ROLE OF BRASSINOSTEROID CATABOLISM IN

ARABIDOPSIS DEVELOPMENT

By

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ROLE OF BRASSINOSTEROID CATABOLISM IN ARABIDOPSIS DEVELOPMENT

Abstract

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BAS1 (phyB-4 ACTIVATION TAGGED SUPPRESSOR 1) and SOB7 (SUPPRESSOR OF phyB-4 7) are brassinosteroid-catabolizing P450s in Arabidopsis thaliana that synergistically/redundantly modulate photomorphogenic traits such as flowering time. This study investigates the role of BAS1 and SOB7 in photomorphogenesis by studying null-mutant genetic interactions with the photoreceptors phyA, phyB and cry1 with regard to seed-germination and flowering time. The removal of BAS1 and/or SOB7 rescued the low germination rate of the phyA-211 phyB-9 double-null mutant. With regard to floral induction, bas1-2 and sob7-1 showed a complex set of genetic interactions with photoreceptor-null mutants. Histochemical analysis of transgenic plants harboring BAS1:BAS1-GUS and SOB7:SOB7-GUS translational fusions revealed overlapping and distinct expression patterns. BAS1’s expression in the shoot apex increased during the phase transition from short-to-long-day growth conditions and requires phyB in red light.

Application of this kind of genetic analysis approach to study other BR catabolic genes required availability of the loss-of-function mutants. Our analysis shows that the ben1-1 (bri1 enhanced l-1) mutant (T-DNA intronic insertion mutant of BEN1) was not suitable for multiple mutant analyses which involve combining two or more T-DNA insertion mutations. We
generated a genetic triple-mutant from a cross between the *bas1-2 sob7-1* double-null (T-DNA exonic insertion mutants) and *ben1-1*. The single *ben1-1* line behaved as a transcript null. However, in the triple-mutant background *ben1-1* was reverted to a partial loss-of-function allele. The enhanced expression of *BEN1* remained stable when the *ben1-1* single-mutant was re-isolated from a cross with the wild-type. In addition, the two genetically identical pre-triple and post-triple *ben1-1* mutants also differed phenotypically. Restriction endonuclease analysis with methylation sensitive enzymes demonstrated that the recovered *ben1-1* mutant was epigenetically different from the original *ben1-1* allele.


*In vitro* biochemical analyses demonstrate that ATST4a has catalytic activity with BRs. Taking a genetic approach in this study, we show that *atst4a1-1* (a T-DNA insertion null mutant of *ATST4a*) did not display reduced seedling hypocotyl growth inhibition in white light. Additionally, overexpression of the *ATST4a* gene in transgenic lines did not result in any characteristic BR-deficient phenotypes. These observations suggest that *ATST4a* is an atypical BR catabolic gene.
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Dedicated to my Parents…
CHAPTER ONE

INTRODUCTION

Brassinosteroids (BRs): BRs are growth-promoting hormones essential for normal development of plants (Sasse, 2003; Kutschera and Wang, 2012). Genetic screens for Arabidopsis seedlings with de-etiolated morphology when grown in the total darkness (short, thick hypocotyl, expanded cotyledons, young primary leaves and high levels of anthocyanins) have led to the identification of many BR biosynthetic genes. *DE-ETIOLATED2 (DET2)* (Chory et al. 1991) and *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD)* (Szekeres et al. 1996) were among the first genes isolated in the above-mentioned screens. Other genes such as *DWARF 1 (DWF1)* (Feldmann et al. 1989; Takahashi et al. 1995; Klahre et al. 1998; Choe et al. 1999a), *DWF4* (Choe et al. 1998), *DWF7/STE1* (Choe et al. 1999b) and *HYPERSENSITIVE TO ABSISIC ACID AND AUXIN (SAX1)* (Ephritikhine et al. 1999a; Ephritikhine et al. 1999b) were isolated in similar screens. Isolation of these genes in screens suggests that BRs are involved in photomorphogenesis. Figure 1 shows important enzymes and the reactions they catalyze in the BR biosynthesis pathway. For review on BR biosynthesis pathways and enzymes see: Fujioka and Yokota, 2003; Zhao and Li, 2012.

Initially, the BR biosynthetic pathway was elucidated by analysis of endogenous BRs and the conversion products of potential intermediates in *Catharanthus roseus* cell cultures. After the identification of BR biosynthetic mutants in Arabidopsis, pea and tomato, these alleles became the main tool for understanding BR biosynthetic pathways (Fujioka and Yokota, 2003). Genetic and molecular studies of BR-insensitive mutants and their modifiers led to the identification of several key components of the BR signal-transduction pathway, including the
receptor. For review on recent advances in BR signaling see: Ye et al. 2012; Kim and Wang 2010; Li 2010; Yang et al. 2011.

Various studies demonstrate that BR biosynthetic and BR perception genes are ubiquitously expressed with increased transcript accumulation in actively growing tissues (Shimada et al. 2003; Bancos et al. 2002; Friedrichsen et al. 2000). The expression of DWF4 and CPD has been demonstrated to play an important role in controlling tissue-specific BR levels (Kim et al. 2006; Mathur et al. 1998). These genes are also important targets of feedback regulation (Tanaka et al. 2005; Guo et al. 2010). The receptor of brassinosteroids, BR-INSSENSITIVE-1 (BRI-1) is also ubiquitously expressed, with increased transcript accumulation in actively growing tissues (Friedrichsen et al. 2000).

Hormone levels in a given tissue can be affected by the relative rates of de novo synthesis, destruction, inactivation, and transportation within the plant (Bandurski et al. 1995). The balance of these processes determines the endogenous level of any hormone in plant tissues. Recent evidence suggests that unlike other plant hormones, BRs do not undergo long distance transport within plants. For example, grafting experiments in pea and tomato demonstrate that BRs are not transported long distances within plants (Reid and Ross, 1989; Symons and Reid, 2004; Montoya et al. 2005). Experiments by Salvadi-Goldstein et al. (2007) involving tissue-layer-specific expression of BR biosynthetic and perception genes in Arabidopsis demonstrate that BRs are not efficiently transported even short distances. These studies suggest that every tissue maintains its levels of BRs by autonomously regulating biosynthesis and catabolism.

**Role of BRs in growth and development in plants:** BRs are known to play diverse roles in plant growth and development. Growth promotion is the most characteristic function of BRs in
plants. The most classical demonstration of this property is the second-internode growth response induced in *Phaseolus vulgaris* by application of exogenous BRs (Grove et al. 1979). These growth responses are partially accomplished by the regulation of the downstream genes involved in the various aspects of cell expansion (Goda et al. 2002; Shigeta et al. 2011). For example early targets of BR responses include *TCH4* which encodes xyloglucan endotransglycosylase (XET) and is implicated in cell wall loosening during cell expansion (Xu et al. 1995). Other mechanisms, such as reorientation of cortical microtubules in *Arabidopsis* hypocotyl cells (Wang et al. 2012) and regulation of plasma membrane ion channels (Zhang et al. 2005) are also part of BR-induced cell expansion. BRs are also known to stimulate cell division. BR treatment of cell cultures of *Chlorella vulgaris* accelerates cell division and causes an increase in the levels of nucleic acids and proteins (Bajguz 2000).

Apart from a general role in cell division and expansion, recent studies show that BRs are also involved in various developmental aspects of plants including: determination of lateral organ boundaries (Gendron et al. 2012; Bell et al. 2012), stomata development (Kim et al. 2012; Gudesblat et al. 2012; Kong et al. 2012), shoot gravitropism (Gupta et al. 2012; Vandenbussche et al. 2013), fruit development (Symons et al. 2006), vascular differentiation (Yamamoto et al. 1997), cell wall homeostasis (Wolf et al. 2012) and biosynthesis (Xie et al. 2011), leaf development (Nakaya et al. 2002; Zhiponova et al. 2013), seed germination (Steber and McCourt, 2001; Xi et al. 2010; Sandhu et al. 2012), sex determination (Hartwig et al. 2011), fertility (Ye et al. 2010; Xi and Yu 2010), determination of the root meristem size (Gonzalez-Garcia et al. 2011; Hacham et al. 2011), plant circadian clock (Hanano et al. 2006) and chromatin modification (Shigeta et al. 2011; Sui et al. 2012). BRs also play a role in plant responses to environmental stresses through their involvement in immunity (For review see:
Plants with altered BR levels or signaling, also display photomorphogenic phenotypes such as altered seed germination, light-mediated hypocotyl growth and flowering (Turk et al. 2005; Sandhu et al. 2012). A brief introduction to the photomorphogenesis as relevant to this study is given in the next section.

**Photomorphogenesis:** Because of the sessile nature of plants, adaptation to changing environmental conditions is critical. One of the most important abiotic factors affecting plant growth is light. Light regulates multiple aspects of plant growth such as germination, plant architecture and transition to flowering. Not only quantity and quality, but also direction of light is very important for plants. Plants perceive light quality and quantity via a suite of signal-transducing photoreceptors to facilitate adaption to their ambient environment. In *Arabidopsis* there are five red/far-red-absorbing phytochromes (phyA through phyE), two blue-light-absorbing cryptochromes, (cry1 and cry2) and two blue/UV-light-absorbing phototropins (phot1 and phot2) (For review see: Chory 2010).

Koornneef (1980) pioneered research in light-signaling by employing large scale mutation screens in *Arabidopsis* using hypocotyl growth assays to detect mutants compromised for light-perception (Koornneef et al. 1980). In these studies, mutants were placed into five unique complementation groups (*hy1*, *hy2*, *hy3*, *hy4* and *hy5*). These mutants display elongated
hypocotyls by virtue of their reduced sensitivity to white light when compared to the wild type. Detailed analysis of these mutants by various laboratories associated these mutations with chromophore biosynthesis (hy1 and hy2) (Parks and Quail, 1991), PHYB (hy3) (Somers et al. 1991; Reed et al. 1993; Reed et al. 1994) and CRY1 (hy4) (Ahmad and Cashmore, 1993) and a downstream transcription factor (hy5) (Oyama et al. 1997; Ang et al. 1998; Chattopadhyay et al. 1998).

Based on the observation that hy3 displays normal hypocotyl inhibition in far-red light and that hy1 and hy2, which lack activity of all phytochromes, were insensitive to both white and far-red light, it was suggested that there was at least one yet unidentified phytochrome dedicated to the perception of far-red light. Based on this knowledge, PHYA mutants were identified by virtue of their elongated hypocotyls in continuous far-red light (Nagatani et al. 1993; Parks and Quail, 1993; Whitelam et al. 1993). Subsequently, PHYC, PHYD and PHYE were also identified based on their homology to the already identified phytochromes (Sharrock and Quail, 1989; Clack et al. 1994). Later CRY2 was also cloned on the basis of its homology to CRY1 (Lin et al. 1998). CRY2 loss-of-function mutants confer wild-type hypocotyl phenotypes under high intensity blue light but exhibit elongated hypocotyls in dim blue light when compared to the wild-type (Lin et al. 1998). These Arabidopsis photoreceptors have overlapping and distinct functions, which help to fine-tune plant responses to dynamic light conditions (Neff and Chory, 1998; for review see, Franklin and Quail, 2010).

phyA is the most important far-red-light sensor in Arabidopsis. A role of phyA as the major far-red sensor is supported by the observation that far-red-light grown PHYA loss-of-function mutants phenocopy dark-grown wild-type plants (Nagatani et al. 1993; Parks and Quail, 1993; Whitelam et al. 1993; Neff and Chory, 1998). phyB is a major regulator of seedling deetiolation
in response to both white and red light, with null alleles conferring elongated hypocotyls in both conditions (Somers et al. 1991; Reed et al. 1993; Reed et al. 1994). cry1 and cry2 regulate deetiolation in response to medium and low intensity blue-light, respectively (Ahmad and Cashmore, 1993; Lin et al. 1998). In addition to seedling deetiolation, some of these photoreceptors also mediate floral induction in responses to changing light conditions.

*Arabidopsis* is a facultative long-day-plant and employs photoperiodic flowering pathways to accelerate flowering under long-day conditions. phyA plays a vital role in the photoperiodic flowering pathway by perceiving changes in day length. *PHYA*-null mutant plants are insensitive to floral induction by day-length extensions or night-break light treatments for short-day-grown plants, both of which mimic long-day growth conditions (Johnson et al. 1994; Reed et al. 1994). In addition, under long-day growth conditions, *PHYA*-null mutant plants display a late-flowering phenotype when compared to the wild type (Johnson et al. 1994; Neff and Chory, 1998). phyB, on the other hand, inhibits flowering in *Arabidopsis*. Loss of phyB accelerates flowering under both long- and short-day conditions (Goto et al. 1991; Halliday et al. 1994; Whitelam and Smith, 1991).

**BR- and photomorphogenesis-mediated development:** As mentioned before, BRs have been implicated in the modulation of photomorphogenic traits. For example, a study examining genome wide targets of PIL5 (*PHYTOCHROME INTERACTING FACTOR-3 Likelike 5*), a major negative regulator of phytochrome regulated seed germination, have identified genes involved in BR signaling, indicating a connection between BR signaling and phytochromes during seed germination (Oh et al. 2004; Oh et al. 2009). PIFs (*PHYTOCHROME INTERACTING FACTORS*) are nuclear transcription factors which are known to interact with phytochromes (Monte et al. 2007; Bae and Choi, 2008). Recent evidence also suggests that brassinosteroids
play a role in flowering, a developmental process predominately controlled by light. For example, BRs affect flowering by repressing the expression of $FLOWERING\ LOCUS\ C\ (FLC)$, a negative regulator of the autonomous flowering pathway (Domagalska et al. 2007). In addition, $bril$-$EMS\ SUPPRESSOR\ 1$ (BES1), a transcription factor required for BR-dependent gene expression, interacts $in\ vivo$ with $EARLY\ FLOWERING\ 6\ (ELF6)$ and $RELATIVE\ OF\ EARLY\ FLOWERING\ 6\ (REF6)$ (Yu et al. 2008). ELF6 and REF6 are transcription factors and were initially isolated in a genetic screen for mutants with altered flowering phenotypes (Noh et al. 2004).

The link between BRs and light-mediated morphogenesis has also been suggested based on the deetiolated morphology of dark-grown BR biosynthetic mutants, suggesting that BR signaling is essential for normal growth in the dark (skotomorphogenesis) (Li et al. 1996; Szekeres et al. 1996; Nagata et al. 2000; Asami et al. 2004). It has also been suggested that light inhibits skotomorphogenesis and promotes photomorphogenesis in part by inhibiting BR levels or signaling (Li et al. 1996; Chory and Li, 1997; Kang et al. 2001). However, studies of BR measurements during deetiolation do not indicate any changes in BR levels during this response (Symons et al. 2008). Light-induced decrease in BR sensitivity has also been suggested as a complementary mechanism for light-regulation of BR signaling (Turk et al. 2003). In fact, light-dependent reduction in BR sensitivity has been reported in the case of $Arabidopsis$ and rice (Bancos et al. 2006; Jeong et al. 2007).

Microarray-based studies that targeted the effects of BRs on transcript profiles in $Arabidopsis$ suggest that BRs may be acting upstream in addition to acting downstream of light-signaling pathways (Goda et al. 2002; Song et al. 2009; Sun et al. 2010). In addition, BRs and photomorphogenesis may interact by converging on common target genes and pathways (Lee et
One such recent study has shown that the BR-activated transcription factor BZR1 (BRASSINAZOLE-RESISTANT 1) and PIF-4 (PHYTOCHROME-INTERACTING FACTOR 4) interact \textit{in vivo} to regulate hundreds of common target genes and are genetically interdependent in regulating cell expansion in the \textit{Arabidopsis} hypocotyl in response to BRs, darkness and heat (Oh \textit{et al.} 2012). For review on BR signaling and its role in photomorphogenesis see: Wang \textit{et al.} 2012.

One of the goals in the Neff lab is to study the role of phytohormones in photomorphogenic development of \textit{Arabidopsis}. For this purpose, we focus our studies on the phyB-mediated development. In addition to the early-flowering phenotype, \textit{phyB} mutants are severely pleiotropic, demonstrating widespread importance of phyB in \textit{Arabidopsis} development (for review see: Franklin and Quail, 2010). To identify downstream components of phyB signaling, various loss-of-function genetic approaches have been employed, including the identification of mutants that either mimic or suppress \textit{phyB}-null phenotypes (Reed \textit{et al.} 1998; Reed \textit{et al.} 2000). To complement these loss-of-function approaches, we utilized an activation-tagging screen to identify gain-of-function downstream components that may act in a redundant manner (Weigel \textit{et al.} 2000). \textit{PHYB-4 ACTIVATION-TAGGED SUPPRESSOR #1-DOMINANT} (bas1-D) and \textit{SUPPRESSOR OF PHYB-4 #7-DOMINANT} (sob7-D) were both identified in a gain-of-function activation-tagging screen for suppressors of the photomorphogenic phenotypes conferred by a weak mutation of \textit{phyB}, \textit{phyB-4} (Neff \textit{et al.} 1999; Turk \textit{et al.} 2005).

\textbf{BAS1 and SOB7:} BAS1 and SOB7 are members of the cytochrome P450 monooxygenase superfamily (P450s). Members of the P450 superfamily catalyze oxidation of a diverse array of plant metabolites. Reactions catalyzed by P450s are highly substrate specific, to an extent that
even close P450 family members may have widely diverse biochemistries as well as substrate requirements (for review see: Schuler et al. 2006).

Over-expression of either BAS1/CYP734A1 or SOB7/CYP72C1 suppresses the long-hypocotyl phenotype of phyB-4 and also confers a BR-deficient phenotype typified by detiolated 2-1 (det2-1), a BR biosynthetic mutant. Molecular, biochemical and genetic analyses have shown that in spite of the sequence similarity at both DNA and protein levels, BAS1 and SOB7 each catabolize their own specific substrates with unique biochemistries. BAS1 hydroxylates brassinolide (BL), the most active BR in Arabidopsis, and its immediate precursor castasterone (CS) to their respective inactive C-26 hydroxy products (Turk et al. 2005). SOB7, on the other hand, is not a C-26 hydroxylase and seems to act on precursors of BR biosynthesis (Turk et al. 2003; Turk et al. 2005; Thornton et al. 2010). BR levels are elevated in the bas1-2 sob7-1 double-null mutant when compared to the wild type or either single null allele (Turk et al. 2005). BAS1 and SOB7 also affect developmental processes, such as hypocotyl growth in response to light and flowering (Figure 2), in a synergistic/redundant fashion. In a manner quantitatively similar to phyB-null mutants, the bas1-2 sob7-1 double null flowers earlier than the wild type in both long- and short-day growth conditions, demonstrating a role for BR inactivation in floral induction (Turk et al. 2005). It is possible that the effect of BRs on flowering is independent of light. However, another possibility is that, the light is regulating flowering by modulating BR levels directly through BR-inactivation.

Other BR inactivating pathways:

**BR Glucosylation:** UGT73C5 was the first of two steroid glucosyltransferases to be identified in Arabidopsis (Poppenberger et al. 2005; Husar et al. 2011). UGT73C5 belongs to a subset
of the UDP-glucosyltransferases family. Members of this family are known to be involved in glucosylation of plant hormones such as auxins, cytokinins and abscisic acid (for review see: Bowles et al. 2006). In most cases the conjugation of hormones with sugars makes them inactive. Steroid glucosylating UGTs are also found in mammals and insects. UGT73C5 (At2g36800) was identified by gene over-expression conferring a characteristic BR-deficient morphology. Plants overexpressing UGT73C5 accumulate 23-O glucosides of BRs to a much higher levels than control plants. No 23-O-glucosides of BRs were detectable in antisense RNAi lines of UGT73C5 demonstrating that UGT73C5 is required to convert BRs to glucosides. Antisense RNAi line seedlings are less sensitive to hypocotyl growth inhibition by red and blue light but not to white light, suggesting a role for UGT73C5 in photomorphogenesis. UGT73C5 transcript accumulation is up regulated in the presence of DON (a fungal toxin). In fact, UGT73C5 was initially identified in a yeast cDNA expression screen for identifying genes in Arabidopsis that are capable of metabolizing DON (Poppenberger et al. 2003). The Arabidopsis over expression lines of UGT73C5 also display BR deficient phenotype as adults in addition to expressing tolerance to DON. This suggests a broader catalytic activity of UGT73C5 with a function in detoxification.

A second glucosyltransferase, UGT73C6 (At2g36790) was recently identified in Arabidopsis as having a BR catabolic activity (Husar et al. 2011). UGT73C6 is the closest homologue of UGT73C5 (Li et al. 2001). UGT73C6 was previously characterized as a flavonol-3-O-glycoside-7-O-glucosyltransferase (Jones et al. 2003). Over-expression of UGT73C6 also results in dwarf plants typical of a BR deficiency. Similar to the case of UGT73C5, CS and BL fed plants overexpressing UGT73C6 accumulate 23-O-glucosides of these BRs to a much higher levels than control plants. Further analysis also detected
modification of BL-23-\textit{O}-glucosides to BL-malonylglucosides. Interestingly, malonylation is catalyzed by acyltransferases of the BAHD family and is considered to protect glucosides from degradation.

Transcript accumulation of \textit{UGT73C5} and \textit{UGT73C6} is not induced in response to externally applied BL in plants (Husar \textit{et al.} 2011). However, both these genes show transcriptional response to toxins and various xenobiotics (Poppenberger \textit{et al.} 2003). The physiological relevance of \textit{UGT73C6} in BR metabolism still remains to be fully determined (Husar \textit{et al.} 2011).

**BR Acylation:** Recently, two members of the putative BAHD (for \textit{benzylalcohol O-acetyltransferase, anthocyanin O-hydroxycinnamoyltransferase, anthranilate N-hydroxycinnamoyl/benzoyltransferase, and deacetylvindoline 4-O-acetyltransferase}) acyltransferase family showing BR catalytic activity were also identified among the gain-of-function mutants generated by activation-tagging (Ahn \textit{et al.} 2007; Yu \textit{et al.} 2008; Roh \textit{et al.} 2012; Wang \textit{et al.} 2012) and full-length cDNA-overexpressor screens (FOX) (Ichikawa \textit{et al.} 2006; Schneider \textit{et al.} 2012; Choi \textit{et al.} 2012) in \textit{Arabidopsis}. Members of BAHD family are involved in acylation modification of a diverse array of secondary metabolites in plants (D’Auria, 2006). The modification may alter one or more of the substrate properties such as volatility, solubility and activity. The \textit{Arabidopsis} genome is known to contain 64 BAHD family members, which can be classified into five major clades (D’Auria, 2006; Yu \textit{et al.} 2009). As with the P450s, BAHD family members are highly diverse in their substrate preferences and sequence similarity provides poor predictability of the substrates.

\textit{BIA1 (BRASSINOSTEROID INACTIVATOR1); Roh \textit{et al.} 2012}, also known as \textit{ABS1}
(ABNORMAL SHOOT; Wang et al. 2012) is encoded by the At4g15400 gene in Arabidopsis. Two activation-tagging alleles of BIA1, bia1-1D and bia1-2D exhibit typical BR deficiency phenotypes. BR measurement in bia1-1D plants shows reduced endogenous levels of various BRs including CS. The expressions of the known BR-feedback regulated genes are also altered in bia1-D plants in accordance with reduced BR levels. The near RNA-null line of At4g15400, bia1-3 confers increased hypocotyl length in the dark when compared to the wild type (Col-0). Quantitative transcript accumulation analysis of BR-related genes in dark grown seedlings of bia1-3 show altered expression of BR-related genes when compared to the wild type, suggesting higher BR levels in dark grown bia1-3 seedlings. BIA1 transcript accumulation in seedlings is also higher in the dark when compared to continuous white light. The BIA1 protein is expressed mainly in the root elongation zone with increased expression in darkness compared to growth in light. The sub-cellular localization of BIA1 protein is cytosolic. In addition, the BIA1 gene expression is feed-back regulated in response to exogenously applied BL as well as in mutants with altered endogenous BR levels. Genetic analysis of the overexpression as well as knockdown of BIA1 in Arabidopsis strongly suggests its involvement in BR homeostasis.

PIZ (PIZZA; Schneider et al. 2012), also known as BAT1 (BR-RELATED ACYLTRANSFERASE; Choi et al. 2012) is encoded by At4g31910. Overexpression of PIZ causes characteristic BR-deficient phenotypes. The dwarf phenotype in PIZ overexpressors can be partially rescued with the application of exogenous BL indicating that the 35S:PIZ phenotypes are caused by BR deficiency. Alteration in the expression of many BR-feedback regulated genes in 35S:PIZ plants confirms lower BR levels. Interestingly, 35S:PIZ plants show more reduction in the concentration of earlier BR precursor than the more active downstream BRs, CS and BL. Feeding experiments with heterologously expressed HIS-tagged PIZ shows
that PIZ can use BL, CS and TY (Typhasterol) as substrates for acylation. PIZ is mainly expressed in roots as detected by the QRT-PCR. However, PIZ1 T-DNA insertion knockout lines piz1 and piz2 do not display any defects in development of roots or other organs. Additionally, PIZ expression is not feedback regulated by alteration of endogenous BR concentration or signaling.

At4g31910/PIZ was also characterized as BAT1 by Choe et al. 2012. Similar to 35S:PIZ, the endogenous BR levels of CS precursors are also reduced in BAT1 overexpression line. BR levels in null allele bat1-1 are not significantly different than the wild type. The bat1-1 allele confers a larger inflorescence stem as well as increased number of vascular bundles when compared to the wild type. BAT1 is localized in the endoplasmic reticulum. GUS transcriptional fusions with the BAT1 promoter show activity in the root tip, root maturation zone, lateral root primordial, cotyledon and leaf vein vasculature, and phloem of the inflorescence stem. BAT1 transcript accumulation is significantly induced by exogenous auxin treatment and not BL treatment in phytohormone response assays. Chromatin immuno-precipitation shows that the BAT1 promoter is bound by ARF19 (Auxin Response Factor 19). The overall genetic and biochemical analysis suggests that BAT1 is involved in BR homeostasis and may also have a role in auxin signaling.

**BR Sulfonation:** A soluble sulfotransferase ATST4a (At2g14920) capable of catalyzing O-sulfonation of BRs has also been identified in Arabidopsis (Table 1; Marsolais et al. 2007). Soluble sulfotransferases form a superfamily of enzymes whose members are found in plants, mammals, and bacteria. Mammalian steroid sulfotransferases are known to be involved in hormone transport and inactivation (Tong et al. 2005). The Brassica napus soluble sulfotransferases BNST3 and BNST4 have broad catalytic activity toward plant steroids (Rouleau et al. 1999; Marsolais et al. 2004). Sulfotransferase genes from Brassica napus are
induced by salicylic acid, ethanol and other xenobiotics, which suggest a role in detoxification. ATST4a was cloned on the basis of homology to ATST1. ATST1 is an ortholog of BNST proteins and displays similar catalytic activities and substrate specificity.

Recent evidence shows that ATST1 is also involved in the sulfonation of salicylic acid in response to pathogen stress (Baek et al. 2010). In vitro protein expression and sulfotransferase assay studies demonstrate that ATST4a has catalytic activity with BRs. Catalytic activity of ATST4a is specific for biologically active end products of the BR biosynthetic pathway, including CS, BL, related 24-epimers and the naturally occurring (22R, 23R)-28-homoBRs. The expression of *ATST4a* is largely root specific and is down regulated by the cytokinin hormone *trans*-zeatin. However, no genetic study involving loss of function or over expression was done to show that BR inactivation is the *in vivo* function of ATST4a. Biochemical data clearly demonstrates the role of ATST4a in BR inactivation. But genetic studies coupled with *in vivo* biochemical data are essential to test the hypothesis that BR inactivation is the endogenous function of ATST4a.

**BR Reduction:** Another putative BR deactivating gene, *BEN1* (*At2g45400*), was identified in an activation-tagging screen for extragenic modifiers of *bri1-5*, a weak mutant allele of BR receptor encoding gene *BRI1*. BEN1 belongs to a small family of proteins in *Arabidopsis* that also includes the dihydroflavonol reductase, DFR, and the anthocyanidin reductase, BAN, both involved in the flavonoid biosynthesis pathway. The over-expressor mutant *BRI1-5 ENHANCED1-IDOMINANT (ben1-1D)* has a characteristic BR deficient phenotype (Yuan et al. 2007). Molecular, genetic and physiological characterization of BEN1 demonstrates a role in BR metabolism. BR levels are slightly elevated in the *ben1-1* null allele when compared to the wild type. Adult *ben1-1* plants have organ-elongation phenotypes when compared to the wild type.
BEN1 is mainly expressed in the root elongation zone in a pattern similar to SOB7. BEN1 expression is up-regulated 4-5 times in white light when compared to growth in the dark. Seedlings lacking BEN1 are also less responsive to light inhibition of hypocotyl growth suggesting a role in seedling photomorphogenesis and BR inactivation. However, feeding experiments failed to detect any metabolic product of BEN1 activity (Yuan et al. 2007). Genetic data strongly favors the hypothesis that BEN1 is involved in BR inactivation though the substrates and products of BEN1 activity are not known. Figure 3 shows the reactions catalyzed by various BR catabolic genes discussed above.

This work: Independent evolution of multiple BR inactivating pathways indicates the importance of this process in plant growth and development. Therefore, identifying the contributions of enzymes and pathways related to the inactivation of these hormones is important for understanding BR-mediated development. The research presented in this dissertation is concerned with the genetic analysis of the role of four BR catabolic genes in growth and development of Arabidopsis.

Chapter Two, which has been published in the journal “G3: Genes, Genomes, Genetics”, investigates the role of BAS1 and SOB7 in photomorphogenesis (Sandhu et al. 2012). Genetic interactions for seed germination and flowering time were studied between BAS1, SOB7 and the photoreceptors PHYA, PHYB and CRY1 using null-mutant combinations. Our results indicate that both BAS1 and SOB7 contribute to the rate of seed germination in a manner that is genetically independent and/or downstream of PHYA and PHYB. BAS1 and SOB7 also show complex genetic interactions with PHYA and PHYB for flowering time. For example, bas1-2 and sob7-1 single-nulls have a mutually distinct pattern of genetic interactions with a phyA null. In contrast, the early-flowering phenotype of the bas1-2 sob7-1 double null requires functional
phyB. Furthermore, we show that BAS1 and SOB7 have both unique and overlapping expression patterns in Arabidopsis, and that BAS1 expression in the shoot apex in red light is dependent on the presence of functional phyB. Recent scientific evidence has shed some light on the molecular and genetic basis of the association between BRs and photomorphogenesis. Research conducted in Chapter Two is also a step in this direction. Research in this area will also lead to the better understanding of photomorphogenesis itself.

Chapter Three reports the genetic analysis of the T-DNA insertion mutant ben1-1. Our study, which has been submitted to the journal “G3: Genes, Genomes, Genetics”, shows that the ben1-1 mutation is unstable due to epigenetic modifications of the intronic T-DNA insertion. As a part of our study, we generated a genetic triple-mutant from a cross between the bas1-2 sob7-1 double-null (T-DNA exonic insertion mutants of BAS1 and SOB7) and ben1-1. Our results show that, the complete loss-of-function ben1-1 mutation was transformed to a partial loss-of-function mutation in the bas1-2 sob7-1 ben1-1 (triple-mutant) background showing enhanced levels of the wild-type-spliced transcript. Interestingly, the enhanced expression of BEN1 remained stable when the ben1-1 single-mutant was re-isolated from a cross with the wild-type. In addition, the two genetically identical pre-triple and post-triple ben1-1 mutants also differed phenotypically in terms of seedling-development characteristics. The size of the T-DNA insertion and the NPTII gene sequence did not change in the pre-triple and post-triple ben1-1 mutants, indicating that it did not play a role in the transformation. However, a previously functional NPTII T-DNA marker gene (which encodes kanamycin resistance) was no longer functional in the triple mutant nor in the recovered ben1-1 allele. Restriction endonuclease analysis with methylation sensitive enzymes followed by genomic PCR showed that the methylation status of the T-DNA is different between the original and the recovered ben1-1.
Chapter four reports the results of genetic manipulation of *ATST4a* in *Arabidopsis*. In this study, we have used both loss-of-function and over-expression genetic approaches to further explore the role of *ATST4a* in *Arabidopsis*. Our results show that *atst4a1-1*, a T-DNA insertion null mutant of *ATST4a* does not display reduced seedling hypocotyl growth-inhibition in white light. Additionally, overexpression of *ATST4a* gene in transgenic lines does not result in characteristic BR-deficient phenotypes despite the lack of feed-back regulation of *DWF4* and *BASI* expressions. These observations suggest that *ATST4a* is an atypical BR catabolic gene.

Chapter Two shows that molecular genetic approach can help in understanding complex processes such as flowering as well as interactions between biological pathways. Chapter Three shows the need to continue the effort of generating additional genetic resources for basic studies. Chapter Four suggests the need for further study to fully understand the role of *ATST4a* in *Arabidopsis* development. Delineating the overall role of BRs and its catabolism in plant physiology and development is likely to include a similar molecular genetic approach as described in this study.

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Table 1. Biochemical analysis of ATST4a BR catalytic activity. Marsolais et al. 2007.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (pkatal mg$^{-1}$)</th>
<th>$V_{\text{max}}K_m^{-1}$ (pkatal mg$^{-1}$ μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassinolide</td>
<td>16</td>
<td>5</td>
<td>0.3</td>
</tr>
<tr>
<td>Castasterone</td>
<td>14</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>24-Epibrassinolide</td>
<td>43</td>
<td>10</td>
<td>0.2</td>
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<tr>
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<td>19</td>
<td>34</td>
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<tr>
<td>(22R, 23R)-28-Homocastasterone</td>
<td>7</td>
<td>19</td>
<td>2.7</td>
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</table>
Figure 1: BR biosynthesis pathway. Copied from Bishop and Koncz, 2002.
Figure 2. Fluence rate response shows genetic redundancy between *BAS1* and *SOB7* for hypocotyl growth repression in white light (A) and flowering time (B). Copied from Turk *et al.* 2005.
Figure 3: Reactions catalyzed by the known BR catabolic enzymes.
CHAPTER TWO

Genetic interactions between brassinosteroid-inactivating P450s and photomorphogenic photoreceptors in Arabidopsis thaliana


Other people who contributed to this work: Genetic mutant combinations generated by Dr. Ed Turk, flowering analysis done by Katherine Hagely (co-author), and GUS constructs generated by Dr. Leeann Thornton.
ABSTRACT

Plants use light as a source of information via a suite of photomorphogenic photoreceptors to optimize growth in response to their light environment. Growth-promoting hormones such as brassinosteroids can also modulate many of these responses. BAS1 and SOB7 are brassinosteroid-catabolizing P450s in Arabidopsis thaliana that synergistically/redundantly modulate photomorphogenic traits such as flowering time. The role of BAS1 and SOB7 in photomorphogenesis has been investigated by studying null-mutant genetic interactions with the photoreceptors phyA, phyB and cry1 with regard to seed-germination and flowering time. The removal of BAS1 and/or SOB7 rescued the low germination rate of the phyA-211 phyB-9 double-null mutant. With regard to floral induction, bas1-2 and sob7-1 showed a complex set of genetic interactions with photoreceptor-null mutants. Histochemical analysis of transgenic plants harboring BAS1:BAS1-GUS and SOB7:SOB7-GUS translational fusions under the control of their endogenous promoters revealed overlapping and distinct expression patterns. BAS1’s expression in the shoot apex increases during the phase transition from short-to-long-day growth conditions and requires phyB in red light. In summary, BAS1 and SOB7 displayed both simple and complex genetic interactions with the phytochromes in a plant-stage specific manner.
INTRODUCTION

In angiosperm plant species, the timing of flowering plays an important role in the success of sexual reproduction. Precision in the timing of reproduction requires a flowering mechanism that is both flexible and robust. Flexibility ensures that the timing of flowering leads to good seed set and survival under a variety of circumstances. Robustness ensures that the mechanism is strong enough to trigger flowering in the majority of members in a population. Cues that regulate floral induction include both environmental (external) and developmental (internal) factors. Being sessile and photosynthetic, light is a major environmental factor for plants. Light-mediated development, or photomorphogenesis, plays an important role in the optimization of flowering time (Mockler et al. 2003). Internal factors that affect flowering time include hormones such as brassinosteroids (BRs), as well as developmental factors, which include age. In plants, these external and internal cues are perceived by various reproductive pathways, which ultimately converge to ensure a proper flowering response (Srikanth and Schmid, 2011). One of the fundamental questions in plant biology relates to how plants integrate light and hormone signals to optimize growth and development in constantly changing environmental conditions.

Plants perceive light quality and quantity via a suite of signal-transducing photoreceptors to facilitate adaption to their ambient environment. In Arabidopsis there are five red/far-red-absorbing phytochromes (phyA through phyE), two blue-light-absorbing cryptochromes, (cry1 and cry2) and two blue/UV-light-absorbing phototropins (phot1 and phot2) (Chory 2010). phyA is the most important far-red-light sensor in Arabidopsis. A role of phyA as the major far-red sensor is supported by the observation that far-red-light grown PHYA loss-of-function mutants phenocopy dark-grown wild-type plants (Nagatani, Reed and Chory, 1993; Parks and Quail, 1993; Whitelam et al. 1993; Neff and Chory, 1998). phyB is a major regulator of seedling
deetiolation in response to both white and red light, with null alleles conferring elongated hypocotyls in both conditions (Somers et al. 1991; Reed et al. 1993; Reed et al. 1994). cry1 and cry2 regulate deetiolation in response to medium and low intensity blue-light, respectively (Ahmad and Cashmore, 1993; Lin et al. 1998). In addition to seedling deetiolation, some of these photoreceptors also mediate floral induction in responses to changing light conditions.

*Arabidopsis* is a facultative long-day-plant and employs photoperiodic flowering pathways to accelerate flowering under long-day conditions. phyA plays a vital role in the photoperiodic flowering pathway by perceiving changes in day length. *PHYA*-null mutant plants are insensitive to floral induction by day-length extensions or night-break light treatments for short-day-grown plants, both of which mimic long-day growth conditions (Johnson et al. 1994; Reed et al. 1994). In addition, under long-day growth conditions, *PHYA*-null mutant plants display a late-flowering phenotype when compared to the wild type (Johnson et al. 1994; Neff and Chory 1998). phyB, on the other hand, inhibits flowering in *Arabidopsis*. Loss of phyB accelerates flowering under both long- and short-day conditions (Goto et al. 1991; Halliday et al. 1994; Whitelam and Smith 1991).

In addition to the flowering phenotype, phyB mutants are severely pleiotropic, demonstrating its widespread importance in *Arabidopsis* development (for review see Franklin and Quail, 2010). To identify downstream components of phyB signaling, various loss-of-function genetic approaches have been employed, including the identification of mutants that either mimic or suppress phyB-null phenotypes (Reed et al. 1998; Reed et al. 2000). To complement these loss-of-function approaches, we utilized an activation-tagging screen to identify gain-of-function downstream components that may act in a redundant manner (Weigel et al. 2000). *PHYB-4 ACTIVATION-TAGGED SUPPRESSOR #1-DOMINANT (bas1-D)* and *SUPPRESSOR OF*
PHYB-4 #7-DOMINANT (sob7-D) were both identified in a gain-of-function activation-tagging screen for suppressors of the photomorphogenic phenotypes conferred by a weak mutation of PHYB (Neff et al. 1999; Turk et al. 2005). BAS1 and SOB7 are members of the cytochrome P450 monooxygenase superfamily (P450s). Members of the P450 superfamily catalyze oxidation of a diverse array of plant metabolites. Reactions catalyzed by P450s are highly substrate specific, to an extent that even close P450 family members may have widely diverse biochemistries as well as substrate requirements (For review see, Schuler et al. 2006).

Over-expression of either BAS1/CYP734A1 or SOB7/CYP72C1 suppresses the long-hypocotyl phenotype of phyB-4 and also confers a BR-deficient phenotype typified by detiolated 2-1 (det2-1), a BR biosynthetic mutant. Molecular, biochemical and genetic analyses have shown that in spite of the high sequence similarity at both DNA and protein levels, BAS1 and SOB7 each catabolize their own specific substrates with unique biochemistries. BAS1 hydroxylates brassinolide, the most active BR in Arabidopsis and its immediate precursor castasterone to their respective inactive C-26 hydroxy products (Turk et al. 2005). SOB7, on the other hand, is not a C-26 hydroxylase and seems to act on precursors of BR biosynthesis (Turk et al. 2003; Turk et al. 2005; Thornton et al. 2010). BR levels are elevated in the bas1-2 sob7-1 double-null mutant when compared to the wild type or either single null allele (Turk et al., 2005). BAS1 and SOB7 also affect developmental processes, such as flowering, in a synergistic/redundant fashion. In a manner quantitatively similar to phyB-null mutants, the bas1-2 sob7-1 double null flowers earlier than the wild type in both long- and short-day growth conditions, demonstrating a role for BR inactivation in floral induction (Turk et al. 2005).

BRs are growth-promoting hormones essential for normal development of plants (Sasse 2003). BRs affect plant growth and development by altering the expression of hundreds of BR
responsive genes (Goda et al. 2002). In addition to their general role in cell division and expansion (Bajguz 2000), BRs are also involved in tissue-specific development (Symons et al. 2006; Yamamoto et al. 1997). Unlike most plant hormones, however, BRs are not transported within or between plant tissues, implying that levels of BRs are regulated locally through both biosynthesis and catabolism (Reid and Ross, 1989; Symons and Reid, 2004; Montoya et al. 2005; Salvadi-Goldstein et al. 2007). BR catabolism, therefore, can play a significant role as a regulatory point for BR-mediated development. In fact, apart from BAS1 and SOB7, there are at least five more enzymes with unique biochemistries leading to BR inactivation in Arabidopsis (Husar et al. 2011; Masrsolais et al. 2007; Poppenberger et al. 2005; Yuan et al. 2007). Independent evolution of multiple BR inactivating pathways further indicates the importance of this process in plant growth and development. Therefore, identifying the contributions of enzymes and pathways related to the inactivation of these hormones is important for understanding BR-mediated development.

The observation that photomorphogenic photoreceptors, along with and BAS1 and SOB7, affect common developmental processes suggests that in at least some cases these pathways act in an interdependent manner. In the present work, we describe the contribution of BAS1 and SOB7 in the modulation of seed germination and flowering time in Arabidopsis. Genetic interactions for seed germination and flowering time were studied between BAS1, SOB7 and the photoreceptors PHYA, PHYB and CRY1 using null-mutant combinations. Our results indicate that both BAS1 and SOB7 contribute to the rate of seed germination in a manner that is genetically independent and/or downstream of PHYA and PHYB. BAS1 and SOB7 also show complex genetic interactions with PHYA and PHYB for flowering time. For example, bas1-2 and sob7-1 single-nulls have a distinct pattern of genetic interactions with a phyA null. In contrast,
the early-flowering phenotype of the bas1-2 sob7-1 double null requires functional phyB. Furthermore, we show that BAS1 and SOB7 have both unique and overlapping expression patterns in Arabidopsis, and that BAS1 expression in the shoot apex in red light is dependent on the presence of functional phyB.

MATERIALS AND METHODS

Plant material: All mutants used in this study, phyA-211 (Reed et al. 1994), phyB-9 (Reed et al. 1993), cry1-103 (Liscum and Hangarter 1991), bas1-2 and sob7-1 (Turk et al. 2005), were in the Columbia (Col-0) background. The phyA-211 mutation was identified by amplifying genomic DNA with primers 5'-GTC ACA AGA TCT GAT CAT GGC-3', 5'-AAC AAC CGA AGG GCT GAA TC-3', 5'-TTA TCC ACA GGG TTA CAG GG-3', and 5'-GCA TTC TCC TTG CAT CAT CC-3', followed by resolution of 1243- and 1136-bp fragments for the wild type and a 1243-bp fragment for the phyA-211 mutant. The PCR-based markers used to identify the phyB-9 and cry1-103 mutants are described by Ward et al. (2005). Identification of bas1-2 and sob7-1 is described in Turk et al. (2005). Photoreceptor mutants were crossed with bas1-2 sob7-1, and multiple mutant combinations were isolated in F2 populations.

Due to the use of a large number of higher order null-mutant combinations in this study, it was not appropriate to use a sibling wild-type Col-0 derived from any one cross as a control. Therefore, a common Col-0 strain was used as a wild-type control in all the experiments for uniformity. To address variation due to environmental growth conditions all genotypes were grown at the same time under the same growth chamber conditions, and seeds harvested for phenotypic analysis.
For generating BAS1:GUS translational fusion lines, the BamHI/NcoI fragment from pED10 (described in Turk et al. 2003) was cloned into the BamH1/Nco1 site of pCAMBIA1305.1 vector (CAMBIA, CANBERRA, AUSTRALIA). This construct was transformed into bas1-2 sob7-1 double-null plants. Multiple transgenic lines segregating at a 3:1 ratio (hygromycin resistant/sensitive ratio) in the T2 generation were identified as single insertion lines. The entire SOB7 gene from ~ 2.1-Kb upstream of the start codon to the last base before the stop codon was cloned in frame with uidA gene in pCAMBIA1305.1 vector. This construct was also transformed into bas1-2 sob7-1 double-null plants. The segregation ratio was studied to identify multiple single locus T-DNA insertion lines.

**Seed sterilization, plating, and growth conditions:** Seeds were surface sterilized by 15 minute agitation in 70% (v/v) ethanol with 0.05 % (v/v) TritonX-100 followed by 5 minutes of agitation in 95% (v/v) ethanol with 0.05 % (v/v) TritonX-100 before being air-dried on 90-mm filter paper in a sterile petri dish. Sterilized seeds were plated on growth media plates containing 1% (w/v) phytagel (Sigma Life Sciences, St. Louis, MO) with 1.5% (w/v) sucrose and 1/2X Linsmaier and Skoog basal media (Phytotechnology Laboratories®; Shawnee Mission, KS). Plates were incubated in darkness at 4°C for four days. Germination was induced with a red-light treatment at a fluence rate 60-70 µmol m² sec⁻¹ for 16 h at 25°C before being transferred to the appropriate light or dark conditions for a total of five days at the same temperature. White light was provided by 8 fluorescent tubes (F17T8 17WT; GE Fairfield, CT) and two incandescent tubes (T10 FR25 130V; Satco, Brentwood, NY) in a temperature-controlled growth chamber (Model E-30B; Percival Scientific, Perry, IA).

**Flowering-time analysis:** Since transplanting seedlings to soil can cause stresses that alter flowering time, all seeds were directly sown in pots containing a pre-watered soil mix (Sunshine
Mix4 (Aggregate) LA4, Green Island Distributers Inc.; Riverhead, N.Y). These pots were then incubated in darkness for four days at 4ºC to induce near-uniform germination. Pots were then transferred to growth chambers with white light (200 µmol m⁻² sec⁻¹) set at 21ºC and 60-70 % humidity. After a week of growth, seedlings were thinned to one per pot by clipping using small scissors, since removal of whole seedlings causes root damage to neighboring seedlings, which in turn can also lead to altered flowering time. We have found that this approach gives the most uniform and repeatable flowering time results for each genotype. Flowering time for both long-day and short-day grown plants was calculated by the number of days until the floral stem was 0.5 cm above the basal rosette. In long-day grown plants, flowering time was also calculated by the total number of primary rosette and cauline leaves present at bolting. This latter approach was not usable with short-day grown plants due to senescence of older leaves during the prolonged growth period for some genotypes.

**Statistical analysis:** All statistical results were obtained from at least three independent experiments. Each independent experiment showed the same statistical trend. Results are presented as mean values for the combined data. Error bars represent the standard error of the mean (SE). A student’s unpaired two-tailed t-test was used to calculate p-values that allowed identification of statistically significant differences between two genotypes in a given experiment.

**Histochemical GUS analysis:** Plant material was incubated overnight in a GUS staining solution containing 100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 % v/v Triton X-100, 0.5 mM potassium ferri- and ferrocyanide and 1 mM X-GlucA (Research Product International Corp.; Mount Prospect, IL). After staining, plant material was treated with 70% ethanol for 1h, followed by 100% ethanol overnight, to remove chlorophyll before photographing. For
histological GUS analysis of BAS1 expression in the shoot apex, shoot apices of transgenic plants were dissected with twin blades and GUS stained as described above. For embedding, tissues were rinsed in 0.1M phosphate buffer (pH 7.0) and embedded in Tissue-Tek® OCT compound for sectioning. Embedded tissues were stored at -20ºC until sectioned. Longitudinal sections were cut using a Leica Cryocut 1800 (Leica Reichert-Jung 1800 Cryostat; Holly, MI) and image analyzed by Olympus BH-2 Light microscope at the Washington State University Francschi Microscopy and Imaging Center.

**Transcript analysis:** Total RNA was isolated, using the RNeasy Plant Kit (Qiagen, Valencia, CA), from four-day-old seedlings grown in continuous white light (45 µmol m⁻² sec⁻¹). On-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen, Valencia, CA), to eliminate genomic DNA contamination. Total cDNA was synthesized using SuperScriptIII First-Strand Synthesis System (Invitrogen, Carlsbad, CA). BAS1 transcript was amplified using primers 5’-GCT TAA AAC GTT GAG TAT GAT C-3’ and 5’- TCC TCA TGA TTG GTC AAT CTC -3’. SOB7 transcript was amplified using primers 5’- CCT GAA AGT CGT AAC AAT GAT C -3’ and 5’- GTT TTC GGA TGA TCA AAT GAG C -3’. ACTIN2 was used as an internal control in RT-PCR. ACTIN2 transcript was amplified using primers 5’-GGT CGT ACA ACC GGT ATT GTG CTG G-3’ and 5’-CTG TGA ACG ATT CCT GGA CCT GCC-3’. The linear range of amplification for each gene transcript was determined by comparing samples obtained using different numbers of cycles. Lack of genomic and foreign DNA contamination was ascertained by using all RNA samples and water as a template in a PCR reaction.

**RESULTS**
Removal of BAS1 and SOB7 rescues the low germination rate of a phyA phyB double-null mutant: Flowering-time analysis required seeds to be directly sown on the soil. Since the number of days to flowering were calculated based on the day of planting, it was important to know if the genotypes in this study conferred any germination phenotypes. For this purpose, a seed germination study was conducted.

In both light and dark conditions the *bas1-2* and *sob7-1* single and *bas1-2 sob7-1* double null did not show a significant difference in germination rates when compared to the wild type Col-0 (in white light, \( p = 0.18, 0.64 \) and 0.27 respectively; in dark, \( p = 0.45, 0.33 \) and 0.29 respectively). The *phyA-211 phyB-9* double mutant conferred a lower germination percentage rate, both in darkness and after a prolonged white light treatment (Table 1). In contrast, the *phyA-211 phyB-9 bas1-2* and *phyA-211 phyB-9 sob7-1* triple-null mutants displayed significantly higher germination rates than *phyA-211 phyB-9* in both white light (\( p = 0.0025 \) and 0.0018 respectively) and dark treatments (\( p = 0.0001 \) and 0.0002, respectively). The *phyA-211 phyB-9 bas1-2 sob7-1* quadruple-null displayed the highest germination rate, which was similar to the wild type in both dark and light treatments (\( p = 0.79 \) and 0.52 respectively). These results suggest that BAS1 and SOB7 are acting downstream and/or in parallel with phyA and phyB for modulating seed germination.

**BAS1 and SOB7 show genetic interactions with photoreceptors for flowering time:** To further examine genetic interactions between the BR-inactivating enzymes and photomorphogenic photoreceptors, we measured flowering time in multiple null mutant combinations of *bas1-2* and *sob7-1* with *phyA-211, phyB-9, cry1-103*, and *phyA-211 phyB-9* grown in both long- (16 h light: 8 h darkness) and short-day (8 h light: 16 h darkness) conditions.
Adult plant phenotypes of the various genotypes grown for three weeks under long-day conditions are shown in Figure 1.

Flowering time analysis demonstrated complex genetic interactions between these various genes for determination of flowering time. These genetic interactions were maintained in both long- and short-day growth conditions (Figure 2). The genetic state of CRY1 did not have any impact on floral induction or the early-flowering phenotype conferred by the loss of BAS1 and SOB7. In contrast, the bas1-2 mutation suppressed the phyA-211 late-flowering phenotype in both long and short days. However, the sob7-1 mutation had the opposite effect on flowering time in combination with phyA-211. In addition, phyB-9 bas1-2 sob7-1 triple-null and phyA-211 phyB-9 bas1-2 sob7-1 quadruple-null plants did not flower earlier than the phyB-9 single-null and phyA-211 phyB-9 double-null controls respectively, suggesting that the bas1-2 sob7-1 early-flowering phenotype requires functional phyB.

**BAS1 and SOB7 have distinct and overlapping expression patterns:** To gain further insight into the function of BAS1 and SOB7, we generated translational GUS fusion lines of BAS1 and SOB7 under the control of their native promoters. These BAS1:BAS1-GUS and SOB7:SOB7-GUS transgenes were expressed in the bas1-2 sob7-1 double-null mutant background. Single locus insertion lines were isolated and characterized via molecular and genetic analyses (Figure S1). Transformation into the bas1-2 sob7-1 null background provided transgene stability by reducing the chance of co-suppression from the endogenous transcript. In addition, it also provided an opportunity for functional analysis of the transgenic lines by complementation analysis of the double-null hypocotyl elongation phenotype.
The hypocotyl elongation phenotype of *bas1-2 sob7-1* was rescued by *BAS1*:BAS1-GUS translational fusions in accordance with the expression level from the transgene (Figure S1A, B). However, all *SOB7*:SOB7-GUS lines except one have a similar hypocotyl-elongation phenotype as the double mutant regardless of the level of gene expression (Figure S1C, D). This observation can be explained on the basis that *BAS1* and *SOB7* are not completely redundant for hypocotyl growth (Turk *et al.* 2005). Since the p*SOB7*:SOB7-GUS was transformed into *bas1-2 sob7-1* double-null background, the p*SOB7*:SOB7-GUS transgenic lines shown in Figure S1C are still lacking BAS1 activity. Therefore, the restoration of *SOB7* to its wild-type-expression level by the transgenic construct does not rescue the *bas1-2 sob7-1* hypocotyl phenotype completely. Only when the expression from the transgenic p*SOB7*:SOB7-GUS construct is significantly higher than the wild type level, as is the case in line #3.3, does it significantly shorten the hypocotyl of the *bas1-2 sob7-1* double-null line.

Histochemical GUS analysis was performed on a set of representative lines. In white-light-grown seedlings, BAS1 expression was observed in the shoot apex and root tip (Figure 3). In contrast, SOB7 expression was seen only in the root elongation zone (Figure 3). In juvenile and adult plants, BAS1 expression was present in the shoot apex before flowering, and in the flowers and developing embryos after flowering (Figure 3). SOB7 expression was present in the transition zone between the root and the shoot, as well as in developing anthers, the vasculature of rosette leaves and hydathodes of cauline leaves (Figure 3). Overall, BAS1 and SOB7 had some overlapping but mostly distinct expression patterns.

The early-flowering phenotype of *bas1-2 sob7-1* and expression of BAS1-GUS in the shoot apex suggests that BAS1 is involved in regulating BR levels in the shoot apical meristem, which may in turn affect the vegetative to floral phase transition. To test this hypothesis transgenic
plants carrying the pBAS1:BAS1-GUS construct were grown in short-days for four weeks. After four weeks, half of the plants were shifted to long days while the remaining half stayed in the short days. After two additional days, tissue from plants that stayed in the short days for the entire time and plants that were shifted to the long days were collected together. As a result, at the time of collection, all plant tissues were the same age. These harvested tissues were immediately used for histological analysis. Histochemical GUS analysis demonstrated a change in the expression pattern of BAS1 during this phase transition from short-day to long-day growth conditions (Figure 4). In short-day-grown plants (Figure 4A), expression was only visible at the base of the shoot apex, whereas after floral induction via transfer to long-day growth conditions BAS1-GUS expression was present throughout the shoot apex (Figure 4B). This observation, that the change in BAS1 expression pattern is correlated with floral induction suggests a role for BAS1 in flowering.

**BAS1 expression in the shoot apex in red light is dependent on the presence of functional phyB:** Based on the observation that BAS1-GUS expression is present in the shoot apex and that the early-flowering phenotype of bas1-2 sob7-1 is dependent on the presence of functional phyB, we hypothesized that phyB signaling is regulating BAS1 expression in the shoot apex which in turn affects flowering time. To test this hypothesis multiple pBAS1:BAS1-GUS transgenic lines in the bas1-2 sob7-1 background were crossed with the phyB-9 bas1-2 sob7-1 triple null to isolate BAS1-GUS translational fusions in both the wild type PHYB and phyB-9 mutant genetic backgrounds. BAS1-GUS fusions in the bas1-2 sob7-1 and phyB-9 bas1-2 sob7-1 background were grown in continuous red-light for five days. BAS1-GUS expression was examined in the shoot apex by histochemical GUS analysis in the genotypes. Using this approach, it was observed that the BAS1-GUS expression in the shoot apex was much more prominent in the wild
type \textit{PHYB} background than the \textit{phyB-9} background (Figure 5). This observation suggests that BAS1 expression at the shoot apex is modulated by the presence of functional phyB. It also suggests a possible cause for the dependence of the early-flowering phenotype of \textit{bas1-2 sob7-1} on functional phyB.

\section*{DISCUSSION}

As previously reported, the \textit{phyA-211 phyB-9} double mutant displayed a lower germination rate than the wild type (Poppe and Schafer 1997), which was rescued by the removal of BAS1 and SOB7 (Table 1). This implies that both are acting either downstream of or in parallel with \textit{PHYA} and \textit{PHYB} to modulate seed germination presumably by changing levels of active BRs. In comparison to germinating seeds, flowering-time phenotypes in adult plants cannot be interpreted solely based on the changing of overall BR levels.

Flowering-time analysis of mutants blocked in BR biosynthesis (e.g. \textit{det2}, \textit{dwf4} and \textit{cpd}) or BR perception (\textit{bri1}) suggests a positive role for BRs in floral induction (Azpiroz \textit{et al.} 1998; Chory \textit{et al.} 1991; Domagalska \textit{et al.} 2007; Li and Chory 1997). However, transgenic \textit{Arabidopsis} plants having constitutive overexpression of \textit{DWF4}, with higher levels of active BRs and increased organ size, do not display early flowering, suggesting that the interplay between BRs and flowering is not simply a matter of altering whole-plant hormone levels (Choe \textit{et al.} 2001). The \textit{bas1-2 sob7-1} double mutant contains higher BR levels and flowers earlier than the wild type, demonstrating a role for these BR-inactivating enzymes in floral induction (Turk \textit{et al.} 2005).
Our results show that the bas1-2 mutation suppresses the late-flowering phenotype of phyA-211, whereas the sob7-1 mutation does not (Figure 2), suggesting that these two BR-inactivating genes can have distinct roles in plant development. Unlike BAS1, SOB7 is not expressed in the apical meristem in seedlings as well as adult plants (Figure 3). Since the transition to flowering occurs at the shoot apical meristem, these differences in expression can, in part, explain the distinct genetic interactions that bas1-2 and sob7-1 have with phyA-211.

In addition, we also observed that in short-day grown-plants that have not been induced to flower, BAS1 expression is confined mainly to the basal region of shoot apex (Figure 4A). In contrast, after floral induction, BAS1 expression is seen throughout the shoot apex (Figure 4B). The expression of BAS1 at the base of the shoot apex may be involved in excluding BRs from the shoot apical meristem to prevent an early transition to flowering. Interestingly, a GA catabolic enzyme encoding gene, OsGA2ox1, implicated in vegetative to floral phase transition in rice, also shows a similar expression pattern (Sakamoto et al. 2001).

The bas1-2 sob7-1 double mutant also displayed a genetic interaction with phyB-9 with regard to flowering time (Figures 2A, B). The observation, that both the phyB-9 single-null and phyB-9 bas1-2 sob7-1 triple-null flower at the same time, suggest that the early-flowering phenotype of bas1-2 sob7-1 requires functional phyB. Loss of BAS1 expression in the phyB-9 background in red-light (Figures 5B, D) also suggests a possible molecular basis for the genetic interaction of BAS1 and SOB7 with PHYB in regard to floral induction. Another possible explanation is that BAS1 expression in the shoot apex is dependent on certain morphological and physiological attributes that are lacking in the phyB-9 mutant background. In this case, the requirement of functional phyB would be indirect rather than direct. Examples of two such morphological attributes that are altered in phyB-9 seedlings grown in red light are smaller
cotyledons and petioles when compared to the wild type (Neff and Chory, 1998; Neff and Van Volkenburgh, 1994), a phenotype that is obvious in the Figure 5. In contrast to PHYA and PHYB, the genetic state of CRY1 had no impact on the early-flowering phenotype conferred by the bas1-2 sob7-1 double null. These results demonstrate that higher-order null-mutant analysis can be used to differentiate the roles of different photoreceptors in BAS1- and SOB7-mediated development.

To fully understand the role of BAS1 in flowering, it will be necessary to study the changes in its expression pattern in relation to cellular or tissue-specific BR levels in the shoot apex, during floral induction, in both the wild type and phyB-9 mutant backgrounds. However, we currently lack the technology to accurately measure BR levels in small tissues and organs such as the shoot apical meristem. Such an advancement, which could include a DR5-like reporter system for BR levels (Ulmasov et al. 1997), would further help in understanding the overall role of BR catabolism in plant growth and development.

A simplified model describing the suggested roles of BAS1 and SOB7 in seed germination and floral induction is shown in Figures 6A and 6B respectively. BRs are known to have a positive effect on seed germination (Steber et al. 2001). Therefore, germination may include an increase in BR levels via the regulation of BR catabolism. phyA and phyB also affect germination (Poppe and Schafer 1997). These two processes are either acting independently, interdependently or both. As shown in the Figure 6A, BAS1 and SOB7 may act downstream of and/or in parallel to phyA and phyB to promote germination.

In the shoot apex, the transition to flowering includes changes in BAS1’s expression pattern (Figure 4), suggesting that there might be a cause and/or effect relationship between the two
events. phyB plays an inhibitory role in flowering (Goto et al. 1991; Halliday et al. 1994; Whitelam and Smith 1991). phyB is required for the early flowering phenotype conferred by the

bas1-2 sob7-1 double mutant (Figure 2) and alters the expression pattern of BAS1 (Figure 5). Therefore, it is possible that phyB modulates BAS1 expression to inhibit phase transition in the shoot apex. Figure 6B depicts a possible mechanism for the inhibition of flowering at the shoot apex with regard to genetic interactions between phyB and BAS1. In addition to BAS1 and SOB7, at least five more genes (BEN1, UGT73C5, UGT73C6, ATST4a and BIA1) have been suggested to play a role in BR catabolism (Husar et al. 2011; Masrsolais et al. 2007; Poppenberger et al. 2005; Yuan et al. 2007). Delineating the overall role of BRs and BR catabolism in plant physiology and development is likely to include a similar molecular genetic approach as described in this study.

ACKNOWLEDGMENTS

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LITERATURE CITED


Marsolais, F., J. Boyd, Y. Paredes, A. M. Schinas, M. Garcia et al., 2007 Molecular and biochemical characterization of two brassinosteroid sulfotransferases from *Arabidopsis*, AtST4a (At2g14920) and AtST1 (At2g03760). Planta **225**: 1233-1244.


Yuan, T., S. Fujioka, S. Takatsuto, S. Matsumoto, X. P. Gou *et al.*, 2007 *BEN1*, a gene encoding a dihydroflavonol 4-reductase (DFR)-like protein, regulates the levels of brassinosteroids in *Arabidopsis thaliana*. Plant J. **51**: 220-233.
### Table 1: Percentage of seed germination in white light or darkness.

Imbibed seeds were incubated at 4°C in the dark for four days before being treated with white light for six days (three replicates) or with white light for one day followed by five days in darkness (six replicates) at 25°C. Values in brackets represent the se of the mean.

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<th>sob7-1</th>
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Figure 1: Adult phenotype of the genotypes used in this study. Wild-type and single-, double- and multiple-mutant genotypes were grown in long-day conditions for three weeks before being photographed. Scale bar= 1cm.
Figure 2: Genetic interactions between BAS1, SOB7, PHYA and PHYB to control flowering time. BAS1 but not SOB7 mutant suppresses the late-flowering phenotype of PHYA mutants in both long day (A and B) and short days (C). phyB-9 bas1-2 sob7-1 triple- and phyA-211 phyB-9 bas1-2 sob7-1 quadruple-null did not flower earlier than the phyB-9 single- and phyA-211 phyB-9 double null respectively suggesting that the bas1-2 sob7-1 early-flowering phenotype requires functional phyB (A and B). Three replications with ten plants per replication were used for flowering analysis. Error bars represent SE.
Figure 3: Expression patterns of BAS1 and SOB7.

GUS staining patterns of the seedlings or tissues from BAS1:BAS1-GUS and SOB7:SOB7-GUS transgenic plants at various developmental stages. Scale bar = 1.0 mm.
Figure 4: Histochemical GUS analysis of BAS1 expression in shoot apex during transition to flowering. Longitudinal section through the shoot apex of four-week-old transgenic plant expressing \textit{BAS1}: BAS1-GUS grown in short day conditions (A). Longitudinal section through the shoot apex of four-week-old transgenic plants expressing \textit{BAS1}: BAS1-GUS after shifting to long day conditions for two days (B). Scale bar = 0.1 mm.
Figure 5: BAS1 expression in the shoot apex in red-light is dependent on the presence of functional phyB. Transgenic seedlings carrying identical transgenic event of pBAS1: BAS1-GUS construct in the bas1-2 sob7-1 background (A and C) and phyB-9 bas1-2 sob7-1 triple-null background (B and D). Seedlings were grown in 45 µmol m$^{-2}$ sec$^{-1}$ of red light for five days before histochemical GUS analysis. Scale bar = 1 mm.
Figure 6: Model based on the interpretation of the genetic interactions between photomorphogenic photoreceptors and *BASI* and *SOB7*. (A) In the seed, *BASI* and *SOB7* act downstream and/or in parallel of phyA and phyB to promote germination. (B) In the shoot-apex, phyB modulate BAS1 expression to inhibit phase transition. The dashed arrows indicate the suggested interactions based on this study.
Figure S1: Genetic and molecular analysis of *BAS1*:BAS1-GUS and *SOB7*:SOB7-GUS lines. Genetic and molecular analysis of transgenic plants expressing *BAS1*:BAS1-GUS (A and B) and *SOB7*:SOB7-GUS (C and D) in bas1-2 sob7-1 background. Seedlings were grown in 45 \mu\text{mol m}^{-2} \text{ sec}^{-1} of white light for four days before being digitized and measured. For transcript analysis total RNA was isolated from four-day-old seedlings grown in similar conditions as used for hypocotyl growth analysis.

(A) **pBAS1**:Bas1-GUS lines

(B) *BAS1* and *ACTIN2*

Col-0  bas1-2  sob7-1  15.3  41.4  72.11  107.6

25 cycles
18 cycles

no template
(C) pSOB7:SOB7-GUS lines

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(D) SOB7

ACTIN2

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25 cycles
18 cycles

no template
CHAPTER THREE

The *ben1-1* brassinosteroid-catabolism mutation is unstable due to epigenetic modifications of the intronic T-DNA insertion

(Sandhu, K.S., Sharma, P., Neff, M.M., The *ben1-1* brassinosteroid-catabolism mutation is unstable due to epigenetic modifications of the intronic T-DNA insertion (Submitted))
ABSTRACT

Loss-of-function genetic analysis plays a pivotal role in elucidating individual gene function as well as interactions among gene networks. The ease of gene tagging and cloning provided by T-DNA insertion mutants have led to their heavy use by the Arabidopsis research community. However certain aspects of T-DNA alleles require caution, as highlighted in this study of an intronic insertion mutant (ben1-1) in the BEN1 (BRII-5 ENHANCED 1) gene. As a part of our analysis of brassinosteroid catabolic enzymes, we generated a genetic triple-mutant from a cross between the bas1-2 sob7-1 double-null (T-DNA exonic insertion mutants of phyB-4 ACTIVATION TAGGED SUPPRESSOR 1 and SUPPRESSOR OF phyB-4 7) and ben1-1. As previously described, the single ben1-1 line behaves as a transcript null. However, in the triple-mutant background ben1-1 was reverted to a partial loss-of-function allele showing enhanced levels of the wild-type-spliced transcript. Interestingly, the enhanced expression of BEN1 remained stable when the ben1-1 single-mutant was re-isolated from a cross with the wild-type. In addition, the two genetically identical pre-triple and post-triple ben1-1 mutants also differed phenotypically. The previously functional NPTII T-DNA marker gene (which encodes kanamycin resistance) was no longer functional in the recovered ben1-1 allele, though the length of the T-DNA insertion and the NPTII gene sequence did not change in the pre-triple and post-triple ben1-1 mutants. Restriction endonuclease analysis with methylation sensitive enzymes followed by genomic PCR showed that the methylation status of the T-DNA is different between the original and the recovered ben1-1. These observations demonstrate that the recovered ben1-1 mutant is epigenetically different from the original ben1-1 allele.
INTRODUCTION

Loss-of-function mutants play an essential role in genetic analysis. Mutation-tagging approaches, such as mutagenesis by T-DNA insertion, takes advantage of Agrobacterium-mediated plant transformation where a modified Ti plasmid is integrated into the plant genome (Krysan et al. 1999). In plant species that are amenable to transformation by Agrobacterium, community projects have been undertaken to generate large collections of genomic T-DNA insertion libraries (Sessions et al. 2002; Alonso et al. 2003; An et al. 2005; Thole et al. 2011). The ease of gene tagging and cloning provided by T-DNA insertion mutants have led to their heavy use in Arabidopsis research. However certain aspects of T-DNA-insertion-generated alleles require caution, as highlighted in this study of an intronic insertion mutant (ben1-1) in the BEN1 (BRI1-5 ENHANCED 1) gene.

BEN1 (At2g45400) was identified in an activation-tagging screen for extragenic modifiers of the semi-dwarf phenotype of bri1-5, a weak mutant allele BRI1 (BRASSINOSTEROID INSENSITIVE-1), which encodes the brassinosteroid (BR) receptor. Though the mechanism of BEN1 activity is not known, genetic data supports the hypothesis that BEN1 is involved in BR inactivation. The BEN1 over-expressor mutant BRI1-5 ENHANCED1-IDOMINANT (ben1-1D) has a characteristic BR-deficient phenotype (Yuan et al. 2007). A transcript-null mutant ben1-1 with a T-DNA insertion in the 2nd intron of BEN1 has elevated BR levels when compared to the wild type. BEN1 expression is up-regulated in seedlings grown in white light when compared to those grown in the dark. ben1-1 seedlings are also less responsive to light-mediated inhibition of hypocotyl growth, suggesting a role in seedling photomorphogenesis (Yuan et al. 2007).
BR inactivation also involves members of the cytochrome P450 gene family, **BAS1** (*phyB-4 ACTIVATION TAGGED SUPPRESSOR 1*) and **SOB7** (*SUPPRESSOR OF phyB-4 7*) (Neff et al. 1999; Turk et al. 2003; Turk et al. 2005). Over-expression of either **BAS1/CYP72B1/CYP734A1** or **SOB7/CYP72C1** suppresses the long-hypocotyl phenotype of *phyB-4* and also confers a BR-deficient phenotype (Neff et al. 1999; Turk et al. 2003; Turk et al. 2005). **BAS1** hydroxylates brassinolide, the most active BR in *Arabidopsis*, and its immediate precursor castasterone to their respective inactive C-26 hydroxy products (Turk et al. 2005). **BAS1** transcript accumulation is strongly feed-back regulated in a positive manner by BR levels (Tanaka et al. 2005). **SOB7**, on the other hand, is not a C-26 hydroxylase and seems to act on precursors of BR biosynthesis (Turk et al. 2005; Thornton et al. 2010). BR levels are elevated in the **bas1-2 sob7-1** double-null mutant (first-exon T-DNA insertion alleles) when compared to the wild type or either single null allele. **BAS1** and **SOB7** also affect developmental processes, such as hypocotyl elongation and flowering, in a synergistic/redundant fashion (Turk et al. 2005). Independent evolution of multiple BR-inactivating pathways indicates the importance of this process in plant growth and development. Therefore, identifying the contributions of enzymes and pathways related to the inactivation of these hormones is important for understanding BR-mediated development (Sandhu et al. 2012). Genetic interactions among the mutants of **BAS1**, **SOB7** and **BEN1** would indicate their specific roles in *Arabidopsis* development.

As a part of our analysis of BR catabolic enzymes, we generated a genetic triple-mutant from a cross between the **bas1-2 sob7-1** double-null and **ben1-1**. Our results show that the full loss-of-function **ben1-1** mutation was transformed to a partial loss-of-function mutation in the **bas1-2 sob7-1 ben1-1** (triple-mutant) background showing enhanced levels of the wild-type-spliced transcript. Interestingly, the enhanced expression of **BEN1** remained stable when the **ben1-1**
single-mutant was re-isolated from a cross with the wild-type. In addition, the ben1-1 single-mutant isolated back from the triple mutant was phenotypically different from the original ben1-1 allele in terms of seedling-development. The size of T-DNA insertion and the NPTII gene sequence did not change in the pre-triple and post-triple ben1-1 mutants. However, the previously functional NPTII T-DNA marker gene (which encodes kanamycin resistance) was no longer functional in the recovered ben1-1 allele. Restriction endonuclease analysis with methylation sensitive enzymes followed by genomic PCR showed that the methylation status of the T-DNA is different between the original and the recovered ben1-1. Our study shows that the ben1-1 BR-catabolism mutation is unstable due to epigenetic modifications of the intronic T-DNA insertion.

MATERIALS AND METHODS

Plant material: All mutants used in this study, ben1-1 (Yuan et al. 2007), bas1-2 and sob7-1 (Turk et al. 2005), were in the Columbia (Col-0) background. ben1-1 was crossed with bas1-2 sob7-1, and multiple mutant combinations were isolated from populations of F2 individuals derived from self-pollinated F1 plants. The ben1-1 allele was characterized by amplifying genomic DNA with gene specific PCR primers GSP1 and GSP2, and T-DNA specific PCR primers LBb1.3, and PRT2 (Table S1). Molecular-genetic analysis of the bas1-2 and sob7-1 alleles is described in Turk et al. (2005).

ben1-1 was isolated from the triple-mutant bas1-2 sob7-1 ben1-1 background by crossing with the wild-type (Col-0) and allowing the F1 population to self-pollinate. The subsequent F2
population was screened with PCR markers specific for the \textit{bas1-2}, \textit{sob7-1} and \textit{ben1-1} T-DNA insertions. An individual F2 plant homozygous for the wild-type \textit{SOB7} allele and heterozygous for \textit{bas1-2} and \textit{ben1-1} T-DNA insertions was thus identified. The F3 progeny of this individual was further screened to identify and recover multiple single \textit{ben1-1} F3 lines (#11.4 and #11.5) as well as a \textit{bas1-2 sob7-1 ben1-1} triple-mutant line. The homozygous F4 seeds derived from the identified individual F3 lines were used for quantitative RT-PCR and hypocotyl growth analysis.

**Exogenous hormone treatment:** The stock solution of brassinolide (BL) was dissolved in 95\% ethanol (v/v). BL treatment was performed by adding the BL stock solution to the seedling growth media to a final concentration of 100 nM. An equal amount of 95\% ethanol were added to the negative-hormone control media.

**Hypocotyl measurement:** Procedures for seed sterilization, plating, growth conditions and hypocotyl measurement were as described in Turk \textit{et al.} (2003).

**Transcript analysis:** Total RNA was isolated, using the RNeasy Plant Kit (Qiagen, Valencia, CA), from five-day-old seedlings grown in continuous white light (25 \(\mu\text{mol m}^{-2}\ \text{sec}^{-1}\)). On-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen, Valencia, CA), to eliminate genomic DNA contamination. Total cDNA was synthesized using SuperScriptIII First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The complete \textit{BEN1} transcript was amplified using primers PRT1 and PRT2. \textit{ACTIN2} was used as an internal control in RT-PCR (Table S1). The linear range of amplification for each gene transcript was determined by comparing samples obtained using different numbers of cycles. Lack of genomic and foreign DNA contamination was ascertained by using all RNA samples and water as a template in a PCR reaction.
Real-time quantitative RT-PCR analysis: For real-time quantitative RT-PCR analysis, Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Foster City, CA) was used. *BEN1* transcript was amplified using primers PQ1 and PQ2. *BAS1* transcript was amplified using primers PQ3 and PQ4 (Table S1). The internal control ubiquitin gene (*At5g15400*) was amplified using PCR Primers UBQ1 and UBQ2 (Table S1). PCR thermocycling program profile used was as following: initial denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s. Melt curve profile used melt curve analysis was as following: 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60°C for 15 s.

Amplification of T-DNA insertion: The T-DNA insertion fragment was amplified using PrimeSTAR® GXL polymerase (Clonetech, Mountain view, CA). PCR thermocycling program profile used was as following: 35 cycles of 98°C for 10 s, 64°C for 15 s, and 68°C for 10 min.

RESULTS

The *ben1-1* T-DNA insertion mutation is unstable in the *bas1-2 sob7-1* background: A *bas1-2 sob7-1 ben1-1* triple mutant line was isolated from a cross between a *bas1-2 sob7-1* double- and a *ben1-1* single-mutant (Figure 1A, B and C). Transcript levels of all three genes were examined to confirm that the triple mutant was a transcript-accumulation-null at all three loci. Surprisingly, quantitative RT-PCR analysis showed that the triple mutant had significantly enhanced levels of *BEN1* transcript when compared to the single *ben1-1* parental line, which showed very low levels of *BEN1* transcript accumulation (Figure 1D). Sequencing confirmed that the *BEN1* cDNA from both the wild type and the triple mutant was identical, indicating that
the T-DNA-containing 2\textsuperscript{nd} intron in the \textit{ben1-1} allele in the triple mutant had spliced in a wild-type manner. These observations show that the \textit{ben1-1} null mutation is unstable in the \textit{bas1-2 sob7-1} background.

**The \textit{BEN1} transcript levels stay steady in the re-isolated \textit{ben1-1} single mutant lines:** The \textit{bas1-2 sob7-1 ben1-1} triple mutant was back crossed to the wild type and in the segregating F3 progeny single \textit{ben1-1} mutant lines (# 11.4 and # 11.5) were re-isolated (Figure 2A). Interestingly, \textit{BEN1} transcript levels in the two re-isolated \textit{ben1-1} lines were still enhanced relative to the original \textit{ben1-1} line and similar to the \textit{bas1-2 sob7-1 ben1-1} triple mutant (Figure 2B). The unstable nature of the \textit{ben1-1} mutation in the \textit{bas1-2 sob7-1 ben1-1} background, and the transmission of this phenotype in the re-isolated \textit{ben1-1} single-mutant lines, suggests that the \textit{ben1-1} locus was modified either genetically or epigenetically in a heritable manner during the creation of the triple mutant.

**Re-isolated \textit{ben1-1} single-null lines are phenotypically different from the original \textit{ben1-1} lines:** To test the hypothesis that the re-isolated \textit{ben1-1} mutant lines are also phenotypically different from the original \textit{ben1-1} allele, fluence-rate response analysis of the hypocotyl-elongation response was studied in these lines (Figure 3A). The two re-isolated \textit{ben1-1} lines showed attenuated hypocotyl-elongation phenotypes when compared to the two original \textit{ben1-1} lines (Figure 3A). At all three fluence rates of white light, the re-isolated lines displayed significantly shorter hypocotyls than the original \textit{ben1-1} lines (P< 0.05). There were no significant differences in hypocotyl-length between the original and the re-isolated \textit{ben1-1} lines in the dark. Hypocotyl-elongation responses to exogenous BL treatments were also examined in these lines (Figure 3B and C). In white-light, two original \textit{ben1-1} lines showed reduced hypocotyl-elongation when compared to the wild type, whereas the behavior of the two re-
isolated ben1-1 lines were similar to the wild type (Figure 3B). The two original and re-isolated ben1-1 lines did not respond differently to BL treatment in darkness (Figure 3C).

**BEN1 transcript accumulation is not feed-back regulated by BRs:** It is possible that the original ben1-1 was a leaky mutation to start with and that the elevated BR levels in the bas1-2 sob7-1 genetic background further increased BEN1 transcript accumulation which amplified the leakiness of ben1-1. To test this hypothesis, first, a modified RT-PCR approach was used to detect the rare wild-type-spliced BEN1 transcripts in the original ben1-1 line. As was previously published, after 35 PCR cycles no product was detected in the ben1-1 line (Yuan et al. 2007; Figure 4A). However after 50 PCR cycles, a BEN1 amplification product could be detected, showing the presence of low level wild-type-spliced BEN1 transcript in the original ben1-1 line (Figure 4A).

To further test the hypothesis that the BR feed-back regulation in the bas1-2 sob7-1 background was causing the instability of the ben1-1 mutation, BEN1 transcript levels were examined in the wild-type and the bas1-2 sob7-1 double-null line using quantitative RT-PCR (Figure 4B). BEN1 wild-type-spliced transcript levels were also examined in response to exogenous BL treatment in the wild type and the original ben1-1 line (Figure 4D). Results showed that when compared to the wild type, BEN1 transcript levels were not significantly enhanced in the bas1-2 sob7-1 background (Figure 4B). Moreover, unlike BAS1 (Figure 4C) exogenous BL treatment did not result in any increase in BEN1 transcript accumulation levels in the wild type (Figure 4D). In addition, exogenous BL treatment also did not result in an increased accumulation of wild-type spliced BEN1 transcript in the original ben1-1 line. These observations clearly show that BR-mediated feed-back regulation in the bas1-2 sob7-1 background is not responsible for the ben1-1 mutation instability in the triple mutant.
**T-DNA trans-interactions likely cause ben1-1 instability:** Given that *BEN1* transcript accumulation is not feed-back regulated by changing levels of BRs, it is possible that T-DNA trans-interactions between T-DNA insertions located in the *bas1-2, sob7-1* and *ben1-1* alleles can also lead to *ben1-1* mutation instability in the triple-mutant. Epigenetic silencing of the T-DNA-located resistance markers often indicates the presence of T-DNA trans-interactions (Daxinger et al. 2008).

To test this hypothesis, *NPTII* resistance marker gene function in the *ben1-1* T-DNA was examined by planting seeds of the wild type, original *ben1-1*, triple-mutant and the re-isolated *ben1-1* (# 11.4) lines on kanamycin containing plant growth media. Seedlings of all the lines except the original *ben1-1* line showed complete kanamycin sensitivity (Figure 5A). This observation suggests the presence of T-DNA trans-interactions resulted in silencing of the resistance marker in the triple- and the re-isolated *ben1-1* single-mutant lines. Another possible explanation for these observations is that the *NPTII* locus had been mutated during the crossing and recombination process. To test this second possibility, the *NPTII* gene was amplified and sequenced from both the original and the re-isolated *ben1-1* lines (Figure 5B and C). The *NPTII* gene sequence and primary structure was found to be unaltered in the original and the re-isolated *ben1-1* lines. These observations confirm that the loss of the kanamycin resistance marker in the triple-mutant and the re-isolated *ben1-1* lines is the effect of the presence of T-DNA trans-interactions in the triple-mutant.

**The *ben1-1* T-DNA is differentially methylated in the original and the re-isolated *ben1-1* lines:** Silencing of the *NPTII* resistance marker gene in the triple-mutant and re-isolated *ben1-1* lines suggest that this genomic region has been differentially modified by methylation in the triple-mutant and the re-isolated *ben1-1* T-DNAs. To test this hypothesis, genomic DNA from
the wild type, triple-, original and re-isolated *ben1-1* single-mutant lines was digested with CpG methylation sensitive and insensitive restriction enzymes (REs). The restriction-site-specific sequences for the CpG methylation sensitive enzymes *Sac*II and *Sma*I are present in the *NPTII* promoter and in proximity to the left border of the *ben1-1* inserted T-DNA respectively (Figure 6A). The restriction-site-specific sequence for the CpG methylation insensitive enzyme *Eco*RI is located adjacent to the *Sma*I RE site (Figure 6A). Following digestion with REs, genomic DNA was used as a PCR template in which primers flanking the corresponding RE sites were used to prime the amplification (Figure 6A). Results of the PCR amplification showed that the triple mutant and re-isolated *ben1-1* T-DNA were more resistant to RE digestion by both the CpG methylation sensitive REs when compared to the original *ben1-1* T-DNA (Figure 6B), whereas there were no differences among these genotypes with regard to *Eco*RI digestion (6B). These observations demonstrate that the *ben1-1* T-DNA in the triple- and the re-isolated *ben1-1* single-mutant is differentially methylated in both the promoter region of *NPTII* gene as well as in the adjacent regions.

**T-DNA size and structure remains unchanged in the original and the re-isolated *ben1-1* lines:** In another scenario, a shortening of T-DNA insertion due to unequal recombination during the creation of the triple-mutant could also result in enhanced *BEN1* transcript levels when compared to the original *ben1-1* line. To test this hypothesis, the inserted T-DNA was amplified from the original and the re-isolated *ben1-1* lines using primers anchored in the 2nd and 3rd exons of the *BEN1* gene (Figure 7A). The PCR results show that, using wild-type DNA as a PCR template gave a band of the size expected in the case of no T-DNA insertion (Figure 7B). On the other hand, both the original and re-isolated *ben1-1* lines showed a large band of the same size, indicating the presence of approximately two T-DNA insertions in each case (Figure 7B). To
further test the possibility of any re-arrangement of the T-DNA in original versus the re-isolated ben1-1, the large T-DNA containing amplification product of the extended PCR (Figure 7B) was purified and cut using two different restriction enzymes. Identical restriction pattern of the two amplification products showed the absence of any T-DNA re-arrangement between the original and the re-isolated T-DNA insertions (Figure 7C). These experiments suggest that the T-DNA size and structure have not changed between the original and the re-isolated ben1-1 lines.

**DISCUSSION**

As a part of our analysis of BR catabolic enzymes, we generated a genetic triple mutant from a cross between the bas1-2 sob7-1 double-null and ben1-1. Surprisingly, the originally stable ben1-1 mutation was converted to a partial loss-of-function mutation in the triple mutant background showing increased levels of the wild-type-spliced transcript (Figure 1D). To study the genetics of ben1-1 instability, we re-isolated the ben1-1 mutation by back crossing the triple mutant with the wild type (Figure 2). Molecular analysis of re-isolated ben1-1 lines showed that the unstable state of the ben1-1 mutation in the triple mutant is maintained in an otherwise wild-type background. The original and the re-isolated ben1-1 single lines also differed phenotypically (Figure 3). The original ben1-1 single-mutant line displays an aberrant hypocotyl-elongation phenotype in white light (Yuan et al. 2007). When compared to the original ben1-1 allele, the re-isolated ben1-1 lines displayed significantly different hypocotyl-elongation phenotypes in white-light fluence-rate response experiments as well as in a BL dose-response treatment (Figure 3A, B and C). These genetic and molecular observations suggest that the
changes in the re-isolated *ben1-1* are of an epigenetic nature. However, the exact mechanism leading to the enhanced T-DNA-containing intron-splicing is unknown.

In the case of intronic T-DNA insertion mutations, the presence of wild-type-spliced transcript is not uncommon (Wang 2008). Additionally, conditional mutant instability has been observed in an intronic T-DNA insertion mutation in the *OPR3* (*OPDA REDUCTASE 3*) gene (Chehab *et al.* 2011). *opr3* is an intronic T-DNA insertion mutant of *OPR3* which does not produce any detectable jasmonic acids (JAs) and acts as a complete loss-of-function allele under normal conditions. However, the *opr3* allele becomes unstable when mutant plants are infected with a fungus (Chehab *et al.* 2011). This suggests that environment may play a role in the phenomenon of T-DNA insertion mutant instability.

To test the hypothesis that the epigenetic transformation of *ben1-1* in the triple mutant could be due to elevated BR content in the *bas1-2 sob7-1* genetic background, we studied *BEN1* expression in the *bas1-2 sob7-1* double-null. *BEN1* expression was not significantly affected in the *bas1-2 sob7-1* background (Figure 4B), suggesting that *BEN1* is not strongly feed-back regulated by changes in BR levels. This suggests that elevated BR levels in *bas1-2 sob7-1* (Turk *et al.* 2005) did not result in *ben1-1* instability in the triple-mutant background (Figure 4B). Another possible explanation is that *BEN1* is not expressed in the same tissues where both *BAS1* and *SOB7* are expressed. *bas1-2* and *sob7-1* single-null mutants do not have altered BR levels. As *BAS1* and *SOB7* expression patterns do not overlap completely (Sandhu *et al.* 2012), it is possible that all tissues in the *bas1-2 sob7-1* double-null do not have altered BR levels. Analysis of the publically available microarray data suggests that *BEN1* transcript accumulation is not significantly affected by BL treatment (Winter *et al.* 2007). To complement the microarray data analysis, we also studied *BEN1* expression in the wild type and the original *ben1-1* single-mutant
in response to exogenous BL (Figure 4D). BAS1 expression was also studied in the same experiment to compare BEN1 gene expression response to a known BR feedback regulated gene. The results of gene expression analysis also showed that BEN1 expression is not affected by BL treatment and hence it is highly unlikely that BR feed-back regulation is responsible for the ben1-1 instability (Figure 4C and D).

Since the mutagenic T-DNA is the same in bas1-2, sob7-1 and ben1-1 (Alonso et al. 2003), a possible cause of ben1-1 instability could be the effect of T-DNA trans-interactions. As documented previously, T-DNA trans-interactions can result in the loss of antibiotic resistance (Daxinger et al. 2008). In fact, the original ben1-1 mutant seedlings showed kanamycin resistance, whereas the re-isolated lines as well as the triple were completely sensitive to kanamycin (Figure 5A). Interestingly, the loss of kanamycin resistance also showed an epigenetic pattern of inheritance. Similar loss of kanamycin resistance is observed in the case of yuc1-1 (yucca1-1) and ag-TD (agamous-TDNA insertion mutant) interactions (Gao and Zhao, 2012). Silencing of homologous genes is known to be an effect of T-DNA trans-interactions (Daxinger et al. 2008). Homology induced silencing is also associated with a phenomenon known as RNA-directed DNA methylation in the region of RNA-DNA homology (Wasseneger 2000). In fact, the promoter region of the re-isolated ben1-1 line T-DNA showed cytosine methylation, which is not the case with the original ben1-1 T-DNA (Figure 6). The T-DNA methylation was also observed in the region adjacent to the NPTII locus (Figure 6). The spread of DNA methylation to adjacent parts of the RNA-DNA homology region has previously been documented (Wassenger 2000). Another possibility is that apart from the NPTII gene, the mRNA transcribed from other parts of the T-DNA is also present in the ben1-1 mutant.
T-DNA trans-interactions have also been associated with instability of an intronic T-DNA insertion mutant (Gao and Zhang, 2012). However, currently there is no evidence which supports that the mutant instability is caused directly by T-DNA trans-interactions. Plausibly, one of the factors involved in mutant instability is the efficient splicing of the T-DNA containing intron. The splicing efficiency could be affected by a change in the size of T-DNA insertion due to events such as unequal recombination. However, the size of T-DNA insertion and structure is unchanged in the pre- and post-triple ben1-1 mutant lines (Figure 7). The most apparent difference in the original and the re-isolated ben1-1 lines is the presence of methylation in the re-isolated ben1-1 T-DNA region (Figure 6). RNA-induced DNA methylation has been associated with chromatin modification (Aufsatz et al. 2002a; Aufsatz et al. 2002b). Chromatin modifications in turn have been linked to the regulation of pre-mRNA splicing by many studies (for review see: Hnilicova and Stanek, 2011). For example, alternative splicing in the CD44 gene required proper recruitment of argonaute RNAi proteins to the CD44 transcribed regions (Ameyar-Zazoua et al. 2012). Therefore, it is possible that the methylation of the T-DNA region is altering the splicing efficiency of the T-DNA containing intron (Figure 8).

However, more studies are required to test the association between T-DNA trans-interactions, T-DNA methylation and the T-DNA containing intron splicing. To further analyze the role of T-DNA trans-interactions, ben1-1 mutation stability can be studied in the genetic background of non-BR related transgenics (harboring T-DNAs expressing kanamycin resistance). Further studies can also analyze the nature of chromatin modifications in the original vs the recovered ben1-1 T-DNA. Primarily, this study indicates the need for more caution while using T-DNA insertional mutants. Future directions for this study will include isolation of
additional mutations in the BEN1 locus. An EMS mutagenesis approach (Street et al. 2008) using the activation-tagged mutant ben1-D will be used to isolate true null-alleles of BEN1.

ACKNOWLEDGMENTS

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TABLES, FIGURES AND LEGENDS

Figure 1: ben1-1 mutation is unstable in the bas1-2 sob7-1 background. (A) Graphical depiction of BEN1 gene structure and location of T-DNA insertion in the ben1-1 allele. (B) Depiction of the crossing scheme used to isolate bas1-2 sob7-1 ben1-1 triple-null. (C) Example of the genetic analysis of ben1-1 allele. (D) Quantitative RT-PCR analysis of BEN1 expression in the wild type, ben1-1 and triple null. Error bars indicate the standard error of the mean (SE).
Figure 2: The \textit{BEN1} transcript levels stay steady in the re-isolated \textit{ben1-1} single-null lines.

(A) Depiction of the crossing scheme used to re-isolate \textit{ben1-1}. (B) Quantitative RT-PCR analysis of the \textit{BEN1} transcript in the five-day-old seedlings of Col-0, original \textit{ben1-1}, \textit{bas1-2 sob7-1 ben1-1}, F3\# 11.4 (re-isolated line) and F3\# 11.5 (re-isolated line) shows that the transcript accumulation levels in the re-isolated lines (F3\# 11.4 and F3\# 11.5) remain stable at the same level as that of triple null line. Error bars indicate the standard error of the mean (SE).

(A) \hspace{1cm} Col-0 \hspace{.5cm} X \hspace{.5cm} \textit{bas1-2 sob7-1 ben1-1}

\hspace{1cm} \uparrow

\hspace{1cm} \textit{Triple heterozygous} \hspace{.7cm} \textit{F1}

\hspace{1cm} \downarrow \hspace{1cm} \textit{Selfing}

\hspace{1cm} \textit{Segregating progeny} \hspace{.7cm} \textit{F2}

\hspace{1cm} \downarrow \hspace{1cm} \textit{Selfing}

\hspace{1cm} \textit{Segregating progeny} \hspace{.7cm} \textit{F3}

\hspace{1cm} (selection by genomic PCR)

\hspace{1cm} (re-isolated \textit{ben1-1} single null)

(B) 

\begin{tikzpicture}
  \begin{axis}[
    ybar, 
    bar width=10pt, 
    ymin=0, ymax=1.2, 
    ylabel={Fold expression}, 
    xlabel={Col-0 \hspace{1cm} \textit{ben1-1} \hspace{1cm} \textit{bas1-2 sob7-1} \hspace{1cm} 11.4 \hspace{1cm} 11.5 \hspace{1cm} \text{Recovered \textit{ben1-1} lines}}, 
    symbolic x coords={Col-0, \textit{ben1-1}, \textit{bas1-2 sob7-1}, 11.4, 11.5}, 
    xtick=data, 
    nodes near coords, 
    every node near coord/.append style={yshift=0.1cm}, 
  ]
    \addplot coordinates {(Col-0, 1) (\textit{ben1-1}, 0.6) (\textit{bas1-2 sob7-1}, 0.4) (11.4, 0.6) (11.5, 0.4)};
  \end{axis}
\end{tikzpicture}
Figure 3: Re-isolated *ben1-1* single-null lines are phenotypically different from the original *ben1-1* lines. (A) Fluence-rate analysis of the Col-0, original *ben1-1* line #1 and #2, and re-isolated *ben1-1* lines at three different white-light fluence rates. The hypocotyl length of the re-isolated *ben1-1* is not significantly different from original *ben1-1* (p > 0.5) in the dark. At all three white-light fluence rates, the hypocotyl length of the re-isolated *ben1-1* is significantly different from original *ben1-1* lines (p < 0.05 in all cases). (B) Hypocotyl-elongation response of the re-isolated and original *ben1-1* lines to the exogenous BL treatment (100 nm BL) in white-light. In white light (24 µmol m$^{-2}$ sec$^{-1}$) the hypocotyl-elongation response of the re-isolated *ben1-1* is similar to the wild-type. The hypocotyl elongation is suppressed by BL treatment in the original *ben1-1* lines when compared to the wild type. (C) In the dark the hypocotyl-elongation response of the re-isolated *ben1-1* is not significantly different from original *ben1-1* (p > 0.5). To calculate the percentage of BL-negative-control hypocotyl length (as in B and C), each seedling value in BL treatment experiment was normalized to the average of the same genotype in BL negative control experiment. The resulting group of values was used to calculate standard error of the mean (SE).
Figure 4: The *ben1-1* is a leaky mutant based on RT-PCR analysis and the enhanced T-DNA-containing-intron splicing in recovered *ben1-1* is not induced by BR feedback regulation. (A) Primer pair, PRT1 and PRT2 was used to amplify full-length coding region of *BEN1* transcript. Amplification of *ACTIN2* transcript was used as a cDNA loading control (Lane 1 and 2). At 35 cycles no product is detected in the *ben1-1* lane (Lane 5) as compared to the Col-0 lane (Lane 4). After 50 cycles *BEN1* transcript is detectable in the *ben1-1* lane (Lane 8). Primer sequence information is given in Table S1. (B) *BEN1* expression is not affected in the *bas1-2 sob7-1* double-null background relative to the wild type background. (C) Exogenous BL treatment induces *BAS1* expression in Col-0 and *ben1-1* background. (D) Exogenous BL treatment did not induce *BEN1* expression in Col-0 and *ben1-1* background. Error bars indicate the standard error of the mean (SE).
Figure 5: The kanamycin resistance trait, conferred by the T-DNA resistance marker NPTII, is absent in the 
bas1-2 sob7-1 ben1-1 and the recovered ben1-1 line. (A) Seedlings were grown on the plant media containing kanamycin for seven days before photographing. (B) The graphic depiction of the location of the primers used to amplify NPTII from the T-DNA. (C) The gel image shows that genomic PCR amplifies bands of equal size from original ben1-1 and the recovered ben1-1 lines.
Figure 6: Genomic DNA in the NOS Promoter region of the T-DNA insertion in the recovered ben1-1 shows methylation. (A) Graphic depiction of restriction enzyme sites and the primer pair locations in the T-DNA used for the methylation study. (B) Genomic PCR amplification from the undigested (-) and the restriction digested (+) DNA of the Col-0, original ben1-1, recovered ben1-1 (11.4), and the bas1-2 sob7-1 ben1-1 (triple). Primer pairs MT1 and MT2 were used to test methylation at the SacII site. Primer pairs MT3 and MT4 were used to test methylation at the Smal site. Primer pairs MT3 and MT4 were also used to amplify genomic DNA digested with non-methylation sensitive EcoRI. PCR amplification, by using SacII site and Smal/EcoRI flanking PCR primers (Table S1), is seen only from the digested genomic DNA of the recovered ben1-1.
Figure 7: The size and sequence of the T-DNA insertion remains unchanged in the pre-triple and post-triple ben1-1 lines. (A) Graphic depiction of the location of the 2nd intron flanking primer pair used for amplification in the ben1-1 allele. (B) The genomic PCR using the 2nd intron flanking primers amplify equal size band from the original ben1-1 and the recovered ben1-1 line. (C) The original and the recovered ben1-1 alleles show identical restriction digestion pattern. The PCR amplification product from gel image (B) was gel purified and restriction digested with HindIII and KpnI.
Figure 8: Hypothetical model for RNAi and chromatin structure dependent intron splicing. Argonaute protein is guided by the associated siRNA and recruited by chromatin component proteins associated with chromatin marks (H3K9me3). This complex slows down the movement of RNA polymerase II and possibly affects the splicing decisions by the spliceosome. Figure adapted from Ameyar-Zozoua et al. 2012.
Supplemental Information:

**Table S1.** Table of PCR primer sequences used in the study.

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<tr>
<td>GSP2</td>
<td>5'-ACCTTTTGCAACTGCTTTTTT-3'</td>
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<tr>
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<td>UBQ2</td>
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CHAPTER FOUR

The *Arabidopsis* gene *ATST4a* is not a typical brassinosteroids catabolic gene.
ABSTRACT

Brassinosteroids (BRs) homeostasis is maintained in part by this hormone’s catabolism. Presence of multiple brassinosteroids (BRs) catabolic pathways in Arabidopsis suggests the importance of this process in plant growth and development. In vitro biochemical analyses demonstrate that ATST4a has catalytic activity with BRs. However, unlike other BR catabolic pathways the genetic evidence to suggest such a function for the ATST4a pathway in planta is scant. In this study, we have used both loss-of-function and over-expression genetic approaches to further explore the role of ATST4a in Arabidopsis. This study shows that atst4a1-1, a T-DNA insertion null mutant of ATST4a did not display reduced seedling hypocotyl growth inhibition in white light. Additionally, overexpression of ATST4a gene in transgenic lines did not result in any characteristic BR-deficient phenotypes. These observations suggest that ATST4a gene encodes an atypical BR catabolic enzyme.
INTRODUCTION

Catabolism is an important aspect of brassinosteroid (BR) homeostasis. Discovery of several independent BR catabolic pathways and their enzymes in *Arabidopsis* also suggest the importance of this process in plant growth and development (Neff *et al.* 1999; Turk *et al.* 2003; Turk *et al.* 2005; Poppenberger *et al.* 2005; Yuan *et al.* 2007; Husar *et al.* 2011; Roh *et al.* 2012; Schneider *et al.* 2012). These BR catabolic pathways were mainly discovered and latter characterized using genetic manipulation of the genes involved. In some cases, *in vivo* and *in vitro* biochemical assays of the enzymes encoded by these genes were also used to confirm their role in BR catabolism. Genetic analyses of knockout or knockdown mutants of most of these BR catabolic genes show a reduction in hypocotyl-elongation inhibition in white light in *Arabidopsis* seedlings (Neff *et al.* 1999; Turk *et al.* 2005; Poppenberger *et al.* 2005; Yuan *et al.* 2007; Roh *et al.* 2012). The knockout or knockdown approach may in some cases fail to provide any evidence of involvement in BR catabolism due to genetic redundancy or yet other unknown reasons (Husar *et al.* 2011). However, in all cases, *in planta* overexpression of BR catabolic genes leads to characteristic BR-deficient phenotypes at both seedling and adult stages of *Arabidopsis* (Neff *et al.* 1999; Turk *et al.* 2003; Turk *et al.* 2005; Takahashi *et al.* 2005; Nakamura *et al.* 2005; Poppenberger *et al.* 2005; Yuan *et al.* 2007; Husar *et al.* 2011; Roh *et al.* 2012; Schneider *et al.* 2012). These BR-deficient phenotypes typified by a BR biosynthetic mutant *de-etiolated 2-1* (*det2-1*), include small organ size, small round and dark green leaves, and delayed flowering (Chory *et al.* 1991; Azpiroz *et al.* 1998; Domagalska *et al.* 2007; Li and Chory 1997).

Members of the sulfotransferase protein superfamily are known to perform various metabolic functions in plants (Klein and Paponbrock, 2004; Kopriva *et al.* 2012) and some family members
are also known to be metabolically active in hormonal pathways (Gidda et al. 2003; Baek et al. 2010). ATST4a (AT2G14920) encodes a sulfotransferase member in Arabidopsis (Klein and Papenbrock, 2004). ATST4a was cloned on the basis of homology to ATST1 (Marsolais et al. 2007). ATST1 is an ortholog of BNST (steroid sulfotransferase from Brassica napus) proteins and displays similar catalytic activities and substrate specificity. Recent evidence shows that ATST1 is also involved in the sulfonation of salicylic acid in response to pathogen stress (Baek et al. 2010). In vitro protein expression and sulfotransferase assay studies demonstrate that ATST4a has catalytic activity with BRs (Marsolais et al. 2007). Catalytic activity of ATST4a is specific for biologically active end products of the BR biosynthetic pathway, including castasterone, brassinolide, related 24-epimers and the naturally occurring (22R, 23R)-28-homobrassinosteroids. The addition of a sulfate group to the steroid molecules may cause a change in their activity and therefore play a role in steroid homeostasis (Strott et al. 2002). These observations suggest that ATST4a is involved in BR homeostasis via catabolism in Arabidopsis.

Although biochemical analysis can provide important clues, use of genetic approaches can potentially further our understanding of endogenous gene functions. In this study, we have used both loss-of-function and over-expression genetic approaches to further explore the role of ATST4a in Arabidopsis. This study shows that atst4a1-1, a T-DNA insertion null mutant of ATST4a did not display reduced seedling hypocotyl growth inhibition in white light. Additionally, overexpression of the ATST4a gene in transgenic lines did not result in any characteristic BR-deficient phenotypes, and did not cause a predicted feed-back-regulation change in the transcript accumulation of DWF4 and BAS1 (Tanaka et al. 2005). These observations suggest that ATST4a gene encodes an atypical BR catabolic enzyme.
MATERIAL AND METHODS

Plant Material: All plant material used in this study was in Col-0 background. For loss-of-function analysis of ATST4a, a T-DNA insertion line GK-177E08 with an insertion in the ATST4a ORF was obtained from GABI-Kat (Kleinboelting et al. 2012). The T-DNA line was backcrossed twice to the Col-0 to clean the background of any unwanted mutations. The T-DNA insertion was followed through the crosses using the following set of primers: gene specific primers- 5'-ATG GAT GAA AAA GAT AGA CC-3' and 5'-TTA GAA TTT CAA ACC GGA ACC-3' and T-DNA specific primer 5'-ATA TTG ACC ATC ATA CTC ATT G-3'. For over-expression analysis, ATST4a coding sequence including start and stop codon were cloned in pENTR/D/TOPO vector (Invitrogen, Carlsbad, CA). The cloned DNA was sequenced to make sure that there were no mutations. The coding sequence containing fragment was further subcloned into a binary vector pEARYLGATE100 by gateway® cloning using LR clonase (Invitrogen). The binary vector was transformed into atst4a1-1 to increase the frequency of transgenic over expressers by avoiding RNAi interference from the endogenous transcript. Single locus insertion lines based on a 3:1 ratio of resistant to susceptible were selected for further analysis. For generating ATST4a:GUS translational fusion lines, the 3.0 Kb genomic fragment containing the ATSt4a promoter and gene was cloned in frame with uidA gene in the pCAMBIA1305.1 vector (CAMBIA, CANBERRA, AUSTRALIA). This construct was transformed into st4a1-1 plants. Multiple transgenic lines segregating at a 3:1 ratio (hygromycin resistant/sensitive ratio) in the T2 generation were identified as single insertion lines.

Transcript analysis: Total RNA was isolated, using the RNeasy Plant Kit (Qiagen, Valencia, CA) from five-day-old seedlings grown in continuous white light (25 µmol m⁻² sec⁻¹). On-
column DNase digestion was performed using the RNase-Free DNase Set (Qiagen, Valencia, CA), to eliminate genomic DNA contamination. Total cDNA was synthesized using SuperScriptIII First-Strand Synthesis System (Invitrogen, Carlsbad, CA). *ATST4a* transcript was amplified using primers 5′-ATG GAT GAA AAA GAT AGA CC-3′ and 5′-TTA GAA TTT CAA ACC GGA ACC-3′. *ACTIN2* was used as an internal control in RT-PCR. *ACTIN2* transcript was amplified using primers 5′-GGT CGT ACA ACC GGT ATT GTG CTG G-3′ and 5′-CTG TGA ACG ATT CCT GGA CCT GCC-3′. The linear range of amplification for each gene transcript was determined by comparing samples obtained using different numbers of cycles. Lack of genomic and foreign DNA contamination was determined by using all RNA samples and water as a template in a PCR reaction.

**Real-time quantitative analysis of transcript levels:** Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Foster City, CA) was used for real-time quantitative RT-PCR analysis. *ATST4a* transcript was amplified using primers 5′-TCA CTC GAG CGG AGG ATT AC-3′ and 5′-GAG TAC GAG GCT CCG CTT T-3′. Internal control ubiquitin gene (*AT5G15400*) was amplified using PCR Primers 5′-GAA ATG CAT GGA GAC GGA TT-3′ and 5′-TTG GTC TCT GCT CCC ACT CT-3′. PCR thermocycling program profile used was as following: initial denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s. Melt curve profile, which was used for melt curve analysis was as following: 95°C for 15 s, 60°C for 1 min, 95°C for 30 s and 60°C for 15 s.

**Exogenous hormone treatment:** The stock solution of brassinolide (BL) was dissolved in 95 % ethanol (v/v). BL treatment was given by adding BL stock solution to the seedling growth media to a final concentration of 100 nM.
Hypocotyl measurement: Procedures for seed sterilization, plating, growth conditions and hypocotyl measurement were done as described in Turk et al. (2003).

RESULTS

The T-DNA insertional mutant of ATST4a displayed a wild-type response to light-induced inhibition of hypocotyl growth: A T-DNA insertion line (designated atst4a1-1) with an insertion in ATST4a gene was obtained from GABI-Kat (Kleinboelting et al. 2012). Genetic analysis by gene- and T-DNA-specific primers indicated that the T-DNA is inserted in the coding sequence of the ATST4a gene (Figure 1A and B). Histochemical analysis of the ATST4a-GUS translational fusion lines indicated that ATST4a is expressed in both white-light and dark grown seedlings (Figure 2A). This observation suggested that ATST4a may play a role in hypocotyl growth. To test this hypothesis, hypocotyl growth was studied in the wild type and the atst4a1-1 lines. Hypocotyl growth in dark and two white-light fluence rates showed no significant differences between wild type and atst4a1-1 lines (Figure 2B). This suggests that ATST4a does not play a role in light-induced hypocotyl inhibition.

Arabidopsis plants overexpressing ATST4a cDNA display wild-type phenotypes at both seedling and adult stages: To test the hypothesis that the constitutive overexpression of ATST4a will lead to BR-deficient phenotypes; multiple transgenic lines overexpressing ATST4a cDNA were generated. These ATST4a-overexpression transgenic lines were characterized by quantitative RT-PCR analysis. Three independent transgenic lines displaying high levels of ATST4a expression (Figure 3A) were selected for further phenotypic analysis. Adult ATST4a-Ox

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transgenic lines did not display any BR-deficit phenotypes (Figure 3B). Hypocotyl-growth was also studied in the homozygous T3 ATST4a-Ox, wild-type and the atst4a1-1 seedlings in both dark and white-light (Figure 4A and B). The hypocotyl length in the atst4a1-1 line was not significantly different than the wild type (p>0.5). The three overexpression lines displayed significantly shorter hypocotyl lengths than the wild type (p<0.01). The hypocotyl-growth response of wild type, ATST4a-OX and atst4a1-1 seedlings was not altered by BL treatment in the dark (Figure 5A). In white light, the hypocotyl length in the atst4a1-1 line was not significantly different than the wild type (p>0.5) (Figure 5B). The T3-52 and T3-64 overexpression lines displayed significantly shorter hypocotyl lengths than the wild type (p<0.01) (Figure 5B.)

Expression of the known BR-feedback regulated genes is not affected in ATST4a loss-of-function and ATST4a-OX lines: Overexpression of BR catabolic genes often causes alterations in the transcript levels of BR-feedback regulated genes. In these conditions, the transcript accumulation of BR biosynthesis genes, such as DWF4, is enhanced whereas that of catabolic genes, such as BAS1, is reduced. This response is often vectorially opposite in the loss-of-function lines. To test this hypothesis, gene expression of BR-feedback regulated genes, BAS1 and DWF4 was studied in wild type, atst4a1-1 and T3 ATST4a-Ox seedlings. Quantitative RT-PCR analysis showed that transcript accumulation of both BAS1 and DWF4 was not significantly different in these lines (p>0.05 for all pair-wise comparisons)(Figure 6A and 6B).

DISCUSSION
Biochemical in vitro analysis of ATST4a suggests its role in BR catabolism (Marsolais et al. 2007). However, not enough genetic data is available to support this role of ATST4a’s in BR catabolism in Arabidopsis. This study uses both loss-of-function and gain-of-function genetic approaches to further analyze the role of ATST4a in Arabidopsis. Mutants of BR catabolic genes in most cases are compromised in light-mediated hypocotyl growth inhibition (Neff et al. 1999; Turk et al. 2005; Poppenberger et al. 2005; Yuan et al. 2007; Roh et al. 2012). However, despite the observation that ATST4a is expressed in the seedling hypocotyl (Figure 2A), atst4a1-1 did not display any defect in light-mediated hypocotyl inhibition (Figure 2B). This is not surprising since mutants of some known BR catabolic genes such as UGT73C6 and PIZ1 also do not display hypocotyl phenotypes (Husar et al. 2011; Schneider et al. 2012).

More surprisingly, the overexpression of ATST4a cDNA in Arabidopsis did not result in BR-deficient phenotypes at adult plant stages (Figure 3A and B). Moreover, seedlings of the transgenic lines overexpressing ATST4a cDNA did not show any dramatic increase in the light-induced inhibition of hypocotyls (Figure 4). This observation is significant since all the known BR catabolic genes characterized so far result in dramatic BR-deficient phenotypes both at seedling and at adult stage when overexpressed in Arabidopsis (Husar et al. 2011; Poppenberger et al. 2005; Roh et al. 2012; Turk et al. 2003; Turk et al. 2005; Yuan et al. 2007; Schneider et al. 2012). This suggests that ATST4a overexpression did not reduce BR content in the transgenic lines. In addition, quantitative transcript analysis of the feed-back regulated genes DWF4 and BAS1 also suggests that BR content is not reduced in the ATST4a-OX lines when compared to wild type (Figure 6). Overall, this study shows that despite its in vitro preference for BR substrates, ATST4a does not behave like a typical BR catabolic gene in planta.
In vitro biochemical analysis can give important clues to the function of an enzyme. However enzyme activity and function at a tissue or cellular level may be dependent on certain in vivo conditions and therefore difficult to assess. For example, the unique metabolic environment within a cell or a tissue may provide crucial cofactors or in another case the substrate preference may be different in an in vivo vs. the in vitro environment. Sulfotransferases can display preference for a diverse range of substrates under in vitro conditions; however, this may not fully reflect their endogenous functions (Kopriva et al. 2012). For example, biochemical analysis of BNST3 suggests that it is involved in catabolizing 24-epibrassinosteroids; however, over-expression of BNST3 do not lead to BR-deficient phenotypes in Arabidopsis, suggesting that BR catabolism is not its endogenous function (Marsolais et al. 2004). Another explanation for lack of BR-deficient phenotypes in overexpression lines is that of post-translational regulation of ATST4a. It is also possible that the overexpressed ATST4a protein is not properly localized in the plant cells. Given these observations, more studies are required to assign biological function to ATST4a. The Brassica napus steroid sulfotransferases are inducible by ethanol, xenobiotics and low oxygen stress and may be involved in plant stress response (Marsolais et al. 2004). Therefore, it is possible that ATST4a also has a similar role in Arabidopsis. This can be studied by observing growth of wild type, ATST4a loss-of-function and overexpression lines in response to various stresses. Another direction would be to identify the transcriptional regulators of ATST4a by using its promoter in a yeast-one-hybrid screen. They may help identify the biological pathways in which ATST4a might be involved.

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FIGURES AND LEGENDS

Figure 1: Genetic analysis of the *atst4a1-1* T-DNA insertion line. (A). Graphical representation of the location of T-DNA insertion in the *ATST4a* ORF. Arrows show the location of the gene and T-DNA specific primers used for genetic screening. (B). A gel image showing results of genomic PCR amplification using *ATST4a* gene specific and T-DNA specific primers.

(A)

![Graphical representation of the location of T-DNA insertion in the ATST4a ORF. Arrows show the location of the gene and T-DNA specific primers used for genetic screening.](image)

(B)

![A gel image showing results of genomic PCR amplification using ATST4a gene specific and T-DNA specific primers.](image)
Figure 2: The *atst4a1-1* mutant does not show a hypocotyl-elongation phenotype in both dark and continuous white light conditions. (A) ATST4a is expressed in the hypocotyls of the white-light and dark grown seedlings as shown by the histochemical analysis of the pATST4a: ATST4a-GUS expressing lines. Scale bar= 2mm. (B) Fluence rate analysis of hypocotyl growth of *atst4a1-1* in dark and at two different fluence rates, WL-3.5 (white light=3.5 µmol m$^{-2}$ sec$^{-1}$) and WL-10 (white light=10 µmol m$^{-2}$ sec$^{-1}$) did not show any altered hypocotyl-elongation response when compared to Col-0. Three replications of five-day-old seedlings were used to measure hypocotyl growth. Error bars indicate standard error (SE).
Figure 3: Overexpression of *ATST4a* does not lead to BR-deficient phenotypes in *Arabidopsis*. (A) The *ATST4a* over-expression lines show ~1000 fold levels of *ATSt4a* transcript as compared to the Col-0. Error bars indicate SE. (B) Adult phenotypes of the three-week-old *Arabidopsis* plants overexpressing *ATST4a* cDNA.
Figure 4: Hypocotyl-elongation response of *atst4a1-l* and *ATST4a-OX* T3 lines to dark and continuous white light conditions. Fluence rate analysis of hypocotyl growth of *ATST4a-OX* T3 lines in dark (A) and in continuous white light (10 µmol m$^{-2}$ sec$^{-1}$) (B) did not show dramatic differences in hypocotyl-elongation response when compared to Col-0. Three replications of five-day-old seedlings were used to measure hypocotyl growth. Error bars indicate SE.
Figure 5: Hypocotyl-elongation response of *atst4a1-1* and *ATST4a-OX* T3 lines to BL treatment. Seedlings were grown in continuous light for five days on media containing BL at 100 nM concentration in Dark (A) and white light (25 µmol m\(^{-2}\) sec\(^{-1}\)). Hypocotyl-elongation response of *atst4a1-1* and *ATST4a-OX* T3 lines do not show dramatic differences when compared to Col-0 in both dark and light conditions. Three replications of five-day-old seedlings were used to measure hypocotyl growth. Error bars indicate SE.
Figure 6: *ATST4a-OX* T3 lines did not display feedback regulation of BR biosynthesis and catabolic genes *DWF4* and *BAS1*. Quantitative RT-PCR analysis of the *DWF4* (A) and *BAS1* (B) transcript in Col-0, *atst4a1-1*, and three single locus insertion *ATST4a-OX* T3 lines show that *ATST4a* over-expression lines did not show significantly altered levels of *DWF4* and *BAS1* transcript as compared to the Col-0. Error bars indicate SE.
CHAPTER FIVE

SUMMARY AND FUTURE DIRECTIONS

BR inactivation is an emerging field in plant biology. Biochemically, there are more possible ways to modify or inactivate a BR molecule than the ones discovered to date (Bajguz 2007). Therefore, it is likely that we will continue to discover novel mechanisms for BR inactivation in the future. In addition, not much is known about the regulation of BR inactivation and how plants use BR inactivation in their development and adaptation. The goal of my doctoral research was to study the unique and overlapping roles in Arabidopsis development played by some of the known BR catabolic genes.

To achieve this goal, Chapter Two explored the role of BAS1 and SOB7 in photomorphogenic development of Arabidopsis. This study uses a molecular genetic approach to delineate the role of BRs and BR catabolism in plant development. In summary, BAS1 and SOB7 displayed both simple and complex genetic interactions with the phytochromes in a plant-stage specific manner (Sandhu et al. 2012). The removal of BAS1 and/or SOB7 rescued the low germination rate of the phyA-211 phyB-9 double-null mutant. With regard to floral induction, bas1-2 and sob7-1 showed a complex set of genetic interactions with photoreceptor-null mutants. Histochemical analysis of transgenic plants harboring BAS1:BAS1-GUS and SOB7:SOB7-GUS translational fusions under the control of their endogenous promoters revealed overlapping and distinct expression patterns. BAS1’s expression in the shoot apex increases during the phase transition from short-to-long-day growth conditions and requires phyB in red light.

This study is important for the following reasons. Firstly, this is the first kind of study which specifically examines the role of BR catabolism in Arabidopsis developmental. It has been
suggested that light promotes photomorphogenesis in part by inhibiting BR levels or signaling (Li et al. 1996; Chory and Li, 1997; Kang et al. 2001). However, this hypothesis lacks support by direct evidence. A major contribution of this study is that it shows the regulation of a BR catabolic gene \( \textit{BAS1} \) by a photomorphogenic pathway (phyB) in the shoot apex. A key future experiment will be to study whether this is a direct regulation of \( \textit{BAS1} \) transcript accumulation or if the change in \( \textit{BAS1} \) expression is caused by cell-specific changes in BR content. Such studies will require technical improvements in the ability to measure BRs in small tissues and organs.

Another contribution of this study lies in the evolution of redundant biological pathways. Previous analysis from the Neff lab has shown that \( \textit{BAS1} \) and \( \textit{SOB7} \) affect developmental processes, such as flowering, in a synergistic/redundant fashion (Turk et al. 2005). However, the existence of multiple BR catabolic pathways suggests that it is unlikely that these pathways are completely redundant. Research in Chapter Two shows divergence of \( \textit{BAS1} \) and \( \textit{SOB7} \), in terms of both gene expression patterns and their interactions with other developmental pathways. For example, \( \text{phyA bas1-2} \) flowers early, whereas \( \text{phyA sob7-1} \) flowers later than the single \( \text{phyA} \). The study therefore provides the critical evidence for the notion that the multiple BR catabolic pathways have functional diversity in \textit{Arabidopsis}.

The application of the genetic analysis approach used in Chapter Two requires availability of loss-of-function mutants of the BR catabolic genes. Chapter Three focuses on the characterization of the T-DNA insertion mutant \( \textit{ben1-1} \). In summary, our analysis shows that the \( \textit{ben1-1} \) mutant is not suitable for multiple mutant analyses which involve combining two or more T-DNA insertion mutations.
As a part of our analysis of brassinosteroid catabolic enzymes, we generated a genetic triple-mutant from a cross between the *bas1-2 sob7-1* double-null (T-DNA exonic insertion mutants of *

* and *

*) and *ben1-1*. The single *ben1-1* mutant behaves as a transcript null for *BEN1*. However, in the triple-mutant background *ben1-1* was reverted to a partial loss-of-function allele showing enhanced levels of the wild-type-spliced transcript. Interestingly, the enhanced expression of *BEN1* remained stable when the *ben1-1* single-mutant was re-isolated from a cross with the wild-type. In addition, the two genetically identical pre-triple and post-triple *ben1-1* mutants also differed phenotypically. The previously functional *NPTII* T-DNA marker gene (which encodes kanamycin resistance) was no longer functional in the recovered *ben1-1* allele though the length of the T-DNA insertion and the *NPTII* gene sequence did not change in the pre-triple and post-triple *ben1-1* mutants. Restriction endonuclease analysis with methylation sensitive enzymes followed by genomic PCR showed that the methylation status of the T-DNA is different between the original and the recovered *ben1-1*. These observations demonstrate that the recovered *ben1-1* mutant is epigenetically different from the original *ben1-1* allele.

Future work in this direction will involve isolation and characterization of at least one novel loss-of-function mutant of *BEN1*. EMS mutagenesis of the activation tagging line *ben1-D* can be used for generating novel mutations in the *BEN1* locus. The *ben1-D* line displays a semi-dwarf seedling phenotype. The M2 progeny of the EMS mutagenized *ben1-D* line can be screened for suppressors of the semi-dwarf phenotype. The identified suppressors will be test crossed with the WS-2 line to identify the intragenic suppressors. Progeny of a cross between an intra-genic suppressors and the wild-type parent will display a wild-type phenotype. On the other hand, the progeny from a cross between an extra-genic suppressors and the wild-type parent will display a semi-dwarf seedling phenotype. The future goal of the research described in Chapter Three is to
isolate and characterize a true loss-of-function mutation in the \textit{BEN1} gene, which can be used for creation of multiple mutants lacking two or more BR catabolic genes. The isolation of true loss-of-function mutant of \textit{BEN1} will also aid in examining its full role in other biological pathways.

Another important future direction will be to isolate transcription factors involved in the regulation of BR catabolic genes. Using a yeast-one-hybrid approach Dr. Hao Peng has isolated a transcription factor ATAF2, which binds to promoters of both \textit{BAS1} and \textit{SOB7}. Molecular and genetic analysis shows that ATAF2 is involved in the regulation of \textit{BAS1} and \textit{SOB7} expression in \textit{Arabidopsis}. Similar approaches can be used to isolate and characterize transcription factors, which regulate expression of other BR catabolic genes. \textit{BEN1} will be a high priority for yeast-one-hybrid analysis. The \textit{ben1-1} single-mutant has elevated BR levels, which indicates its importance in maintaining BR homeostasis in \textit{Arabidopsis} (Yuan \textit{et al.} 2007). \textit{BAS1} gene expression is up-regulated by BL application (Bancos \textit{et al.} 2002). However, \textit{BAS1} expression is not up-regulated in the \textit{ben1-1} background, which has elevated BR levels (Chapter Three, Figure 4C). This suggests that these two genes have distinct spatio-temporal expression patterns. Therefore, these two genes are likely to be part of different biological pathways.

This study also contributes to the further characterization of the \textit{ATST4a}. \textit{In vitro} biochemical analyses demonstrate that \textit{ATST4a} has catalytic activity with BRs (Masolais \textit{et al.} 2007). However, unlike other BR catabolic pathways, the genetic evidence to suggest such a function for \textit{ATST4a} pathway \textit{in planta} is scant. In this study, we have used both loss-of-function and over-expression genetic approaches to further explore the role of \textit{ATST4a} in \textit{Arabidopsis}. This study shows that \textit{atst4a1-1}, a T-DNA insertion null mutant of \textit{ATST4a} did not display reduced seedling hypocotyl growth inhibition in white light. Additionally, overexpression of the \textit{ATST4a} gene in transgenic lines did not result in any characteristic BR-deficient phenotypes.
Overexpression of ATST4a did not cause a predicted feed-back-regulation change in the transcript accumulation of DWF4 and BAS1. These observations suggest that ATST4a is an atypical BR catabolic gene. Given these observations, more studies are required to assign as in planta biological function to ATST4a. The detailed analysis of atst4a1-l mutant under various growth conditions might give a hint to its functions. Previous reports on steroid sulfotransferase suggest their role in environmental stress responses (Marsolais et al. 2004; Marsolais et al. 2007).

Understanding BR inactivation will impact many fields of basic sciences including hormone biology, role of redundant biological pathways, and evolution of biological pathways. Detailed understanding of BR inactivation and its role in plant growth and development will also enable crop scientists to use this growth-promoting hormone as a plant biotechnology tool.

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