bud to suppress outgrowth. NCED3 is a key enzyme in the ABA biosynthesis pathway, and ABA2 is another important ABA biosynthesis gene. ABA accumulated to significantly higher levels in lower, more dormant buds compared to less dormant buds at higher rosette positions. Additionally, bud ABA content and the correlative inhibition index, which is a measure of systemic branching suppression, were increased in plants grown under low R:FR compared to those grown under high R:FR. Under low R:FR the NCED3 deficient mutant *nced3-2* and the ABA2 deficient mutant *aba2-1* had significantly more branches and lower correlative inhibition index than WT. The results indicate that the suppression of branching by low R:FR may be mediated, at least in part, by elevated levels of ABA in the buds.

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1. INTRODUCTION

Branching (tillering in monocots) is relevant to maximizing the utilization of resources and production of vegetative and/or reproductive structures of crops. Axillary meristems contribute to plant secondary growth by allowing the plant to change its architecture to respond to environmental conditions. Studies on axillary meristem outgrowth and development may help us understand how to modify plants to improve yield and/or other characteristics.

Many factors are involved in the regulation of axillary branching, including developmental, genetic, hormonal, and environmental factors. Plant hormones play an important role as endogenous factors regulating bud outgrowth and development. Abscisic acid (ABA) is a hormone that has been implicated in the regulation of branching, but until recently a definitive role had not been proven. One recent study showed that ABA plays an important role in axillary bud outgrowth (Reddy et al., 2013). However, little is known about the mechanisms of ABA regulation of axillary bud outgrowth. It seems that the effects depend on transport sites and paths inside plant.

Correlative inhibition (CI) of branching describes the capacity of some positive primary growth process to suppress the secondary growth including the outgrowth of lateral buds and shoot. The correlative inhibition index (CII) can be used to quantify branching strength using the differences in inter-branch size (Finlayson et al., 2010; Su et al., 2011). The correlative inhibition index (CII) is the negative slope of the lengths of the top three rosette branches [branch n (uppermost branch), branch n-1 (branch

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immediately below branch n) and branch n-2 (branch immediately below branch n-1)].
Larger CII values mean weaker branching.

Light signals are among the key environmental factors influencing plant growth
and development. Plant responses to competition- derived light signals are mediated by
phytochromes (phy). Previous studies in our lab revealed that low R:FR or phyB loss of
function increases the CII (Finlayson e al., 2010). Furthermore, increasing the red light:
far red light ratio (R:FR) on plants grown under low R:FR activated the outgrowth of
lower position buds that otherwise remain arrested and was associated with reduced bud
ABA content and decreased expression of ABA-related genes (Reddy et al., 2013).
Plants deficient in the ABA biosynthesis enzymes NCED3 or ABA2 have significantly
more branches than WT. As a result, it was concluded that ABA plays an important role
in the R:FR regulation of bud outgrowth.

Much remains to be discovered regarding the role of ABA in regulating
Arabidopsis branching. Moreover, the relationship between ABA and other branching
regulators are still unknown. The primary objective of this study was to define the role
of ABA in regulating bud outgrowth and development, extending the study of its
involvement in R:FR responses, and examining how ABA may play a more general role
in branching. Wild type Columbia (Col-0), the NCED3 deficient mutant *nced3-2* and the
ABA2 deficient mutant *aba2-1* were used to quantify branching responses under
continuous exposure to high and low red light: far-red light ratios (R:FR). The
hypothesis tested was that ABA plays an important role in regulating branching under
both light regimens. To thoroughly understand the interaction between ABA and light

competition signals in branching regulation, the pattern of ABA accumulation in axillary buds of plants grown under high and low R:FR was investigated using Col-0. The main hypothesis for this objective was that low R:FR will result in elevated bud ABA accumulation. Moreover, it was also predicted that lower buds will accumulate more ABA which may act locally in the bud. Beyond the ABA role in the R:FR responses, the interaction between ABA and auxin and how they regulate bud outgrowth was also investigated. It was hypothesized that ABA acts downstream of auxin to inhibit bud outgrowth. Finally, to further investigate the mechanism of ABA regulation of branching, the relative location of ABA in the branching pathway with respect to other known components including *Branched 1*(*BRC1*) and *MAX2* was investigated. The hypothesis was that *MAX2* or *BRC1*, or both, act downstream of the ABA-mediated branching pathway.

2. LITERATURE REVIEW

The high plasticity of plant development allows plants to respond to changes in the environment. The production and activity of axillary meristems contributes to this plasticity of plants (Bennett et al., 2006). Axillary buds are initiated in leaf axils and may then form a branch or remain dormant depending on what signals the bud receives. The signals can be endogenous or exogenous, or both (Domagalska and Leyser, 2011). Plant hormones and light are endogenous and exogenous signals, respectively, involved in the regulation of the outgrowth and development of axillary buds.

2.1 Axillary meristem/bud development

Axillary bud meristems form on the adaxial surface of the joint of the leaf and the stem. Cell division is essential for the development of axillary meristems. Initially, the cells produced by cell division in the meristem will grow larger which leads to a larger meristem. Once established, the axillary meristem will begin producing leaf primordia, then form small leaves and a short stem at which point the axillary meristem has become an axillary bud. The buds then may remain dormant, or grow out to form branches. These different behaviors of axillary buds result from different patterns of branching development control (Sussex and Kerk, 2001). In Arabidopsis grown under conditions promoting extended vegetative growth, or in late flowering mutants, axillary meristem initiation can occur in an acropetal wave at lower positions during the vegetative phase, and then in the typical basipetal wave in the upper positions following the floral transition (Grbic and Bleecker, 2000). Under long days, plants will transit from

vegetative growth to reproductive growth, and start producing flower. Once plant has gone through the floral transition, it stops producing vegetative leaves and therefore the number of rosette and cauline leaves and the number of potential axillary buds will be fixed at this stage.

Arabidopsis is a widely used model plant for biological studies. Because of the small size and short life cycle, it is possible to grow it in growth chambers with controlled humidity, temperature, etc. It has been used frequently in plant architecture studies. The axillary bud of Arabidopsis will grow out to form axillary shoot and then to form lateral inflorescence during prolonged vegetative growth (Stirnberg et al., 1999). In most cases, some of the axillary buds will remain dormant because of apical dominance which results from the suppression of shoot apex on the axillary buds development.

2.2 Apical dominance

Apical dominance is a phenomenon whereby the main shoot dominates the growth of axillary buds below it. Previous studies showed that the removal of the Arabidopsis shoot tip can lead to the release of the outgrowth of axillary buds (Cline, 1996; Beveridge et al., 2000). The communication between the shoot apex and axillary buds on the plant can partially regulate the fate of axillary buds (whether they form branches or remain dormant temporarily or permanently) (Stafstrom et al., 1998; Shimizu-Sato and Mori, 2001). The communication depends on various signals, transporters and regulators. Auxin is a phytohormone produced in the shoot apex and young leaves and transported in the polar auxin transport stream to influence bud outgrowth at more basal positions. In some species, exogenous auxin can repress axillary

buds outgrowth (Thimann and Skoog, 1933). The auxin transported down in the shoot from shoot apex was the reason which resulting in the transition of the buds between dormancy and growth. Decapitation is to remove the shoot tip which is the source of auxin. It eliminates the source of auxin production which may allow axillary buds to export auxin into the main shoot Polar Auxin Transport Stream (PATS) and transition from dormancy to growth. It was found that exogenous auxin applied to decapitated peas allows the plants to regain the apical dominance. Decapitated peas showed a stronger response to exogenous auxin than *Arabidopsis* (Cline, 1996; Beveridge et al., 2000; Cline et al., 2001). These results indicated that auxin participates in branching regulation. However, the regulation of bud outgrowth in *Arabidopsis* is not determined by auxin alone. It has been suggested that auxin may regulate branching indirectly by influencing the supply of CK to the axillary buds (Cline, 1994). Using pea, King and Van Staden (1988) showed that axillary buds at different positions have different responses to CK treatment. This result also indicated that auxin is not the only hormone that involving in branching regulation. In summary, studies on apical dominance revealed that hormone networks plays an important role in determining the status of axillary buds.

2.3 Shoot branching

The process of shoot branching can be divided into development into five stages (Schmits and Theres, 2005). The first stage is the establishment of axil identity. Then the process goes through the axillary meristem initiation, the organization of the meristem, the formation of the axillary bud, and finally the outgrowth of the bud. Auxin and

strigolactone are major branching regulators that act in the last stage (Lincoln et al., 1990; Arumingtyas et al., 1992; Rameau et al., 2002; Stirnberg et al., 2002; Takeda et al., 2003; Snowden et al., 2005).

Branched1 (*BRC1*) is also an important branching regulator which acts locally in the axillary bud (Aguilar-Martínez et al., 2007; Finlayson et al., 2007). *BRC1* encodes a TCP domain protein that is closely related to the transcription factor TEOSINTE BRANCHED1 (*TB1*) that represses axillary bud outgrowth in grasses. *BRC1* is a negative regulator of axillary bud outgrowth. It has been shown that *BRC1* acts downstream of auxin and the strigolactone-mediated pathway to suppress bud outgrowth (Aguilar-Martinez et al. 2007; Finlayson 2007). *BRC1* deficient mutant Arabidopsis has significantly more branches than WT. Recently, it was found that *BRC1* is required to inhibit axillary bud outgrowth in response to low R:FR (Finlayson et al., 2010; Eduardo González-Grandío et al., 2013).

Breaking bud dormancy is a complex process that requires environmental stimuli, hormone networks and branching-regulated gene activation. Among these factors, hormone networks have been studied and discussed in great detail. Abundant evidence has shown how hormones including auxin, cytokinins, strigolactone and abscisic acid are involved in regulating branching.

2.4 Hormones involved in branching regulation

2.4.1 Auxin

The role of auxin in branch development is well established. In peas and *Arabidopsis*, it has been shown that decapitation can lead to the release of apical

dominance and rapid outgrowth of axillary buds (Beveridge et al., 2000; Cline, 1996).

Studies have shown that the fate of the axillary buds in the transition stage is partially

regulated by communication among axillary buds, and between the shoot apex and

axillary buds (Stafstrom et al., 1998; Shimizu-Sato and Mori, 2001). Auxin and its

signaling pathway play important roles in this communication. AUXIN-RESISTANT1

(AXR1) is an Arabidopsis protein that acts in the auxin signaling pathway to regulate

branching and other auxin responses. It promotes auxin signaling by enabling

destabilization of the Aux/IAA transcriptional repressors (Gray et al., 2001). The

Arabidopsis *axr1-12* mutant has a hyperbranching phenotype because of reduced auxin

signaling and thus loss of apical dominance (Lincoln et al., 1990; Stirnberg et al., 1999).

The hyperbranching phenotype of *axr1* mutants and related physiological evidence has

been taken as support for a role of auxin in the regulation of shoot branching.

2.4.2 Cytokinins

CK is also involved in the regulation of many aspects of plant development, such

as seed germination, meristem formation, apical dominance, and stem growth and

differentiation (Mok and Mok, 2001; Heyl and Schulling, 2003). CK is known to be

produced in the root (Chen et al. 1985). However, it was later found that the synthesis of

CK can also occur in aerial parts, especially in the young developing leaves with active

cell division (Nordstrom et al., 2004). Besides the biosynthesis of CK, its signaling

pathway also regulates meristem growth and development. The function of cytokinins

(CK) in the regulation of branching has been considered to be closely related to auxin.

Sachs and Thimann (1967) suggested that endogenous auxin inhibits cytokinin

production in the buds. It was recently found that auxin negatively regulates the level of CK in pea by suppressing a CK biosynthesis enzyme both at nodes and in roots (Tanaka et al., 2006). Evidence has clearly shown that cytokinin (CK) is involved in the promotion of bud outgrowth. Previous studies have proved that exogenous CK can partially weaken apical dominance and promote outgrowth of the axillary buds (Wickson and Thimann, 1958; Cline et al., 1997; Faiss et al., 1997), especially when CK was directly applied to the bud. Cytokinin appears to interact with auxin in controlling apical dominance (Bangerth 1994; Li et al., 1995; Cline et al., 1997; Dun et al., 2012). Moreover, it was recently shown that low concentrations of cytokinin applied to the main stem vascular system below a specific axillary bud node can stimulate the outgrowth of the bud. It was concluded that cytokinin can enhance axillary bud development from a distance (Dun et al., 2012).

2.4.3 Strigolactone

Strigolactone, which is a carotenoid-derived hormone, has been identified as another regulator of shoot branching. In *Arabidopsis,* MORE AXILLARY GROWTH1-4 [MAX1-MAX4] are proteins which are involved in the carotenoid-derived hormone biosynthesis and signaling pathways. Deficiency in those proteins will lead to hyperbranching and resistance to exogenous auxin (Stirnberg et al., 2002; Bainbridge et al., 2005, McSteen and Leyser, 2005, and Bennett et al., 2006). This suggests that these proteins are involved in branching repression and they need auxin to influence bud outgrowth. Arabidopsis *MAX3* and *MAX4* have been shown to encode divergent members of the carotenoid cleavage dioxygenase (CCD) family that can act on multiple

linear and cyclic carotenoid substrates and generate a mobile signal (Sorefan et al., 2003; Booker et al., 2004). *MAX3* encodes for CCD7 (Booker et al., 2004; Schwartz et al., 2004), and *MAX4* encodes for CCD8 (Sorefan et al., 2003). Studies have provided evidence that exogenous auxin can enhance the expression of *MAX4* (Sorefan et al., 2003; Agusti et al., 2011). The negative effect of auxin on branching may require the up-regulation of *MAX4,* which was detected in the root, especially in the root tip (Sorefan et al., 2003).

MAX1 is a cytochrome p450 family member (Booker et al., 2005). The MAX-dependent signal generated by MAX3 and MAX4 requires further modification by MAX1 to synthesize the strigolactone. MAX2 has been identified as an F-box LRR containing member of the SCF family of ubiquitin ligases that functions in regulating protein degradation (Stirnberg et al., 2002). It acts in the MAX-dependent hormone signal transduction pathway (Stirnberg et al., 2002; Booker et al., 2005). Over-expression of *MAX2* rescues the hyperbranching phenotype of *max2* mutants (Stirnberg et al. 2007). This supports the contention that axillary bud outgrowth is controlled the SCF$_{MAX2}$ complex.

The interactions between auxin and strigolactone have been studied. Though *max* mutant buds are resistant to the inhibitory effects of apically applied auxin, the AXR1-mediated auxin signaling pathway was found not to be directly involved in the MAX-dependent regulation of branching (Bennett et al., 2006). An auxin efflux facilitator termed PIN1 has been suggested as the direct regulator in branching suppression in the auxin signaling pathway. *max* mutants were found to have increased auxin transport

capacity resulting from increased abundance of PIN1 (Bennett et al., 2006). The accumulation of PIN1 in the stem may allow axillary buds to export auxin out to the stem and thus decrease the negative effects of auxin (Mader et al., 2003; Tanaka et al., 2006). This may allow axillary buds release from dormancy and lead to hyperbranching phenotype of *max* mutants (Bennett et al., 2006).

However, another hypothesis described MAX-dependent hormone as a second messenger whom produced long-range transmissible signals in root and shoot and the signals may interact with auxin and involve in branching inhibition. To prove this hypothesis, grafting experiments were conducted on several species. Genes involved in in the carotenoid-derived hormone biosynthesis and signaling pathways have been identified in pea (*RAMOSUS1-5* [*RMS1-RMS5*]) and petunia (*DECREASED APICAL DOMINANCE1-3* [*DAD1-DAD3*]), and the orthologs to *MAX4* in pea and petunia have already been identified as *RMS1* and *DAD1*, respectively (Snowden et al., 2005; Sorefan et al., 2003; Foo et al. 2005). Auxin-independent signal was considered involved in branching inhibition through tracing the expression of *RMS1* in rms mutants (Bainbridge et al., 2005).The hyperbranching phenotype of *max1* and *max3* mutants in Arabidopsis, the *rms1*, *rms2* and *rms5* mutants in pea, and the *dad1* mutant in *Petunia* all suggested that the long-range signal may be produced to inhibit branching (Beveridge, 2000; Beveridge et al., 1997; Morris et al., 2001; Napoli, 1996; Turnbull et al., 2002).

In summary, it is already clear that the MAX-dependent hormone plays a role in inhibiting branching. However, the relationship between the MAX-dependent hormone this novel carotenoid-derived hormone and auxin still needs further investigation.

2.4.4 Abscisic acid

Various studies have provided conflicting evidence regarding the role of abscisic acid (ABA) in branching. Eliasson (1975) described that the abscisic acid-like inhibitor can strongly inhibit lateral buds outgrowth in *Populus tremula* L. and *Pisum sativum* L. Zieslin (1978) reported the similar inhibitor by providing the evidence showing that the inhibitive effects on axillary bud outgrowth was induced by old stem and leave tissues. Also, it has been proven that ABA can inhibit bud outgrowth by applying it directly to active buds (Rogan and Smith, 1976; Wareing and Phillips, 1983; Tamas, 1995; Taylor et al., 1995). However, studies with ABA insensitive *Arabidopsis* mutants (*abi1-1* and *abi2-1*) showed that auxin can inhibit axillary bud outgrowth independently without ABA activity, thus it was concluded that ABA cannot be a second messenger for indoleacetic acid (IAA) in apical dominance responses in *Arabidopsis* (Chatfield et al., 2000). A subsequent study on the interaction of auxin and ABA of pea showed that applying ABA below the axillary bud position moderately repressed axillary bud outgrowth (Cline and Oh, 2006). Moreover, additive repression of axillary bud outgrowth in *Ipomoea nil* was observed when combining apical auxin with basal ABA treatments. These results proved that basally applied ABA partially restored apical dominance via acropetal transport up in the shoot, which suggests that ABA might interact with auxin and other unidentified inhibitors to regulate branching (Cline and Oh, 2006). The inhibitory effects directly depended on the application site and transport direction because only basally applied, and acropetal transported, ABA suppressed axillary bud outgrowth. In summary, the relationship between ABA and auxin is still

unknown. But it is now clear that ABA inhibits bud outgrowth in response to low R:FR (Reddy et al., 2013).

2.5 Environmental factors influencing shoot branching

Environmental factors including parameters such as light intensity and the red: far red ratio (R:FR), which is a competition signal, also contribute to the control of shoot branching.

2.5.1 Light signals

Photosynthetic photo flux density (PPFD) is a light signal that can affect bud outgrowth. Increasing the PPFD reduces the correlative inhibition (CI), which is a measure of systemic inhibition of branching, and releases the outgrowth of lower branches (Su et al., 2011). Far red light (FR) is an early signal reflected by neighbor plants eliciting shade-avoidance responses. The red light far-red light ratio is another important branching influencing factor.

2.5.1.1 Red:Far-red light

The R:FR is the ratio of red light and far-red light, with the photon irradiance between 655 and 665 nm and between 725 and 735 nm, respectively. In a high density growing conditions, plants will absorb the red light while reflect the far-red light to neighbor plants thus decrease the ratio of R:FR of their competitors. Neighbor plants who received the reflected far-red light will then show "shade avoidance syndromes" (SAS) as a response to the decrease of R:FR during their whole life including germination, subsequent growth and development (Franklin et al., 2005). The SAS describes plants avoiding shade and maximizing light resource occupation when

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growing in a population (Smith and Whitelam, 1997; Ballare, 1999). Studies on *Lolium multiflorum* showed that tillering activity can be regulated by the R;FR, with tillering elevated by high R:FR (Deregibus et al*.,* 1983; Casal et al., 1986). It was concluded that phytochrome was involved in the process. This conclusion was further supported by the work by Wan and Sosebee (1998) demonstrating that high R:FR was able to stimulate both basal and aerial tiller production in *Eragrostis curvula*. Phytochromes are known to be responsible for sensing the R:FR (Kebrom et al., 2006; Finlayson et al., 2010). Among the five members in *Arabidopsis* (phyA-phyE), phytochrome B (phyB) plays the major role in sensing the R:FR (Franklin et al. 2003; Chen et al. 2004).

To investigate the potential role of ABA in regulating branching, wild type Arabidopsis and various mutants were used. The specific objectives are to:

1) Define the role of ABA in regulating branching under continuous high and low R:FR. Hypothesis- ABA plays an important role in regulating branching under both light regimens.

2) Define the pattern of ABA accumulation in axillary buds of plants grown under high and low R:FR. Hypothesis- Low R:FR will result in elevated bud ABA accumulation. Younger buds will accumulate more ABA which acts locally in the bud.

3) Characterize the interaction between ABA and auxin and how they regulate bud outgrowth. Hypothesis- ABA acts downstream of auxin to inhibit bud outgrowth.

4) Determine where in the branching pathway ABA operates with respect to other known components. Hypothesis- MAX2 or BRC1, or both, act downstream of the ABA-mediated branching pathway.

3. MATERIALS AND METHODS

3.1 Plant materials and growth conditions

Arabidopsis thaliana was used as plant material. Seeds were stratified in distilled water for 2-4 days at 4°C and then planted on a commercial growth medium (LC1). Plants were grown in trays (30 x 60 cm) with 36 cells and 1 plant per cell in a growth chamber with 18 h photoperiod (long days) at 24°C during the day and 18°C during the night. Each cell was fertilized with 7 ml of 1X Hoagland's solution once a week until harvest. Diodes emitting far-red light were used to decrease the R:FR. Light was measured with a Licor Li-1800 spectroradiometer and the R:FR was calculated as the photon flux from 655 to 665 divided by the photon flux from 725 to 735.

3.2 Branching analysis

First, the roles of NCED3 and ABA2 in regulating branching in *Arabidopsis* were determined by growing WT, *nced3-2*, *aba2-1* under both low (0.08) and high R:FR (4.0) and then conducting an architectural analysis on the 10th day after anthesis. Thirty six individual plants of each genotype were examined. The architectural analysis included the number of primary and secondary rosette and cauline leaves, numbers and lengths of primary rosette and cauline branches (shoots > 3mm), numbers of secondary branches (shoots > 3mm), and numbers of primary and secondary rosette and cauline buds or meristems (shoots < 3mm). The number of rosette leaves was taken as the branching potential of the plant. The value of primary branches/ rosette leaves was used to compare the differences in bud outgrowth taking variations in rosette leaf number into

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account. Similarly, the value of cauline branches/cauline leaves was calculated to compare the differences in inflorescence branching.

3.3 Bud ABA content measurement

WT (Col-0) was grown under 185 μmol m^{-2} s^{-1} PPFD, both low R:FR (0.08) and high (4.0) R:FR. Low R:FR application started 2 days after planting. The top three rosette axillary buds were harvested just before the predicted onset of elongation of the top bud. Four biological replicates were collected and kept in liquid nitrogen immediately with approximately 12 to 14 buds in each sample. Each sample was weighed immediately after collection and then frozen at -80°C. 10μL of labeled standard phytohormone isotopes 2H^{2}ABA were added into each sample replicate. Tissue was grounded in 1.5mL microfuge tube with a plastic pestle and extracted by methanol for at least twice and then by 80% ethanol for once. Both methanol and ethanol were hot (55°C). The extracts were collected in a large culture tube and dried down to about 100μL in the Speedvac at medium temperature. 800μL chloroform and 1mL water together with one drop of 2% NH$_4$OH were used two times for removing contaminants including chlorophyll. Acidic hormones were attracted into chloroform phase. Then 1mL ethyl acetate together with one drop of 2.6% acetic acid was used for purifying the hormones further. Cryopumping was used for separating aqueous phases on the Speedvac. The upper ethyl acetate phase was transferred into Reactival after purification. Methylation were used as analytical derivation of hormones. For methylation (ME), 10μL methanol and a half drop of ethereal diazomethane were used for both isotope labeled and unlabeled samples. 7μL ethyl acetate was used for resuspending the sample

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dried under N_2 in Reactival and all the samples were transferred into ALS vials. The combination of derivation and ionization were sent for gas chromatography - mass spectroscopy- selected ion monitoring (GC-MS-SIM) measurement: negative chemical ionization (NCI) combined with ME. The GC-MS machine was an Agilent 7890 GC-5975 MSD. The retention time together with the areas of both labeled standard selected ion (mass 284) peak and unlabeled sample selected ion (mass 278) peak were collected. ABA abundance was calculated according to the formula below:

$$Abundance = (((Area_{unlabeled}/Area_{labeled})*1)/fresh\ weight)/Mass_{ABA}*1000$$

3.4 ABA application treatment

Wild type, *axr1-12*, *max2* and *brc1* were grown with high R:FR (7.0) under 195 $\mu mol\ m^{-2}\ s^{-1}$ PPFD. At 2 approximately days before anthesis, 1 μL of 100 μM ABA together with 0.03% of Silwet was applied to top five rosette axillary buds [bud n (uppermost bud), bud n-1 (bud immediately below bud n) and bud n-2 (bud immediately below bud n-1), etc.] for each genotype every day with 24h intervals untill harvest. 0.03% Silwet was used as control. A 10μL glass syringe with a long narrow needle was used to apply ABA or pure silwet onto the meristem of the buds. Twenty four individual plants were treated and examined. Plants were harvested at 5 Days Post Anthesis (DPA) and branching analysis was conducted as described in 3.2.

3.5 Gene expression test

The roles of abscisic acid in branching were further assessed by measuring the expression of the ABA biosynthesis gene *NCED3* in the three topmost rosette axillary buds from WT, *brc1* and *nced3-2* by quantitative real-time PCR (QPCR). Axillary buds

of various genotypes from the three topmost rosette leaf axils were collected by position before the onset of the outgrowth. Four biological replicates were collected, with approximately 12 buds in each replicate. Harvested buds were immediately frozen at - 80°C. Total RNA was extracted with TRIzol (Invitrogen). RNA concentration and purity were estimated by spectrophotometry. Gel electrophoresis was used to verify RNA quality and ensure similar concentrations among samples. Three and a half units of RQ1 DNAse was added to 5 μ g of RNA from each sample to digest DNA according to the manufacture's protocol (Promega) followed by re-extraction of the RNA with TRIzol (Invitrogen). The concentration of each RNA sample after re-extraction was measured by spectrophotometery and each RNA sample was suspended to the same concentration. cDNA was synthesized from the RNA using the Superscript III kit according to the manufacture's protocol (Invitrogen). Controls (minus RT) were also prepared by substituting water for the reverse transcriptase. RNAseH was added afterwards to remove the remaining RNA. The cDNA was then diluted 1:10 for further use. QPCR was performed using 3 replicates of plus RT sample and 1 of minus RT sample (used to verify that genomic DNA contamination did not substantially affect results). 10 μL QPCR reactions were run using the SYBR Green Jumpstart kit (Sigma) on an ABI 7900 HT SDS instrument (ABI), following the manufacturer's recommendations. *NCED3* and *18S* primers were used at 100 nM each forward and reverse. A standard curve for each primer set was generated from a dilution series of known concentrations of cloned fragments. Cycle threshold values of the target genes were determined and converted to the actual transcript number per reaction using the *18S* ribosomal RNA as a control. The

dissociation curve of each reaction was checked to verify primer specificity. For

NCED3, the primer combination ACGGATTTCACGGTACATCATCG (forward) and

ATTCCGGGGACGTATATGCAGA (reverse) was used. For *18S* rRNA, the primer

combination AAACGGCTACCACATCCAAG (forward),

ACTCGAAAGAGCCCGGTATT (reversed) was used. The expression levels of the

various genes were compared in buds from different positions of individual genotypes,

as well as between buds from the same position of various genotypes.

3.6 Statistical analysis

Eighteen biological replicates of each genotype/light treatment were used for

architectural analysis, and each experiment was performed twice. The data from the two

experiments were combined giving a total of 36 observations per genotype/light

treatment. Four biological replicates of each genotype/light treatment were collected for

the gene expression analysis, with approximately 12 buds in each replicate. All statistics

analyses were run using JMP software and Analysis of Variance (ANOVA). A Tuckey

HSD test was used for *post hoc* comparisons with significance at $\alpha = 0.05$. Student's t-

test was used for comparisons between treatments within genotypes with significance at

$\alpha = 0.05$. Fischer's exact probability test was used for comparisons of branch outgrowth

frequency between treatments with significance at $\alpha = 0.05$.

4. RESULTS

4.1 Objective 1: Defining the role of ABA in regulating branching under high and low R:FR
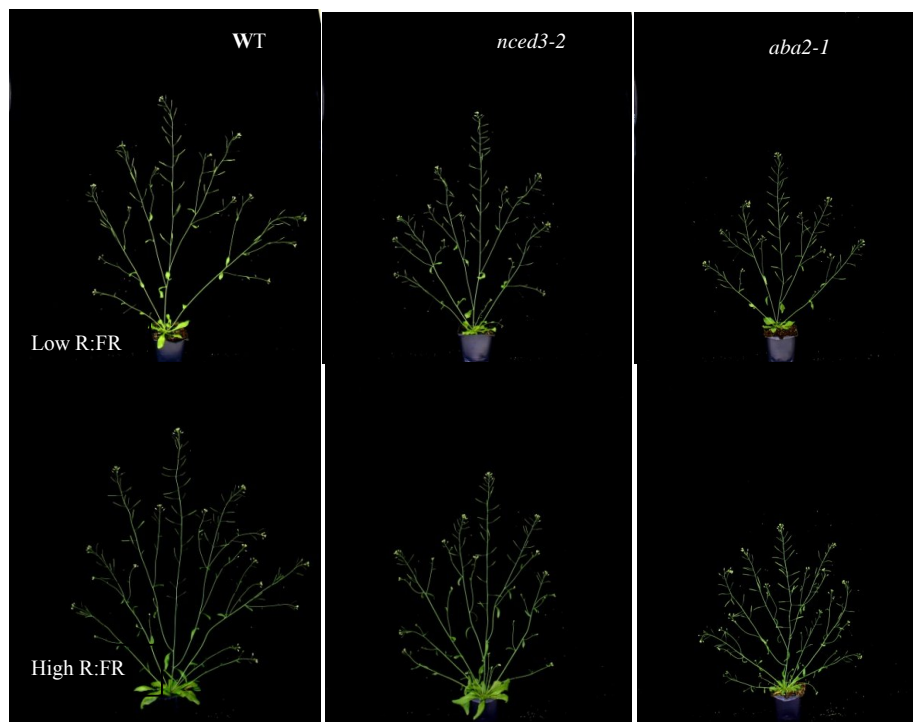

Figure 1 Phenotypes of WT, *nced3-2* and *aba2-1* grown under low and high R:FR.

Figure 1 shows the phenotypes of three genotypes grown under low (0.08) and high R:FR (4.0). Branching analysis revealed that all three genotypes had significantly less rosette leaves under low R:FR than under high R:FR (Fig. 2A). The number of

rosette branches of *nced3-2*, *aba2-1* and WT are significantly smaller under low R:FR comparing with the branches number under high R:FR (Fig. 2B). Also, WT and *aba2-1* had significantly greater correlative inhibition indices under low R:FR than under high R:FR. The differences between the correlative inhibition under low R:FR and high R:FR in WT was much larger than the differences in *aba2-1*. However, the correlative inhibition index in *nced3-2* was not significantly different under low and high R:FR (Fig. 2C). Additionally, a decrease in the R:FR also led to a decrease in plant height of WT and *nced3-2* but not in the height of *aba2-1* (Fig. 2D). All three genotypes showed a significant decrease in cauline branches numbers, sum of rosette branch lengths and sum of cauline branches lengths (Fig. 2 E, F, G).
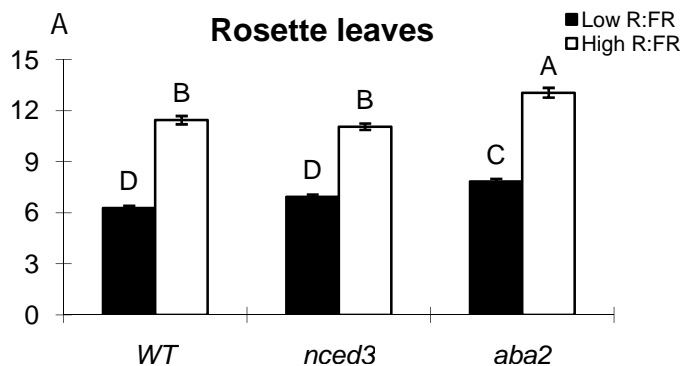
Figure 2 Number of rosette leaves (A), number of rosette branches (B), correlative inhibition (C), plant height (D), number of cauline leaves (E), sum of rosette branch lengths (F) and sum of cauline branch lengths (G) of WT, *nced3-2* (*nced3*) and *aba2-1* (*aba2*) under low and high R:FR. Results are means ± SE, and different letters indicate a significant difference between light treatments within genotypes at $\alpha = 0.05$.
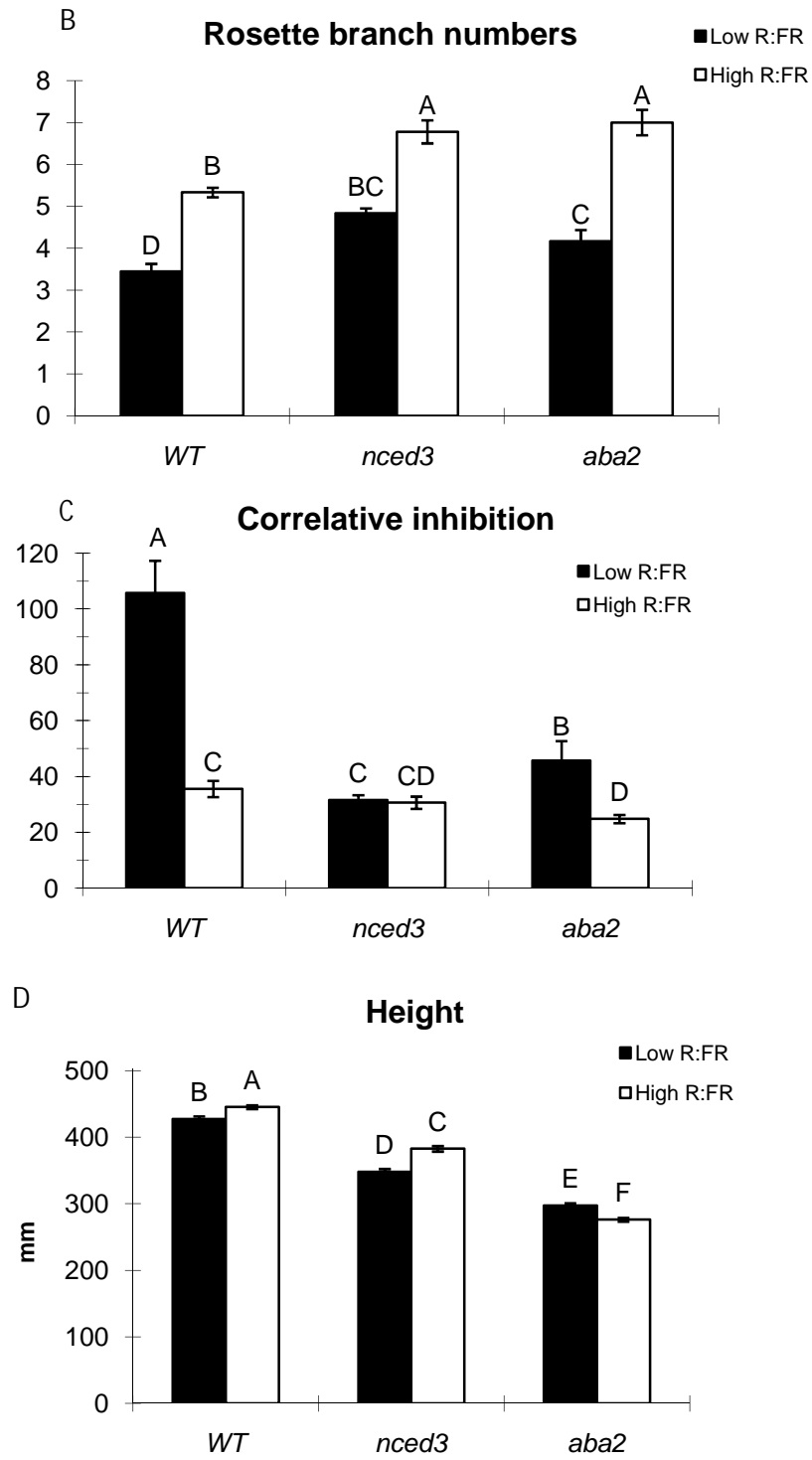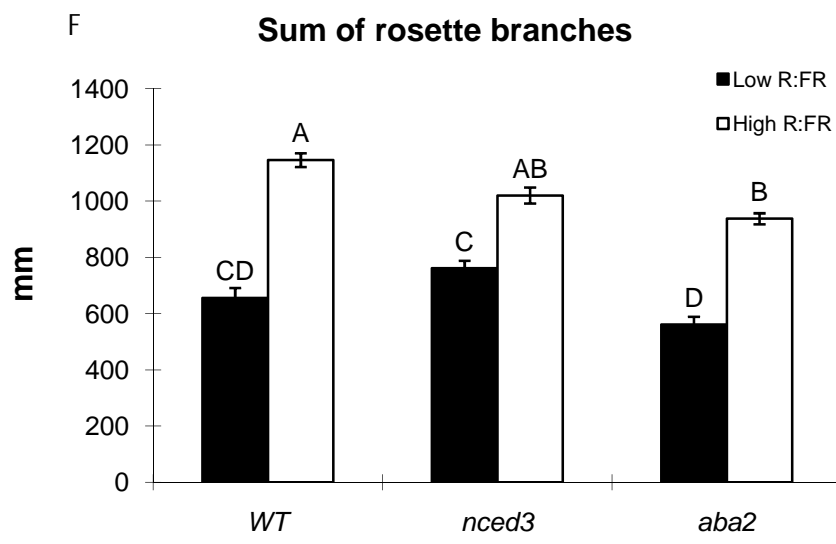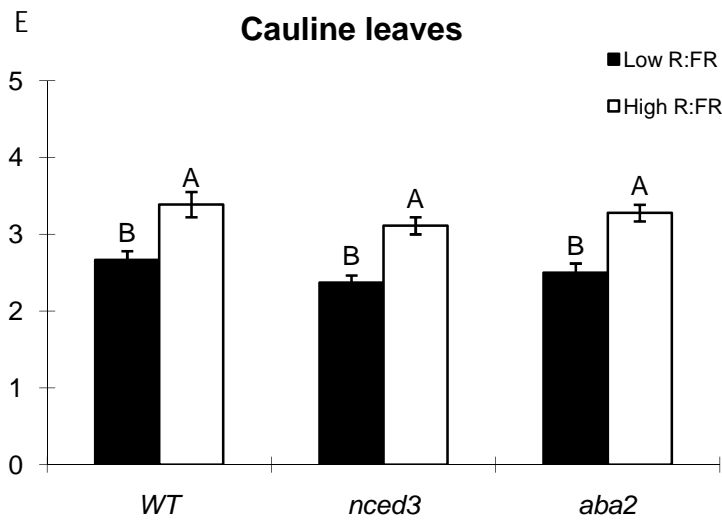
Figure 2 Continued.

Figure 2 Continued.

Sum of cauline branches
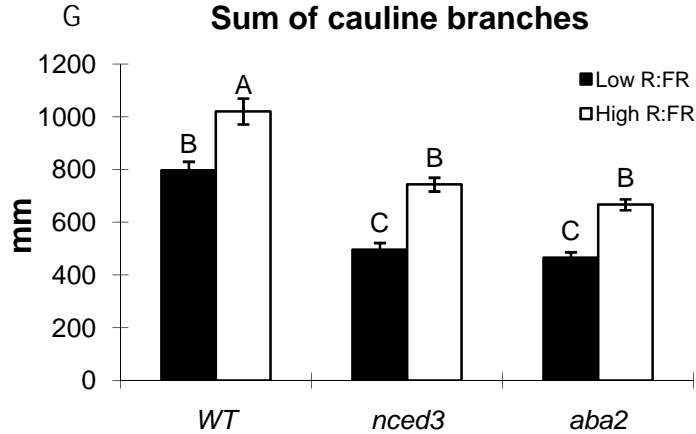
Figure 2 Continued.

When making comparisons among genotypes, it was found the plant height and cauline branch lengths were significantly smaller in the two ABA deficient mutants compared to WT. The rosette leaf numbers and rosette branch numbers were significantly greater in the mutants than WT. The correlative inhibition index value was significantly lower in mutants than in WT both under low and high R:FR. *aba2-1* produced smaller plants with reduced plant height and cauline branch lengths than *nced3-2,* while *nced3-2* was smaller than WT. Thus, the order of plant size from the smallest to largest was: *aba2-1< nced3-2*<WT. Under high R:FR, *aba2-1* had the most rosette leaves and rosette branches, *nced3-2* had intermediate rosette branch numbers and WT had the fewest rosette branches. There were no significant differences in rosette branch numbers between genotypes under low R:FR.

The numbers of primary cauline branches of the various genotypes were analyzed to determine the roles of *NCED3* and *ABA2* in the regulation of cauline

branching (Fig. 2G). All three genotypes had almost 100% bud initiation (cauline

branches+axillary buds)/ axil (data not shown) and elongation. Therefore, *NCED3* and

ABA2 seems have no effects on cauline axillary meristem initiation or outgrowth.

Moreover, the number of cauline branches of all three genotypes showed significant

differences under low and high R:FR. This suggests that ABA does not play a necessary

role in determining the number of cauline leaves under low R:FR.

4.2 Objective 2: Defining the pattern of ABA accumulation in axillary buds of plants grown under high and low R:FR

Figure 3 Top three rosette [bud n (Rn), bud n-1 (Rn-1), bud n-2 (Rn-2)] ABA content of WT under

low and high R:FR. Results are means ± SE, and different letters indicate a significant

difference at $\alpha = 0.05$.

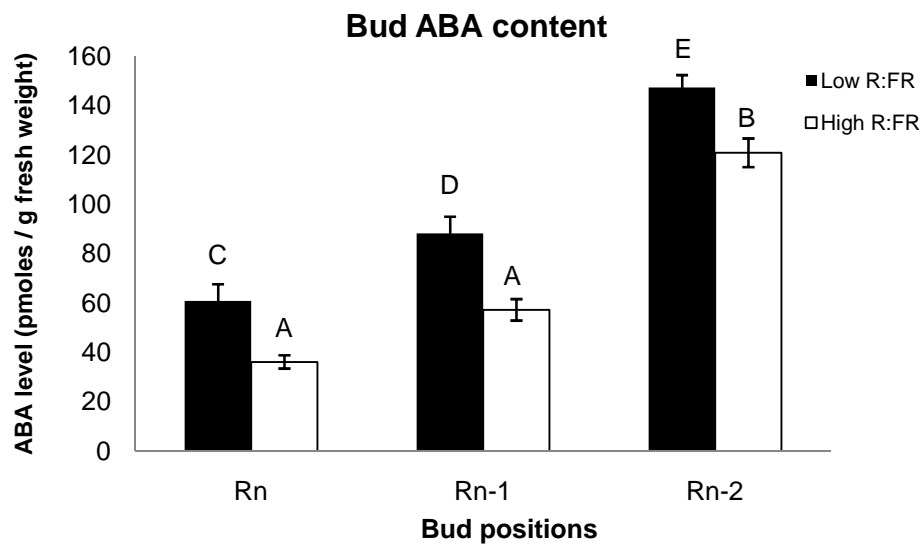Figure 3 shows the ABA content of the top three rosette buds of WT under low and high R:FR. Bud n-2 had highest ABA content both under low and high R:FR, while bud n had the lowest ABA content. Under low R:FR, the ABA level in bud n-1 was significantly higher than the level in bud n while lower than the level in bud n-2. However, there was no significant difference between the ABA level in bud n and in bud n-1 under high R:FR. In general, all three buds showed significantly higher ABA content under low R:FR comparing to the ABA content under high R:FR. The difference between the ABA level of bud n-2 and bud n was larger under low R:FR than under high R:FR, although bud n had an elevated ABA level under low R:FR compared to the level under high R:FR. The order of bud ABA content from smallest to largest was the same under low and high R:FR, which is bud n< bud n-1< bud n-2.

4.3 Objective 3: Characterizing the interaction between ABA and auxin and how they regulate bud outgrowth

Figure 4 shows the phenotypes of WT and *axr1-12 Arabidopsis* genotypes with and without exogenous ABA treatment. Branching analysis revealed that both genotypes had significantly smaller rosette branch lengths after ABA application (Fig. 5A). Differences in rosette branch lengths were only significant in n-1 to n-4 branches in WT while significant differences were observed in all top five branches in *axr1-12* after treating with ABA (Fig. 5B). Also, WT had a significantly greater correlative inhibition index with and without exogenous ABA treatment compared to *axr1-12*. However, the correlative inhibition index in *axr1-12* was not significantly different after treating with ABA (Fig. 5C). Moreover, it seems ABA application does not change the rosette or

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cauline branch numbers in WT (Fig. 6A, B). However, the rosette branch number was significantly decreased in *axr1-12* after ABA treatment. The differences of the values between the rosette and cauline leaf numbers were not significant. The plant height was similarly not affected significantly after ABA treatment (Fig. 6C).
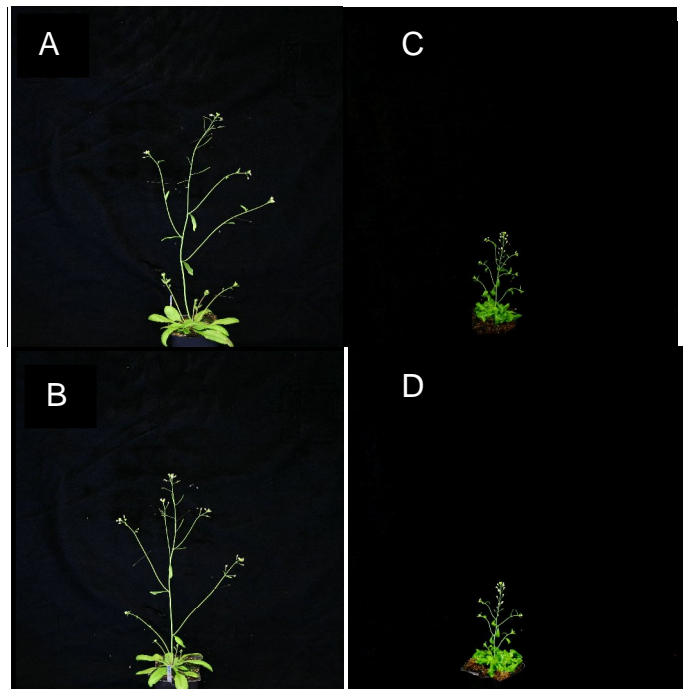
Figure 4 Phenotypes of WT not treated with ABA (A), WT treated with ABA (B), *axr1-12* not treated with ABA (C) and *axr1-12* treated with ABA (D).
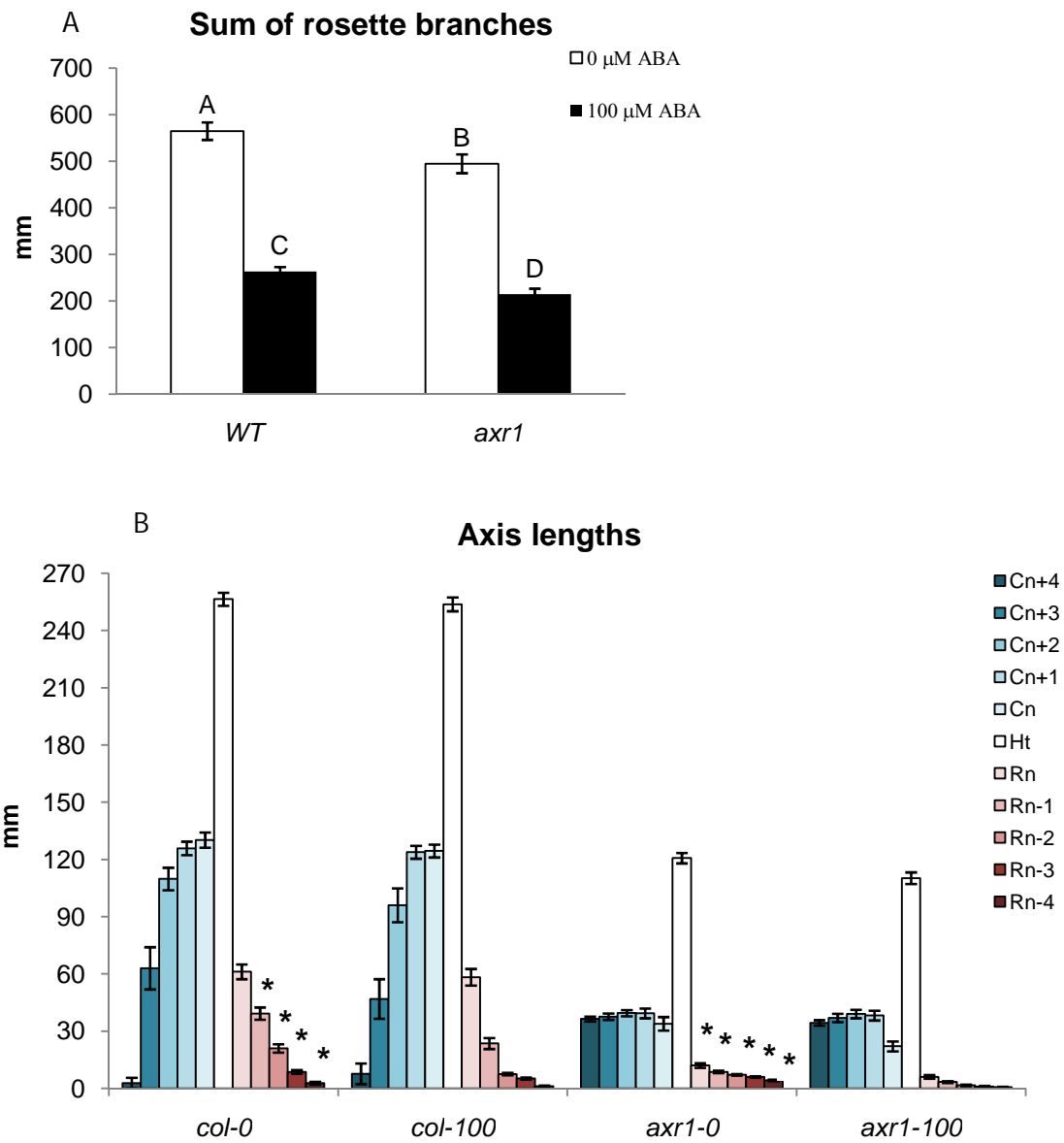
Figure 5 Sum of rosette branch lengths (A), axis lengths (B), correlative inhibition (C) of WT and

axr1-12 (*axr1*) with (100 μM ABA) and without ABA treatment (0 μM ABA). Results are

means ± SE, different letters indicate a significant difference at α = 0.05, and asterisks indicate

a significant difference between ABA treatments within genotypes at α = 0.05.

C **Correlative inhibition**
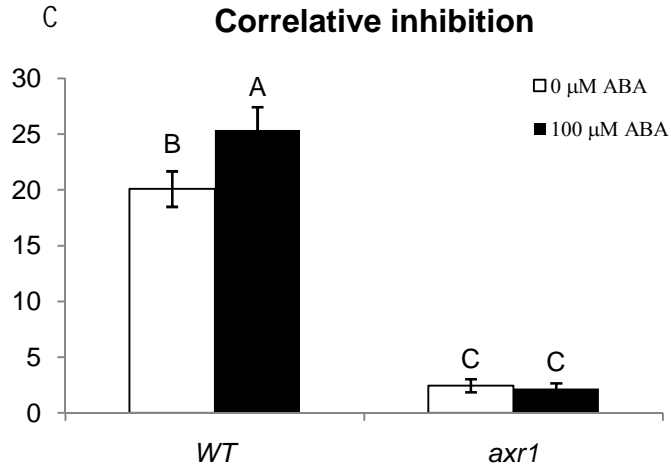
Figure 5 Continued.

The rosette branch outgrowth frequency is the average of the frequency of rosette branches elongated longer than 3mm. The outgrowth frequency of the top five rosette branches was significantly suppressed in *axr1-12* after treating with 100 pmoles ABA. All five rosette branches have significantly lower outgrowth frequency in the treatment group compared to control. In contrasting, no significant differences of the outgrowth frequency of the top three rosette branches were found in WT. Significant differences in outgrowth frequency were only found in n-3 and n-4 branches (Fig 6. D).
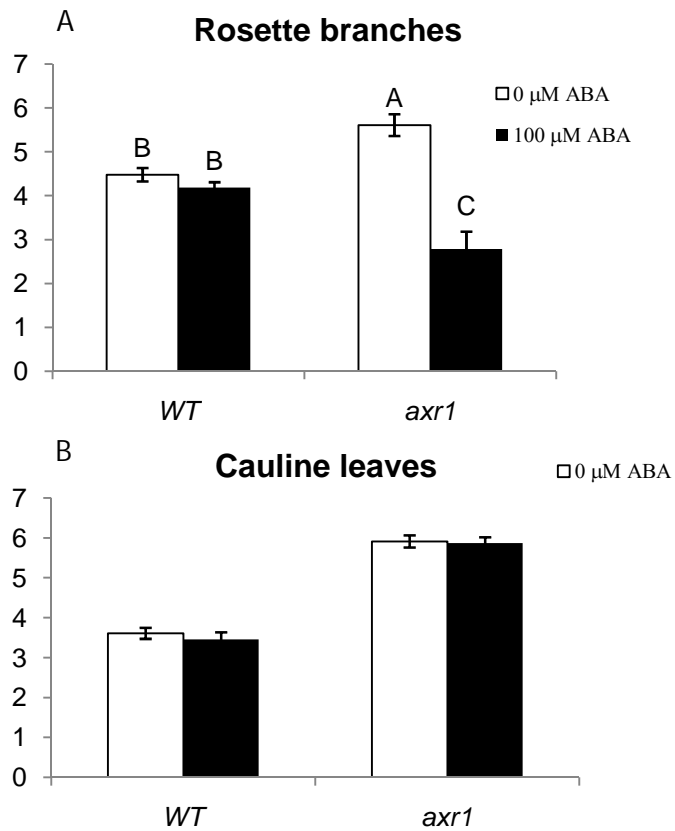
Figure 6 Number of elongated rosette branches (A), number of cauline leaves (B), plant height (C)

and the rosette branch outgrowth frequency (D) of WT and *axr1-12* (*axr1*) with (100 μM

ABA) and without ABA treatment (0 μM ABA). Results are means ± SE, different letters

indicate a significant difference at α = 0.05, and asterisks indicate a significant difference

between ABA treatments within genotypes at α = 0.05.

C

Height

D

Rosette branch Growth Frequency
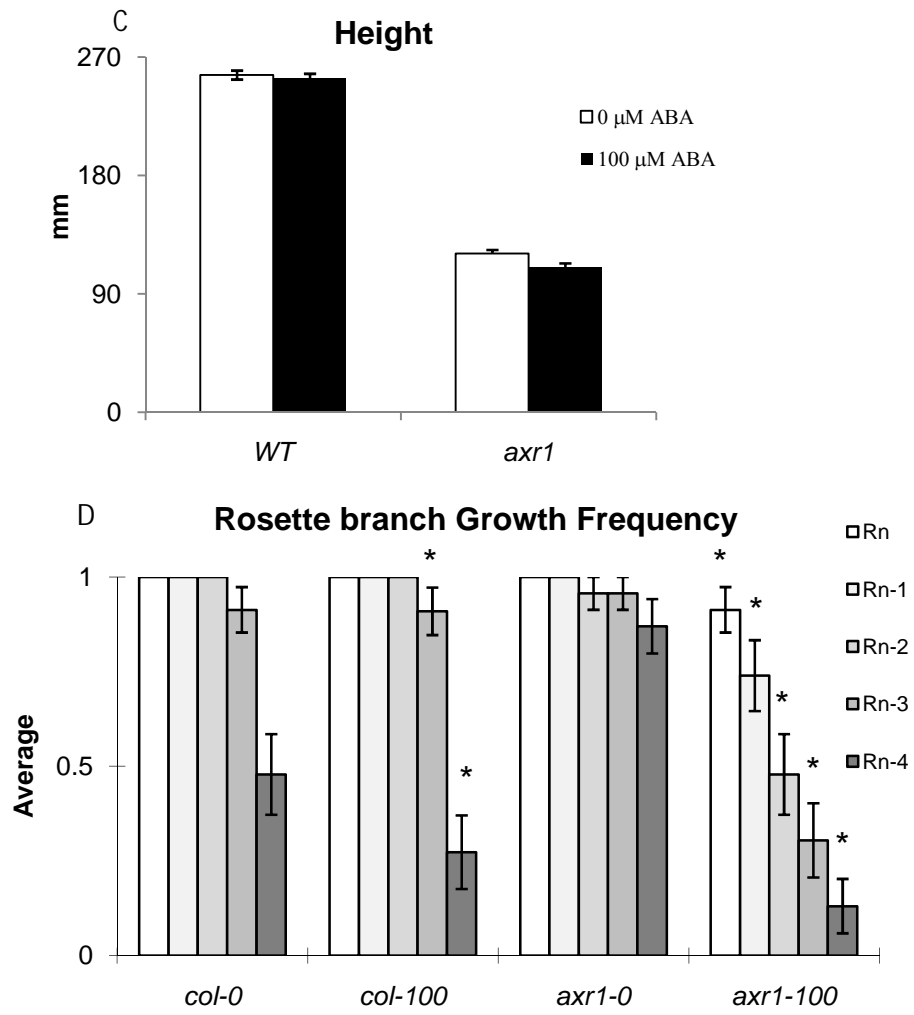
Figure 6 Continued.

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4.4 Objective 4: Determining where ABA operates in the branching pathway with respect to other known components

*4.4.1 Objective 4.1: Defining the role of exogenous ABA on MAX2 deficient mutant branching*

Figure 7 Phenotypes of WT not treated with ABA (A), WT treated with ABA (B), *max2* not treated with ABA (C) and *max2* treated with ABA (D).

Figure 7 shows the phenotypes of WT and *max2* with and without exogenous ABA treatment. Branching analysis revealed that WT had a significantly reduced sum of

rosette branch lengths after ABA application while *max2* had no significant differences

(Fig. 8A). Differences in the lengths of individual WT rosette branches were observed

from n-1 to n-4 branches after treating with ABA, which is consistent with the results

shown in objective 3. However, for *max2*, the differences in rosette branches length were

only apparent in the topmost branch (branch n, Fig. 8B). WT also had a significantly

greater correlative inhibition index with and without exogenous ABA treatment, but

similar differences were not seen in *max2* (Fig. 8C).

A
Sum of rosette branches

Figure 8 Sum of rosette branch lengths (A), lengths of top five rosette branches (B), correlative

inhibition (C), number of elongated rosette branches (D), number of cauline leaves (E) and

the rosette branch outgrowth frequency (F) of WT and *max2* with (100 μM ABA) and

without ABA treatment (0 μM ABA). Results are means ± SE, different letters indicate a

significant difference at $\alpha = 0.05$, and asterisks indicate a significant difference between

ABA treatments within genotypes at $\alpha = 0.05$.

B

Top five branch lengths

C

Correlative inhibition

Figure 8 Continued.

D **Rosette branch number**

E **Cauline leaves**

Figure 8 Continued.

F

Branches outgrowth frequency

Legend: □Rn □Rn-1 □Rn-2 □Rn-3 ■Rn-4

Y-axis: Average (0, 0.5, 1)

X-axis: WT-control, WT-ABA, max2-control, max2-ABA

Figure 8 Continued.

Again, ABA application changed the rosette branch numbers but not cauline branch numbers (Fig. 8D, E). The differences between the control and the ABA treatment in the rosette branch numbers and cauline leaf numbers were not significant. The plant height was also not affected significantly by ABA treatment. The outgrowth frequency of the top five rosette branches was not different in *max2* after treating with 100 pmoles ABA (Fig. 8F). In contrast, significant differences in outgrowth frequency were observed in n-2, n-3 and n-4 branches of WT.
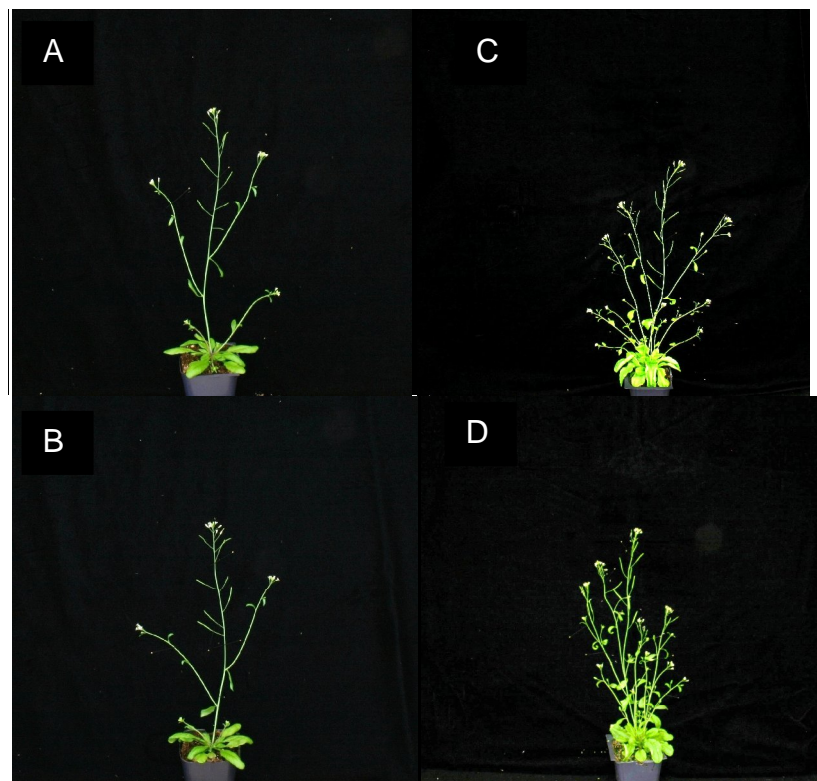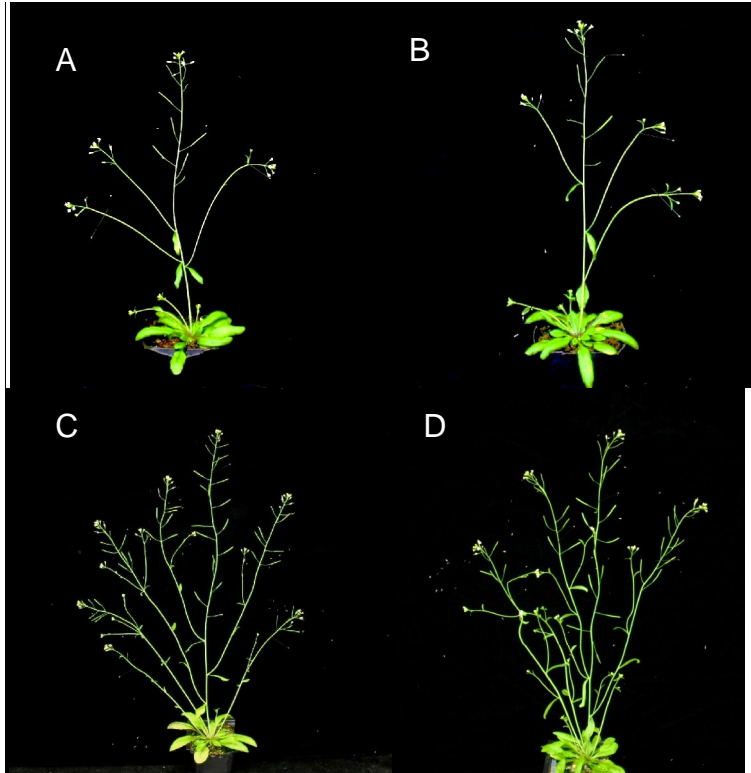
Figure 9 Phenotypes of WT not treated with ABA (A), WT treated with ABA (B), *brc1* not treated
     with ABA (C) and *brc1* treated with ABA (D).

Figure 9 shows the phenotypes of WT and *brc1* with and without exogenous

ABA treatment. Branching analysis revealed that both genotypes had a significantly

reduced sum of rosette branch lengths after ABA application (Fig.10A). Differences in

the lengths of individual rosette branches were observed in n-1, n-2 and n-3 branches of WT which were treated directly with ABA (Fig. 10B). Significant differences were found in the branch lengths of all five branches in *brc1* with ABA treatment.
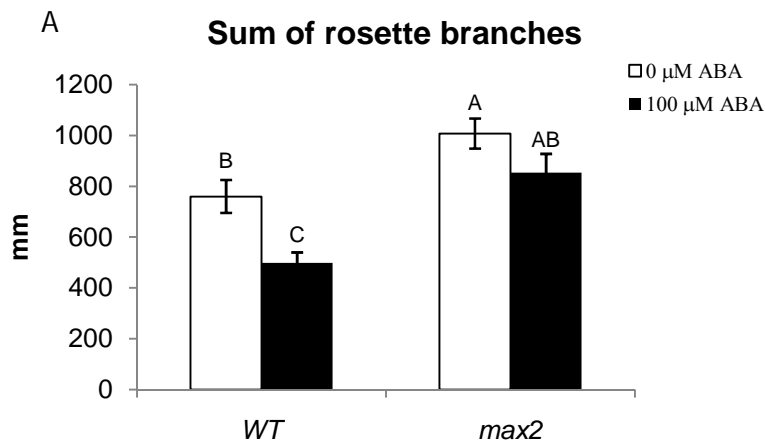
A

Sum of rosette branches
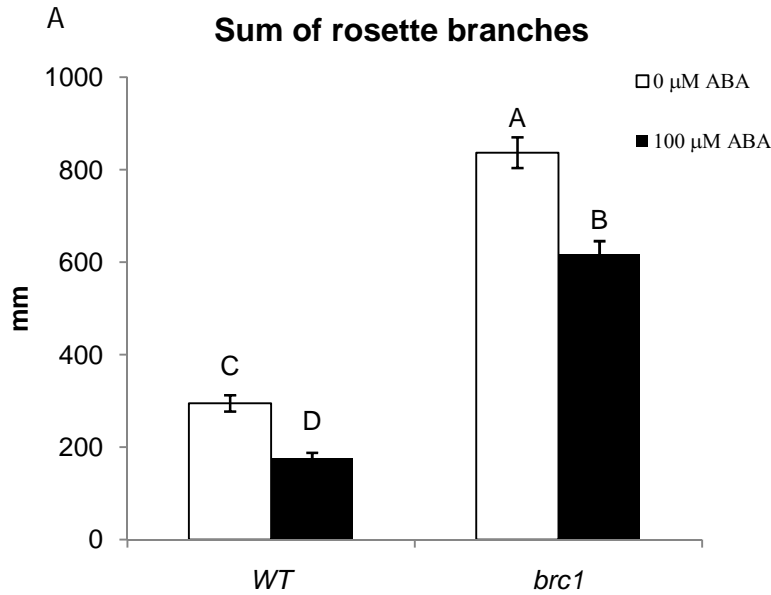
Figure 10 Sum of rosette branch lengths (mm) (A), lengths of top five rosette branches (B), correlative inhibition (C), number of elongated rosette branches (D), number of cauline leaves (E) and the rosette branch outgrowth frequency (F) of WT and *brc1* with (100 µM ABA) and without ABA treatment (0 µM ABA). Results are means ± SE, different letters indicate a significant difference at $\alpha = 0.05$, and asterisks indicate a significant difference between ABA treatments within genotypes at $\alpha = 0.05$.
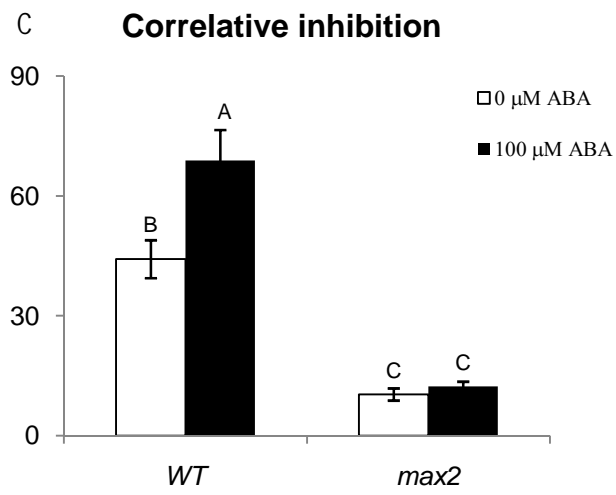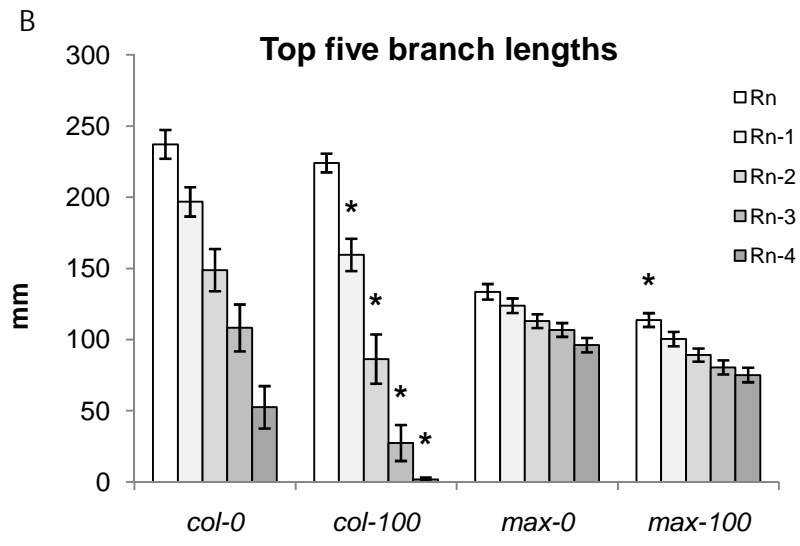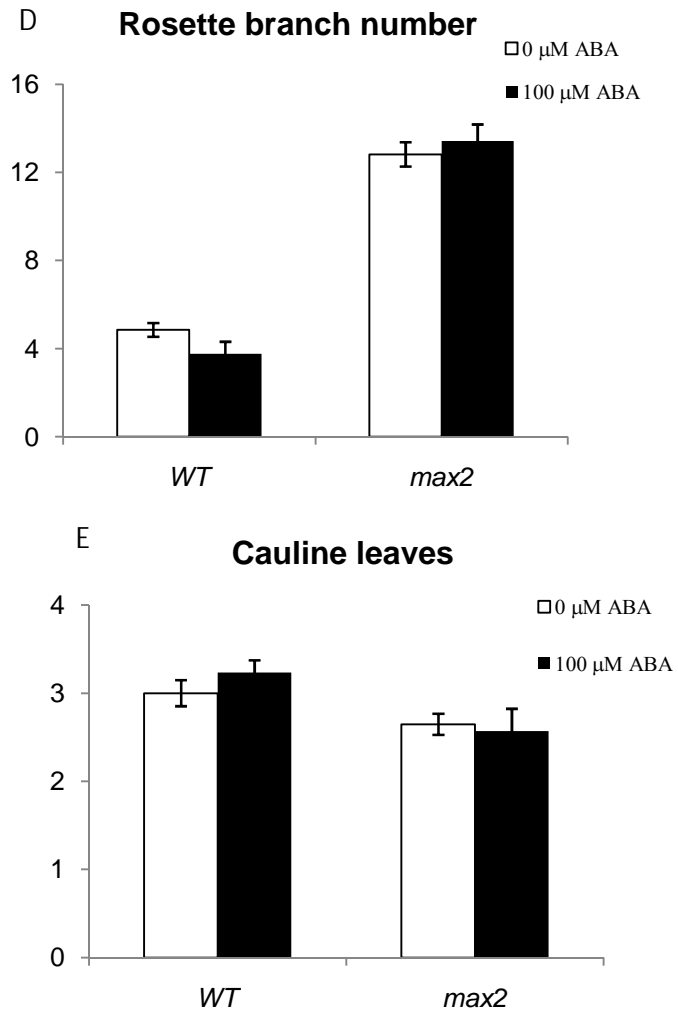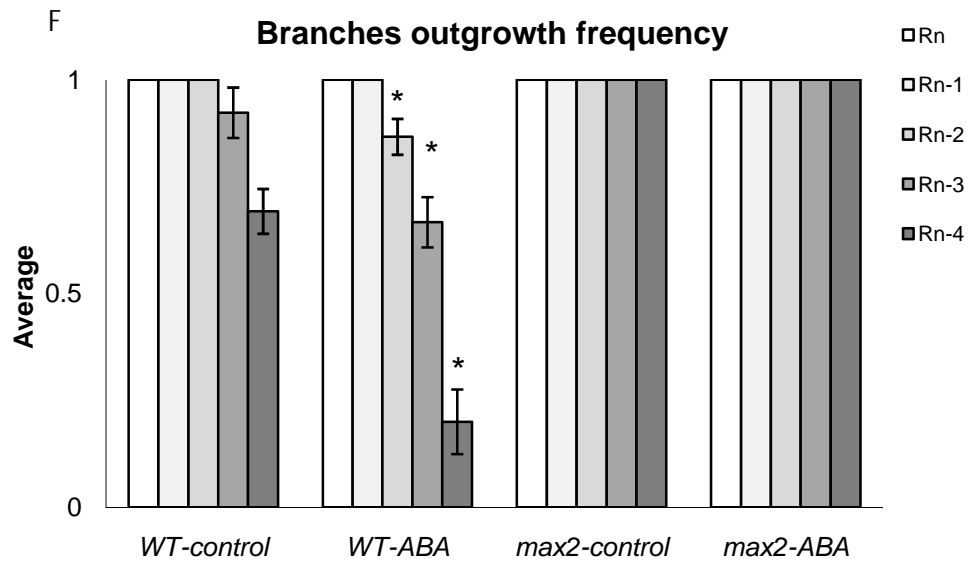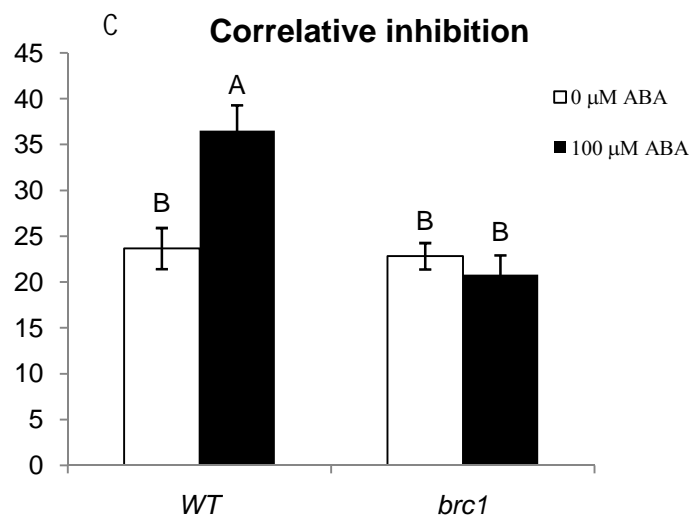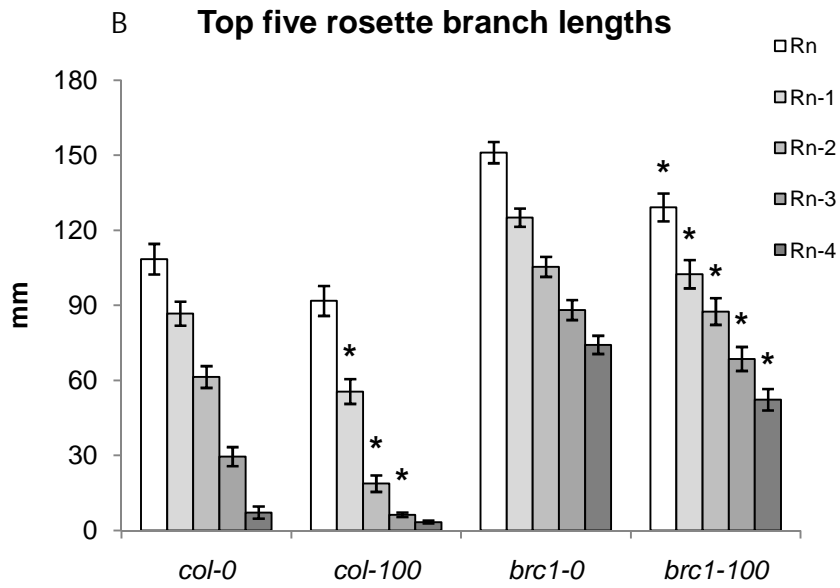
Figure 10 Continued.
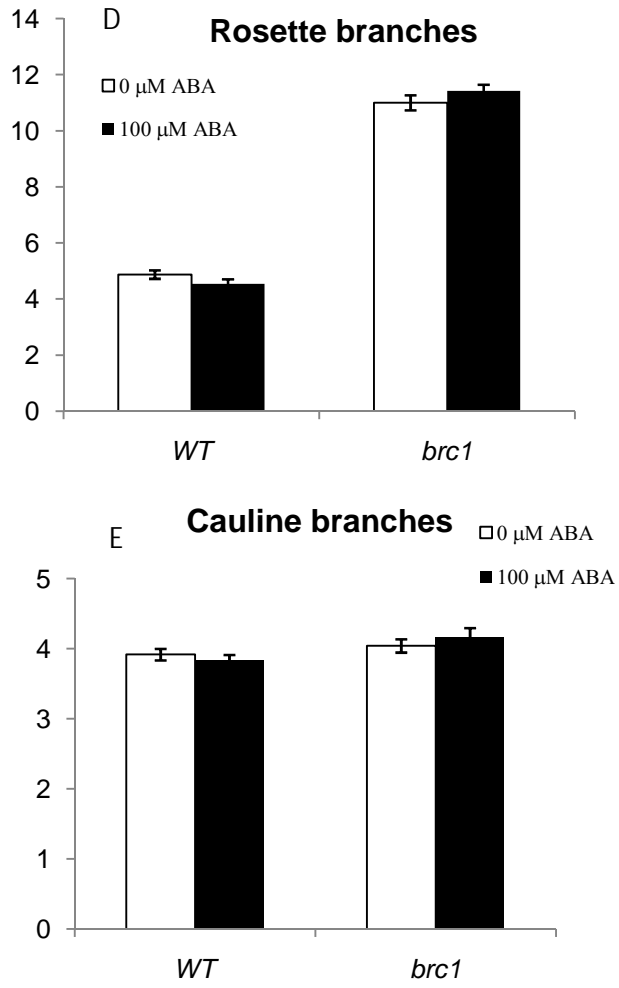
Figure 10 Continued.
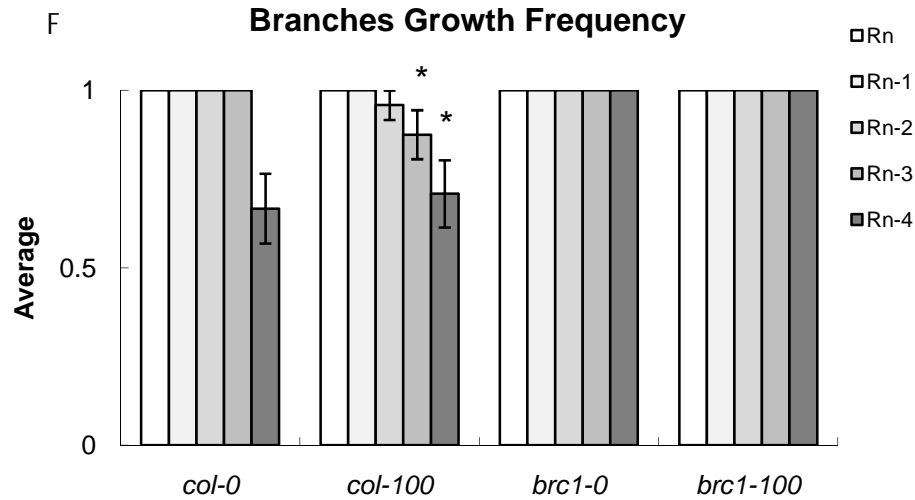
Figure 10 Continued.

As with previous comparisons, WT had a significantly greater correlative inhibition index with exogenous ABA treatment than the control. For *brc1*, the correlative inhibition index was not significantly different (Fig. 10C). Moreover, it seems ABA application did not change either the rosette or cauline branch numbers (Fig. 10D, E). The differences in rosette and cauline leaf numbers with ABA treatment were not significant. The plant height was also not affected significantly by ABA treatment. The outgrowth frequency of the top five rosette branches in *brc1* was not affected by ABA treatment (Fig. 10F). The sum of rosette branch lengths was reduced after treatment with ABA in both *brc1* and WT (Fig. 10A). This indicates that ABA still has a negative effect on branching of *brc1*.

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5. CONCLUSIONS AND DISCUSSIONS

5.1 Objective 1: Defining the role of ABA in regulating branching under continuous high and low R:FR

The results showed that ABA plays a role in rosette bud outgrowth suppression and correlative inhibition in *Arabidopsis*. In WT, both rosette bud outgrowth and rosette leaf initiation were significantly repressed under low R:FR compared to high R:FR. For cauline branches, however, ABA plays a weaker role in branch initiation and elongation suppression comparing with rosette branches.

Taken together, these branching analysis results suggest that ABA has a negative role in regulating the correlative inhibition of branching under low R:FR. This result matches with a previous study that showed that ABA deficiency can cause increased bud outgrowth in plants grown under low R:FR (Reddy et al., 2013). ABA2 seemed to have a greater effect on regulating the number of rosette leaves than NCED3 compared to WT, as the *aba2-1* mutant had more branches and rosette leaves than the *nced3-2* mutant. This matches with Reddy's result which showed that the ABA level in lower position buds of ABA2 deficient mutants was lower than the ABA level in NCED3 deficient mutants, compared to WT.

5.2 Objective 2: Defining the pattern of ABA accumulation in axillary buds of plants grown under high and low R:FR

In summary, low R:FR had a significant role in elevating the bud ABA content, especially in lower position buds. This can partially explain the increase of the

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correlative inhibition index of WT under low R:FR. Because the difference between the value of the correlative inhibition index under low and high R:FR in bud n-2 was larger than that in bud n, bud n-2 seems grow comparatively more slowly under low R:FR. According to the definition of CII, larger differences among top three axillary branches lead to larger slope. That is why there is a significant increase in CII of WT under low R:FR. This result provides additional evidence that ABA plays a negative role in determining the rosette branching potential under low R:FR.

5.3 Objective 3: Characterizing the interaction between ABA and auxin and how they regulate bud outgrowth

Overall, these results suggest that exogenous ABA can partially inhibit branching. It seems that exogenous ABA has a stronger inhibitory effect on the branching pattern of *axr1-12* than WT. The reason why there were no significant differences in the correlative inhibition index of *axr1-12* may be because the mutant plants were too small with short branches which led to minor differences between upper position branches and lower ones. However, it was found that direct application of exogenous ABA can inhibit the outgrowth of *axr1-12* rosette buds. The results suggest that ABA may act locally in the bud, not systematically in the whole plant. Reduced rosette branch lengths also indicated that ABA negatively regulates branch outgrowth and elongation. Since *axr1-12* is auxin insensitive, stronger suppression on the outgrowth of branches of *axr1-12* than WT indicated that ABA may act downstream of auxin. However, further evidence needs to be provided to fully support this view. Additionally, because the inhibitory effects of exogenous ABA did not last longer than

24h (data not shown), it further indicates that ABA may only act locally in the bud to regulate branching.

5.4 Objective 4: Determining where in the branching pathway ABA operates with respect to other known components

In summary, the results suggested that exogenous ABA can partially inhibit branching of *max2*. It seems that exogenous ABA has a weaker inhibitory effect on the branching pattern of *max2* than WT. ABA application did not suppress the outgrowth of rosette branches of *max2* but it partially suppressed branch elongation. These results suggest that MAX2 may act partially downstream of ABA-mediated negative branching regulation. However, the evidence was not clear enough to confirm that MAX2 acts downstream of ABA. If MAX2 acts downstream, then there should be no difference in branching suppression with ABA treatment when the function of MAX2 is lost. However, an inhibitory effect of exogenous ABA was observed in *max2* which means MAX2 may only partially act downstream of ABA. One possibility is that the MAX-mediated pathway may be parallel to the ABA-mediated pathway and they share the same downstream regulator. Another possibility is that ABA may increase carotenoid biosynthesis as a feedback while carotenoid increases MAX activity. Previous studies have suggested that strigolactone acts downstream of auxin (Brewer et al., 2009). Taken together, it can be postulated that ABA acts downstream of auxin to negatively regulate branching. However, the relationship between ABA and the MAX-related hormone is still unknown.

Exogenous ABA has a minor inhibitory effect on the branching pattern of *brc1*.

Significant differences between treatment and control were found in the sum of rosette branch lengths and in all ABA treated individual branch lengths. The rosette branch numbers and the rosette bud outgrowth frequency were not affected by the application. Compared with WT, exogenous ABA played a weak role in inhibiting the branching of *brc1*. Unlike in *max2*, *brc1* showed significant decrease in branch lengths after ABA treatment in all top five branches. Thus, the data indicate that *BRC1* may partially act downstream of ABA in regulating branching. Moreover, the phenotype of *brc1* looks more similar to WT than the phenotype of *max2* or *axr1* mutants. Because the outgrowth frequency had not been influenced, it suggests that ABA acts locally in the bud to suppress branching which is consistent with previous results.

All in all, the data support the hypothesis that ABA plays a role in bud outgrowth suppression and correlative inhibition of *Arabidopsis* branching. It has been shown that ABA tends to accumulate in the buds with low outgrowth potential. Moreover, another conclusion has been reached that *BRC1* may partially act downstream of ABA while ABA may act downstream of auxin to regulate branching.

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