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MOLECULAR CLONING AND CHARACTERIZATION OF TWO GENETIC MODIFIERS OF AN INTERMEDIATE *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) MUTANT

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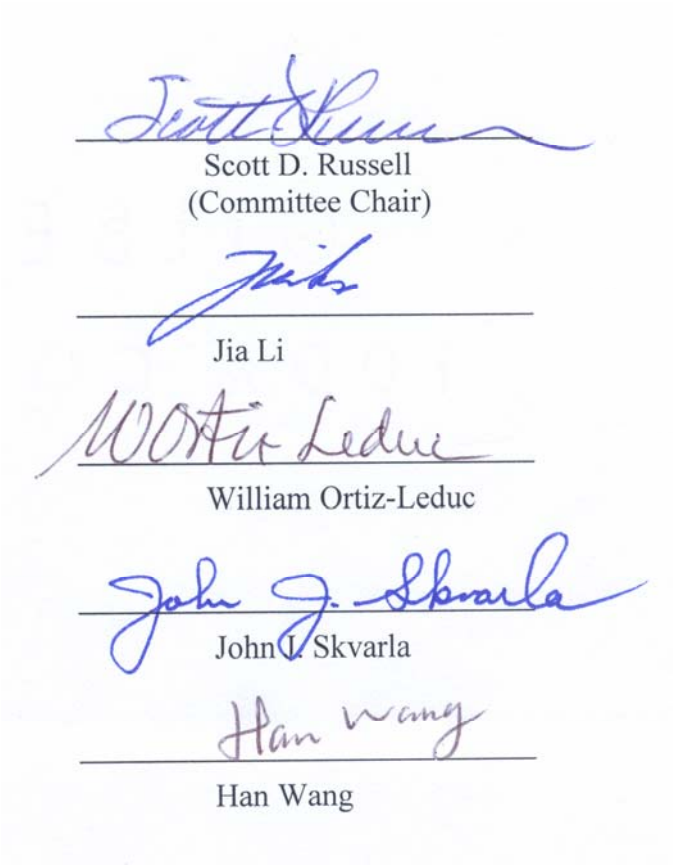
MOLECULAR CLONING AND CHARACTERIZATION OF TWO GENETIC MODIFIERS OF AN INTERMEDIATE *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) MUTANT

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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This dissertation is dedicated to my parents,

Wensui Yuan and Jinmei Cui

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LIST OF ABBREVIATIONS

aa: amino acid

BAK1: BRI1 ASSOCIATED RECEPTOR KINASE 1

BAN: anthocyanidin reductase

bas1-D: *phyB activation-tagged suppressor 1-dominant*

BES1: BRI1-EMS-SUPPRESSOR1

BKI1: BRI1 KINASE INHIBITOR1

BKK1; BAK1-LIKE 1

ben1-1D: *bri1-5 enhanced 1- 1dominant*

ben2-1D: *bri1-5 e*nhanced *2-1d*ominant

ben2-2D: *bri1-5 e*nhanced *2-2d*ominant

ben3-1D: *bri1-5 enhanced 3- 1dominant*

bin2: *brassinosteroid insensitive2*

BL: brassinolide

BR: brassinosteroid

BR6ox1: *BR-6-oxidase1*

bri1: *brassinosteroid insensitive 1*

BRL1: BRI1-LIKE 1

BRS1: *bri1 SUPPRESSOR1*

BRZ: brassinazole

bZIP: basic domain-leucine zipper proteins

BZR1: BRASSINAZOLE-RESISTANT1

BSU1: *bri1* SUPPRESSOR1

CaMV 35S: Cauliflower mosaic virus 35S

CHI2: *CHIBI2*

CO: *CONSTANS*

CPD: CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM

CS: castasterone

CT: cathasterone

DAG1: *Dof Affecting Germination*

*det*2: *de-etiolated*2

DFR: dihydroflavonol 4-reductase

Dof: DNA one finger binding

DWF4: *DWARF4*

EMS: ethylmethanesulfonate

FLC: *FLOWERING LOCUS C*

GA: gibberellin

GC-MS: gas chromatography-mass spectrometry

GFP: green fluorescent protein

GSK3: glycogen synthase kinase-3-like protein

GUS: β-glucuronidase

LRR: leucine-rich repeat

mRNA: messenger RNA

MRR: methionine-rich repeat

ORF: open reading frame

26-OHBL: 26-hydroxybrassinolide

PCR: polymerase chain reaction

RLK: receptor-like kinase

ROT3: *ROTUNDFOLIA3*

RT: reverse transcription

tRNA: transfer RNA

SEM: scanning electron microscopy

SOC: *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*

TAIL-PCR: thermal asymmetry interlaced-PCR

TE: teasterone

TRIP1: TGF-β receptor interacting protein

TTL: TRANSTHYRETIN-LIKE

TY: typhasterol

SHK1: *SHRINK1-D*

SOB7: *SUPPRESSOR* of *phyB-4 7*

UGT: UDP-glycosyltransferase enzyme

Chapter I. History of brassinosteroid research

1. Brassinosteroids: Discovery, Activity, Biosynthesis, and Signal Transduction

1.1. The discovery of BRs

In the 1930's to 1940's, USDA researchers discovered that pollen extracts can promote plant growth and first reported that the application of maize pollen extracts to the internodes of young bean seedlings could greatly stimulate their elongation (Mitchell and Whitehead, 1941). At that time, most people attributed this effect to gibberellins (GAs), but additional research cast doubt on this conclusion. In the 1960's, scientists, after comparing pollen extracts from 60 different plant species, found that rape (*Brassica napus* L.) pollen extracts produced the most response on bean second internode elongation. Unlike the effect of GAs, the pollen extracts not only can promote cell elongation, they also can have additional effects such as causing the treated internode to swell and curve. The compounds from rape pollen were proposed to be a new group of lipidic plant hormones and were named *brassins* (Mitchell et al., 1970). In 1972, Mitchell and Gregory showed that the application of brassins was able to increase plant growth and crop productivity (Mitchell and Gregory, 1972). After that, the functions of brassins on plant growth and productivity in different crops, such as wheat, corn, soybean, potato and other plant species, were investigated; brassins were able to increase the yield of all these crops (Mandava, 1988). In 1975, USDA decided to identify the chemical structure(s) of the active compound(s) in brassins. To achieve this goal, about 250 kilograms of bee-collected rape pollen were extracted with isopropanol (Mandava et al., 1978). The extracts were purified using a series of silica, chromatography and high performance

liquid chromatography columns. Ultimately, 4 milligrams of a pure crystalline substance were obtained, and it was designated as brassinolide (BL). The chemical structure of BL was determined based on spectroscopic methods including X-ray analysis. The full chemical name of brassinolide is (22R, 23R, 24S)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one (Grove et al., 1979; Figure 1).

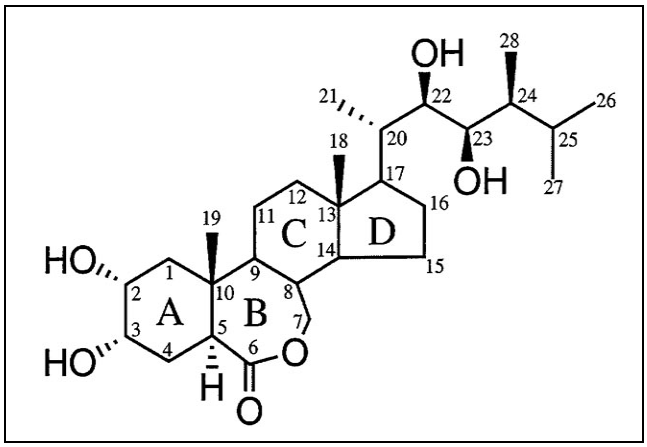

Figure 1. Brassinolide (BL) chemical structure. The capital letters indicate the four typical rings in BL and the numbers represent the positions of carbons.

In the early 1980's, Japanese scientists isolated the second bioactive plant steroid, called castasterone (CS), from chestnut gall tissues (Yokota et al., 1982). CS was soon identified as an immediate precursor of BL and has biological activity similar to BL (Ikekawa et al., 1984; Fujioka and Yokota, 2003). Since then, additional steroid compounds, such as dolicholide, 28-homodolicholide, dolichosterone, 28-homodolichosterone and typhasterol, have been extracted from different plant species. To

date, more than sixty phytosteroids have been identified and are collectively called brassinosteroids (BRs). Among them, BL is the most active and the final product of the BR biosynthesis pathway (Fujioka and Yokota, 2003).

In the 1980's, studies of BR were mainly focused on defining their physiological functions and seeking their practical application in crop improvement. Although BRs proved to be important in the regulation of plant growth and development, they were not demonstrated to be essential in mediating plant growth and development and, therefore, BRs were not widely accepted as new class of plant hormones (Kende and Zeevaart, 1997). In the middle of the 1990's, investigators turned their attention to the biosynthetic pathway of BL (Fujioka and Sakurai, 1997). At the same time, the essential role of BRs on plant growth was demonstrated by the characterization of a number of BR biosynthetic and signal transduction mutants (Clouse et al., 1996; Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996; Li and Chory, 1997). The phenotypes of the BR-deficient mutants indicated that BRs were indeed essential hormones, a view that is widely accepted since then.

1.2. Biological activity of BRs

BRs are essential for plant growth and development (Figure 2). The biological activity of BRs was initially determined by two bioassays: the bean second internode bioassay and the rice lamina inclination test (Grove et al., 1979; Thompson et al., 1981; Thompson et al., 1982; Wade et al., 1984; Mandava, 1988). The test of rice lamina inclination is considered as a specific assay for BRs, although it was a test originally developed for

auxins (Maeda, 1965). The detection limit for auxins was 50 ppm, whereas the limit for BL was 0.1 to 0.5 ppb (Wada et al., 1981; Takeno and Pharis, 1982; Wada et al., 1984). When brassins were first found, their functions were considered as promoting plant growth (Mitchell et al., 1970). Since then, the activity of BRs has been detected under laboratory, greenhouse, and field conditions. The results of these investigations have been summarized in several reviews and books (Maugh et al., 1981; Fujita, 1985; Mandava, 1988; Takeuchi, 1992; Clouse and Sasse, 1998; Kamuro and Takatsuto, 1999; Khripach et al., 1999; Sakurai, 1999; Sasse, 1999; Khripach et al., 2000). In this dissertation, some of the research results, including the physiological properties of BL and their applications in agriculture, are presented.

Figure 2. Phenotypes of several representative BR response and biosynthetic mutants. Both signal transduction mutants (*bri1-5, bri1-9, bri1-8, bri1-1,* and *bri1-4*) and biosynthetic mutant (*det2*) show pleiotropic growth and developmental defects. All the mutants shown are in the ecotype WS2 background.

1.2.1. Agricultural benefits

In the early 1980's, scientists in the USA, Japan, China, and Russia proved the economic value of BL (Maugh, 1981; Fujita, 1985; Takeuchi, 1992; Kamuro and Takatsuto, 1999; Khripach et al., 2000). In the next two decades, numerous experiments on applications of BLs as agricultural chemicals have been carried out all over the world. BL can affect many aspects of plant development, including germination, maturation, stress tolerance, senescence, and biological yield.

1.2.1.1. Increasing crop yield

In bean crops, after using BL, the weight of seeds per plant increased by 41%, and leaf weight showed an increase of 51% (Meudt et al., 1983). By treating rice plantlets with a 5 ppm solution of BL, an increase of 22% in fresh weight and 31.5% in dry weight of seeds per plant was reported (Lim, 1987). In corn, ear fresh weight increased by 7% and seed dry weight increased by 11% to 14% after the treatment of BRs (Lim and Han, 1988). The application of 24-epiBL on wheat increased panicle weight by 25-33% and seed weight by 4-37% (Takeuchi, 1992). From 1986 to 1988, BL treatment experiments at the Instituto Agronomico (*Campinas, SP, Brasil*) showed crop yields of wheat increased by up to 18%; the yields of soybean increased by up to 22%; and yields of bean increased by up to 83% (Zullo and Adam, 2002).

Applications of BL not only increased crop yield, but also increased the quality of crops (Prusakova et al., 1999). For example, BL added to potato plants in a dose of 10±20

mg ha^{-1} resulted in a 20% increase in productivity along with a better quality of crop, as indicated by reduced nitrate content and increased in starch and vitamin C content (Khripach et al., 1996).

1.2.1.2. Increasing stress tolerance

As a new class of phytohormones, BRs have been found to increase the ability of plants to deal effectively with different environmental stresses (Kamuro and Takatsuto, 1999; reviewed in Krishna, 2003). Hence, the anti-stress activity of BRs suggests another important role in agriculture, particularly in adverse conditions.

-- Chilling tolerance and thermotolerance

Temperature stress, one of the main stress factors occurring in nature, can be remedied by treating plants with BRs. For example, both maize and cucumber are chilling-sensitive species, but their seedling can grow normally with the treatment of BL at low temperatures (He et al., 1991; Katsumi 1991; Yu et al., 2002; Yu et al., 2004). 24-epiBL can strongly protect spring rape (*Brassica napus* L. cv. Lycosmos) from a 2 °C cold treatment (Janeczko et al., 2007). BR-treatment can significantly reduce ion leakage; retain chlorophyll and carotenoids content, and alleviate chill-induced inhibition of photosynthesis in chilled leaves (Yu et al., 2004; Janeczko et al., 2007). Application of BRs could also improve plants tolerance to heat stress. When grown under lethal heat treatment conditions, *Brassica napus* and tomato seedlings are significantly tolerant to high-temperature stress in the presence of BRs. Expression of heat shock proteins was higher in BR-treated seedlings than in untreated seedlings, and cell damage was

significantly reduced in the BR-treated seedlings (Dhaubhadel et al., 1999) This process is associated with the induction of enzymatic antioxidants and the protection of the photosynthetic apparatus (Mazorra et al., 2002; Ogweno et al., 2007). BL treatment also helps maintain protein synthesis in heat stress (Kulaeva et al., 1991).

--Salt injuries and heavy metal stress

Studies on the seed germination of rice and *Eucalyptus camaldulensis* in the presence of 150 mM salt indicate that seed germination is normally inhibited, but with the addition of BL, seed germination was found to be enhanced (Sasse, 1999; Anuradha and Rao 2001; Özdemir et al., 2004). BL treatment was also shown to protect the cell ultrastructure of barley leaves with significantly reduced damage to nuclei and chloroplasts induced by salt stress (Kulaeva et al., 1991).

Along with the industrial development and utilization of fungicides, fertilizers and pesticides, heavy metals have become increasingly hazardous in pollutants of agricultural soils. The application of BRs at a certain stage of crop development can regulate the uptake of ions into the plant cells and reduce the accumulation of heavy metals and radioactive elements in plants (Bajguz, 2000; Janeczko et al., 2005; Sharma and Bhardwaj, 2007). For instance, 24-epiBL can significantly block heavy metal (copper, lead, cadmium, zinc) absorption in *Chlorella vulgaris***,** barley, tomatoes, and sugarbeet (Volynets et al., 1997; Bajguz, 2000). In addition, 24-epiBL can reduce the toxic effect of copper and cadmium by maintaining efficient photosynthetic electron transport and photochemical processes in cucumber, winter rape, and *Brassica juncea* (Burzyński and Żurek, 2007; Sharma and Bhardwaj, 2007). As a result, the treatment of 24-epiBL can improve plant biomass production and quality under heavy metal stress.

--Water stress

BL treatment increased acid invertase activity in the young leaves of sugar beet plants and led to the restoration of normal taproot biomass in drought stress (Schilling et al., 1991). When BL was applied to drought-tolerant and non-drought-resistant wheat varieties, both showed increased growth under stress conditions (Sairam, 1994). Cucumber plants also displayed improved drought tolerance with the addition of BL (Pustovoitova et al., 2001). In leguminous crops, BRs have the ability to increase root nodulation, improve endogenous abscisic acid and cytokinin contents, maintain nitrogenase activity and enhance pod yield under water stress (Upreti and Murti, 2004).

--Pathogen infection

BR-induced disease resistance has been studied extensively for many years (reviewed in Clouse and Sasse, 1998; Khripach et al., 1999; Khripach et al., 2000; Krishna 2003). Results indicated that BL could enhance plant resistance against different pathogen infections. For example, potato plants sprayed with BR solution were shown to resist infection by *Phytophthora infestans*. This BR-induced resistance was associated with increases in abscisic acid and ethylene levels in BR-treated potato tubers (Krishna, 2003). In cucumber plants, BL treatments increased the activity of peroxidases and polyphenoloxidases associated with increased disease resistance. Tomato seedling roots grown for 14 days in the presence of BL were inoculated with *Verticillium dahliae*. After inoculation, BR-treated plants showed no disease or exhibited symptoms with the lowest disease scores, whereas the untreated plants showed moderate to severe symptoms (Krishna, 2003). In addition, BL also showed increases in antiviral activity against the measles virus, herpes simplex virus type 1 and arena virus replication in cell cultures

(Wachsman et al., 2000; Wachsman et al., 2002). Furthermore, treatments with BL could induce resistance against tobacco mosaic virus, the bacterial pathogen *Pseudomonas syringae*, and the fungal pathogen *Oidium* in tobacco plants. In rice, BL application improved resistance to rice blast and bacterial blight diseases caused by *Magnaporthe grisea* and *Xanthomonas oryzae*, respectively (Nakashita et al., 2003).

1.2.1.3. Insect control

In studies of the bioactivity of BRs and their analogues in insects, striking structural similarities were found between BRs and insect arthropod hormones (Richter and Koolman, 1991); it was also found that their biosynthetic pathway are highly conserved (Dinan et al., 2001). In addition, BRs had inhibiting and antiecdysone effects in insects (Hetru et al., 1986; Lehmann et al., 1988; Richter and Koolman, 1991; Khripach et al., 2000; Thummel and Chory, 2002; Zullo and Adam, 2002). For example, the first antiecdysones, 22, 23-diepi-28-homocastasterone, could bind competitively to ecdysteroid receptors from larvae of the *Calliphora vicina* blowfly (Lehmann et al., 1988) and produce morphological effects and inhibit chitin synthesis similar to the molting hormones (Voigt et al., 2001; Luc Decombel, 2005). New strategies to influence ecdysteroid-dependent stages of insect development could lead to new methods for insect pest control. In the future, BR has the potential to be used as a substitute for some traditional pesticides (Khripach et al., 2000; Thummel and Chory, 2002).

1.2.2. Physiological effects

In addition to their benefits in agriculture, BRs play an essential role in plant development, including cell elongation, cell division, vascular development and reproduction (Clouse and Sasse, 1998; Clouse, 2002; Müssig, 2005). Investigations on the physiological roles of BRs were mainly carried out using exogenous applications of BL to plants and the use of BR biosynthetic and signaling mutants. Exogenous BL applications not only showed that BL could stimulate cell elongation, cell division and differentiation (Mandava, 1988; Clouse and Zurek, 1991; Fukuda, 1997), but also showed promotion of senescence and flowering (Clouse and Sasse, 1998). The effects of exogenous BL could be confirmed by examining the phenotype of BR-deficient mutants, such as *bri1, bin2* (*Arabidopsis*; Clouse et al., 1996; Li et al., 1996), *lka* (pea; Nomura et al. 1999) and *curl* (tomato; Bishop et al. 1999).

The above results summarize some of the biological effects of BRs. Many other functions of BRs can be found in a number of reviews (Maugh, 1981; Fujita, 1985; Mandava, 1988; Clouse and Zurek, 1991; Takeuchi, 1992; Clouse, 1996; Kauschmann et al., 1996; Li and Chory, 1997; Noguchi et al., 1999; Prusakova et al., 1999; Friedrichsen et al., 2000; Khripach et al., 2000) and are summarized in Table 1.

1.3. Biosynthesis of BRs

Following the characterization of the physiological effects of BR in the 1980's, an historical turning point in BR research occurred from 1991 to 1996. In this period, the major BR biosynthetic pathways were determined, mainly by Japanese scientists, and

Table 1. Biological activity of brassinosteroids in plants (According to Clouse and Sasse, 1998; 2002; Khripach et al.,2000)	
Cellular level	Entire plant level
Promotion of cell expansion, cell division, and elongation	Stimulate young plants growth
Effect on hormonal balance	Promote fertilization, accelerate senescence and flowering
Effect on enzyme activity, enhance the photosynthetic capacity and translocation of products	Increase the yield of crop and fruits
Activation of protein and nucleic acid synthesis. Effect on the protein spectrum and on the amino acid composition of proteins	Improve the content of nutritive components of crops and fruit
Effect on H^+-pump activation and on the properties of membranes	Modulation of biotic and abiotic stress responses
Effect on the fatty acid composition	Increase resistance to unfavorable environmental factors; improve fruit quality
Regulation of vascular development	Promote plant development
Regulation of gene expression, initiate light-regulated development	Effects on skotomorphogenesis and photomorphogenesis

BR-regulated genes were found by several groups (Clouse et al., 1996; Kauschmann, 1996; Li et al., 1996; Szekeres et al., 1996; Fujioka and Sakurai, 1997). The elucidation of the BR biosynthetic pathway was a key step in understanding how the regulation of endogenous BR levels could promote plant development. The first system used for studying BR biosynthesis involved feeding cell suspension cultures of *Catharantus roseus* with deuterium labeled precursors of BL and then analyzing labeled metabolites using gas chromatography-mass spectrometry (GC-MS; Fujioka et al., 2000). BR biosynthetic pathways were also confirmed using BR biosynthesis deficient mutants in *Arabidopsis thaliana*, *Pisum sativum* and *Lycopersicon esculentum* (Clouse and Sasse, 1998; Clouse and Feldmann, 1999).

1.3.1. Biosynthetic pathway of the plant sterol precursor (acetyl-CoA→ cycloartenol)

A commonly accepted route for the biosynthesis of precursor cycloartenol is described in Figure 3 (Asami and Yoshida, 1999; Clouse, 2002; Zullo and Adam, 2002; and PATHWAY at http://www.arabidopsis.org/). Plant sterols are synthesized by the mevalonic acid pathway via acetyl-CoA, mevalonate, isopentenyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate. Squalene is produced by condensation of two farnesyl pyrophosphate molecules and then converted to squalene-2,3-epoxide (Figure 3). The step from squalene-2,3-epoxide to cycloartenol is unique to plants (Clouse, 2002).

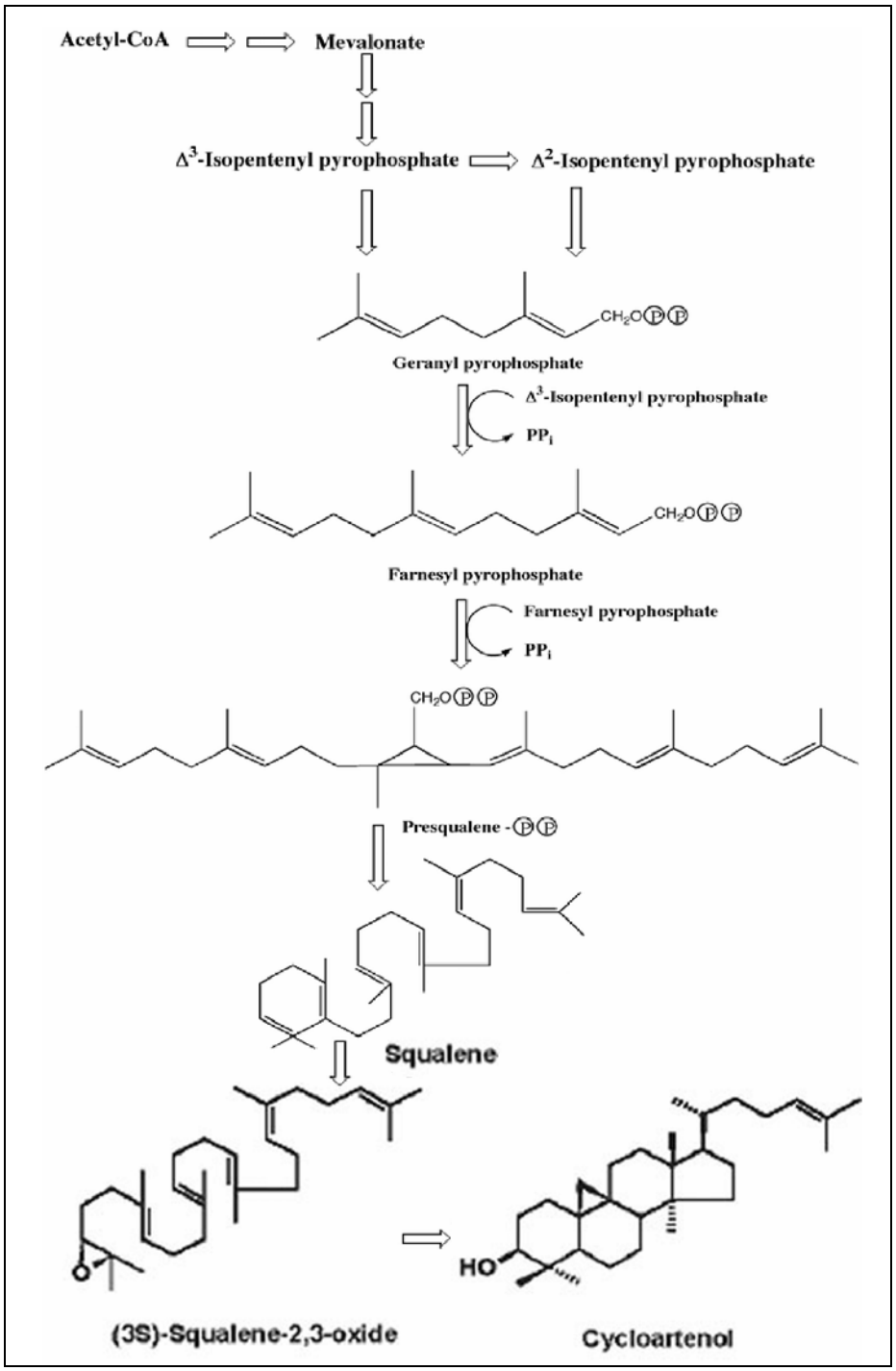

Figure 3. Biosynthetic pathway of the plant sterol precursors: from mevalonate to cycloartenol, according to Clouse (2002), Zullo and Adam (2002) and PATHWAY (http://www.arabidopsis.org/). (Modified from Clouse, 2002; Zullo and Adam, 2002).

1.3.2. General sterol biosythesis pathway (cycloartenol → campesterol)

The steps from cycloartenol to campesterol are considered to be a general sterol biosynthesis pathway (Figure 4). In this pathway, cycloartenol is converted to 24-methylenecycloartenol and demethylated to cycloeucalenol, which is isomerized to obtusifoliol. Demethylated of obtusifoliol forms 4-α-methyl-5-α-ergosta-8,14,24(28)-trien-3-β-ol, which is reduced to 4α-methylfecosterol. Isomerization of the $\Delta^{8(9)}$ double bond to $\Delta^{7(8)}$ generates 24-methylenelophenol, which then forms episterol through demethylation. Episterol is dehydrogenated to 5-dehydroepisterol and hydrogenated to 24-methylenecholesterol by a Δ^{7}-sterol reductase. 24-methylenecholesterol is converted to 24-methyldesmosterol through the isomerization of the $\Delta^{24(28)}$ double bond to $\Delta^{24(25)}$. Finally, 24-methyldesmosterol is reduced to campesterol, a common precursor of plant BL. Several mutants have been isolated in this pathway and labeled in Figure 4. These sterol mutants are classed into two groups (Fujioka and Yokota, 2003). One group, including *SMT1*, *FACKEL*, *HYD1* and *SMT2* mutants, has a lesion in the early steps of sterol biosynthesis. The adult plants show unique defects during embryogenesis that cannot be rescued by BR application; the second group, such as *dwf7*, *dwf5* and *dwf1,* has a lesion in the late steps of the biosynthetic pathway. The phenotypes of these mutants can be rescued by BR application. For example, the *fackel* has a distorted embryo, supernumerary cotyledons, multiple shoot meristems, and stunted roots. Analysis of *fackel* mutants demonstrated that the *FACKEL* gene encodes a sterol C-14 reductase (Jang et al., 2000; Schrick et al., 2000). The BR contents were reduced in the *fackel* mutants, but the 4-α-methyl-5-α-ergosta-8,14,24(28)-trien-3-β-ol accumulated at high

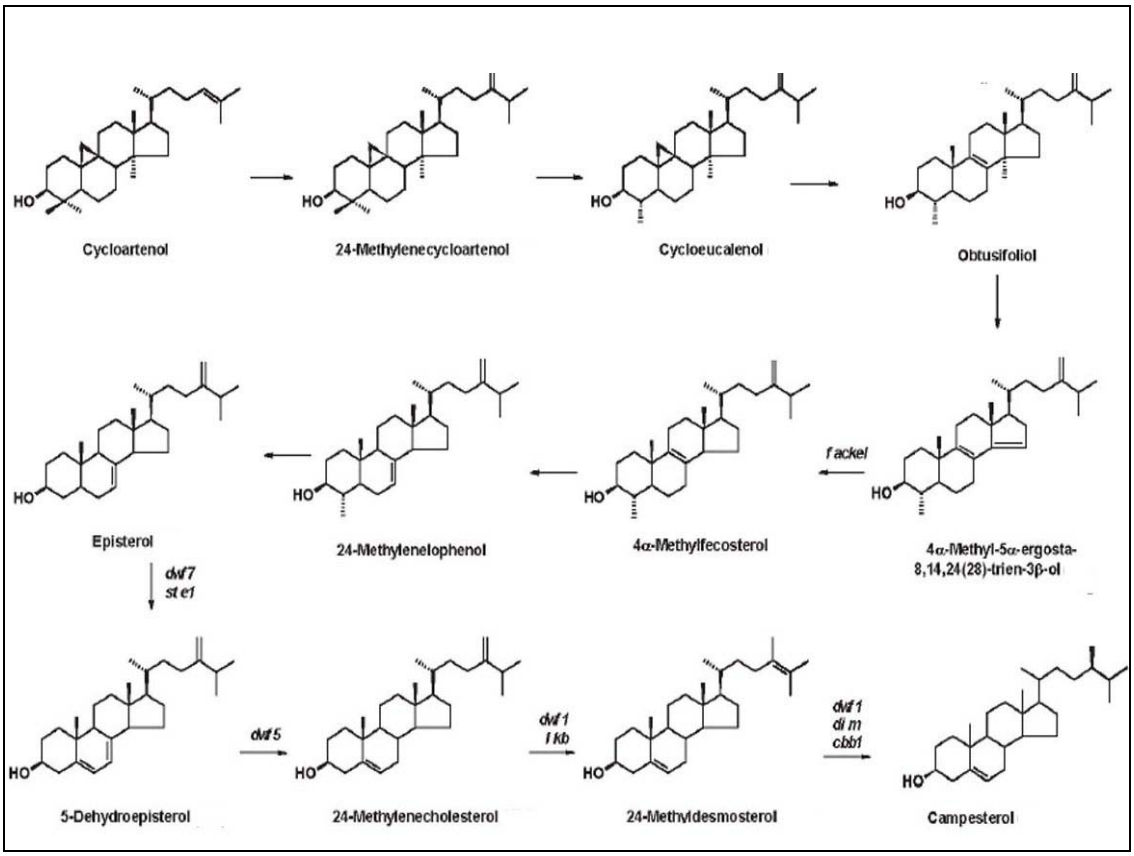

Figure 4. General sterol biosynthesis pathway: from cycloartenol to campesterol, according to Zullo and Adam (2002) and PATHWAY (http://www.arabidopsis.org/). (Modified from Clouse, 2002; Zullo and Adam, 2002).

level, indicating a blockage in the conversion of 4-α-methyl-5-α-ergosta-8,14,24(28)-trien-3-β-ol to 4-α-ethylfecosterol (Jang et al., 2000; Schrick et al., 2000). Two *Arabidopsis* mutants, *dwf7* and *ste1*, were identified because they are unable to convert of episterol to 5-dehydroepisterol (Choe et al., 1999; Catterou et al., 2001); and *dwf5* was blocked in the conversion of 5-dehydroepisterol to 24-methylenecholesterol (Choe et al., 2000). The *Arabidopsis dwf1* mutant was defective in the conversion of the last compound to campesterol (Feldmann et al., 1989; Klahre et al., 1998). Analysis of mutants suggested that sterols alone could play critical roles during plant development, such as cell division and expansion in embryogenesis, in addition to their roles as BR precursors.

1.3.3. BR- specific biosynthesis pathway

The BR-specific biosynthesis pathway is limited to the pathway from campesterol to BL. There are three key steps involved in this process. 1) The pathway begins with the conversion of campesterol to campestanol (Figure 5); 2) This is followed by the conversion of campestanol to castasterone (CS) via the parallel branched BL pathways, namely the early and late C-6 oxidation pathways (Figure 6 and Figure 7); 3) Finally, CS is converted to BL. The first step, the conversion of campesterol to campestanol, makes use of a series of reductions, hydroxylations, epimerizations and oxidations (Figure 5). Oxidation of campesterol to (24R)-24-methyl-4-cholesten-3-one is followed by saturation of the olefinic double bond to (24R)-methyl-5-α-cholestan-3-one and reduction of the carbonyl group to form campestanol.

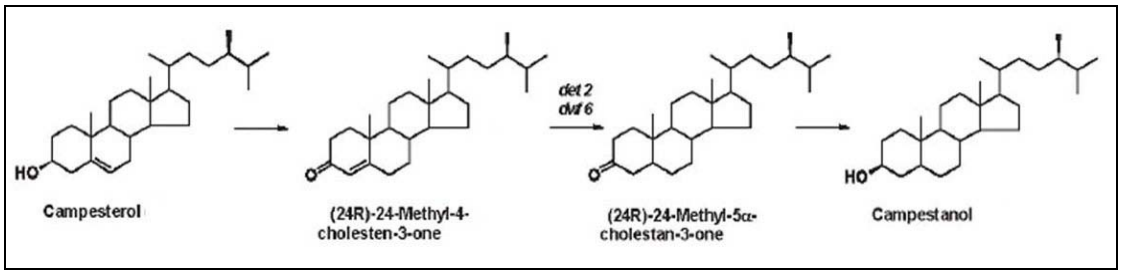

Figure 5. The BR-specific biosynthesis pathway: from campesterol to campestanol according to Zullo and Adam (2002) and PATHWAY (http://www.arabidopsis.org/). (Modified from Zullo and Adam, 2002).

In the early C-6 pathway, campestanol is oxidized to 6-β-hydroxycampestanol and then to 6-oxocampestanol (Suzuki et al., 1995a). The conversion of 6-oxocampestanol to cathasterone was elucidated using feeding experiments in cultured *C. roseus* cells (Fujioka et al., 2000). Parallel experiments showed that cathasterone is the biosynthetic precursor of typhasterol and teasterone (Fujioka et al., 1995). The oxidation of typhasterol to CS and then to BL was fully demonstrated by mutant studies with periwinkle, tobacco and tomato (Suzuki et al., 1994; Suzuki et al., 1995a; Figure 6).

Evidence for the existence of the late C-6 oxidation pathway came to light with the observation that 6-deoxoBRs and BRs are present in pairs in many plant species. At the beginning, 6-deoxoBRs were regarded as inactivation products of 6-oxoBRs. However, as more 6-deoxoBRs were detected in plants, scientists hypothesized the 6-deoxoBRs could also be biosynthetic precursors of CS. Using labeled compounds in cultured periwinkle, tobacco, and rice cells, the conversion of 6-deoxocastasterone to castasterone was found (Choi et al., 1996; Choi et al., 1997; Figure 7). The whole pathway includes the conversion of 6-deoxoteasterone to 3-dehydro-6-deoxoteasterone,

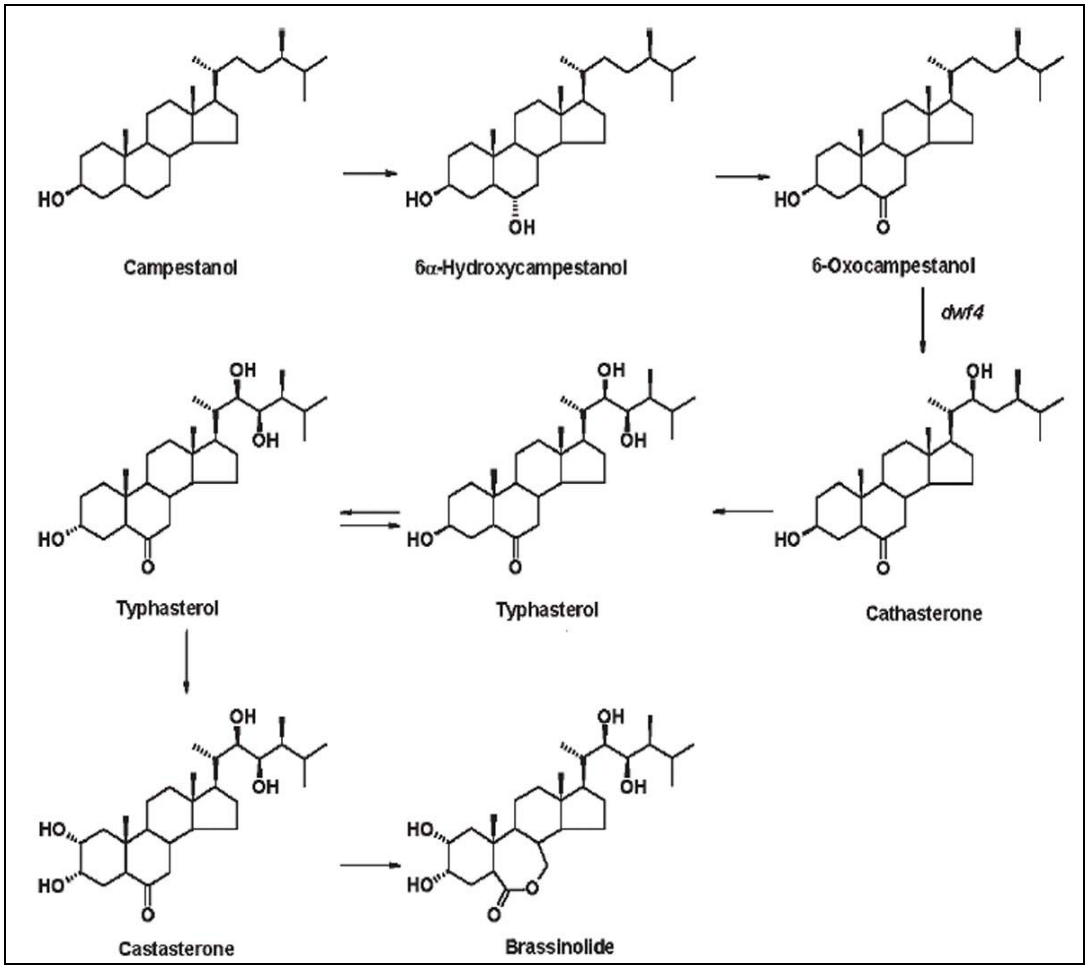

Figure 6. Biosynthesis of brassinolide via the early C-6 oxidation pathway according to Zullo and Adam (2002) and PATHWAY (http://www.arabidopsis.org/). (Modified from Zullo and Adam, 2002).

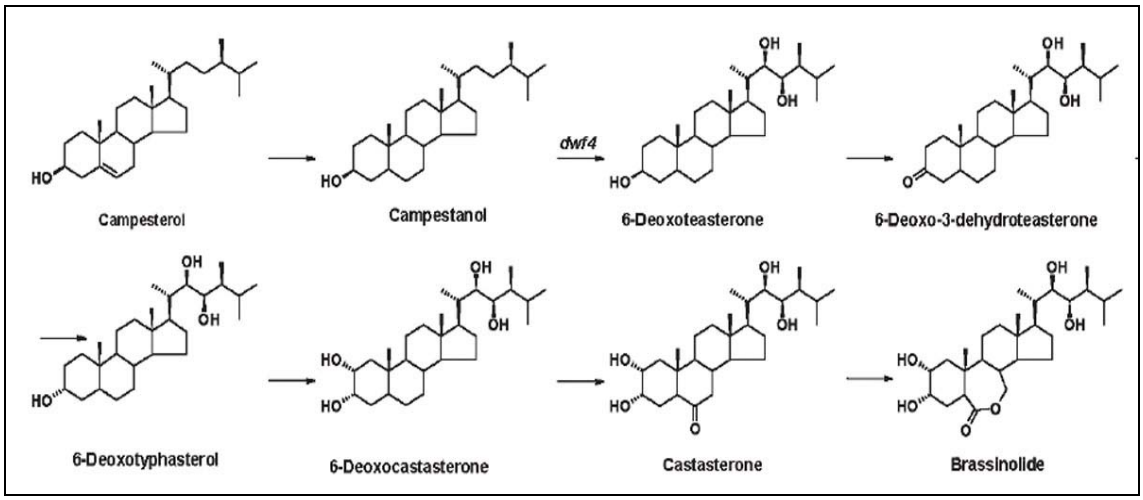

Figure 7. Biosynthesis of brassinolide via the late C-6 oxidation pathway according to Zullo and Adam (2002) and PATHWAY (http://www.arabidopsis.org/). (Modified from Zullo and Adam, 2002).

6-deoxotyphasterol, 6- deoxocastasterone and finally to CS. Recent studies revealed that there exists crosstalk between early and late C-6 oxidation parallel pathways (Fujioka and Yokota, 2003).

The identification of a variety of BR biosynthetic mutants is a powerful means for investigating the BR biosynthesis pathway and for discerning potential alternative steps. For example, the first step in BR-specific biosynthesis pathway, the conversion of campesterol to campestanol, was initially considered as a single reaction (Suzuki et al., 1995b). However, when this pathway was studied using the mutant *de-etiolated*2 (*det*2), it was shown that this conversion consists of several reactions. *DET*2 encodes a 5-α-steroid reductase, which catalyzes the 5-α-reduction of (24*R*)-ergost-4-en-3-one to (24*R*)-5-α-ergostan-3-one (Noguchi et al., 1999). DWARF4, a C-22 steroid hydroxylase, catalyzes both the conversion of campestanol to 6-deoxocathasterone in the late C-6 oxidation pathway and of 6-oxocampestanol to cathasterone in the early C-6 oxidation pathway (Choe et al., 1998). The *dwf*4 mutant resembles other BR-deficient mutants, displaying dwarfism, dark-green round leaves, and male sterility. However, feeding experiments demonstrated that only 22-α-hydroxylated intermediates (and later intermediates) could rescue *dwf*4 to wild type. The full recovery of mutant phenotypes provides evidence that DWF4 functions on C-22 steroid hydroxylation (Choe et al., 1998). In the conversion of 6-deoxocathasterone to 6-deoxoteasterone (late C-6 oxidation) and of cathasterone to teasterone (early C-6 oxidation), the CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) is involved the hydroxylation of the 23-C side chain in both branches of the pathway. The *cpd* mutant exhibits an extreme

dwarf phenotype that could only be rescued by 23α-hydroxylated BRs, indicating that CPD acts as a C-23 steroid hydroxylase (Szekeres et al., 1996).

1.4. Metabolism of BRs

BRs can promote plant development, but the growth-promoting activations depend on optimal endogenous levels. Like other phytohormones, either deficient or excess amounts of endogenous BRs are harmful to normal growth and development. BRs also have some unique features when compared with other plant hormones. For example, BRs are not mobile from one tissue to another through long-distant transport mechanisms (Symons and Reid, 2004), and each tissue of a plant must synthesize and metabolize its own BRs to maintain adequate BR levels.

Feeding experiments showed that exogenously applied excess amounts of BRs are rapidly metabolized suggesting that each biosynthetic intermediate could be inactivated or degraded (Yokota, 1997). So far, more than forty BR metabolites have been identified from various plant species (Fujioka and Yokota, 2003), but only several metabolic processes have been well characterized.

1.4.1. Inactivation of teasterone (TE)

The most common deactivation process for TE is rapid conjugation. TE can conjugate with fatty acids, glucose, disaccharide, 6-*O*-β-glucosylglucose or 4-*O*-β-glucosylglucose (Kolbe et al., 1998; Soeno et al., 2000). In fact, conjugation of TE is an important way to

control the endogenous BR levels in lily pollen. During lily pollen maturation, TE formed fatty acid esters such as TE-3-laurate and TE-3-myristate, which are regarded as the storage forms of BL (Asakawa et al., 1996). Other processes, such as β-epimerization and hydroxylation, also occur during the metabolism of TE (Abe et al., 1994; Kolbe et al., 1998; Fujioka and Yokota, 2003). For example, 3β-OH -24- epiTE converted to 24-epiTE and 24-epityphasterol in cell suspension cultures of tomato (Kolbe et al., 1998). Some processes of TE metabolism are shown in figure 8.

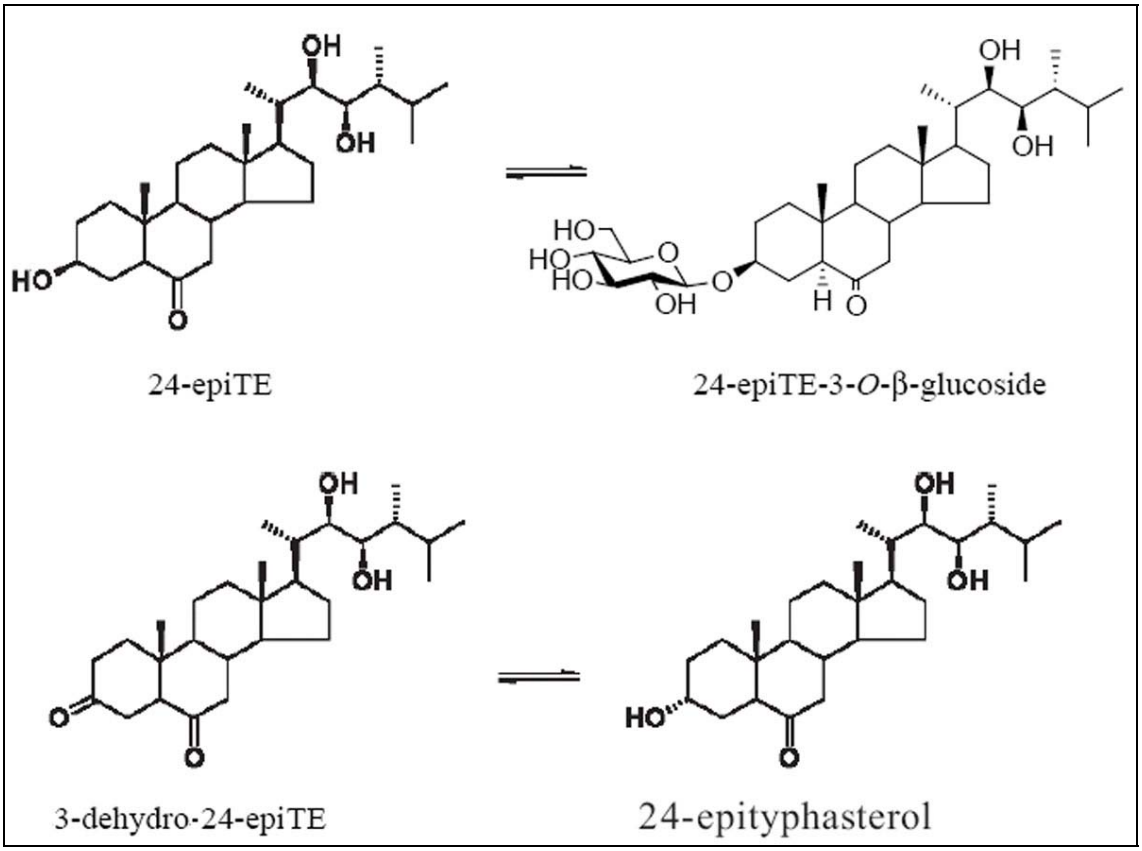

Figure 8. Metabolism of 24-epiteasterone (24-epiTE). (Modified from Fujioka and Yokota, 2003; Zullo and Adam 2002).

1.4.2. Inactivation of cathasterone (CT)

Mammalian sulfotransferase (EC 2.8.2) are widely involved in the metabolism of steroid hormones. The first sulfonation identified during BR metabolism was the inactivation of 24-epicathasterone. A purified *Brassica napus* sulfotransferase catalyses the 24-epiCT to 24-epiCT-22-sulfate at position 22 (Rouleau et al., 1999, Figure 9). Recently, the *AtST1* gene responsible for C-22 sulfonation of 24-apiCT was characterized (Marsolais et al., 2007). Besides sulfonation, β-epimerization has also been found in the metabolism of 3-epi-6-deoxoCT and 3-epi-6-deoxoCT in *Arabidopsis* and *C. roseus* (Fujioka et al., 2002).

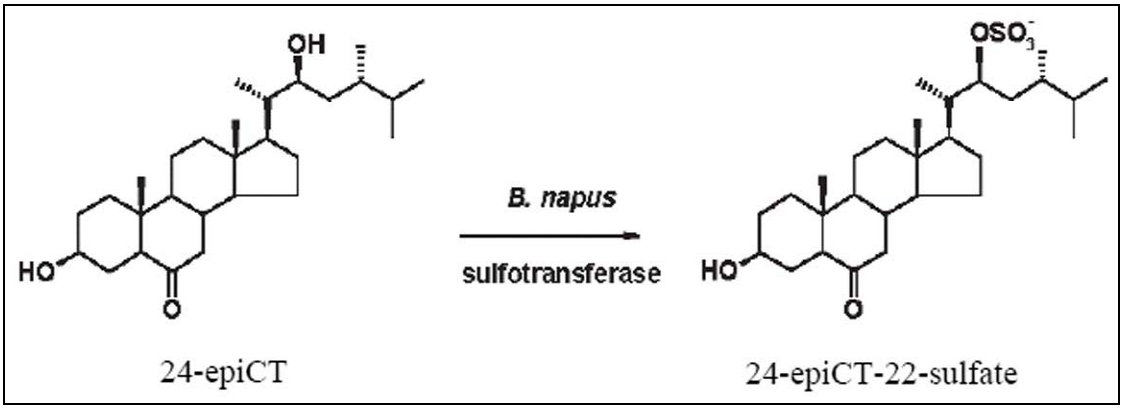

Figure 9. Sulfonation of 24-epicathasterone (24-epiCT). (Modified from Rouleau et al., 1999; Fujioka and Yokota, 2003; Zullo and Adam, 2002).

1.4.3. Inactivation of castasterone (CS) and brassinolide (BL)

The metabolic and catabolic processes of CS and BL have been well characterized because they are the most active forms of the naturally occurring BRs. In 1993, Suzuki et al. demonstrated that BL was deactivated to its 23-*O*-β-glucoside in feeding experiments

using bean explants, which was the first report on BL metabolism (Suzuki et al., 1993; Figure 10A). In 1994, Schneider et al. fed [^{3}H]-24-epiBL to cell suspension cultures of *Lycopersicon esculentum* and found that exogenously 24-*epi*-BL is converted into 25-hydroxy-24-epiBL and 25-hydroxy-24-epiBL-25-*O*-β-glucoside (Schneider et al., 1994; Figure 10B). Two distinct enzymes catalyze these hydroxylations: 25-hydroxylase and 26-hydroxylase (Winter et al., 1997).

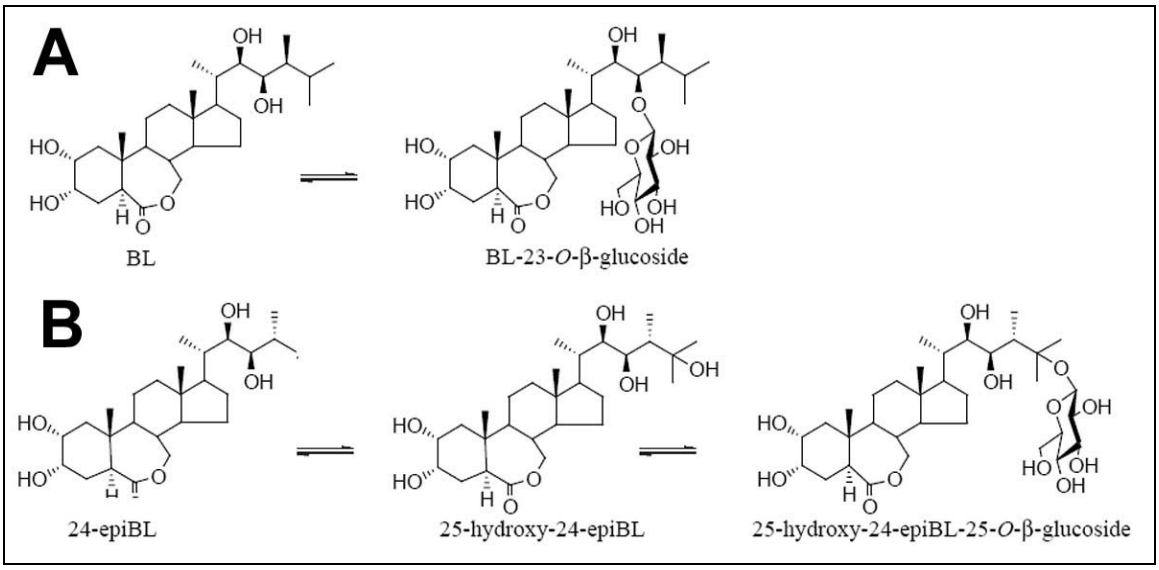

Figure 10. Metabolism of brassinolide (BL) and 24-epibrassinolide (24-epiBL).
A. Conjugation BL with glucose. **B.** Hydroxylation of 24-epiBL. (Modified from Suzuki et al., 1993; Schneider et al., 1994; Fujioka and Yokota, 2003; Zullo and Adam, 2002)

In *Ornithopus sativus* cell cultures, 24-epiCS could be degraded either into 3,24-diepiCS C_{21}-catabolite (Kolbe et al., 1994; Kolbe et al., 1996; Figure 11A) or conjugated

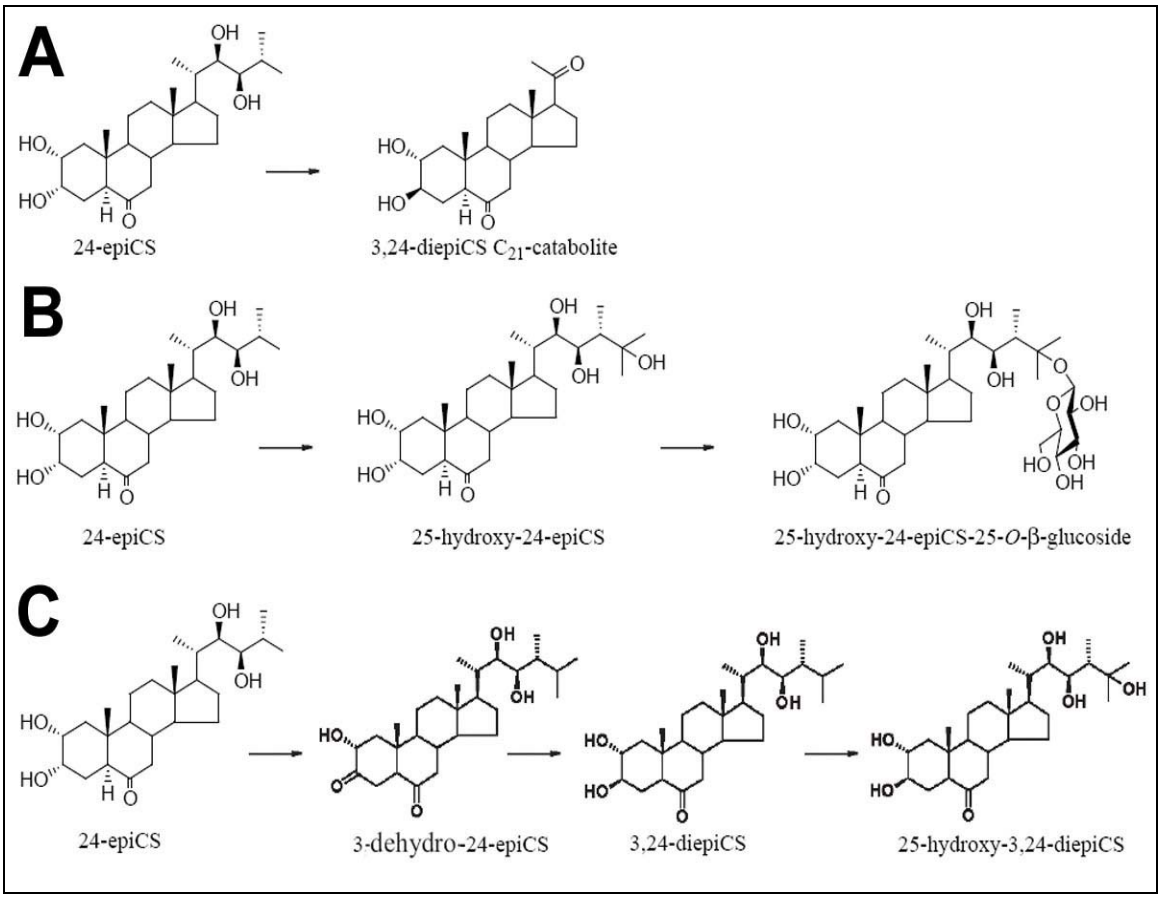

Figure 11. Metabolism of 24-epicastasterone (24-epiCS). **A.** Side chain cleavage on C-20/22 of 24-epiCS. **B.** Hydroxylation and glucosylation of 24-epiCS. **C.** Epimerization and dehydrogenated of 24-epiCS. (Modified from Fujioka and Yokota, 2003; Zullo and Adam, 2002)

with fatty acids (Kolbe et al., 1995). In tomato, 24-epiCS is hydroxylated and glucosylated at C-25 or C-26 yielding 25-hydroxy-24-epiCS and 25-hydroxy-24-epiCS-26-*O*-β-glucoside (Figure 11B), or it is dehydrogenated to 3-dehydro-24-epiCS. The latter compound can be hydroxylated at C-25 resulting in 25-hydroxy-3,24-diepiCS (Hai et al., 1996; Figure 11C). CS, 24-epiCS, BL and 24-epiBL can be inactivated by epimerization at the 2α-OH and 3α-OH positions (Nishikawa et al., 1995, Suzuki et al., 1995b; Fujioka, 1999), by demethylation of C-26 CH_3 and C-28 CH_3 (Fujioka et al., 2000; Kim et al., 2000; Watanabe et al., 2001), by side chain cleavage of C-20/22 (Kolbe et al., 1996), and by oxidation of 23-OH (Watanabe, 2000).

1.4.4. Mutants in BR metabolism

Since the knowledge discussed above is mainly derived from feeding experiments using plant cultured cells, the BR metabolism/catabolism pathways in figures 8-10 may not represent true endogenous BR inactivation mechanisms. The identification of a variety of BR metabolic mutants can help us understand the BR metabolic pathway *in vivo*. Several BR metabolic enzymes have recently been cloned in some research laboratories. *BNST3* and *AtST1*, encoding BR sulfotransferases, were isolated from *Brassica napus* and *Arabidopsis*, respectively. *In vitro* biochemical analysis indicated that BNST3 and AtST1 were stereo-specific for 24-epiBRs, with a substrate preference for the metabolic precursor 24-epiCT. They sulfonated 22-OH of 24-epiBRs to reduce their biological activities (Rouleau et al., 1999; Marsolais et al., 2007; Figure 12A). The *phyB activation-tagged suppressor 1-dominant* (*bas1-D*) was isolated as a suppressor of the

phyB-4 mutant via an activation tagging strategy. *bas1-D* exhibited a dwarf phenotype which was caused by the amplified expression of a cytochrome P450: CYP734A1. *bas1-D* mutant has reduced BR levels and accumulates high levels of 26-hydroxyBL, which suggests that BAS1 is responsible for converting active BL into an inactive form (Neff et al., 1999; Turk et al., 2003; Figure 12B). Using the activation tagged method, three research groups independently found a close homolog of BAS1 (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). This gene was named *SHRINK1-D* (*SHK1*)/*SUPPRESSOR* of *phyB-4 7* (*SOB7*)/*CHIBI2(CHI2),* and it encodes another cytochrome P450 protein, CYP72C1. *SHK1*/*SOB7*/*CHI2* may have a different BR inactivation mechanism compared to BAS1 because feeding experiments could not show the accumulation of 26-hydroxyBL in mutants (Takahashi et al., 2005; Turk et al., 2005). The fourth gene involved in BR metabolism is *UGT73C5*. *UGT73C5* encodes a UDP-glycosyltransferase enzyme (UGT). Transgenic plants overexpressing UGT73C5 showed a BR-deficient phenotype. The phenotype can be rescued by 24-epiBL supplementation. Feeding experiments indicated that UGT73C5 catalyzed BL-23-*O*-glucosylation of the BL and CS converting active CS and BL into glucose-conjugated inactive molecules, CS-23-*O*-glucoside and BL-23-*O*-glucoside, respectively (Poppenberger et al., 2005; Figure 12C). Data from UGT73C5 analyses support the hypothesis that 23-*O*-glucosylation of CS and BL is a BR inactivation pathway *in planta*, consistent with results from feeding experiments in plant culture systems. Recently, we carried out a large-scale gain-of-function genetic screen in *bri1-5* and identified a genetic enhancer of *bri1-5*— *bri1-5 enhanced 1- 1dominant* (*ben1-1D)*. The phenotype of the *bri1-5 ben1-1D* double mutant resembled the phenotypes of null alleles of *BRI1* and *CPD*. BR profile analyses revealed

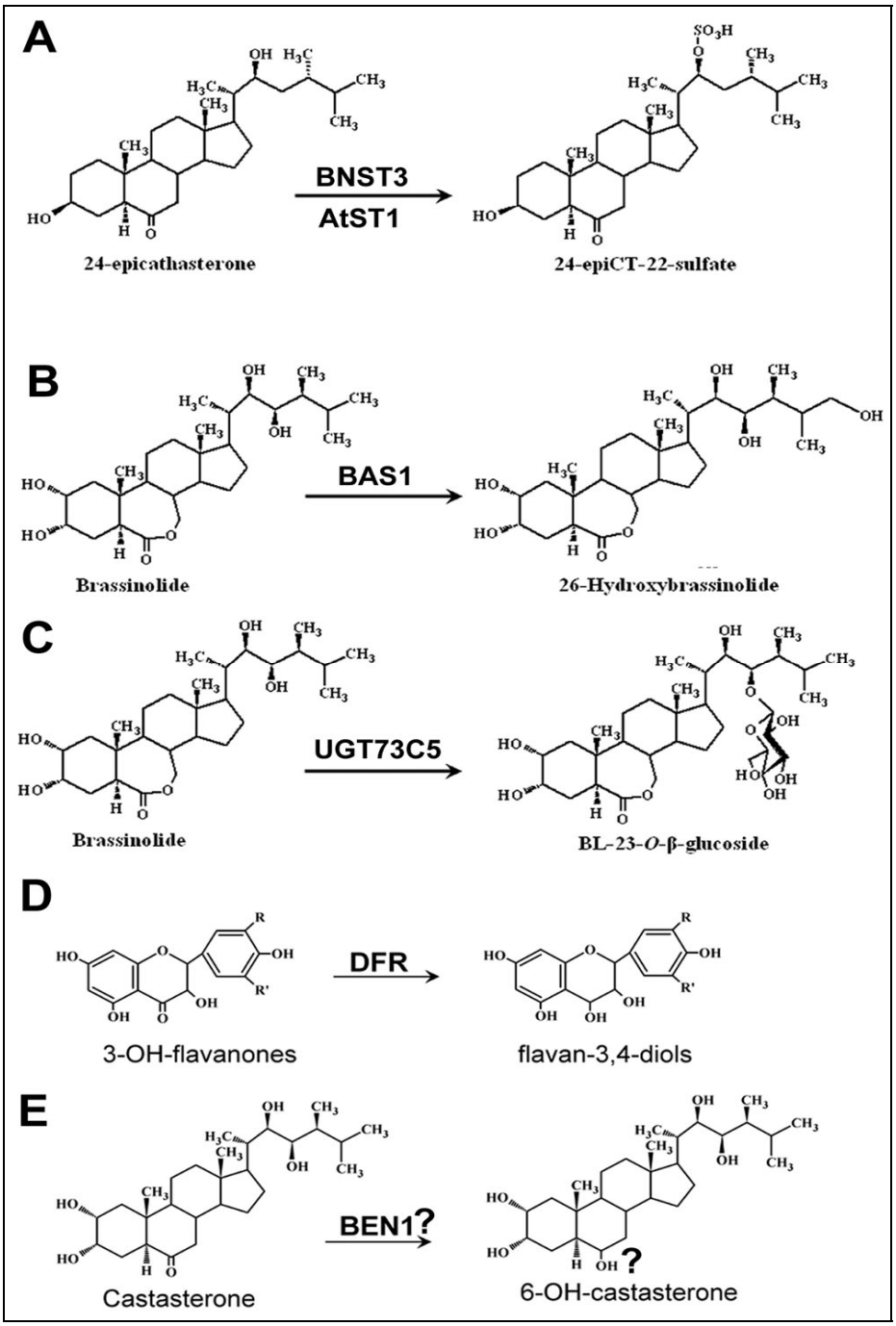

Figure 12. BR inactivation reactions in mutants. **A.** Inactivation through sulfonation by BNST3 and AtST1. **B.** Inactivation through hydroxylation by BSA1. **C.** Inactivation through conjugation with glucose by UGT73C5. **D.** Reaction catalyzed by of dihydroflavonol 4-reductase (DFR). **E.** Proposed role of BEN1 in BR inactivation. (Modified from Li and Gou, 2005; Yuan et al., 2007)

that the endogenous BR levels were dramatically reduced in mutant plants. Feeding experiments suggest that BEN1 may inactivate BRs using a mechanism different from that of BAS1 and UGT73C5. Gene cloning and sequencing revealed that *BEN1* encodes a novel protein homologous to dihydroflavonol 4-reductase (DFR) and anthocyanidin reductase (BAN; Figure 12D). We proposed that BEN1 probably acts as a novel steroid reductase responsible for the inactivation of a number of plant steroids in the BRs biosynthesis pathway (Yuan et al., 2007; Figure 12E).

1.5. Signal transduction of BR

1.5.1. BR signal perception

BRs are perceived by two plasma membrane-localized receptor kinases in *Arabidopsis*. Studies on plant steroid hormonal signal transduction began with the identification of BR insensitive mutants. Although mutants of biosynthesis pathway and signal transduction pathway are morphologically identical, biosynthetic mutants can be rescued to wild-type by supplementation of exogenous BL. On the other hand, signal transduction mutants cannot be restored to wild-type. The first BR insensitive mutant, *brassinosteroid insensitive 1* (*bri1*), was initially identified by screening an ethylmethanesulfonate (EMS) -mutagenized T_2 pool for BR-insensitivity in regulating the inhibition of root growth (Clouse et al., 1996). *BRI1* encodes an 1196 amino acid (aa) leucine-rich repeat (LRR) receptor-like kinase (RLK), which consists of three major domains: an extracellular LRR domain, a single-pass hydrophobic transmembrane domain, and a cytoplasmic kinase domain (Li and Chory, 1997). The BRI1 extracellular region consists of 25 LRRs with a 70-aa island segment located between repeats 21 and 22. Recent bioinformatics analysis

predicted, however, the 22nd LRR could be an unusual methionine-rich repeat (MRR) in stead of a typical LRR (Vert et al., 2005). The intracellular cytoplasmic portion of BRI1 can be subdivided into a juxtamembrane domain, a typical serine/threonine kinase domain and a short C-terminal tail (Li and Chory, 1997; Vert et al., 2005; Figure 13). Point mutations in the kinase domain often result in severe *bri1* phenotypes. The overall molecular structure of BRI1 suggests that it is a typical receptor kinase (Li and Chory, 1997).

The role of BRI1 as a critical component in the BR receptor has been established by several lines of evidence. First, *bri1* loss-of-function mutants are morphologically similar to BR biosynthetic mutants and cannot be rescued by exogenous BR applications (Clouse et al., 1996; Kauschmann et al., 1996; Li and Chory, 1997; Noguchi et al., 1999; Friedrichsen et al., 2000). Second, *BRI1* overexpression increases the number of BL available binding sites, and the BR-binding activity was abolished by mutations in the extracellular domain but not the kinase domain of BRI1 (Wang et al., 2001). Third, chimeric receptor kinases indicated the extracellular domain of BRI1 is required for BR perception. When the extracellular domain of BRI1 was fused to the cytoplasmic kinase domain of rice *XA21* (a gene involved in pathogen defense), and expressed in rice cell suspensions, the extracellular domain of BRI1 could perceive BL and induce defense responses (He et al., 2000). Fourth, microsomal preparations demonstrated that BRs could directly bind to the extracellular domain of BRI1 (Kinoshita et al., 2005).

Plant steroid hormone perception begins when BRs directly bind to BRI1, BRI1 interacts with another LRR-RLK, BRI1 ASSOCIATED KINASE 1 (BAK1) to form a heterodimer. BRI1 and BAK1 can transphosphorylate one another. Transphosphorylation

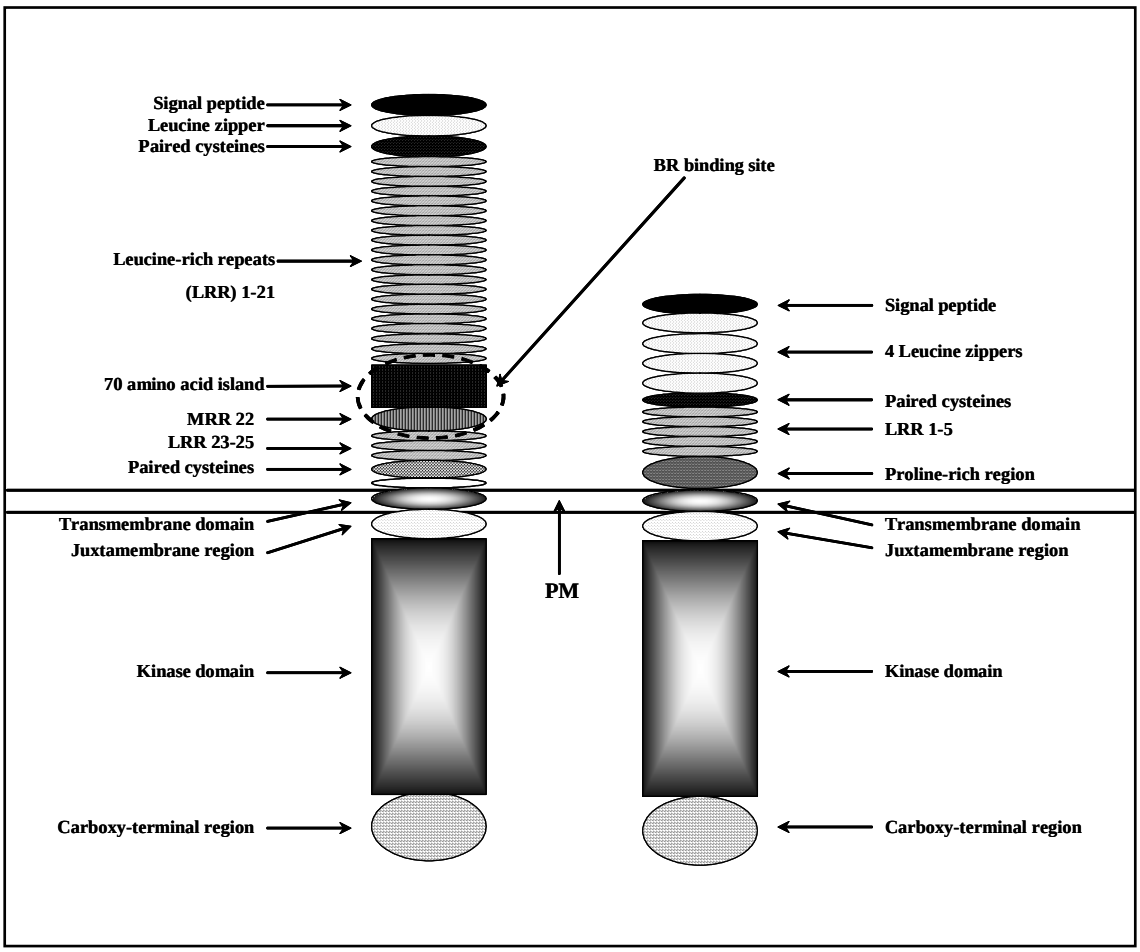

Figure 13. The structures of BRI1 (left) and BAK1 (right). BRs are perceived by a cell surface LRR-RLK, BRI1 (left). BAK1 (right) is also critical in regulating BR signal perception via its dimerization with BRI1. BL interacts with the extracellular portion of BRI1, including the 70-aa "island" and 22nd LRR. The direct interaction of BL with BAK1's extracellular domain has not been demonstrated (Reproduced from Li and Gou, 2005).

subsequently triggers a downstream signaling cascade. BAK1, the second LRR kinase, was independently found as a gain-of-function suppressor of a weak allele of *bri1*, *bri1-5,* and as a BRI1 kinase specific interactor identified by a yeast-two hybrid approach (Li et al., 2002; Nam and Li, 2002). The extracelluar domain of BAK1 only contains five LRRs. BRI1 and BAK1 can physically interact *in vivo* and *in vitro*, and the interaction is BL dependent. The role for BAK1 as a heterodimer of BRI1 in the BR signaling was confirmed both genetically and biochemically (Clouse, 2002; Kinoshita et al., 2005; Wang et al., 2005; Figure 13).

Phenotypes of null *BAK1* mutant plants are rather subtle compared to *BRI1* knockouts, suggesting that there are additional proteins in the *Arabidopsis* genome that are functionally redundant with BAK1. Recently, a paralog of BAK1, BAK1-LIKE 1 (BKK1), was reported to play a redundant role with BAK1 in regulating BR signaling. In addition, *bak1 bkk1* double mutants exhibit a seedling lethality phenotype rather than the expected *bri1*-like phenotype, suggesting that BAK1 and BKK1 have dual physiological roles: positively regulating a BR-mediated plant growth pathway, and negatively regulating a BR-independent cell death pathway (He et al., 2007; Kemmerling et al., 2007). BAK1's involvement in the BRI1-mediated BR signaling pathway is currently unknown. It is not clear whether the signal is transduced solely through BAK1, or it can be transduced through both BAK1 and BRI1. If BAK1 and all its functionally redundant proteins are simultaneously knocked out, and the resulting mutant plant resembles the phenotypes of a null BRI1 plant, it would suggest that the BR signaling can only be transduced through BAK1.

1.5.2. BR signal exportation from BRI1 to cytoplasm

Once BL binds BRI1, the signal will be transduced to downstream components of the BR signal transduction pathway. Several downstream proteins have been identified. BRI1 KINASE INHIBITOR1 (BKI1) is one of such proteins (Wang and Chory, 2006). BKI1 is a substrate of BRI1 and is responsible for limiting the interaction of BRI1 and BAK1. Overexpression of BKI1 resulted in semi-dwarfed plants and reduced BL induced response, whereas reduction of BKI1 by RNAi caused a dosage-dependent long-hypocotyl phenotype and an enhanced sensitivity to brassinazole (BRZ; an inhibitor of BR biosynthesis). Therefore, BKI1 functions as a negative regulator in the BR signaling. Another protein that could function in this process is a TGF-β receptor interacting protein (TRIP1), a WD domain protein. Transgenic plants overexpressing antisense-*TRIP1* showed BR-insensitivity and a broad range of developmental defects similar to BR-deficient mutants, suggesting that TRIP1 is a regulator in the BR signaling (Jiang and Clouse, 2001). TRIP1 was found to interact with BRI1 and could be a substrate of BRI1 kinase by *in vivo* co-immunoprecipitation and *in vitro* phosphorylation analysis (Ehsan et al., 2005). In addition, a TRANSTHYRETIN-LIKE protein (TTL; Nam and Li, 2004) was found to be phosphorylated by BRI1 *in vitro* and could also play a role in regulating the signal transduction from BRI1.

1.5.3. BR signal transduction from cytoplasm to nucleus

Another important downstream component of the BR signaling pathway, *brassinosteroid insensitive2* (*bin2*), was identified in screening an EMS-mutagenized F_2 population. *BIN2* encodes a glycogen synthase kinase-3-like protein (GSK3), which is a constitutively

active kinase that phosphorylates many different substrates in animals (Li et al., 2001b; Choe et al., 2002; Li and Nam, 2002). *bin2* inhibits BR signaling and shows a typical *bri1*-like dwarfed phenotype. Several genetic and biochemical studies have revealed that BIN2 regulates the phosphorylation status of several related downstream nuclear proteins, including BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1; Wang et al., 2002; Yin et al., 2002). This process is similar to the Wingless/wnt signaling pathways in animals, which also involves the interaction of GSK-3 and a nuclear transcription factor β-catenin (He et al., 2002).

When BRs are lacking, BIN2 can enter the nucleus and phosphorylate BES1 and BZR1, deactivating their transcription activity and blocking BR signaling in the cytosol. The targeted BZR1 and BES1 are short-lived proteins which can easily be degraded by 26S proteasome (Vert and Chory, 2006). On the other hand, when BL is present, both BRI1 and BAK1 are activated, but BIN2 is inhibited. As a result, BIN2 is retained in the cytoplasm, and BES1 and BZR1 remain dephosphorylated and able to bind to specific motifs on responsive genes and regulate their expression. The dephosphorylation processes of BES1 and BZR1 are through the action of the nuclear localized seine/threonine phosphatase, *bri1* SUPPRESSOR1 (BSU1), which offsets the action of BIN2 (Mora-Garcia et al., 2004). The most recent BR signaling model was described by Li and Jin (2007) as shown in Figure 14.

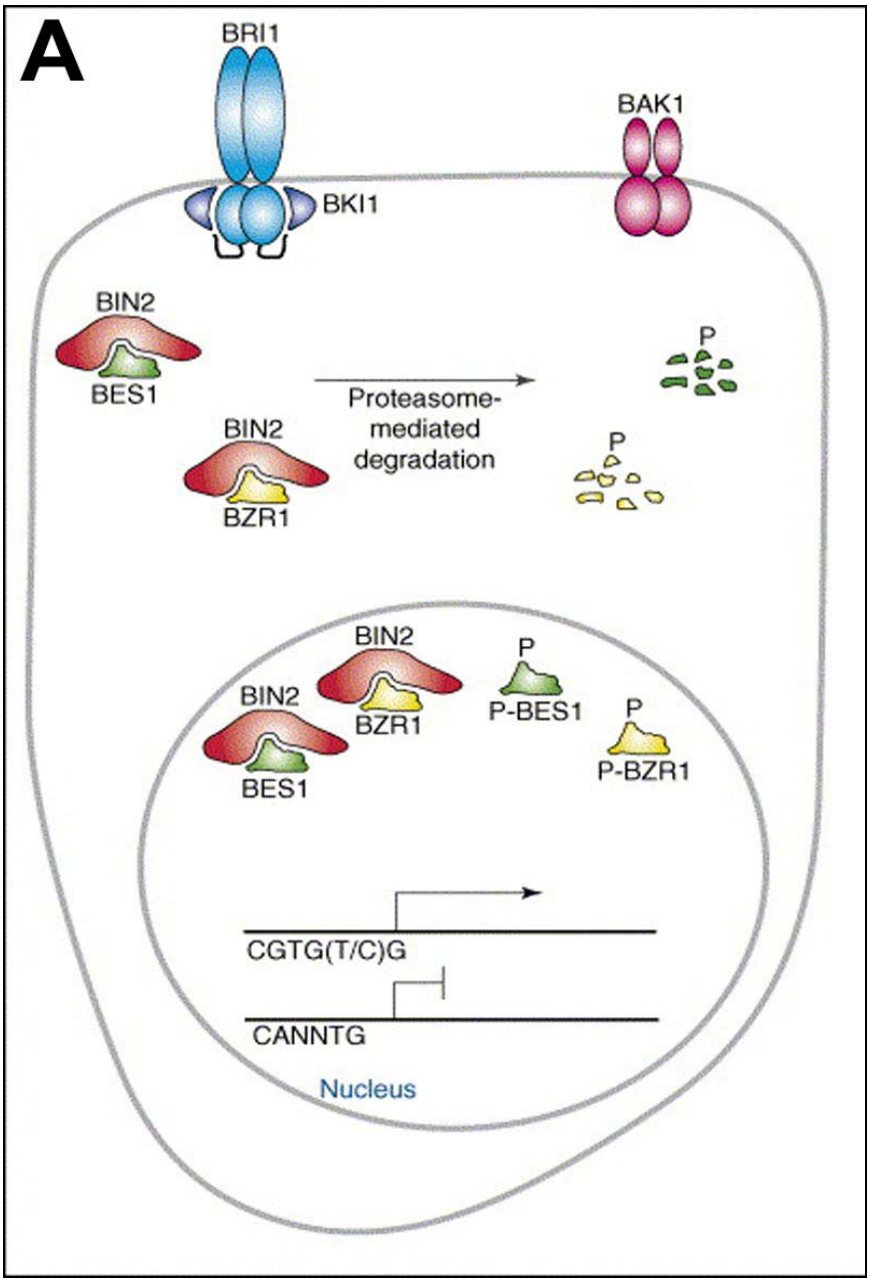

Figure 14. A current model of BR signaling. **A.** In the absence of BR, the BR receptor BRI1 mainly exists as an inactive dimer that associates with BKI1 at the cell membrane, whereas BIN2 is a constitutively active kinase that phosphorylates BES1 and BZR1 to promote their degradation in the cytosol and inhibit their DNA binding activity inside the nucleus (P-BES1 and P-BZR1 denote the phosphorylated forms of the two BIN2 substrates; Reproduced from Li and Jin, 2007).

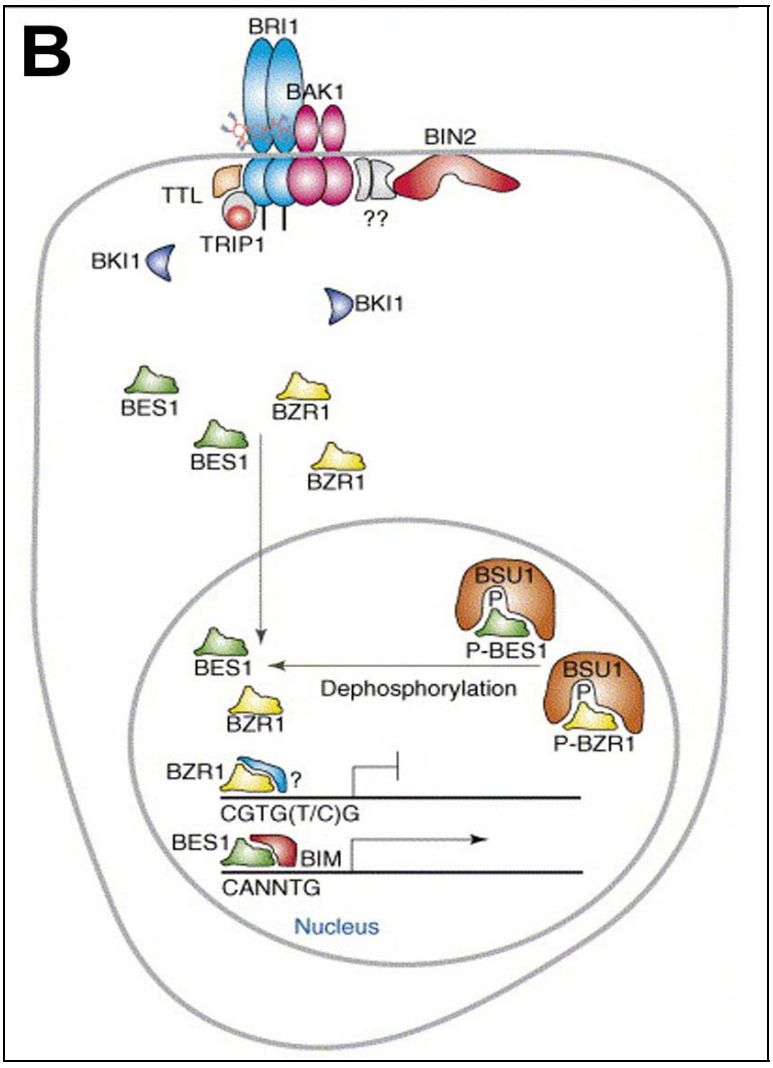

Figure 14 (continued). A current model of BR signaling. **B.** BR binding to the extracellular domain of BRI1 triggers a conformational change in its kinase domain to relieve the autoinhibitory effect of the C-terminal kinase tail and a rapid dissociation of BKI1 from the plasma membrane, accompanied by the association of BRI1 with BAK1 and other BRI1 substrates such as TTL and TRIP1. As a result, the phosphorylation activity of BIN2 is inhibited, possibly through a membrane recruitment mechanism (represented by two question-marked proteins) that physically separates the GSK3-like kinase from its two substrates. The cytosolic nonphosphorylated BES1 and BZR1 translocate into the nucleus, together with their dephosphorylated counterparts produced through the action of a nuclear phosphatase BSU1, to function as DNA-binding transcriptional factors (Reproduced from Li and Jin, 2007).

1.5.4. Comparison of steroid signaling pathways in animals and plants

The widely accepted mechanism for mammalian steroid hormone perception involves binding of the steroid to an intracellular receptor, dimerization, and ligand-dependent transcriptional activation. The ligand-activated receptor recognizes the *cis* elements for steroid-responsive genes in promoters to control the related gene expression (Mangelsdorf et al., 1995). The DNA-binding domains of steroid receptors are highly conserved and consist of two C2-C2 Zn fingers (Evans, 1988). In addition to intracellular receptors, animals may also perceive steroids at the cell surface. But cell surface receptors for animal steroids have not yet been identified. Several cell surface receptors for peptide ligands, however, have been extensively studied. For example, in Wingless/wnt signaling pathway, TGF-β receptor can perceive TGF-β at the cell surface, and transduce the signal through a phosphorylation cascade to the nucleus for gene activation (Massague et al., 1994; Heldin, 1995; Falkenstein et al., 1996). In plants, cell surface recognition is the only experimentally demonstrated form of plant steroid perception. BRs are perceived by plasma membrane receptor kinases, BRI1 and BAK1, with their general structures similar to TGF-β receptors. Because, in nearly all cases the C2-C2 Zn finger is the DNA binding domain of nuclear hormone receptors in animals (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi), C2-C2 Zn fingers may have the same function in plants. DNA-binding-with-one-finger (Dof) proteins represent a unique group of transcription factors in plant, containing the C2-C2 Zn finger domain, whose structure is similar to animal steroid hormone receptors. For instance, the positions of cysteine residues in Dof motif are identical to those of steroid hormone receptors (Figure 15). Furthermore, it has been demonstrated that the Dof motif not only binds DNA, but it

also engages in protein-protein interactions (Zhang et al., 1995; Kang and Singh, 2000). Taken together, Dof transcription factors may act as intracellular steroid receptors in plants. Characterization of this group of transcription factors may lead to the discovery of a new pathway for plant steroid hormone transduction.

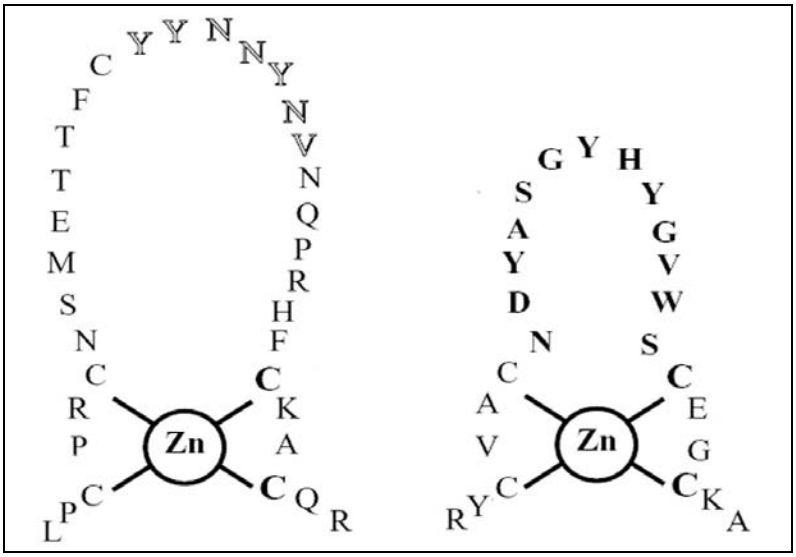

Figure 15. Comparison of the structures of the proposed Dof domain in AOBP of pumpkin (Left) and the zinc-binding units U1 in estrogen receptor (P06212) (Right). Four Cys residues coordinate to zinc ion (Zn). (Modified from Shimofurutani, et al., 1988; Umemura et al., 2004).

2. Activation tagging: A novel approach to identify components in the BR biosynthesis and signal transduction pathways

The first BR receptor, BRI1, was identified by screening an EMS-mutagenized T_2 population for BR insensitive mutants (Clouse et al., 1996). After that, the similar

strategy was used to identify additional components in the BRI1-mediated signaling pathway. However, all the additional *bri1*-like mutants isolated were actually different mutant alleles of *bri1*, which suggests that other regulatory components may have functional redundant proteins in the plant genome, or may be indispensable for normal plant growth and development. In fact, about two-thirds of the genes in the *Arabidopsis* genome contain at least one additional copy (*Arabidopsis* Genome Initiative, 2000). In order to identify other important components in the BR biosynthesis and signaling pathway, redundant-insensitive strategies such as yeast-two-hybrid and gain-of-function screening methods were employed.

Activation tagging is a technique to obtain gain-of-function mutations. Using the *Agrobacterium*-mediated transformation method, large collections of transgenic *Arabidopsis* plants are generated by the random insertion of an enhancer-containing T-DNA. The strong enhancers were obtained from the cauliflower mosaic virus 35S promoter to activate the expression of nearby genes (Weigel et al., 2000). The constructs contain a selectable marker for transgenic plant screening. Because the T-DNA sequences are known, it allows the flanking genomic sequence of the inserted T-DNA to be easily determined by various techniques such as thermal asymmetry interlaced PCR (TAIL-PCR; Liu and Whittier, 1995; Terauchi and Kahl, 2000). Since only the genes near the insertion siteare likely to be activated, confirmation usually focuses only on genes adjacent to the T-DNA insertion. This can be achieved by comparing the expression levels of the same gene from wild-type plants and from the activation tagged plants by Northern blotting or RT-PCR analysis. The true activation tagged gene related to the observed phenotype has to be confirmed by overespressing the candidate gene in

background plants by gene transformation. If the transgenic plants overexpressing that particular gene can recapitulate the phenotypes shown in the original activation tagged line, it would suggest that the right gene has been identified. An outline of approach for the activation tagging is presented in Figure 16.

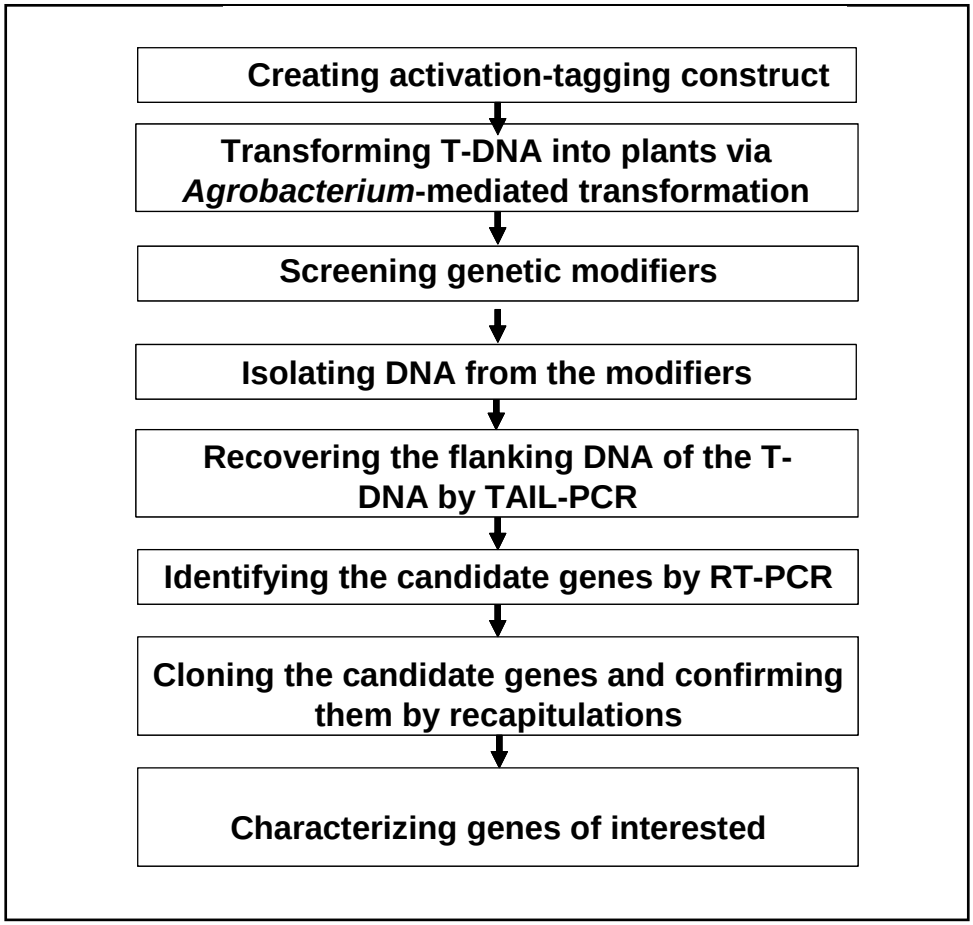

Figure 16. Outline of activation tagging genetic approach.

Previous studies from several labs indicated that activation tagging genetic approaches can successfully reveal a gene's function (Neff et al., 1999; Weigel et al.,

2000; Li et al., 2001a; Li et al., 2002; Mora-Garcia et al., 2004; Zhou et al., 2004; Takahashi et al., 2005). For example, *bri1 SUPPRESSOR1* (*BRS1*) was identified as a suppressor of a weak *bri1* mutant, *bri1-5* (Li et al., 2001a; Zhou and Li, 2005). BRS1 may be involved in a regulation process at an early step of the BR signal transduction. Another example, BAK1, which can interact with BR-BRI1 complex, was identified as a co-receptor of the BR (Li et al., 2002; Nam and Li, 2002; Wang et al., 2005). BRI1-LIKE 1 (BRL1), a BRI1-Like receptor-like kinase was also identified by the activation tagging, BRL1 probably plays a redundant role with BRI1 in mediating the BR signal transduction because it can suppress both weak and null alleles of *bri1* (Zhou et al., 2005). Using this genetic approach, we also identified some *bri1-5* genetic enhancers that show enhanced *bri1* mutant phenotypes. For instance, *bri1-5 ben1-1D* double mutant plants are more severe than *bri1-5* single mutant and mimic the null mutants of *BRI1*. Molecular cloning and characterization of this mutant demonstrated BEN1 is likely to be directly or indirectly involved in BR metabolism (Yuan et al., 2007).

3. Summary of Our Work

The main purpose of our studies was to identify important regulatory components in the BR biosynthesis and signal transduction pathways by gain-of–function genetic screens for weak *bri1* extragenic modifiers. We have carried out a genome-wide activation tagging screen in *bri1-5,* one of the semi-dwarfed *bri1* mutants. Unlike other null *bri1* mutants, *bri1-5* plants are fertile, which made it possible for screening both genetic suppressors and genetic enhancers based on their phenotypic sizes. Our goal was to

screen enough activation tagging lines to activate every single gene in the *Arabidopsis* genome by randomly adding enhancers in the genome of *bri1-5* plants. Toward this end, we have screened over 120,000 activation tagging lines. Preliminary results indicate that over 40 distinct *bri1-5* suppressors have been isolated, which include *BRS1* (Li et al., 2001a), *BAK1* (Li et al., 2002), and *BRL1* (Zhou et al., 2004). Detailed analyses of these genes indicate that they play important roles in the BR signal transduction pathway (Clouse, 2002; Li et al., 2002; Nam and Li, 2002; Wang et al., 2005; Zhou and Li, 2005). Meanwhile, a number of mutants that showed enhanced *bri1-5* defective phenotypes were also identified. One of these mutants, designated *bri1-5 enhanced 1-1dominant* (*ben1-1D*) is described in Chapter II in detail. Using the same strategy, other two mutants, *bri1-5 e*nhanced *2-1d*ominant (*ben2-1D*) and *bri1-5 enhanced 3-1dominant (ben3-1D),* were identified (discussed in Chapter III). Gene cloning results indicated *ben3-1D* is allelic to *ben2-1D.* It was therefore renamed as *ben2-2D. bri1-5 ben1-1D, bri1-5 ben2-1D* and *bri1-5 ben2-2D* all showed enhanced *bri1-5* defective phenotypes, including dwarfism, dark-green round leaves and more compact rosette width. *BEN1* encodes a putative steroid reductase whereas *BEN2* encodes a transcription factor, Dof 1.5. When the double mutant was backcrossed with the wild-type *Arabidopsis* plant and segregated out the *bri1-5* mutation, *ben1-1D* single mutant plants were semi-dwarfed with rounded leaves and shortened petioles suggesting that BEN1 is independent on BRI1-mediated BR signaling pathway. Unlike *ben1-1D*, *ben2-1D* single mutant plants showed opposite leaf phenotypes with the *bri1-5 ben2-1D* double mutant. Instead of more compact and smaller epidermal cells being seen in the double mutant, the *ben2-1D* single mutant shows more elongated leaves and enlarged epidermal cells. Our studies thus indicate that the function

of BEN2 in regulating the cell elongation and plant growth is dependent on the function of BRI1-mediated BR signaling. Preliminary results also suggest that BEN2 may represent a nuclear receptor of BRs and that it may be involved in regulating GA biosynthesis/signaling transduction pathway to control *Arabidopsis* flowering time. Our studies and those of others demonstrate that BEN2 may play a specific role in connecting the phytochrome, cell surface receptor-based BR signal transduction, and GA biosynthesis/signal transduction pathways.

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Chapter II. *BEN1*, a gene encoding a dihydroflavonol 4-reductase (DFR)-like protein, regulates the levels of brassinosteroids in *Arabidopsis thaliana*

Summary

The *ben1-1D* (*bri1-5 enhanced 1-1d*ominant) mutant was identified via an activation-tagging screen for *bri1-5* extragenic modifiers. *bri1-5* is a weak mutant allele of the brassinosteroid receptor gene, *BRI1*. Overexpression of *BEN1* greatly enhances the defective phenotypes of *bri1-5* plants. Removal of *BEN1* by gene disruption in a Col-0 wild-type background, on the other hand, promotes the elongation of organs. Because *BEN1* encodes a novel protein homologous to dihydroflavonol 4-reductase (DFR) and anthocyanidin reductase (BAN), BEN1 is probably involved in a brassinosteroid metabolic pathway. Analyses of brassinosteroid profiles demonstrated that BEN1 is indeed responsible for regulating the levels of several brassinosteroids, including typhasterol, castasterone and brassinolide. *In vivo* feeding and *in vitro* biochemical assays suggest that BEN1 is probably involved in a new mechanism to regulate brassinosteroid levels. These results provide additional insight into the regulatory mechanisms of bioactive brassinosteroids.

Keywords: activation-tagging, BEN1, DFR, BANYULS, brassinosteroids, brassinolide

Introduction

Brassinosteroids (BRs) are steroidal plant hormones that regulate critical plant developmental processes, including cell elongation, cell division, xylem tissue

development, senescence and photomorphogenesis. Within the last decade, significant progress has been made in elucidating both the biosynthetic and signal transduction pathways of BRs. Genetic and biochemical approaches have revealed many key elements in both pathways. Plants bearing mutations in these essential proteins display extreme dwarfism, round and epinastic leaves, delayed senescence, reduced male fertility, and altered light-regulated growth responses (Chory et al., 1991; Clouse et al., 1996; Kauschmann et al., 1996; Li et al., 1996; Szekeres et al., 1996; Li and Chory, 1997; Choe et al., 1998; Noguchi et al., 1999; Friedrichsen et al., 2000). Although biosynthetic and response mutants are morphologically identical, biosynthetic mutants can be restored to wild-type upon the supplementation of brassinolide (BL), the end and most active product of the BR biosynthetic pathway. The response mutants, on the other hand, are generally insensitive to treatment with BL.

BRs share some common features with several other classes of growth-promoting phytohormones such as auxins, gibberellins and cytokinins. For example, plants maintain an extremely low amount of BRs endogenously. Although a low quantity of BRs can invigorate plant growth and development, an excessive amount of BRs is usually detrimental to normal growth and development. Unlike other plant hormones, BRs appear to be unable to travel from one tissue to another using long-distance transport mechanisms (Symons and Reid, 2004), suggesting that each tissue of a plant must possess both biosynthetic and metabolic machinery. Maintenance of adequate physiological BR function is dependent on the equilibrium between BR biosynthesis and BR metabolism. The expression levels of a number of crucial BR biosynthetic genes are principally regulated by the endogenous concentration of BRs via a negative feedback

mechanism (Mathur et al., 1998; Li et al., 2001b; Goda et al., 2002; Mussig et al., 2002; Yin et al., 2002; Tanaka et al., 2005). Five BR-specific biosynthesis genes, *DEETIOLATED2* (*DET2*; Li et al., 1996), *DWARF4* (*DWF4*; Choe et al., 1998), *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* (*CPD*; Szekeres et al., 1996), *BR-6-oxidase1* (*BR6ox1*; Bishop et al., 1999), and *ROTUNDFOLIA3* (*ROT3*; Kim et al., 1998) were found to be upregulated in BR-depleted *Arabidopsis* plants after treatment with a BR biosynthesis inhibitor, brassinazole (BRZ; Asami and Yoshida, 1999; Tanaka et al., 2005). Four of the five genes (*DWF4*, *CPD*, *BR6ox1* and *ROT3*) were downregulated in response to addition of exogenous BL (Tanaka et al., 2005). Fluctuation in the expression levels of these key BR-specific biosynthesis genes can influence the BR biosynthesis rate. The homeostasis of endogenous bioactive BRs, however, is also determined by additional factors, such as their inactivation rate. Several BR metabolic enzymes have recently been identified by various research laboratories. The *BNST3* gene, encoding a steroid sulfotransferase, was isolated from *Brassica napus*. *In vitro* biochemical analysis indicated that the recombinant BNST3 protein was able to sulfonate 22-OH of 24-epimeric BRs and abolish their biological activities (Rouleau et al., 1999). Using an activation-tagging strategy, Neff et al. (1999) isolated a gain-of-function *Arabidopsis* mutant named *phyB activation-tagged suppressor 1-dominant* (*bas1-D*). *BAS1* encodes a cytochrome P450 (CYP734A1, previously called CYP72B1) that appears to possess steroid 26-hydroxylase activity. *bas1-D phyB-4* double mutant plants had undetectable levels of BL. Feeding experiments indicated that the mutant accumulated high levels of 26-hydroxybrassinolide (26-OHBL), suggesting that BAS1 is responsible for turning active BL into inactive 26-OHBL. The 26-hydroxylase activity of

BAS1 has been demonstrated in both yeast and *Arabidopsis* (Turk et al., 2003). A BAS1-related gene, *SHRINK1-D* (*SHK1*)/*SUPPRESSOR of phyB-4 7* (*SOB7*)/*CHIBI2 (CHI2)* was activation-tagged by three research groups independently (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). *SHK1/SOB7/CHI2* encodes another cytochrome P450 protein, CYP72C1, that is a close homolog of BAS1. Interestingly, feeding experiments failed to detect the accumulation of 26-OHBL (Takahashi et al., 2005; Turk et al., 2005). These results suggested that SHK1/SOB7/C HI2 is not a functionally redundant protein with respect to BAS1, and it may have a unique BR inactivation mechanism that is different from that of BAS1. The mechanism of BL inactivation in the *shk1-D/sob7-D/chi2* mutant is unknown. Another gene involved in BR metabolism is *UGT73C5*. *UGT73C5* encodes a UDP-glycosyltransferase enzyme. Overexpression of *UGT73C5* in wild-type *Arabidopsis* plants showed a typical BR-deficiency phenotype that can be rescued by 24-epibrassinolide (24-epiBL) supplementation. LC-MS/MS analysis indicated that UGT73C5 is responsible for converting active BL to a glucose-conjugated inactive molecule, BL-23-*O*-glucoside (Poppenberger et al., 2005).

In an attempt to isolate extragenic modifiers of a weak *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*) mutant allele *bri1-5* through activation tagging, we identified several *bri1-5* genetic suppressor genes including *bri1 SUPPRESSOR1* (*BRS1*; Li et al., 2001a), *BRI1 ASSOCIATED RECEPTOR KINASE 1* (*BAK1*; Li et al., 2002) and *BRI1-LIKE 1* (*BRL1*; Zhou et al., 2004). Detailed analyses of these genes indicated that they play important roles in the BR signal transduction pathway (Clouse, 2002; Li et al., 2002; Nam and Li, 2002; Wang et al., 2005; Zhou and Li, 2005). During a large-scale gain-of-function genetic screen, a number of mutant plants that showed enhanced *bri1-5*-

defective phenotypes were also identified. One of these mutants, designated *bri1-5 enhanced 1-1dominant* (*ben1-1D*), was particularly interesting because the phenotype of the *bri1-5 ben1-1D* double mutant resembled the phenotypes of null alleles of *BRI1* and *CPD*. This indicated that a negative regulator of either BR signal transduction or biosynthesis may have been activation-tagged. Molecular cloning and characterization of this mutant demonstrated, however, that BEN1 is likely to be directly or indirectly involved in BR metabolism. The BR profile and detailed physiological and genetic analyses supported this prediction. Feeding experiments suggest that BEN1 may inactivate BRs using a unique mechanism that is different from that of BAS1 and UGT73C5. Because BEN1 is the most closely related paralog of dihydroflavonol 4-reductase (DFR) and anthocyanidin reductase (BAN), both of which are involved in flavonoid biosynthesis, BEN1 could also indirectly modulate BR levels via flavonoids. These results suggest that bioactive BR can be regulated by complicated mechanisms yet to be identified.

Results

Identification of ben1-1D via an activation-tagging genetic screen

Loss-of-function genetic approaches have been widely used to determine the biological functions of a great number of *Arabidopsis* genes. However, largely due to functional redundancy (*Arabidopsis* Genome Initiative, 2000), the roles of many other genes need to be defined by alternative strategies. Gain-of-function-based activation-tagging genetic

approaches, for example, have been successfully used to reveal the functions of many of these genes within the last few years (Neff et al., 1999; Weigel et al., 2000; Li et al., 2001a; Li et al., 2002; Mora-Garcia et al., 2004; Zhou et al., 2004; Takahashi et al., 2005).

bri1-5 is a weak mutant allele of the BR receptor gene *BRI1*. Unlike most other *bri1* alleles, which usually show severe phenotypes such as extreme dwarfism and male sterility, *bri1-5* plants are semi-dwarfed and completely fertile. The intermediate phenotype severity and unaffected fertility of *bri1-5* make it a unique genetic material for extragenic modifier screens. From about 120, 000 activation-tagging lines generated in *bri1-5*, *ben1-1D* was identified as a *bri1-5* extragenic enhancers. Increasing the expression of *BEN1* in *bri1-5* greatly enhances defective phenotypes of *bri1-5* plants (Figure 1a). The leaf blades of *bri1-5 ben1-1D* double mutants are much smaller and more curled than those of *bri1-5* single mutants (Figure 1b). Scanning electron microscopy (SEM) analysis indicated that the smaller leaf size is mainly caused by reduced expansion of the mutant cells (Figure 1c, 1d). At maturity, both the height and rosette width of the double mutant are about half those of *bri1-5* (Figure 1e, 1f). The overall phenotype of *bri1-5 ben1-1D* resembles that of more severe *bri1* alleles, such as the null mutant *bri1-4* or a *bri1-5 det2-28* double mutant (Noguchi et al., 1999; Li et al., 2002). A *bri1-5 ben1-1D* plant only produces about 50% of the seeds that *bri1-5* produces, mainly because the siliques of the double mutant are significantly shorter than those of the *bri1-5* plant (data not shown). These observations suggested that a gene responsible for negative regulation of BR responses or biosynthesis might have been activation-tagged.

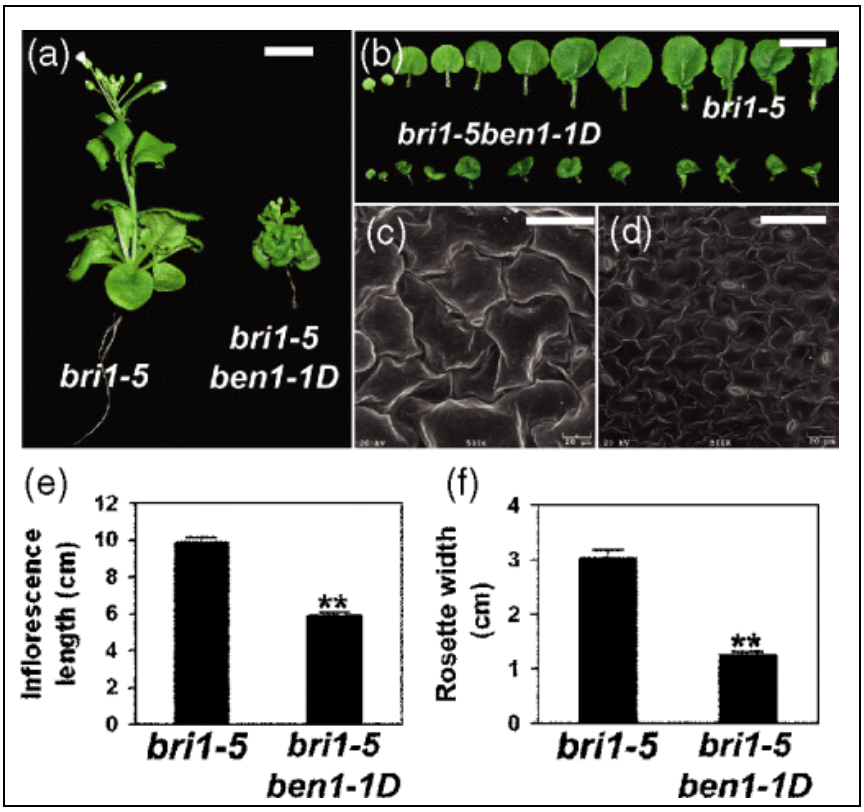


Figure 1. *ben1-1D* enhances *bri1-5*-defective phenotypes. (a) *ben1-1D* was identified through an activation-tagging genetic modifier screen in the *bri1-5* background. Phenotypes of 4-week-old *bri1-5* and *bri1-5 ben1-1D* plants. Scale bar = 1 cm. (b) Leaf phenotypes from 4-week-old plants. Scale bar = 1 cm. (c, d) Scanning electron micrographs of epidermal cells from the third pair of leaves of 4-week-old *bri1-5* (c) and *bri1-5 ben1-1D* (d) plants. Scale bar = 40 μm. (e, f) *ben1-1D* enhances the inflorescence length (e) and rosette width (f) defects of *bri1-5*. Measurements from 5-week-old *bri1-5* and *bri1-5 ben1-1D* plants are shown. The experiments were repeated three times, and the data from one representative experiment are presented as means and standard errors ($n = 40$). **Significantly reduced compared to *bri1-5* using Student's unpaired *t*-test, two-tailed ($P < 0.01$).

ben1-1D single mutant plants are semi-dwarfed with rounded leaves

In order to determine whether the severe phenotype observed in *bri1-5 ben1-1D* is synergistically dependent on the *bri1-5* allele, *bri1-5 ben1-1D* was back-crossed to wild-type (WS2) plants. The *bri1-5* mutation was subsequently segregated out, and a homozygous *ben1-1D* single mutant was generated. As seen in double mutant plants, *ben1-1D* single mutant plants exhibit a phenotype of shortened inflorescences and reduced rosette size (Figure 2a, 2b). The inflorescence length of the *ben1-1D* plants is about 70% that of wild-type plants. In addition, *ben1-1D* plants produce rounded leaves and shortened petioles (Figure 2c). Unlike the leaf blades seen in the *bri1-5 ben1-1D* mutant, which are much smaller in size than those in the *bri1-5* mutant in all dimensions, the width of the leaf blades of the *ben1-1D* mutant is greater than that of wild-type (WS2). Student's *t*-tests indicated that the measurements between the mutant plants and wild-type plants are all significantly different ($P < 0.01$). These results suggest that BEN1, as a negative regulator of either BR signaling transduction or the BR biosynthetic pathway, is independent of the *bri1-5* allele.

RT-PCR and recapitulation analysis demonstrate that At2g45400 is responsible for the ben1-1D phenotype

Our genetic segregation analysis indicated that it is probable that a single T-DNA was inserted in *ben1-1D*, because the observed phenotype always co-segregates with BASTA

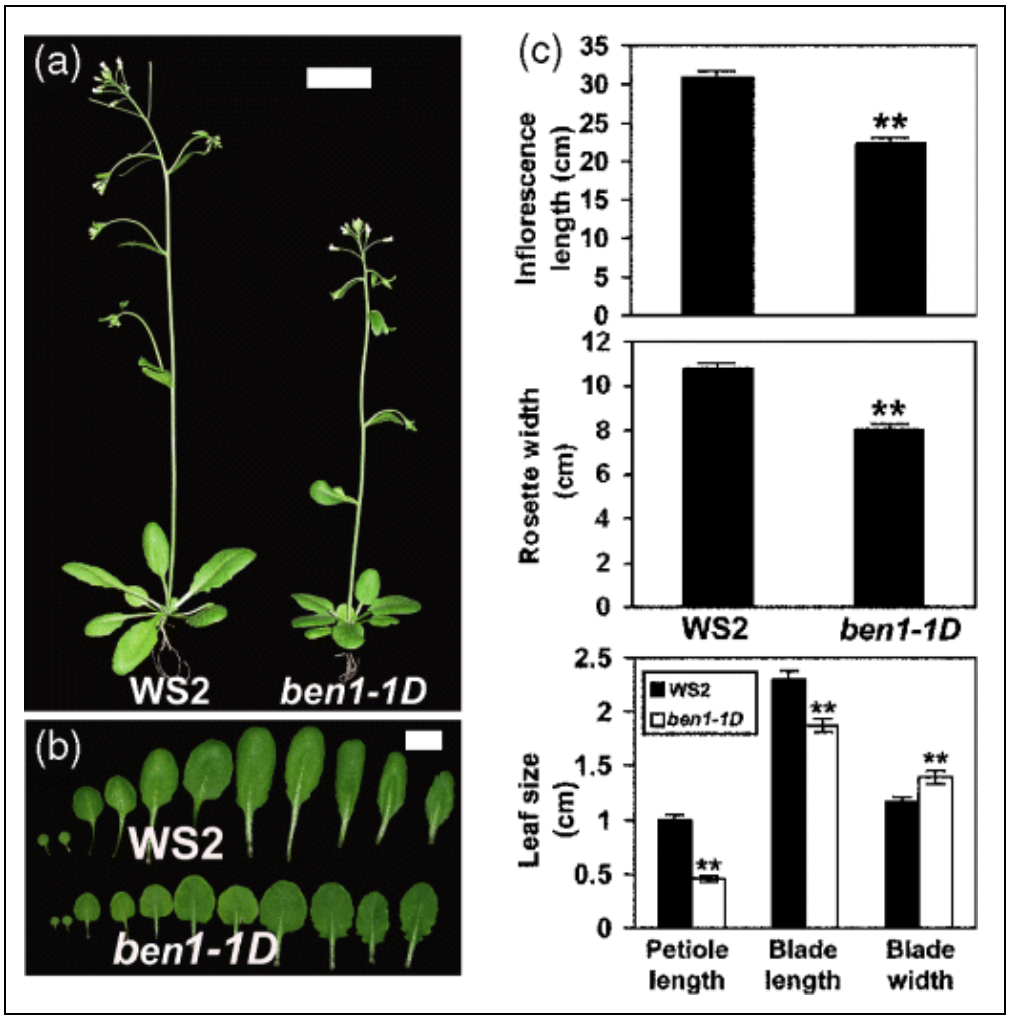


Figure 2. Phenotypes of *ben1-1D* in the WS2 background. (a) The *ben1-1D* single mutant causes a semi-dwarfed phenotype in wild-type plants. Scale bar = 2 cm. (b) Leaf phenotypes from 4-week-old plants. Scale bar = 1 cm. (c) Various measurements of 5-week-old wild-type (WS2) and *ben1-1D* plants. The experiments were repeated three times and the data from one representative experiment are shown. The plants from different genotypes were measured and the data are presented as means ± standard errors ($n = 40$). Student's *t*-tests indicated that the inflorescence length, rosette width, petiole length, leaf blade length and leaf blade width of *ben1-1D* are all statistically different (**) from those of WS2 ($P < 0.01$).

resistance. Thermal asymmetry interlaced PCR (TAIL-PCR) was subsequently used to amplify the genomic sequences flanking the T-DNA insertion (Liu and Whittier, 1995; Terauchi and Kahl, 2000). These analyses showed that the enhancer-containing T-DNA was inserted in chromosome 2. Sequence analysis indicated that the T-DNA was inserted 544 bp downstream of the predicted stop codon of *At2g45400* (Figure 3a). *At2g45400* is therefore a reasonable candidate for the tagged *BEN1*.

To further confirm that overexpression of *At2g45400* is responsible for the enhanced *bri1-5*-defective phenotype, the CaMV 35S promoter was used to drive the expression of *At2g45400* cDNA in the *bri1-5* mutant. Of 60 transgenic plants examined, 12 lines(20%) showed obvious enhanced *bri1-5*-defective phenotypes (Figure 3b). RT-PCR analysis confirmed that *BEN1* was indeed overexpressed in both the activation-tagged *bri1-5 ben1-1D* mutant and the transgenic lines showing enhanced *bri1-5* phenotypes (Figure 3c, 3d). Overexpression of other genes flanking the enhancer-containing T-DNA insertion site was not demonstrated (data not shown). These results verified that elevated expression of *AT2g45400* caused the enhanced *bri1-5*-defective phenotype in the activation-tagged *ben1-1D* plants.

BEN1 encodes a DFR-like protein

Comparison of *BEN1* cDNA and its corresponding genomic sequence indicates that *BEN1* contains four exons and three introns (Figure 3a). The *BEN1* cDNA has an open reading frame of 1098 bp, which putatively encodes a protein with 365 amino acids.

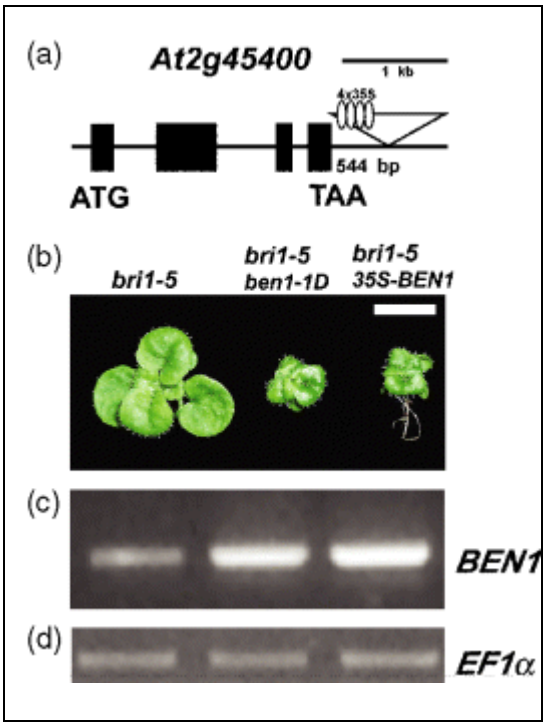


Figure 3. Overexpression of *At2g45400* recapitulates the *bri1-5 ben1-1D* phenotypes. (a) The T-DNA from activation-tagging construct pBASTA-AT-2 (see Experimental procedures) was inserted 544 bp downstream from the *At2g45400* stop codon, with the left border oriented toward to the At2g45400 stop codon. The ovals indicate the four 35S enhancers from pBASTA-AT-2. (b) Phenotypes of *bri1-5*, *bri1-5 ben1-1D* and a representative line of transgenic plants carrying 35S-*BEN1* cDNA. The plants were photographed 3 weeks after germination. Scale bar = 1 cm. (c, d) RT-PCR showing that *BEN1* is overexpressed in *bri1-5 ben1-1D* and 35S-*BEN1 bri1-5* transgenic plants. The numbers of PCR cycles for *BEN1* and the quantitative control gene, *EF1*α, were 36 and 22, respectively.

Database searches indicated that BEN1 belongs to a small gene family that also includes the well-characterized dihydroflavonol 4-reductase (DFR) and anthocyanidin reductase (BANYULS or BAN) (Figure 4a, 4b). Both play key roles in flavonoid biosynthesis pathways. BEN1 shares 42% sequence identity with DFR (At5g42800), 37% with both BAN (At1g61720) and At4g35420, and 34% with At4g27250. The closest homolog of BEN1 is DFR, as shown by phylogenetic analysis and amino acid alignment results (Figure 4a, 4b). Further analysis showed that proteins of this group contain a putative NADPH-binding domain, as well as a domain determining substrate specificity ((Devic et al., 1999; Shimada et al., 2004). Surprisingly, bioinformatic analysis indicated that DFR family proteins share structure and sequence similarities with proteins that would not be expected to be their homologs, such as mammalian 3β-hydroxysteroid dehydrogenases, bacterial cholesterol dehydrogenase and bacterial UDP-galactose-4-epimerase (Baker and Blasco, 1992). These observations suggest that BEN1 could be a reductase; however, it also could be a dehydrogenase or an epimerase. It is also possible that BEN1 or its homologs have dual or even triple enzyme activities. In fact, BAN of *Medicago truncatula* shows both reductase and epimerase activities (Xie et al., 2003).

The ben1 loss-of-function mutant shows an organ-elongated phenotype

Based on the observation that overexpression of *BEN1* can enhance the *bri1-5*-defective phenotype, we hypothesized that a *BEN1* loss-of-function mutant would show an organ-elongated phenotype. To test this hypothesis, T-DNA knockout pools were screened for

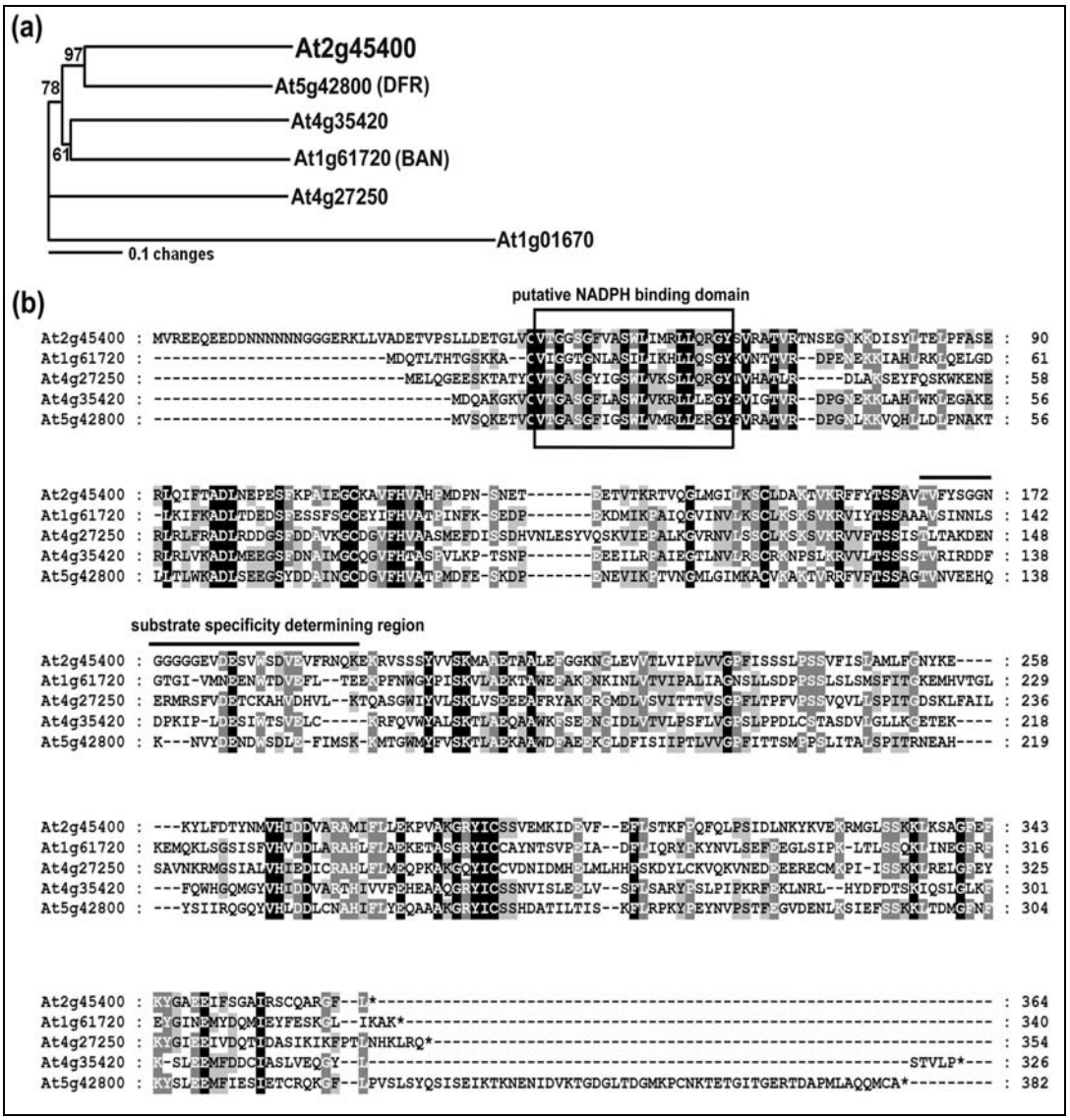


Figure 4. *BEN1* encodes a novel protein homologous to DFR and BAN. (a) Phylogenetic tree generated from the amino acid sequences of At2g45400 (BEN1) and its closest paralogs from the *Arabidopsis* genome. At1g01670 was used as an outgroup sequence. The bootstrap values shown at branch nodes are from 1000 bootstrap replications. (b) Sequence alignment of BEN1 and its closest paralogs. The most similar protein sequence to BEN1 is the well-characterized DFR. Amino acids shared by all members are shaded in black; those shared only by some members are shaded in gray (the darker the color, the more members share the residue). The putative NADPH-binding domain is boxed, and the region determining substrate specificity is underlined.

null insertion alleles, and a *ben1-1* line was identified from SALK T-DNA insertion pools, in which a single T-DNA is inserted in the second intron of the *BEN1* genomic sequence (Figure 5a). Because *BEN1* mRNA was not detectable in the *ben1-1* mutant (Figure 5d), *ben1-1* is a null allele. *ben1-1* homozygous plants show obvious organ-elongated phenotypes, with their inflorescences, leaves and petioles all longer than those of wild-type plants (Figure 5b, 5c, 5e), similar to the phenotypes previously reported when the BR-signaling genes *BRI1* and *BAK1*, or a BR biosynthetic gene *DWF4*, were overexpressed (Choe et al., 1998; Choe et al., 2001; Wang et al., 2001; Li et al., 2002; Nam and Li, 2002). To verify that silenced *BEN1* caused the elongated phenotype, 35S-*BEN1* was transformed into *ben1-1* plants to test whether the elongated phenotype can be reversed to the wild-type-like phenotype by overexpressing *BEN1*. The result indicated that the *ben1-1* phenotype can be complemented by the expression of *BEN1* (Figure 5d, 5e). Our measurements indicated that many individual *ben1-1* 35S-*BEN1* transgenic plants are significantly shorter and smaller than either *ben1-1* or wild-type plants (Figure 5e). The smaller stature of the transgenic lines is probably caused by the overexpression of *BEN1* due to the strong constitutive 35S promoter. These analyses also showed that some transgenic plants were completely restored to the wild-type-like phenotype (Figure 5d), implying that an adequate amount of *BEN1* expression can completely complement the organ-elongation phenotype seen in *ben1-1*. The clear phenotype of *ben1-1* suggests that other members of the DFR family do not play any key redundant roles with BEN1.

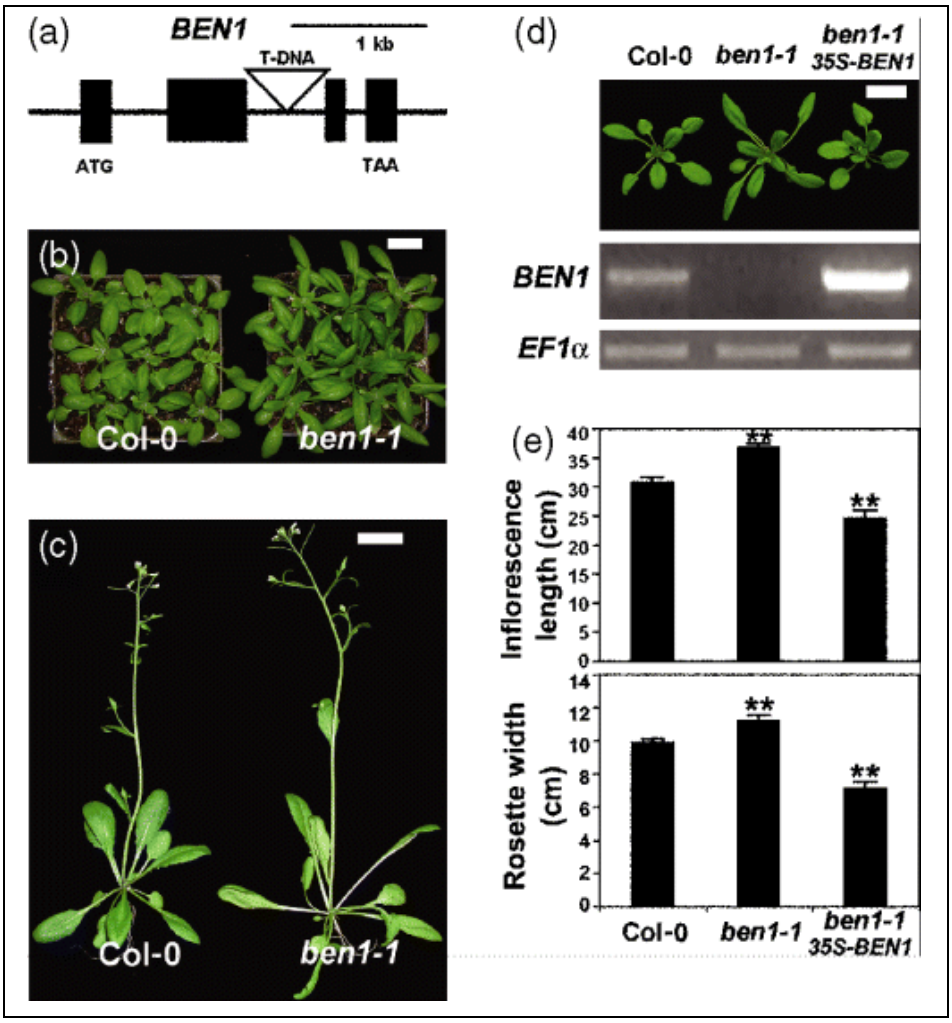


Figure 5. A null allele of *BEN1*, *ben1-1*, shows an elongated organ phenotype that can be complemented by the overexpression of *BEN1* cDNA. (a) In *ben1-1*, a T-DNA was inserted in the second intron of the genomic DNA. (b, c) Phenotypes of wild-type (ecotype Col-0) and *ben1-1* plants at the 3-week-old vegetative growth stage (b) and 4-week-old reproductive growth stage (c). Bar = 1 cm. (d) The *ben1-1* elongated phenotype can be suppressed by overexpression of *BEN1* cDNA driven by a 35S promoter. *ben1-1* plants do not show detectable *BEN1* mRNA by RT-PCR analysis, while 35S-*BEN1 ben1-1* plants show increased levels of *BEN1* transcripts compared to wild-type plants (middle panel). *EF1*α was used as a quantitative control (bottom panel). Bar = 1 cm. (e) *ben1-1* plants show longer inflorescences and larger rosettes, while 35S-*BEN1 ben1-1* plants show shorter inflorescences and compact rosettes compared to wild-type plants. Measurements were taken 5 weeks after germination and are presented as means and standard errors ($n = 40$). The experiments were repeated three times and data from one representative experiment are shown. Student's *t*-tests indicate that the measurements in knock-out plants are significantly different (**) from their wild-type background (Col-0) ($P < 0.01$).

The shortened hypocotyls of the ben1-1D mutants can be rescued by 24-epibrassinolide

bri1-5 ben1-1D mutant plants show an enhanced *bri1-5* dwarf phenotype, similar to those of severe BR-deficient or BR signaling mutants (Clouse and Sasse, 1998). Cloning and recapitulation analyses indicated that the overexpressed *BEN1* is responsible for the observed phenotype. Root inhibition analysis showed that the root growth of *ben1-1* is fully sensitive to 24-epiBL treatment, similar to that of wild-type plants (data not shown), suggesting that BEN1 is unlikely to be involved in modulating the BR signaling pathway. BEN1 is thus more likely to be involved in regulating the levels of BRs by altering either BR biosynthesis or a BR metabolism pathway. To test this hypothesis, WS2 and *ben1-1D* seedlings were planted in half-strength MS medium supplemented with 1% sucrose and 0.1% DMSO (mock) as controls, and *ben1-1D* seedlings were also planted in half-strength MS medium with 1% sucrose and various concentrations of 24-epiBL dissolved in DMSO. Addition of 10 nM 24-epiBL can completely rescue the shortened hypocotyls of *ben1-1D* to wild-type-like hypocotyls (Figure 6a, 6b). Student's *t*-test analysis indicated that the recovered hypocotyls of *ben1-1D* by treatment with 24-epiBL are not statistically different from those of untreated wild-type seedlings (Figure 6b). These results indicate that the reduced cell expansion phenotype of *ben1-1D* might be caused by reduced levels of endogenous BRs.

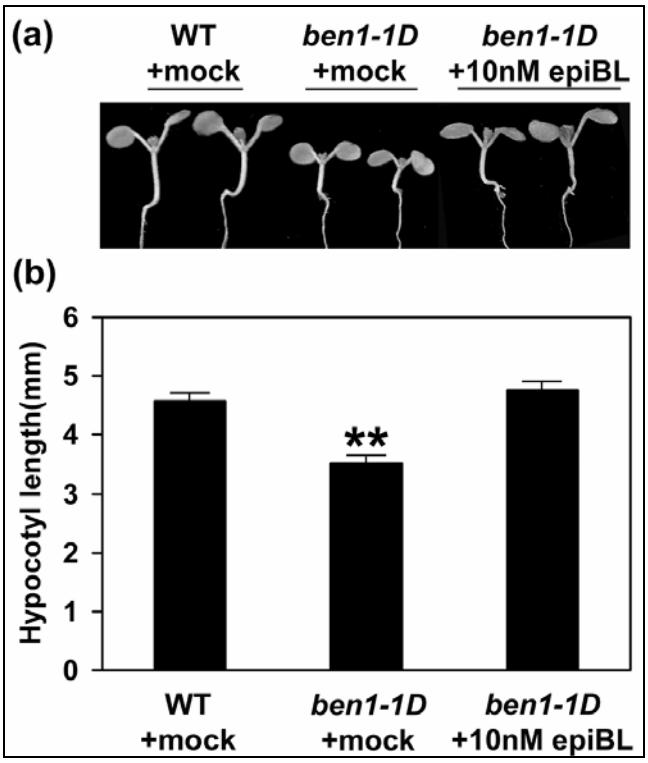


Figure 6. The shortened hypocotyl phenotype of *ben1-1D* can be restored by the supplementation of exogenous BL. (a) Phenotypes of WT (ecotype WS2) or *ben1-1D* (in WS2 background) seedlings grown in half-strength MS medium supplemented with 1% sucrose, and DMSO (mock) or 10 nM 24-epiBL (dissolved in DMSO). (b). Measurements of hypocotyls of the seedlings shown in (a). Measurements were taken 6 days after germination and are presented as means and standard errors ($n = 60$ to 90). The experiments were repeated three times and the data from one representative experiment are shown. **Significantly different from the WS2 mean, based on Student's unpaired *t*-tests, two-tailed ($P < 0.01$).

BEN1 expression is downregulated in the dark

To test whether light alters the growth response of *ben1-1D* and *ben1-1* relative to their wild-type backgrounds, WS2, *ben1-1D* (in WS2 background), Col-0 and *ben1-1* (in Col-0 background) seedlings were grown in constant white light or darkness. Our measurements showed that *ben1-1D* seedlings were much shorter than their wild-type counterparts, whereas *ben1-1* seedlings were significantly taller than their corresponding wild-type seedlings after growth in continuous light for 6 days. However, when grown in continuous darkness for 6 days, *ben1-1D* seedlings were slightly, but not statistically, taller than WS2 seedlings, and *ben1-1* seedlings did not appear to be different from Col-0 seedlings (Figure 7a to 7c). The phenotypic differences between the mutants and their background plants were not significant in dark conditions, based on Student's *t*-test analysis. These interesting results prompted us to test whether the expression of *BEN1* is regulated by light. Interestingly, the expression of *BEN1* is indeed significantly downregulated in darkness (Figure 7d). This result implies that BEN1 may play a greater role in regulating hypocotyl elongation in light than in darkness, which explains why the *BEN1* mutants show more dramatic phenotypic alterations in the light than in the dark.

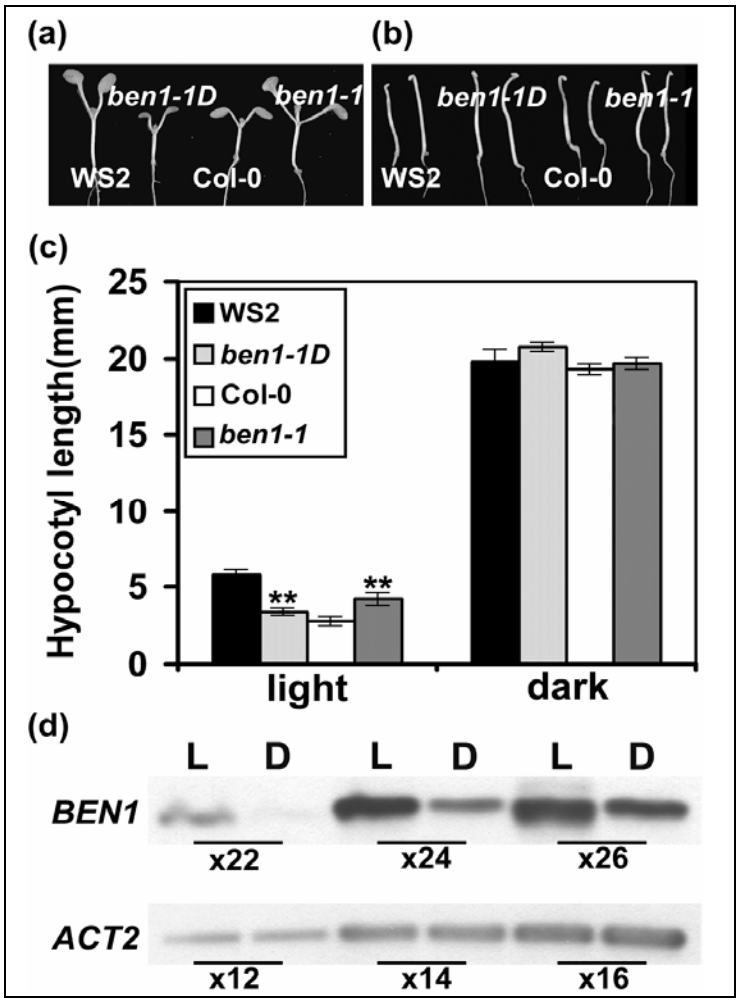


Figure 7. *BEN1* expression is regulated by light. (a, b) Phenotypes of 6-day-old seedlings of WS2, *ben1-1D*, Col-0 and *ben1-1* plants grown under continuous white light (a) or darkness (b) on half-strength MS medium supplemented with 1% sucrose. (c) Measurements of the seedlings shown in (a) and (b). Hypocotyls of 6-day-old light- and dark-grown seedlings were measured, and the data are presented as means ± standard errors (n = 60 to 90). The experiments were repeated three times, and the data from one representative experiment are shown. **Significantly different from their wild-type background, based on Student's unpaired t-tests, two-tailed ($P < 0.01$). (d) Quantitative RT-PCR analysis of *BEN1* and *ACT2* expression in 6-day-old WT seedlings grown under continuous white light (L) or darkness (D). The numbers below each pair of bands indicate the numbers of PCR cycles used. *ACT2* was used as a control to show that the same amount of mRNA was initially used for RT-PCR.

Overexpression of BEN1 downregulates the expression of BAS1 and upregulates the expression of CPD

Several important BR response genes have been identified utilizing genetic, molecular and genomic tools in previous studies (Mathur et al., 1998; Li et al., 2001b; Goda et al., 2002; Mussig et al., 2002; Yin et al., 2002; Tanaka et al., 2005). For example, *BAS1* is upregulated in response to exogenously elevated BR levels, whereas *CPD* is downregulated when BR levels are increased. If overexpression of *BEN1* results in a decrease in endogenous bioactive BRs, the expression of *BAS1* should be reduced and the expression of *CPD* should be increased. To test this scenario, RT-PCR analysis using total RNA from *ben1-1D* and WS2 plants was performed to examine the expression levels of the two BR response genes. The results confirmed that *BAS1* was significantly downregulated, and that *CPD* was significantly upregulated in *ben1-1D* mutant plants, compared to wild-type plants (Figure 8). This result is consistent with the hypothesis that the endogenous levels of BRs are reduced in the *ben1-1D* mutant. RT-PCR analyses also indicated that the expression of *BEN1* in wild-type plants cannot be significantly regulated by exogenously applied BL, consistent with the microarray data previously reported (Goda et al., 2002). It is apparent that *BEN1*, unlike *BAS1* or a number of other BR biosynthetic genes, is not part of the transcriptional feedback pathways that mediate BR homeostasis.

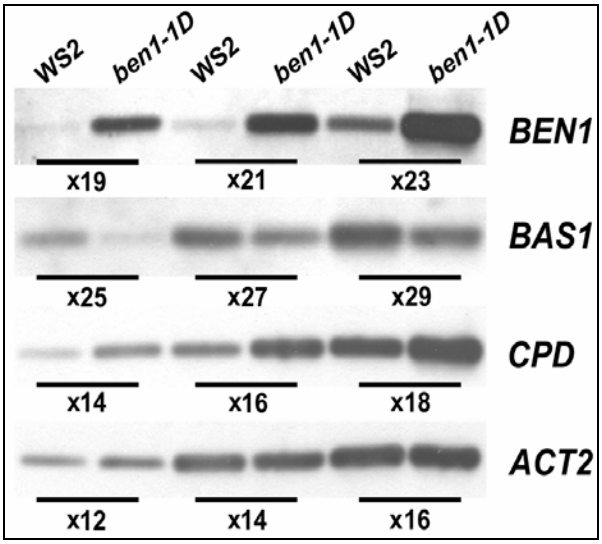


Figure 8. Quantitative RT-PCR analysis of the expression of various BR response genes in WT (WS2) and *ben1-1D* plants. RT-PCR analysis of *BEN1, CPD*, *BAS1* and *ACT2* in 6-week-old WT and *ben1-1D* plants. The numbers below each pair of bands indicate the numbers of PCR cycles used for analyses. *ACT2* was used as a control to show that the same amount of mRNA was initially used for RT-PCR.

Increased expression of BEN1 causes reduction of the endogenous levels of BRs

Comparing expression of the major BR response genes in *ben1-1D* with those in wild-type suggested that the levels of endogenous BRs were probably reduced upon overexpression of *BEN1*. Therefore, the endogenous levels of BRs in *bri1-5 ben1-1D*,

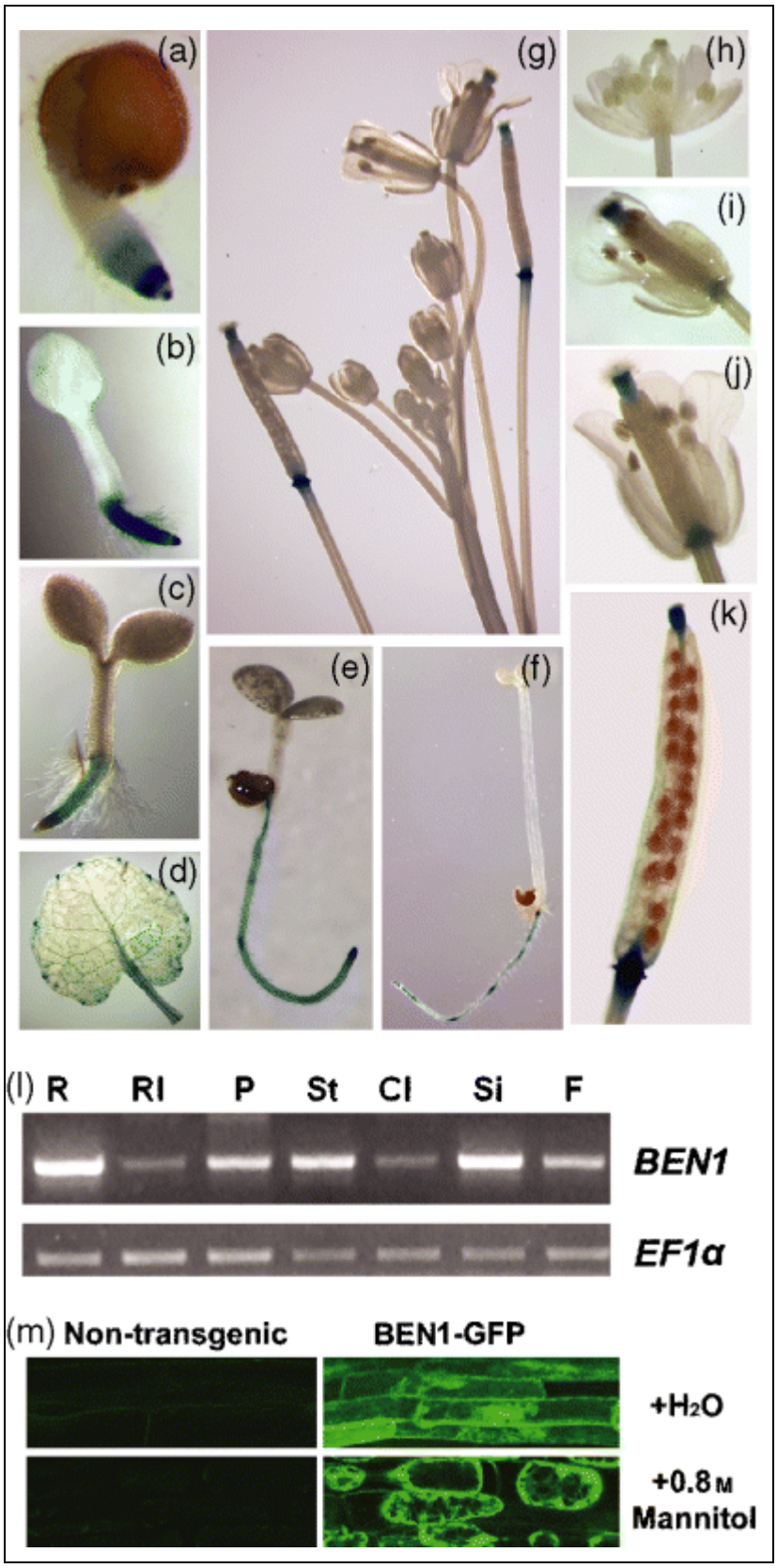
bri1-5, *ben1-1D* and WS2 were determined using GC/MS analysis. Relative to the levels detected in the background line, *bri1-5*, the endogenous levels of several major BRs were drastically decreased in the *bri1-5 ben1-1D* activation-tagged line. For example, both typhasterol (TY) and castasterone (CS) were reduced more than fourfold, and BL was reduced more than twofold (Table 1). Reduction of 6-deoxotyphasterol (6-deoxoTY) and 6-deoxocastasterone (6-deoxoCS) was also detected, but the reductions were not as dramatic as for TY and CS. In the WS2 background, reduction of endogenous TY and CS, but not 6-deoxoTY and 6-deoxoCS, by the overexpressor line, *ben1-1D*, was also observed, suggesting that TY and CS are the preferred targets of BEN1. However, the reductions were not as great as those in the *bri1-5* background. This result may explain why overexpression of *BEN1* in the *bri1-5* background resulted in more striking phenotypic alterations than overexpression of *BEN1* in the wild-type background. However, BL was undetectable in *ben1-1D* and its wild-type WS2 background, consistent with previous results (Takahashi et al., 2005; Turk et al., 2005). The endogenous levels of BRs in the *BEN1* knock-out line were also compared with those in its background line. A significantly increased accumulation of the bioactive BR, CS, was detected. The accumulation of TY, 6-deoxoTY and 6-deoxoCS was not statistically significant. BL levels were too low to be detected. These analyses were repeated three times with plants grown on different occasions and always gave consistent results, supporting our hypothesis that BEN1 is responsible for regulating the endogenous levels of BRs.

Table 1. Endogenous levels of BRs (ng/gFW) [1]						
BR	*bri1-5*	*bri1-5 ben1-1D*	WS-2	*ben1-1D*	Col-0	*ben1-1*
6-DeoxoCT	2.00±0.24	1.70±0.22	1.59±0.16	1.43±0.14	0.96±0.22	0.95±0.27
6-DeoxoTE	0.05±0.01	0.06±0.01	0.06±0.01	0.07±0.01	0.06±0.00	0.08±0.01
6-Deoxo3DT	0.31±0.08	0.22±0.04	0.23±0.04	0.21±0.02	0.15±0.02	0.15±0.01
6-DeoxoTY	1.26±0.23	0.88±0.21*	1.11±0.15	1.20±0.14	0.80±0.13	1.07±0.19
6-DeoxoCS	4.26±0.77	2.85±0.48*	1.71±0.07	2.40±0.13*	1.57±0.11	2.07±0.30
CT	nd	nd	nd	nd	nd	nd
TE	nd	nd	nd	nd	nd	nd
TY	1.05±0.25	0.18±0.06*	0.24±0.05	0.10±0.01*	0.12±0.03	0.13±0.03
CS	9.52±2.55	2.16±0.36*	0.49±0.04	0.30±0.01*	0.29±0.03	0.39±0.02*
BL	1.42±0.39	0.61±0.18*	nd	nd	nd	nd

[1] The aerial parts of 5-week-old plants were used for BR profile analysis. 6-deoxoCT: 6-deoxocathasterone. 6-deoxoTE: 6-deoxoteasterone. 6-deoxo3DT: 3-dehydro-6-deoxoteasterone. 6-deoxoTY: 6-deoxotyphasterol. 6-deoxoCS: 6-deoxocastasterone. CT: cathasterone. TE: teasterone. TY: typhasterol. CS: castasterone. BL: brassinolide. nd, not detected. Values are means ± standard error ($n = 3$). *Significant differences compared with respective wild-type based on paired *t*-test, two-tailed ($P < 0.05$).

BEN1 is expressed strongly in the roots and flowers, and BEN1 is localized in the cytoplasm

Knowing expression patterns would help us to further understand the role of *BEN1* in real physiological settings. Transgenic plants were generated carrying the GUS reporter gene driven by a 2.2 kb *BEN1* promoter to examine tissue-specific expression patterns of *BEN1*. More than 40 independent transgenic lines were analyzed. All showed consistent expression patterns. Representative expression patterns are summarized in Figure 9. *BEN1* can be detected just after seeds germinate. It is mainly expressed in the root cap, and in elongation and maturation zones, but much less in the apical meristem zone (Figure 9a to 9c). In 6-day-old seedlings, GUS expression was detected in roots, but the intensity was much weaker in dark- than in light-grown seedlings (Figure 9e, 9f), consistent with the RT-PCR results (Figure 7d). GUS staining was weak in young flowers. As the siliques develop from young to mature, higher GUS signals can be detected. Strong expression was seen at both ends of siliques (Figure 9g to 9k). In mature rosette leaves, GUS expression was seen mainly in vascular tissues and hydathodes (Figure 9d). RT-PCR results also showed that *BEN1* transcripts can be detected in all major organs tested, with the strongest signals detected in roots and siliques (Figure 9l). The tissue-specific expression patterns of *BEN1* are similar to those of *SHK1*, a gene that is also involved in regulating the internal BR levels (Takahashi et al., 2005). Previous studies have shown that the bioactive BRs, such as CS, and other BL intermediates are lowest in roots. The expression levels of several key BR biosynthetic genes, such as *DWF4* and *ROT3*, however, are relatively high in roots (Shimada et al., 2003). The lower BR levels in roots may be caused by higher expression levels of BR metabolic


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
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(j)
(k)
(l) R RI P St Cl Si F
BEN1
EF1α
(m) Non-transgenic
BEN1-GFP
+H2O
+0.8 M Mannitol

Figure 9. Tissue-specific expression of *BEN1* and localization of BEN1–GFP. (a to k) GUS staining patterns of the seedlings or tissues from *BEN1p*–GUS transgenic plants at various developmental stages, including 1–3-day-old seedlings (a to c), mature leaf (d), 6-day-old seedling grown in the light (e) and in darkness (f), inflorescence (g), flowers from various developmental stages (h–j) and siliques (k). (l) RT-PCR analyses of *BEN1* expression in roots (R), rosette laminae (Rl), rosette petioles (P), stems (St), cauline leaves (Cl), siliques (Si) and flowers (F). The numbers of PCR cycles for *BEN1* and *EF1α* were 26 and 22, respectively. *EF1α* is a quantitative control. (m) Confocal analysis of the transgenic plants harboring a 35S-*BEN1*–GFP construct indicated that BEN1–GFP is mainly localized in the cytoplasm. These transgenic plants also show enhanced *bri1-5*-defective phenotypes (data not shown). Root apices from 6-day-old non-transgenic and transgenic *bri1-5* plants harboring 35S-*BEN1*–GFP were analyzed. Mannitol (0.8 M) was used to induce plasmolysis.

genes such as *BAS1* (Shimada et al., 2003), *SHK1* (Takahashi et al., 2005) and *BEN1*. High levels of BRs are favorable to cell division and unfavorable to root elongation (Clouse et al., 1996), which may explain why *BEN1* is expressed to a greater extent in the root elongation zone but to a lesser extent in the cell division zone. These results suggest that *BEN1* is expressed less in tissues with active cell division such as root meristems and developing embryos (Figure 9a, 9b, 9k).

Transgenic plants harboring 35S-*BEN1–GFP* were generated in the *bri1-5* background and used to determine the subcellular localization of the BEN1 protein. Overexpression of *BEN1–GFP* in *bri1-5* can recapitulate the phenotypes of *bri1-5 ben1-1D*, suggesting that the C-terminal fusion of *GFP* does not alter the biological function of BEN1. The subcellular localization revealed using *bri1-5* 35S-*BEN1*–GFP should thus represent the real *in vivo* site of the BEN1 protein. Confocal analyses indicated that BEN1 is a cytoplasmic protein (Figure 9m). The subcellular localization of BR biosynthesis has not been demonstrated, but it was indicated that at least some steps proceed in the cytoplasm (Stündl and Schneider, 2001). The detected BEN1 localization is consistent with the proposed role of BEN1 as a possible BR inactivation enzyme.

Discussion

BRs are plant growth-promoting hormones that regulate a variety of physiological processes crucial for normal plant growth and development. Within the last decade, a

number of key steps modulating BR biosynthesis and signal transduction have been elucidated by extensive genetic and biochemical analyses (Clouse, 2002; Fujioka and Yokota, 2003; Li, 2005; Vert et al., 2005; Wang et al., 2006). The physiological actions of BRs are largely determined by the amount of bioactive BRs in real physiological settings. Levels of bioactive BRs, such as CS and BL, are mainly controlled by an internal cellular process balancing the rate of their biosynthesis and the speed of metabolism. Feeding experiments have shown that exogenously applied BRs are rapidly metabolized (Yokota, 1999). The detailed molecular mechanism controlling the BR inactivation process, however, is not well understood. So far, more than 40 BR metabolites have either been identified by feeding experiments from various plant species or predicted from naturally occurring metabolic intermediates (Fujioka and Yokota, 2003). Reactions putatively involved in BR inactivation include β-epimerization of 2α-OH and 3α-OH (Nishikawa et al., 1995; Suzuki et al., 1995; Fujioka, 1999), hydroxylation of C-12, C-20, C-25 and C-26 (Voigt et al., 1993; Hai et al., 1996; Kolbe et al., 1996; Yokota et al., 1996; Winter et al., 1997; Neff et al., 1999), demethylation of C-26 CH_3 and C-28 CH_3 (Fujioka et al., 2000b; Kim et al., 2000; Watanabe et al., 2001), side chain cleavage of C-20/22 (Kolbe et al., 1996), oxidation of 23-OH (Watanabe, 2000), sulfonation of 22-OH (Rouleau et al., 1999), conjugation with fatty acids at 3β-OH (Kolbe et al., 1995; Asakawa et al., 1996), conjugation with glucose at 2α-OH, 3β-OH, 23-OH, 25-OH and 26-OH (Suzuki et al., 1993; Hai et al., 1996; Winter et al., 1997; Kolbe et al., 1998; Fujioka, 1999; Soeno et al., 2000), and conjugation with 6-*O*-β glucosylglucose or 4-*O*-β glucosylgalactose at 3β-OH (Kolbe et al., 1998). Only three enzymes have been identified to be responsible for BR-specific inactivation reactions: (1)

a steroid sulfotransferase from *Brassica napus* that is responsible for sulfonation at 22-OH of 24-epicathasterone (Rouleau et al., 1999), (2) an *Arabidopsis BAS1* that participates in a hydroxylation reaction at C-26 (Neff et al., 1999; Turk et al., 2003), and (3) another *Arabidopsis* protein UGT73C5 that catalyzes the conjugation of BL with glucose at 23-OH (Poppenberger et al., 2005). In all these cases, modification of the BRs abolished biological activity. The enzymes responsible for most other predicted reactions have not yet been identified. For example, BL epimers are naturally occurring BR metabolites, but the epimerases catalyzing BL epimerization have not yet been discovered.

Several lines of evidence indicate that BEN1 is possibly involved in a BR metabolic pathway. First, a gain-of-function allele, *ben1-1D*, or overexpression of *BEN1* in the *bri1-5* background, severely enhances the defective phenotypes of *bri1-5*. In fact, the phenotypes of *ben1-1D bri1-5* double mutant plants resemble the phenotypes of null alleles of *bri1*, such as *bri1-3* or *bri1-4* (Noguchi et al., 1999). Second, a loss-of-function mutant of *BEN1*, *ben1-1*, showed an obvious organ-elongation phenotype, opposing the phenotype of the gain-of-function mutant, *ben1-1D*, but similar to the *DWF4* overexpression phenotype (Choe et al., 2001). Third, BR profile analyses indicated that several major BRs such as TY, CS and BL are significantly reduced by twofold to fourfold in 5-week-old *ben1-1D bri1-5* double mutant seedlings compared to *bri1-5* single mutant seedlings. Conversely, in *ben1-1* knock-out seedlings, although BL was not detectable, accumulation of the bioactive CS was significantly elevated compared to the wild-type background. It is assumed that the elongated organ phenotype of *ben1-1* is due to the accumulation of bioactive BRs compared to its background. Fourth, consistent with

its role in BR metabolic pathway, overexpression of *BEN1* was able to upregulate key BR biosynthesis genes such as *CPD* by a feedback gene regulation loop that has been well characterized previously (Tanaka et al., 2005). *BAS1*, known to be upregulated by the exogenous application of BL, was downregulated in *BEN1* activation-tagged plants. These characteristics are similar to those of other gain-of-function BR metabolic genes, such as *bas1-D*, *shk1-D/sob7-D* and *UGT73C5*. Finally, feeding with 24-epiBL can restore the growth of shortened *ben1-1D* hypocotyls.

BL does not appear to significantly regulate the expression of *BEN1* at a transcriptional level, similar to the effect of BL on another BR homeostasis maintenance gene *SHK1/SOB7/CHI2* (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). *BEN1* expression is noticeably upregulated by light as detected by our semi-quantitative RT-PCR analysis (Figure 7d). Seedlings in continuous white light showed a fourfold to fivefold increase in *BEN1* expression levels compared with those grown in darkness. Consistently, both gain-of-function *ben1-1D* and loss-of-function *ben1-1* mutant plants show clear altered growth phenotypes only when they are grown in light. Under light, the phenotypes of *ben1-1D* and *ben1-1* are opposite: whereas *ben1-1D* plants show a weak BR-deficiency phenotype, *ben1-1* plants exhibit a BR-overproducing phenotype. These results suggest that BEN1 may be primarily responsible for the metabolism/inactivation of abundant BRs under illuminated conditions. Another BR inactivation protein, SHK1/SOB7/CHI2, may be responsible for BR inactivation in the dark because *SHK1* is upregulated in the absence of light (Takahashi et al., 2005).

BEN1 is a close paralog of two well-characterized flavonoid biosynthesis enzymes, DFR and BAN. DFR catalyzes the reduction of dihydroflavonols to leucoanthocyanins, which is a key step in the biosynthesis of anthocyanin (Winkel-Shirley, 2001; Shimada et al., 2004; Xie et al., 2004; Lo Piero et al., 2006); whereas BAN reduces anthocyanidins to 2,3-cis-flavan-3-ols, a precursor for condensed tannins (Devic et al., 1999; Xie et al., 2003). The three-dimensional structures of flavonoids and BRs are similar, with the connected rings A and C in flavonoids being analogous to rings A and B in BRs (Winkel-Shirley, 2001; Fujioka and Yokota, 2003). As DFR catalyzes a reduction step at carbon 4 within ring C of dihydroflavonols (structurally equivalent to carbon 6 in ring B of BRs), it is plausible to hypothesize that BEN1 could be a steroid reductase that catalyzes the conversion of TY, CS and BL to biologically inactive 6-OHTY, 6-OHCS and 6-OHBL, respectively (Figure 10). Consistent with this hypothesis, TY, CS and BL appear to be preferred targets of BEN1 relative to 6-deoxoTY and 6-deoxoCS as reflected by the BR profile analysis, especially when *ben1-1D* single mutant was analyzed (Table 1). The only structural differences between 6-deoxoTY and TY, or 6-deoxoCS and CS, are at the carbon 6 position. Feeding experiments using CS, however, did not generate any detectable 6-OHCS. There are a few possible explanations as to why the accumulation of 6-OHCS was not detected by *in vivo* feeding experiments. First, 6-OH BRs may be unstable and may undergo a rapid degradation process or conjugation with other molecules *in vivo*. Second, the resultant 6-OH BRs from feeding experiments may be undetectably low.

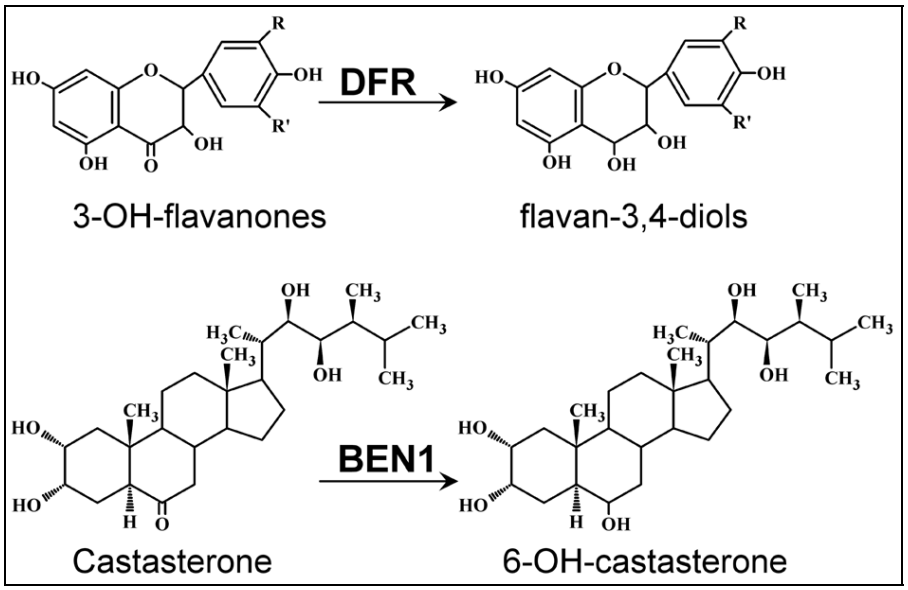

Figure 10. The DFR-catalyzed reaction in flavonoid biosynthesis and one of the possible roles of BEN1 in regulating BR homeostasis.

It is fully possible, however, that BEN1 may have a different enzymatic activity. It has been reported that BAN possesses both reductase and epimerase activities (Xie et al., 2003), and the putative NADPH-binding domain identified in BEN1 is conserved in the mammalian 3β-hydroxysteroid dehydrogenases/epimerases/DFR superfamily (Lacombe et al., 1997). 3β-hydroxysteroid dehydrogenases, epimerases and DFR are probably evolved from the same ancestor. When we tested the possible formation of various BR epimers and 3β-dehydroBL after feeding the *bri1-5 ben1-1D* plants with BL, no epimers or 3β-dehydro BL were identified. It is clear that, in the *bri1-5 ben1-1D* mutant, the levels of TY, CS and BL are significantly reduced relative to those in *bri1-5*

plants. But, because there is no detectable accumulation of teasterone (TE) or 6-deoxoteasterone (6-deoxoTE) in *bri1-5 ben1-1D* plants, BEN1 is unlikely to catalyze the reversible reaction that was previously reported between TE and TY in cultured cells of *Marchantia polymorpha* (Park et al., 1999).

In conclusion, we have discovered a new protein regulating endogenous BR steady-state levels in *Arabidopsis* by an activation-tagging approach for *bri1-5* extragenic modifiers. Although we favor the hypothesis that BEN1 is involved in BR inactivation (Figure 10), it is possible that BEN1 regulates BR levels through an unknown indirect mechanism yet to be identified. For example, it was found that, because the three-dimensional structures of flavonoids are similar to those of steroids, various flavonoids such as genistein, luteolin and coumestrol can regulate the mammalian steroid hormone estrogen signaling pathway through direct interactions with estrogen receptors (Kuiper et al., 1998; Borras et al., 2006). It is possible that overexpression of *BEN1* directly modulates the biosynthesis of flavonoids, which then indirectly regulate BR biosynthetic or metabolic enzymes. As a result, several BRs are reduced. Our attempts to define the biochemical function of BEN1 were mainly based on our current knowledge of BR metabolism. Many metabolites known today have been discovered solely from feeding experiments using exogenously applied BRs, which may not always accurately reflect endogenous metabolism of BRs. The future discovery of new endogenous metabolic pathways of BRs is key to understanding the role of BEN1. Understanding the regulation of bioactive BRs will facilitate our control of plant growth and development by manipulating key metabolic enzymes such as BEN1.

Experimental procedures

Plant material and mutant screening

The *ben1-1D* mutant was obtained by screening extragenic modifiers of the *bri1-5* mutant as described previously (Li et al., 2001a; Li et al., 2002; Zhou et al., 2004). The activation-tagging construct used, pBASTA-AT-2, was a modified version of SKI015. The Pmas promoter and BASTA coding region were PCR-amplified and cloned into the *Bam*HI and *Hin*dIII sites of pBIB-KAN (Becker et al., 1992). The *Bam*HI restriction site was eliminated after *Bam*HI–*Bgl*II ligation. Synonymous mutations were introduced into the BASTA sequence to eliminate all common restriction sites. The resulting vector was named pBASTA. The vector was sequenced, and its resistance to Basta herbicide was confirmed in transgenic plants. Four copies of the CaMV 35S enhancer were subsequently ligated into the *Kpn*I and *Eco*RI sites of pBASTA to create the pBASTA-AT2 activation-tagging construct. The T-DNA region of the pBASTA-AT2 construct is much smaller than that in SKI015, and its transformation efficiency was greatly elevated. Both *bri1-5* and *det2-28* were in *Arabidopsis* ecotype WS-2 background. The *ben1-1* knock-out line was from the SALK pool, which is in an ecotype Col-0 background.

Determination of the ben1-1D locus

TAIL-PCR was used to amplify the flanking genomic sequence of the T-DNA of pBASTA-AT2 as described previously (Liu and Whittier, 1995; Terauchi and Kahl, 2000).The T-DNA insertion site was determined by sequencing the flanking genomic

DNA. The T-DNA was inserted between *At2g45380* and *At2g45400*. The activation-tagged gene was determined by RT-PCR.

Recapitulation analysis

cDNA of *BEN1* was amplified by RT-PCR using primers *BEN1*-fw (5'-TCTGGTACCATGGTGAGAGAAGAACAAGAAGAAGA-3') and *BEN1*-rv (5'-TCTGGTACCTTAAAGAAATCCCCTTGCTTGA-3'), and cloned into pBASTA-35S, which we routinely use for gene overexpression in transgenic *Arabidopsis*. The resulting construct, pBASTA-35S-BEN1, was transformed into *bri1-5* plants. T_1 transgenic plants were selected and analyzed for a *bri1*-5-enhanced phenotype.

RT-PCR analysis

Total RNA was isolated using RNeasy plant mini kits with on-column DNase-treatment (Qiagen; http://www.qiagen.com/). Total RNA (5 μg) was converted to form the first strand of the cDNA in a 50 μl volume using the SuperScript III first-strand synthesis system (Invitrogen; http://www.invitrogen.com/). A 3 μl volume of RT product was used as a PCR template. Preliminary experiments were performed to determine the exponential range of RT-PCR for each individual gene. Three determined cycles were used for each gene. PCR products for the various numbers of cycles were separated by 1% agarose gel electrophoresis and blotted to Biotrans nylon membrane (Amersham Biosciences, http://www.amersham.com). The PCR amplification signals were detected by DNA hybridization with digoxigenin (DIG)-labeled gene-specific probes, and then exposed on X-ray film.

The *BEN1* gene was amplified using primers *BEN1*-fw and *BEN1*-rv; *BAS1* was amplified using primers *BAS1*-fw (5'-ATGGAGGAAGAAAGTAGCAGCT-3') and *BAS1*-rv (5'-TCAATCCTCATGATTGGTCAAT-3'); *CPD* was amplified using primers *CPD*-fw (5'-ATGGCCTTCACCGCTTTTCTCC-3') and *CPD*-rv (5'-TCAAGTAGCA AAATCACGGCGC-3'); *EF1*α was amplified using primers *EF1*α-fw (5'-CAGGCTGA TTGTGCTGTCCT-3') and *EF1*α-rv (5'-TCAAGTAGCAAAATCACGGCGCTT-3'); *ACTIN2* was amplified using primers *ACTIN2*-fw (5'-AGCGCTGAGGCTGA TGATATTCAAC-3') and *ACTIN2*-rv (5'-TCTAGAAACATTTTCTGTGAACGATTC-3'). Both RT-PCR and quantitative RT-PCR for the detection of each transcript were in the linear range of accuracy and repeated four times. All the results shown were repeated four times.

Knock-out mutant screening

The homozygous *ben1-1* T-DNA insertion line was obtained by PCR analysis. Leaves from individual SALK_128043 seedlings were collected and total genomic DNA was isolated. Using the gene-specific primer *BEN1*-rv and a T-DNA-specific primer LBb1 (5'-ATGGTTCACGTAGTGGGCCATC-3') for PCR amplification, the T-DNA insertion lines can be determined. These lines were further analyzed by PCR using a pair of gene-specific primers, *BEN1*-fw and *BEN1*-rv, flanking the T-DNA insertion site to determine homozygous lines.

Hypocotyl measurements

All of the seeds used for hypocotyl analysis were surface-sterilized as described previously (Li et al., 2001a) and planted on semi-solid half-strength MS medium supplemented with 1% sucrose, 0.8% agar and various concentrations of 24-epiBL. The plates were kept at 4°C for 2 days, and then irradiated under white light for 8 h at 22°C to induce uniform germination. Seedlings were grown vertically in continuous white light (for 24-epiBL treatment analysis) or transferred into light or darkness (for light/dark analysis) at 22°C. After 6 days, the seedlings were transferred onto agar plates and scanned using a scanner at the 600 dpi resolution. The hypocotyls were measured using ImageJ 1.29J (National Institutes of Health imaging software; http://rsb.info.nih.gov/ij/java1.3.1). All measurements were repeated three times independently, and 60 to 90 seedlings were measured each time.

Confocal microscopy analysis

The fusion construct 35S-*BEN1*–GFP was constructed by cloning a *BEN1* cDNA fragment, amplified via RT-PCR using primers BEN1-fw and BRE-GFP-rv (5'-TCTGGATCCAAGAAATCCCCTTGCTTGACAGCTCCT-3'), in-frame into a pBASTA-35S–GFP construct modified in the Li laboratory. Transgenic plants were generated by the floral dipping method (Clough and Bent, 1998). T_2 seeds were grown vertically on semi-solid half-strength MS medium containing 1% sucrose and 0.8% agar under continuous light. Non-transgenic seedlings grown under the same conditions were used as negative controls. Root tips of 3–5-day-old seedlings were used for confocal analysis using an Olympus FluoView 500 laser scanning microscope

(http://www.olympus-global.com/) as previously described (Zhou and Li, 2005). Mannitol solutions of 0.8 M were used to induce plasmolysis.

Promoter::GUS analysis

A 2.2 kb *BEN1* promoter region was PCR-amplified using primers BEN1p-fw (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTGGATCTCTCTCATGCTTCTCT-3') and BEN1p-rv (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTCTCTCTGTCTCTTTCTCTCTCT-3') and cloned into the pBIB-BASTA-GUS-GWR vector routinely used in the Li laboratory. Transgenic plants were generated by the floral dipping method (Clough and Bent, 1998). T_2 transgenic seeds harboring *BEN1p*–GUS were germinated either on semi-solid half-strength MS medium containing 1% sucrose and 0.8% agar for root or seedling staining, or in soil for GUS expression analysis of later plant stages. Seedlings or tissues at various developmental stages were vacuum-infiltrated in X-Gluc solution (Robatzek and Somssich, 2001), followed by 6 h incubation at 37°C, and destained using several changes of 70% ethanol. The destained tissues were visualized for blue color using a dissecting microscope.

BR profile analysis

Aerial parts of seedlings (5-week-old, 20–30 g fresh weight) grown under 16 h light (150–200 μmol m^{-2} sec^{-1}) 8 h^{-1} dark were harvested and lyophilized. The tissues were extracted twice with 500 ml of MeOH. Deuterium-labeled internal standards were added to the samples, and BR quantification was performed using GC/MS as described previously (Fujioka et al., 2002).

Feeding experiments

Seedlings were grown on semi-solid half-strength MS medium supplemented with 1% sucrose and 0.8% agar under continuous white light. One-week-old seedling were transferred into 250 ml flasks containing liquid half-strength MS medium supplemented with 1% sucrose, and incubated for 2 days with shaking. CS or BL (5 µl of 1 µg μl^{-1} concentration) or 5 µl of 100% ethanol (mock treatment) were added to the flasks and incubated for 2 days. These samples were extracted twice with MeOH. Metabolites were purified and analyzed by GC/MS as described previously (Fujioka et al., 2000a).

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Note

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Chapter III. Role of *BEN2* in regulating hypocotyl growth is mediated by brassinosteroids

Summary

The *ben2-1D* (*bri1-5 en*hanced *2-1d*ominant) was isolated by an activation tagging in the *bri1-5* background for putative genetic modifiers of *bri1-5*. Detailed morphological analyses show that the *bri1-5 ben2-1D* double mutant exhibited more compact rosette leaves and significantly later flowering phenotypes than *bri1-5*. Molecular cloning and sequencing suggest that a single gene, *BEN2*, was activation tagged in *bri1-5 ben2-1D* plants. Overexpression of *BEN2* in *bri1-5* driven by a constitutive Cauliflower mosaic virus 35S (CaMV 35S) promoter recapitulated the phenotypes of *bri1-5 ben2-1D* plants. BEN2 was previously reported as a DNA-binding-with-one-finger (Dof) transcription factor, which negatively regulates the phytochrome signaling pathway. Interestingly, when the double mutant was backcrossed with a wild-type *Arabidopsis* WS2 plant, and the *bri1-5* mutation was segregated out, the resulting plants showed leaf phenotypes opposite to those of the double mutant. Instead of more compact rosette leaves and smaller epidermal cells in the double mutant, the *ben2-1D* single mutant shows elongated leaves and enlarged epidermal cells. In addition, the regulation of hypocotyl growth by *BEN2* appears to be directly tied to the BR signaling pathway. The *ben2-1D* single mutants grown in the light show more elongated hypocotyls than wild-type plants, whereas the length of hypocotyls in *bri1-5 ben2-1D* plants are similar to those of *bri1-5*. Furthermore, if the endogenous BRs are depleted by the treatment of brassinolide (BRZ), a specific BR biosynthesis inhibitor, the *ben2-1D* and wild-type plants show identical phenotypes. Thus, the function of BEN2 in regulating cell elongation and plant growth is dependent on a functional BR signaling pathway. In addition, BEN2 may be involved in

regulating the gibberellin (GA) biosynthesis/signaling transduction pathway that controls *Arabidopsis* flowering time. The specific role of BEN2 in connecting phytochrome signal transduction, BR biosynthesis/signal transduction, and GA biosynthesis/signal transduction pathways needs to be further examined in the future.

Keywords: activation-tagging, BEN2, Dof domain, flowering time, brassinosteroids, brassinolide

Introduction

Plant growth hormones, like auxins, gibberellins (GAs), cytokinins and brassinosteroids (BRs), are critical for normal plant growth and development. Deficiencies of any of these hormones will cause aberrant growth or even lethality. For example, mutant plants defective in BR biosynthesis or signal transduction display dramatic growth defects, including dwarfism, delayed senescence, reduced organ expansion, and male sterility (Chory et al., 1991; Clouse et al., 1996; Kauschmann et al., 1996; Li et al., 1996a; Szekeres et al., 1996; Li and Chory, 1997; Choe et al., 1998; Noguchi et al., 1999; Friedrichsen et al., 2000; Choe, et al., 2001). Earlier studies suggested that many of these processes are regulated at the transcriptional level (Weaver, 1999). Several transcription factors implicated in BR signal transduction pathway, such as BES1 and BZR1, have been identified via genetic analyses (Wang, et al., 2002; Yin, et al., 2002; Li, and Deng, 2005; Mussig, et al., 2006; Deng, et al., 2007).

Regulation of gene transcription in eukaryotes is more complex than that in prokaryotes. Prokaryotic RNA polymerases can directly bind to promoters, but eukaryotic RNA polymerases rely on general transcription factors to guide them to the promoter regions and make use of gene-specific transcription factors to modulate transcription activation and gene expression in a timely and tissue specific manner. In *Arabidopsis thaliana*, over 5% of the genome encodes approximately 1,500 transcription factors, classified into approximately 50 groups (Riechmann et al., 2000). One of these groups, the Dof-domain transcription factors, are plant-specific transcription factors that regulate a variety of physiological processes such as seed germination and plant responses to various internal and external developmental signals (reviewed by Yanagisawa, 2002; Yanagisawa, 2004).

Like most transcription factors, Dof-domain proteins contain at least two functional domains: a DNA-binding domain and a transcription-activation domain. The DNA-binding domain of Dof proteins is highly conserved and contains a CysX2CysX21CysX2C -type zinc finger motif (where X represents any amino acid) in a 52 amino acid region located at the N-terminus. The transcription-activation domains in the Dof proteins, on the other hand, are divergent, which allows proteins to have numerous functions (Yanagisawa, 2002; Yanagisawa, 2004).

Since the identification of the first Dof protein in maize (Yanagisawa and Izui, 1993), many Dof proteins and their genes have been isolated using a variety of different biochemical and genetic approaches. For example, MNB1, PBF, and StDof1 were identified in maize and potato plants based on sequence homology (Yanagisawa, 1995; Vicente-Carbajosa, et al., 1997; Plesch, et al., 2001). OBP1 was isolated from

Arabidopsis based on its ability to interact with OBF4 protein (Zhang et al., 1995), and NtBBF1 was isolated by its ability to bind domain B, a regulatory domain necessary for expression of a plant oncogene *rolB* in tobacco (DePaolis, et al., 1996). AOBP was isolated from pumpkin using a Southwestern method for its specific binding to the silencer region of an ascorbate oxidase gene (Kisu, et al., 1998). *GmDof4* and *GmDof11* genes were identified in soybean by analyzing their expressed sequence tags (Tian, et al., 2004; Wang, et al., 2007). Similar to defining the functions of many plant genes, mutant analyses were also used to determine the importance of Dof in normal plant growth and development.

Dof mutants were identified mainly by three different genetic approaches. 1) Loss-of-function analysis. For instance, *dag1* was the first Dof mutant isolated by T-DNA insertion analysis. The *dag1* mutant, in which the *Dof Affecting Germination* (*DAG1*) gene was disrupted by the inserted T-DNA, shows a seed nondormant phenotype (Gualberti, et al., 2002; Papi, et al., 2000; Papi, et al., 2002). 2) Constitutive overexpression of Dof transcription factors under the control of the CaMV 35S promoter followed by the phenotypic analysis. Skirycz et al.(2006,2007) carried out a screen of plants constitutively overexpressing every *Arabidopsis* Dof transcription factor under the control of the CaMV 35S promoter. They identified *AtDof1.1* and *AtDOF4.2*. *AtDof1.1* is involved in controlling glucosinolate biosynthesis (Skirycz et al., 2006). *AtDOF4.2* was identified as a gene inducing a bushy plant phenotype and potentially being involved in the regulation of phenylpropanoid metabolism (Skirycz et al., 2007). In addition, plants overexpressing the salicylic acid-inducible Dof domain protein, *OBP3*, displayed yellow leaves and abnormal root growth phenotypes (Kang and Singh,

2000). 3) *Arabidopsis* Dof transcription factor mutants were also obtained by screening an activation tagged pool. Activation tagging uses an *Agrobacterium*-mediated transformation approach to randomly insert a T-DNA fragment, containing four copies of an enhancer element, into *Arabidopsis* genome. The enhancers, obtained from the CaMV 35S promoter, strongly activate the expression of a gene proximity to the insertion (Weigel et al., 2000). This strategy gave us a new way of revealing the function of many genes that could not be found by loss-of–function approaches because their phenotype would be masked by other functional redundant genes in the *Arabidopsis* genome (*Arabidopsis* Genome Initiative, 2000). By screening the long hypocotyl phenotype in the light, an *Arabidopsis* dominant Dof mutant, *cog1*, was identified. COG1 was regarded as a negative regulator of both the phytochrome A and the phytochrome B signaling pathways (Park et al., 2003). In *sob1-D* mutant, a Dof transcription factor, *OBP3*, was activation tagged (Ward et al., 2005).

We carried out a large-scale activation tagging in *bri1-5* to screen for novel BR signaling or biosynthesis regulators. The semi-dwarfed intermediate *bri1-5* mutant was an ideal genetic material for screening its extragenic modifiers. *bri1-5* mutant carries a point mutation in the BR receptor BRI1 that substitutes C to Y at position 69 in the extracellular domain (Noguchi et al., 1999). The *bri1-5* plants exhibit normal male fertility which permits a feasible large scale genetic screen using a simple floral dipping transformation method (Li et al., 2002a; Li et al., 2002b). The semi-dwarfed phenotype made it possible to screen both genetic suppressors and genetic enhancers, which show enlarged plant phenotypes or reduced plant phenotypes, respectively. Using this BRI1 defective background, we could also find genes which may have crosstalk with the BL

signaling pathway, such as the newly identified mutant—*ben2-1D* (*bri1-5 enhanced 2-1 Dominant*), discussed in this chapter. The *ben2-1D* is a dominant Dof mutant, previously named *COG1* (Park et al., 2003). The *cog1* mutant, which was isolated in a wild-type background, displays long hypocotyls in light and exhibits hyposensitive responses to red and far-red light (Park et al., 2003). Our studies uncovered new roles of this gene in regulating both BRI1-mediated cell elongation and GA-mediated flowering time phenotypes. Our studies indicate that BEN2/COG1 may play a significant role in regulating crosstalk among the phytochrome, BR, and GA signaling pathways.

Results

ben2-1D and ben2-2D were identified as genetic enhancers of bri1-5 using large-scale activation-tagging screen

After screening 120,000 *bri1-5* activation tagged plants, we identified two dominant mutants with similar phenotypes: dark green leaves and extremely delayed flowering phenotypes (Figure 1A, 1B). Although at maturity, both mutants are taller than the *bri1-5,* the leaf petioles in mutants are much shorter and blades are much smaller and more curled than those of *bri1-5* (Figure 1C). Because these two lines have a similar phenotype which resembles that of the *bri1-5 ben1-1D* previously described (Yuan et al., 2007), we designated these mutants as *bri1-5 enhanced 2-1Dominant* (*ben2-1D*) and *bri1-5 enhanced 3-1Dominant* (*ben3-1D*). *ben3-1D* was renamed as *ben2-2D* because it was discovered to be allelic to *ben2-1D* after the response gene was cloned and analyzed.

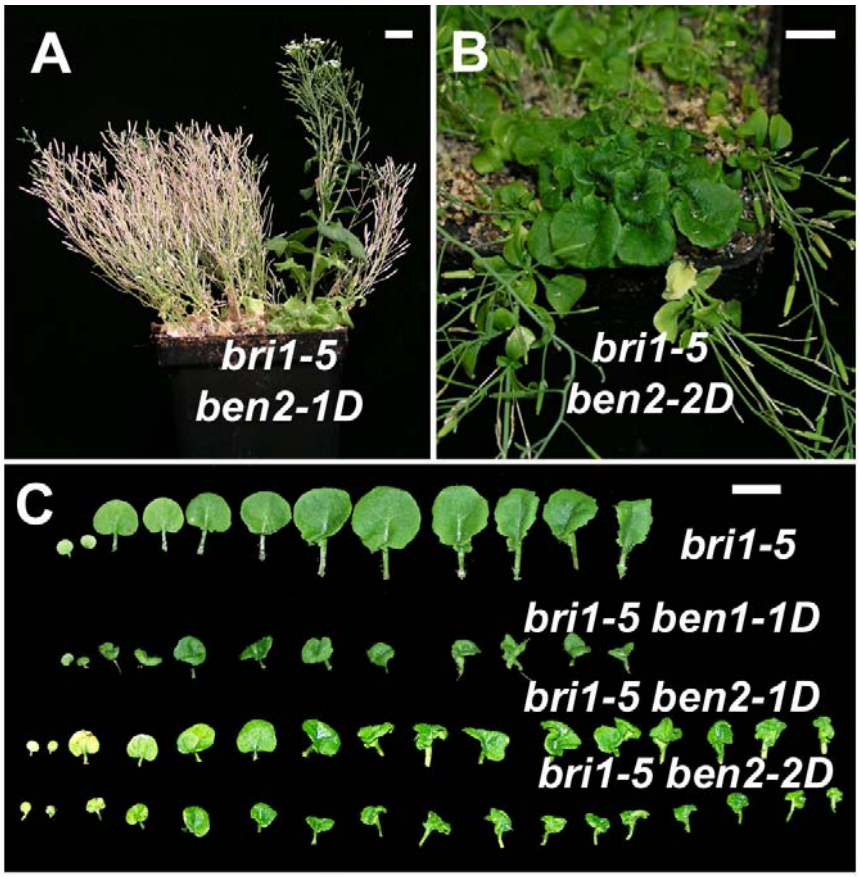

Figure 1. *ben2-1D* and *ben2-2D* were identified by an activation tagging genetic modifier screen in *bri1-5*. **A.** Phenotypes of an 8-week-old *bri1-5 ben2-1D* plant. Scale bar =1 cm. **B.** Phenotypes of a 6-week-old *bri1-5 ben2-2D* plant. Scale bar =1 cm. **C.** Leaf phenotypes from 4-week-old *bri1-5*, *bri1-5 ben1-1D, bri1-5 ben2-1D,* and *bri1-5 ben2-2D* plants. Scale bar = 1 cm.

Activation tagged At1g29160 is responsible for the bri1-5 ben2-1D and bri1-5 ben2-2D phenotypes

The genomic sequences flanking the right border of the activation tagged T-DNA insertions in *ben2-1D* and *ben2-2D* were amplified by thermal asymmetry interlaced-PCR (TAIL-PCR; Figure 2A, 2B).

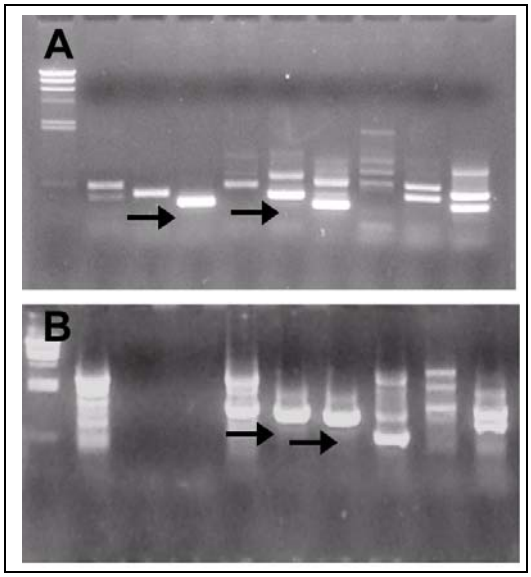

Figure 2. TAIL-PCR for *bri1-5 ben2-1D* (A) and *bri1-5 ben2-2D* (B) mutants. The arrows indicate the ladder PCR products used for sequencing.

The PCR products were sequenced. A blast search revealed that the activation tagged T-DNAs were inserted in the intergenic region between genes *At1g29160* and *At1g29170* in both mutants (Figure 3A, 4A). RT-PCR analysis revealed that *At1g29160* was the only gene overexpressed in both *bri1-5 ben2-1D* (Figure 3B) and *bri1-5 ben2-2D* (Figure 4B) mutants. Other genes (*At1g29150, At1g29170,* and *At1g29180*), which were

physically close to the inserted T-DNA site, were not overexpressed in the activation tagged mutants (Figure 3B, 4B). Thus, *At1g29160* was considered as a reasonable candidate of the tagged *BEN2*.

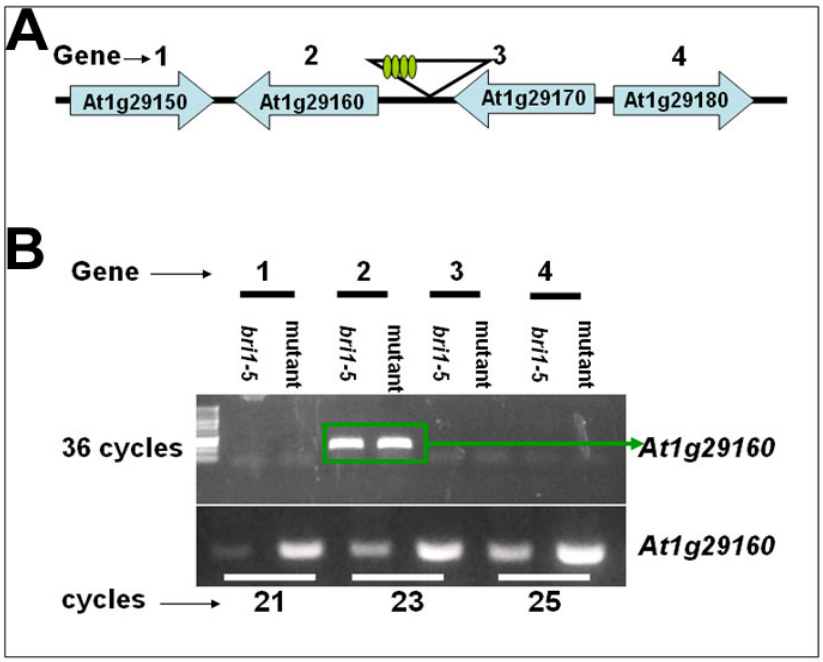

Figure 3. The *ben2-1D* locus and its nearby genes. **A.** The T-DNA was inserted between genes *At1g29160* and *At1g29170* in the *ben2-1D* mutant, with the left border oriented toward to the *At1g29160*. The ovals represent the four 35S enhancers from pBASTA-AT-2. **B.** RT-PCR analysis indicated that *At1g29160* was overexpressed in the tagged mutant. Upper lanes: at 36 cycles, *At1g29160* was overexpressed in *bri1-5* and in the mutant; the expressions of other nearby genes *At1g29150, At1g29170,* and *At1g29180* were not detected. Lower lanes: RT-PCR for *At1g29160* with different cycles, showing *At1g29160* gene was overexpressed in the mutant.

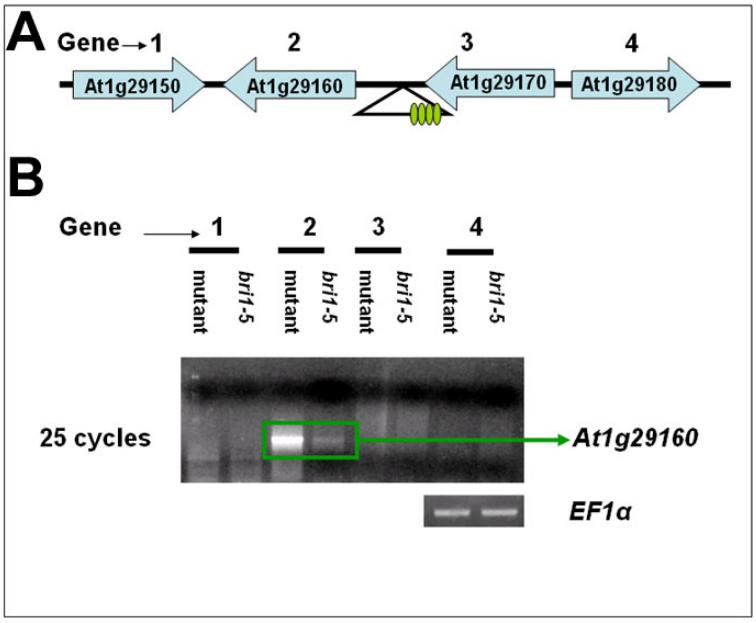

Figure 4. Determination of the *ben2-2D* locus. **A.** T-DNA of *ben2-2D* was also inserted between genes *At1g29160* and *At1g29170* with the left border oriented toward *At1g29170*. The ovals represent the four 35S enhancers from the activation construct, *pBASTA-AT-2*. **B.** RT-PCR analysis of *bri1-5 ben2-2D* mRNA, indicating that *At1g29160* was overexpressed compared to *bri1-5,* but *At1g29150, At1g29170* and *At1g29180* were not expressed. *EF1*α was used as the quantitative control gene.

The activation tagging T-DNA was inserted 1400 bp and 1100 bp upstream of its putative translational start codon of *At1g29160* (Figure 5). Because the same gene was activation tagged in *bri1-5 ben2-1D* and *bri1-5 ben2-2D* mutants, our studies were focused only on the *bri1-5 ben2-1D* mutant.

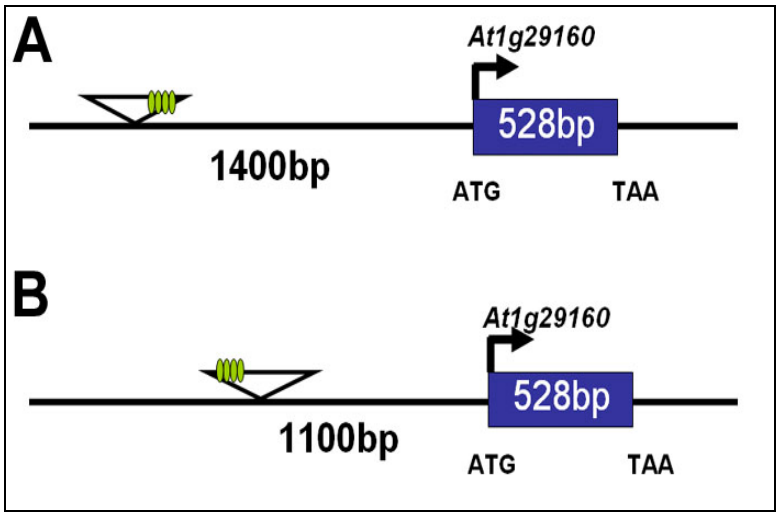

Figure 5. Gene maps for *ben2-1D* (A) and *ben2-2D* (B). **A.** In *ben2-1D*, the T-DNA from activation tagging construct pBASTA-AT-2 was inserted 1400bp upstream from the *At1g29160* start codon, with the left border oriented toward to the *At1g29160* start codon. The ovals indicate the four 35S enhancers from pBASTA-AT-2. **B.** In *ben2-2D*, the activation tagging T-DNA was inserted 1100 bp upstream of *At1g29160* start codon, with the left border oriented away from the start codon.

A single T-DNA insertion was found in bri1-5 ben2-1D; homozygous plants show extremely compact rosettes and delayed flowering phenotype

To test whether a single T-DNA was inserted in the *bri1-5 ben2-1D* mutant, various genetic segregation analyses were employed. The results were summarized in Table 1. The information is important for us to determine whether the phenotypes were from the

overexpression of *At1g29160*. The offspring seedlings from the self-pollinated *bri1-5(-/-) ben2-1D (+/-)* showed a BASTA-resistant to BASTA-sensitive ratio at the expected 3:1 (data not shown) suggesting a single T-DNA insertion in the activation tagged mutant. Further analyses also revealed the dosage effect of the activation tagged gene. For example, the BASTA-resistant mutants could divided into two phenotypic classes: (1) extremely compact and extremely delayed flowering plants and (2) semi-compact plants resembling the originally identified mutant (Figure 6), with an approximately 1:2 ratio (data not shown). We hypothesized that the original mutant that caused the compact phenotype was a dosage effective dominant heterozygote and the three phenotypic classes (BASTA-sensitive, BASTA-resistant semi-compact, BASTA-resistant extremely compact) were consistent with plants containing no T-DNA insertion, a heterozygous insertion, or a homozygous insertion. To confirm this hypothesis, we self-pollinated the semi-compact and extremely compact mutants and analyzed the phenotypes of the resulting progeny. Extremely compact mutants produced only extremely compact-type progeny, and semi-compact mutants segregated into three phenotypic classes with a BASTA-resistant to BASTA-sensitive ratio of 3:1 (Table 1). In addition, we backcrossed the semi-compact *bri1-5 ben2-1D* mutants with wild-type (WS2) plants and obtained wild-type-like F_1 progeny with a BASTA-sensitive to BASTA-resistant plants ratio of approximate 1:1. Each BASTA-resistant F_1 offspring gave rise to an F_2 population with four phenotypes: (1) wild-type-like BASTA-resistant plants, (2) *bri1-5*-like BASTA-resistant plants, (3) wild-type-like BASTA-sensitive plants, and (4) *bri1-5*-like BASTA-sensitive plants at a ratio of approximate 9:3:3:1 (Table 1). To test segregation in the wild-type background, we segregated the *bri1-5* mutation by genotypic screening in the

Table 1. Segregation analysis of *BEN2* mutants								
Plant line	Total	No. of plants				Observed Ratio	χ_c^{2*}	*P**
		BASTA-resistant		BASTA-sensitive				
		WT-like	*bri1-5*-like	WT-like	*bri1-5*-like			
bri1-5 ben2-1D (T3) / from T2 self (predicted ratio 3:1)								
line-1	228		179		49	3.65:1	1.315	0.221
line-2	57		42		15	2.8:1	0.006	0.819
line-3	41		32		9	3.56:1	0.073	0.652
line-4	30		22		8	2.75:1	0.001	0.833
line-5	109		83		26	3.19:1	0.028	0.782
line-6	78		59		19	3.1:1	0.001	0.896
line-7	37		27		10	2.7:1	0.009	0.776
line-8	48		48		0			
line-9	67		67		0			
line-10	52		52		0			
bri1-5 ben2-1D x WS2 (predicted ratio 1:1)								
F1	37	15	0	22	0			
F2 (predicted ratio 9:3:3:1)								
line-1	70	37	16	14	3	12.3:5.3:4.7:1	1.263	0.738
line-2	42	30	4	6	2	15:2.4:3:1	4.222	0.238
line-3	100	60	12	23	5	12:2.4:4.7:1	3.893	0.273
line-4	62	33	15	9	5	6.6:3:1.8:1	2	0.572
line-5	53	28	15	8	2	14:7.5:4:1	3.587	0.310
line-6	182	108	38	27	9	12:4.2:3:1	2.733	0.435
line-7	193	114	28	39	12	9.5:2.3:3.3:1	2.344	0.504
line-8	158	90	30	27	11	8.2:2.7:2.5:1	0.380	0.944
line-9	206	116	44	38	8	14.5:5.3:4.7:1	2.604	0.457
line-10	162	97	30	25	10	9.7:3:2.5:1	1.336	0.721
line-11	120	68	28	19	5	13.6:5.6:3.8:1	2.726	0.436
line-12	267	147	58	45	17	8.6:3.4:2.6:1	1.844	0.605
line-13	68	36	13	14	5	7.2:2.6:2.8:1	0.392	0.942
line-14	59	32	12	11	4	8:3:2.8:1	0.149	0.985
F3-segregated out *bri1-5* by genotypic screening (predicted ratio 3:1)								
line-1	63	49		14		3.5:1	0.132	0.611
line-2	43	31		12		2.58:1	0.070	0.660
line-3	23	18		5		3.6:1	0.014	0.718
line-4	259	202		57		3.54:1	1.082	0.266
line-5	42	32		10		3.2:1	0.001	0.859
line-6	49	37		12		3.1:1	0.007	0.934
line-7	33	25		8		3.1:1	0.010	0.920
line-8	114	114		0				
line-9	63	63		0				
line-10	227	227		0				

* When $\chi_c^2 < \chi_{0.05}^2$, or $P > 0.05$ (df =1, $\chi_{0.05}^2 = 3.840$; df =3, $\chi_{0.05}^2 = 7.810$), there is no statistical differences between actual values and the expected values.

wild-type-like BASTA-resistant plants and examined some of these T3 lines. As expected, the phenotypes of BASTA-resistant to BASTA-sensitive in T3 were 3:1 (Table 1).

Unlike the dominant mutant *bri1-5 ben1-1D*, which has the same phenotype in heterozygous and homozygous plants, *bri1-5(-/-) ben2-1D (-/+)* plants show different phenotypes to those of *bri1-5 (-/-) ben2-1D (-/-)* plants (Figure 6). The *ben2-1D* homozygous plants show more severe phenotypes than those of *ben2-1D* heterozygous plants. The rosette widths of the 4-week-old *bri1-5 ben2-1D (-/+)* and *bri1-5 ben2-1D (-/-)* homozygous plants are about one half and one third of *bri1-5*, respectively (Figure 6A, 6B). After six weeks growth, the *bri1-5 ben2-1D (-/+)* plants are taller than the *bri1-5* plants (Figure 6C, 6D). On the other hand, the *bri1-5 ben2-1D (-/-)* plant greatly enhances the defective phenotypes of *bri1-5* plant at all developmental stages, displaying extremely compact rosettes and short inflorescence length. The inflorescence length of the homozygous plant is about a third that of the wild-type plant even at nine weeks after germination (Figure 6E, 6F).

The leaf sizes of double mutants are much smaller than those of *bri1-5* single mutants, and the size of *bri1-5 ben2-1D* homozygous plants are significantly reduced compared to those of *bri1-5 ben2-1D* heterozygous plants, too (Figure 7A). However, the differences in leaf size between *bri1-5 ben2-1D (-/+)* and *bri1-5 ben2-1D (-/-)* plants could be attributed to blade size, since petioles had almost the same length (Figure 7B). Scanning electron microscopy (SEM) analysis of *bri1-5 ben2-1D (-/-)* blade showed that the epidermal cells are unevenly expanded along with numerous extremely small cells (Figure 7E). By contrast, no obvious differences were observed between epidermal cells in *bri1-5 ben2-1D (+/-)* and *bri1-5,* except that *bri1-5 ben2-1D (+/-)* has some

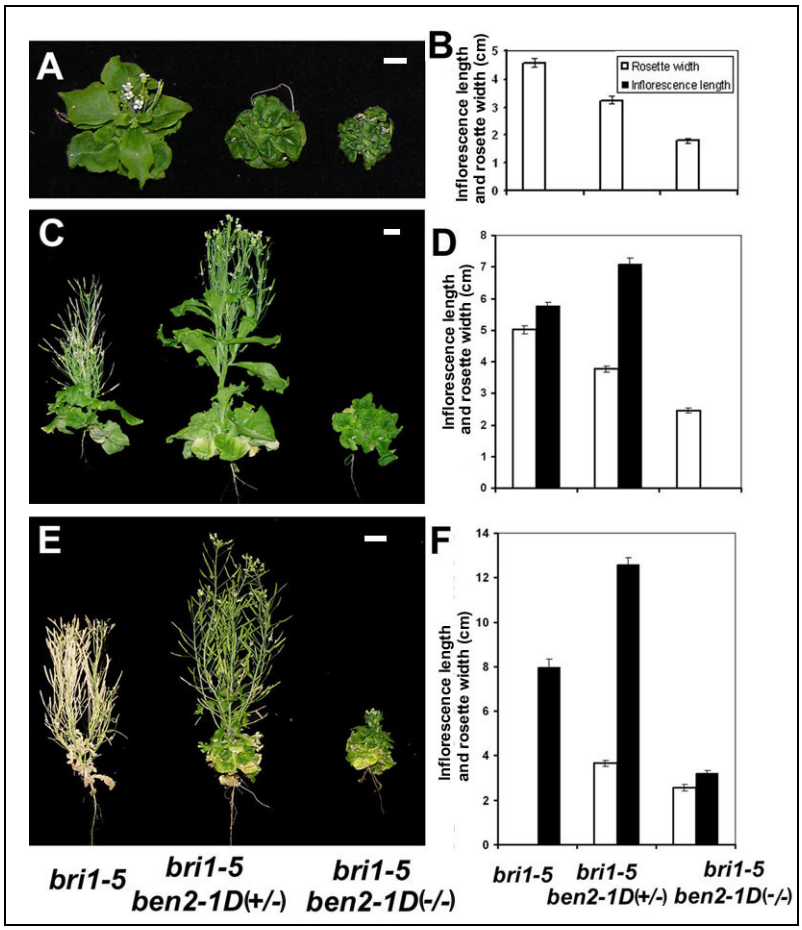

Figure 6. Phenotypic characterization of *bri1-5 ben2-1D* heterozygous and homozygous plants. **A. B.** 4-week-old *bri1-5 ben2-1D (-/+)* and *bri1-5 ben2-1D (-/-)* phenotypes (A) and inflorescence length and rosette width (B). Scale bar = 1 cm. **C. D.** 6-week-old *bri1-5 ben2-1D (-/+)* and *bri1-5 ben2-1D (-/-)* phenotypes (C) and inflorescence length and rosette width (D). Scale bar = 1 cm. **E. F.** 9-week-old *bri1-5 ben2-1D (-/+)* and *bri1-5 ben2-1D (-/-)* (E) and inflorescence length and rosette width (F). Scale bar = 2 cm. White columns show rosette width, and black columns show inflorescence length. The experiment was repeated three times and data from one representative experiment are shown. Inflorescence length and rosette width were presented as means ± standard errors (n = 30). Inflorescence lengths and rosette widths in mutants at different developmental stages were all statistically different (**) from those of *bri1-5,* based on Student's unpaired *t*-test, two-tailed ($P < 0.01$).

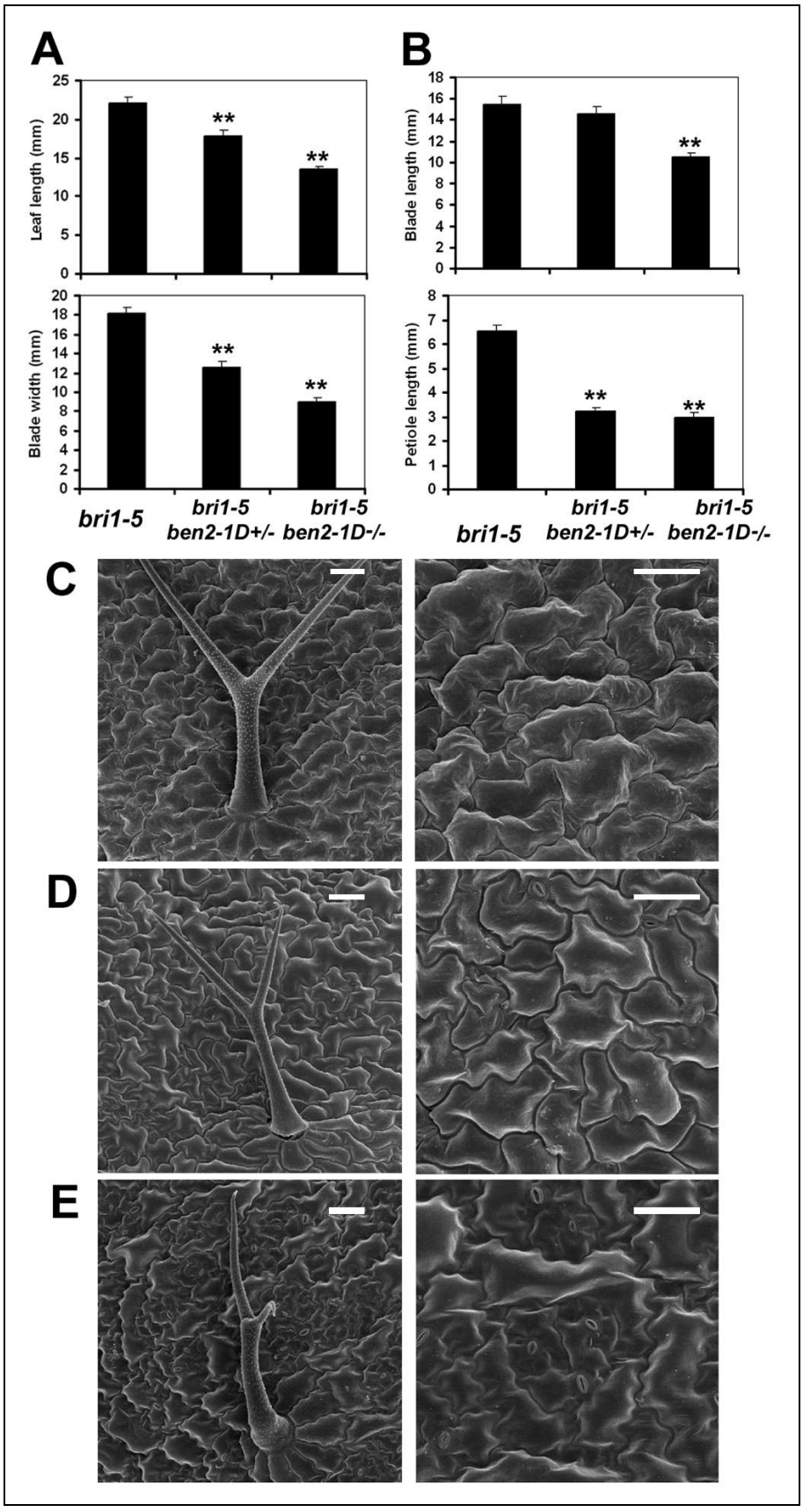
A
B
Leaf length (mm)
Blade length (mm)
Blade width (mm)
Petiole length (mm)
**
bri1-5
bri1-5 ben2-1D+/-
bri1-5 ben2-1D-/-
C
D
E

Figure 7. Leaf phenotypes from 4-week-old *bri1-5 ben2-1D (+/-)* and *bri1-5 ben2-1D (-/-)* plants. **A.** *bri1-5 ben2-1D* produces significantly smaller leaves than *bri1-5* single mutants, and the leaf size of homozygous plants is much smaller than those of heterozygous plants. **B.** Comparison of petiole and blade length. Differences in leaf size between *ben2-1D* heterozygous and homozygous plants were due to differences in blade size (upper panel). Petioles lengths were not significantly different between *ben2-1D* heterozygous and homozygous plants. However, they were significantly smaller than those of *bri1-5* (lower panel). ** Significant differences based on Student's unpaired *t*-test, two-tailed ($P < 0.001$). **C. D. E.** Scanning electron micrographs of epidermal cells from the third pair of leaves of 4-week-old (C) *bri1-5*, (D) *bri1-5 ben2-1D (+/-)* and (E) *bri1-5 ben2-1D (-/-)* plants. Scale bar = 40μm.

extremely small cell regions distributed on the blade (Figure 7C and 7D). The presence of smaller cells perhaps contributes to the smaller, curly leaf phenotype. The overall phenotype of homozygous *bri1-5 ben2-1D* closely resembles that of the more severe *bri1* mutants (Li et al., 2002).

The *bri1-5 ben2-1D* double mutants also show significantly delayed flowering time. Under long-day conditions, mutants have a longer period of vegetative growth and flowering time was delayed 4 to 7 weeks compared to *bri1-5* (Table 2). By the time of the first flower opened, the mutants had produced 14.6 to 31.9 more leaves than *bri1-5*.

Table 2. Flowering in *bri1-5 ben2-1D (+/-), bri1-5 ben2-1D (-/-), ben2-1D (+/-)* and *ben2-1D (-/-)* plants

Line	Bolting time [a] (days)	Number of leaves at the opening of the first flower
bri1-5	25.2±0.14	8.4±0.21
bri1-5 ben2-1D (+/-)	53.15±0.85**	23.0±0.74**
bri1-5 ben2-1D (-/-)	69.7±.69**	40.3±0.73**
WS2	19.6±0.23	7.8±0.17
ben2-1D (+/-)	30.0±0.20**	23.4±1.2**
ben2-1D (-/-)	44.7±0.55**	51.1±3.4**

[a] Estimates of Bolting time (in days) began when the bolt had reached 0.5 cm in length. A total of 30 to 40 plants were analyzed for each genotype. Data is shown as means ± standard errors. ** Significantly different from their background plants using Student's unpaired *t*-test, two-tailed ($P < 0.001$).

Constitutive overexpression of At1g29160/BEN2 recapitulates the bri1-5 ben2-1D phenotypes

Since *At1g29160* is upregulated in two independent activation-tagged lines, *bri1-5 ben2-1D* and *bri1-5 ben2-2D,* we postulated that *At1g29160* is only reason for the enhanced

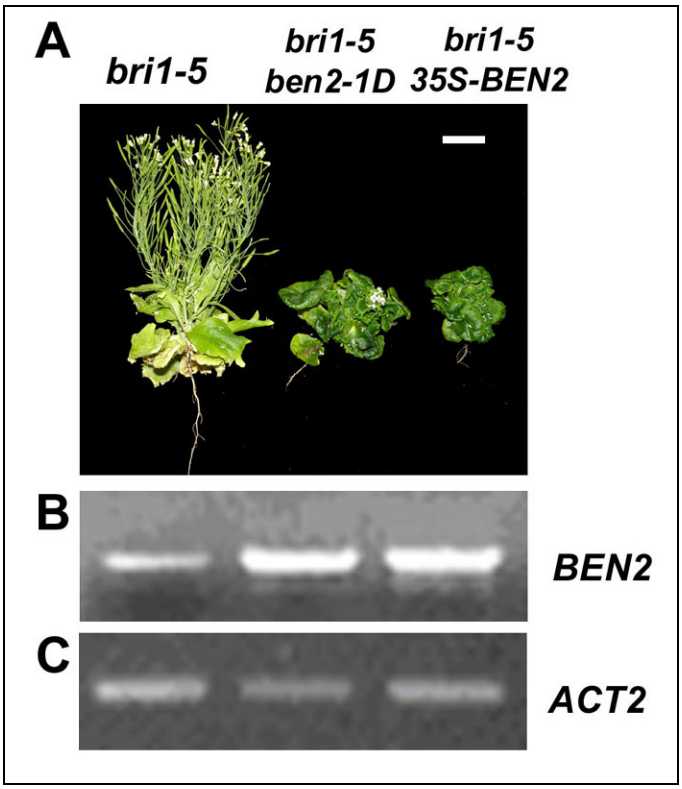

Figure 8. Overexpression of *At1g29160 /BEN2* in *bri1-5* recapitulates the original titivation tagged mutant phenotypes. **A.** Phenotypes of *bri1-5*, *bri1-5 ben2-1D*, and one representative line of *bri1-5* 35S-*BEN2* transgenic plant. The plants were 5-week-old. Scale bar = 1 cm. **B., C.** RT-PCR results show *BEN2* is truly overexpressed in *bri1-5 ben2-1D* and *bri1-5* 35S-*BEN2* plants. The numbers of PCR cycles for *BEN2* and *ACT2,* the quantitative control gene, were 25 and 18, respectively.

bri1-5 defective phenotypes when it is overexpressed. To test this hypothesis, a *35S* promoter was used to drive the expression of *At1g29160* cDNA in *bri1-5* plants. Of about 50 transgenic plants examined, 13% of them recapitulated the compact rosette and extremely delayed flowering time phenotypes (Figure 8A). RT-PCR analysis confirmed that *At1g29160/BEN2* is indeed overexpressed in these transgenic lines (Figure 8B, 8C). The compact and delayed flowering phenotypes are also evident in T_2 and in subsequent generations (data not shown). These results demonstrate that increased expression of *At1g29160 /BEN2* is likely responsible for the *bri1-5 ben2-1D* and *bri1-5 ben2-2D* phenotypes.

BEN2 encodes a putative Dof zinc finger-like transcription factor

Sequence analysis and GeneBank search suggest that BEN2 belongs to Dof transcription factor family. There are 37 putative *Dof* genes identified in the *Arabidopsis* genome (Yanagisawa, 2002). These genes were sequentially named based on their chromosomal localization. BEN2 was also termed Dof 1.5 in the GeneBank. Proteins in this family contain four cysteine residues in their conserved DNA-binding domain, or Dof domain, forming a single C2-C2 type zinc-finger-like motif (Yanagisawa, 1996). Dof specifically binds to a 5'-AA[AG]G-3' consensus core sequence, and it is implicated in the regulation of various physiological processes, such as light, phytochrome and plant hormone responses (reviewed in Yanagisawa, 2004). The closest homolog of BEN2 is At2g34140, which shares 79% sequence identity to BEN2 (Figure 9A, 9B).

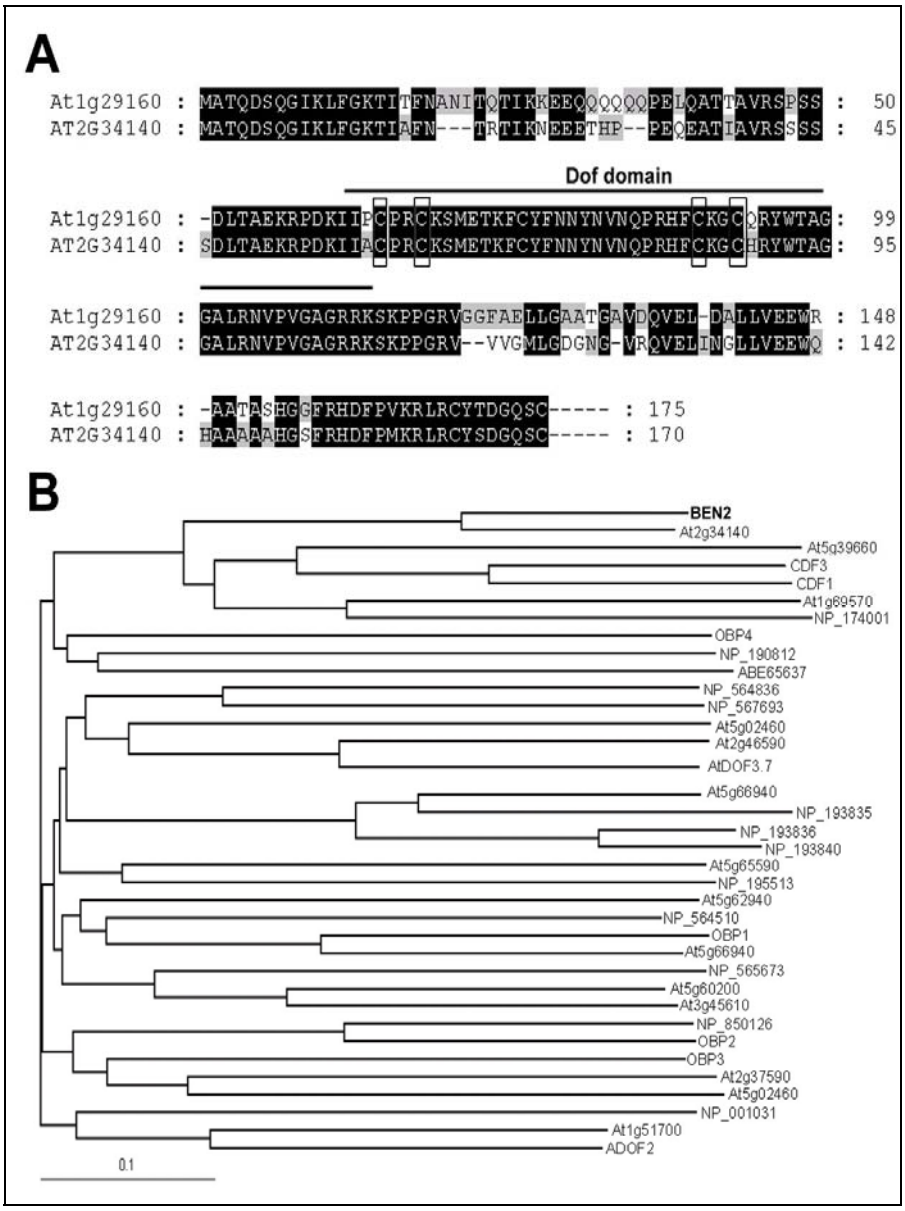

Figure 9. *BEN2* encodes a transcription factor belonging to a Dof zinc finger protein, Dof 1.5. **A.** Alignment of BEN2 with its closest paralog, At2g34140. Common amino acids are shaded with black boxes; putative Dof domain region is underlined; four Cys sites are marked in open-boxes. **B.** Phylogenetic tree generated from the amino acid sequences of At1g29160 (BEN2) and all its 36 Dof paralogs from the *Arabidopsis* genome.

BEN2 is expressed in all tissues and most strongly in seedlings

Sequence analyses showed that the *bri1-5 ben2-1D* mutant phenotype was caused by the overexpression of the *AtDof1.5* transcription factor, which was previously named *COG1* (Park et al., 2003). Onion epidermal cells transiently expressing 35S::green fluorescent protein (GFP)-COG1 fusion protein suggested that the fusion protein was mainly expressed in nucleus (Park et al., 2003). Tissue specific expression pattern of *COG1/BEN2* was not explored. To reveal the expression pattern of *COG1/BEN2*, a multi-tissue RT-PCR was performed (Figure 10A). We examined the *BEN2* RT-PCR products using total RNA from roots, stems, cauline leaves and rosette leaves, flowers, siliques and 8-day-old seedlings. The *BEN2* transcription product could be detected in all tissues examined and relatively strong expression was found in seedlings, leaves and flowers. Weaker expression was observed in stems and siliques (Figure 10A). To confirm the gene expression pattern and its subcellular localization, *35S::BEN2-GFP* cDNA and *BEN2 promoter*::β-glucuronidase (GUS) constructs were generated and transformed into *Arabidopsis*. More than 20 independent transgenic lines were analyzed for each construct. Transgenic plants harboring 35S-*BEN 2-GFP* were examined with a confocal microscope and the result indicated that BEN2 is a nuclear localized protein (Figure 10G). Unfortunately, GUS staining experiments showed inconsistent results. Staining for GUS not always produced a stable signal: sometimes the signal could be detected, but sometimes it could not be detected. In those instances when the signal could be detected, GUS expression patterns were almost the same among the transgenic lines (Figure 10B and 10C, 10E and 10F). In 6-day-old *Arabidopsis* light-grown seedlings, GUS expression was detected in the apex and in cotyledons, but not in hypocotyls or in roots (Figure 10B,

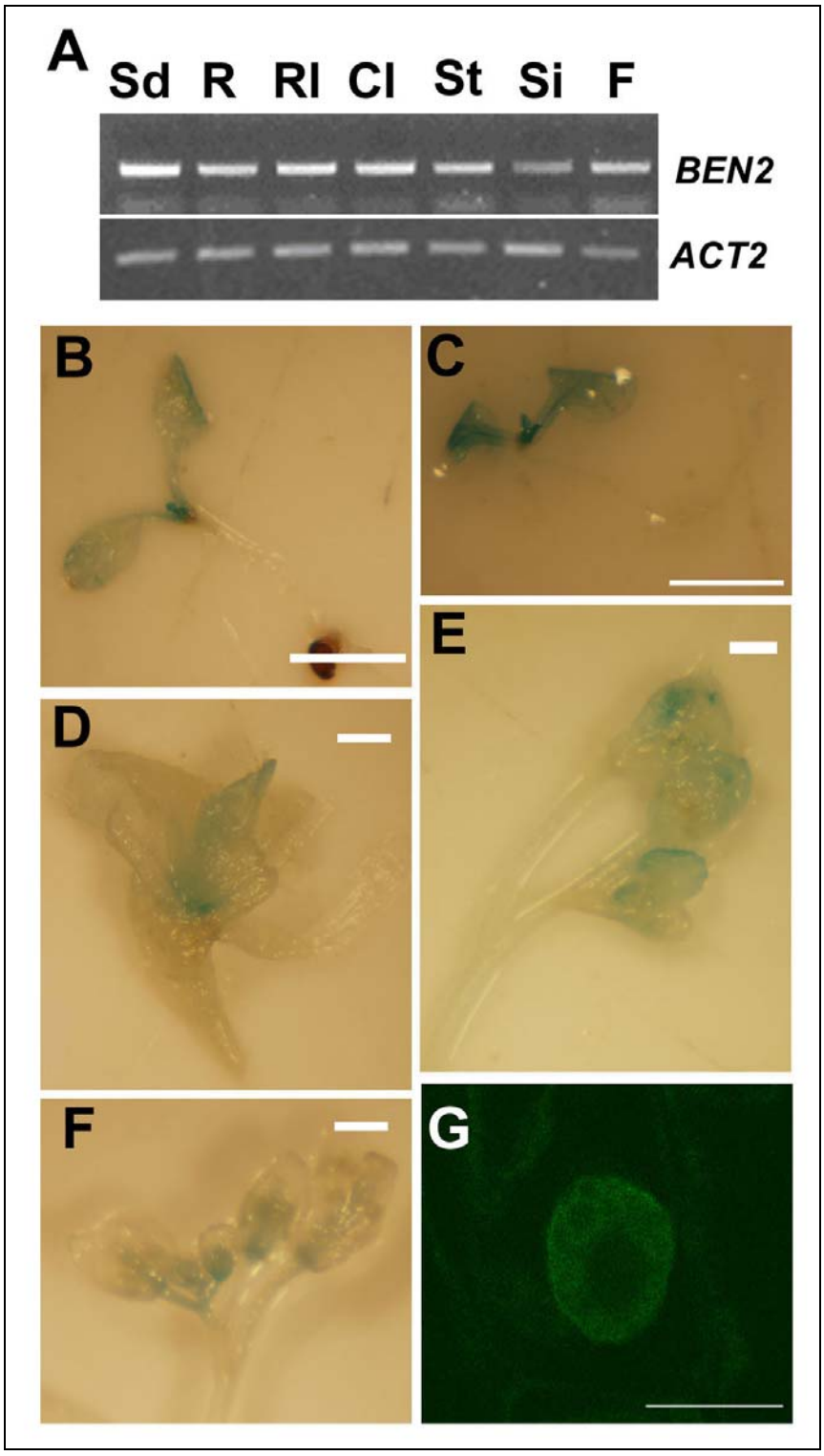
A
Sd R Rl Cl St Si F
BEN2
ACT2
B
C
D
E
F
G

Figure 10. Expression patterns of *BEN2*. **A.** RT-PCR analyses of *BEN2* in seedlings (Sd), roots (R), rosette laminae (Rl), cauline leaves (Cl), stems (St), siliques (Si), and flowers (F). The PCR cycles for *BEN2* and *ACT2* (as a quantitative control) were 26 and 18, respectively. **B** to **F**. GUS staining patterns of the seedlings or tissues from promoter *BEN2:: BEN2-GUS* transgenic plants at various developmental stages. Scale bar = 0.5 cm. **B. C.** Different transgenic lines of 6-day-old seedlings, showing similar GUS expression patterns in the apex and cotyledons; **D.** 3-week-old plant, showing GUS staining at the shoot apex; **E. F.** Inflorescences from different transgenic lines, showing GUS expressed in young flowers. **G.** Confocal microscopy examination of transgenic plants harboring a 35S-*BEN2-GFP* construct, showing one nucleus with BEN2-GFP signal. Scale bar = 10μm.

10C). As plants grew, the signal remained concentrated near the apex, but it was not present in later leaves (Figure 10D). In bolting plants, GUS activity was detected in young flowers, but not in siliques (Figure 10E, 10F). These results showed that *BEN2* mainly expressed in the green growing parts of plants.

The ben2-1D single mutant plants show organ-elongateds and delayed flowering phenotype

Because increased expression of *BEN2* in *bri1-5* plants can cause severely compacted rosettes, we explored whether the phenotype is also dependent on the *bri1-5* mutation. If the severe phenotype is independent of the *bri1-5* mutation, increased expression of *BEN2* should show similar effect in a wild-type background. The *bri1-5 ben2-1D* was therefore backcrossed to wild-type (WS2) plants. The *bri1-5* mutation was subsequently segregated out by genotypic and phenotypic screening, and the *ben2-1D* single mutant was generated (Table I). As seen in double mutant plants, *ben2-1D* single mutant plants exhibit homozygous and heterozygous differences and both of them show delayed flowering (by 25 days and 10 days, respectively). However, to our surprise, overexpression of *BEN2* in a wild-type background not only resulted in enlarged plant size in heterozygous plants, but also dramatically enlarged the plant size in homozygous plants (Figure 11). At 5-week-old stage, rosettes of the homozygous *ben2-1D* plants are 1.6 times wider than wild-types and the leaf number is twice that of the wild-type. Because the mutant and the wild-type plants have different flowering times, we compared their final height. The average inflorescence length of *ben2-1D* (-/-) is 79.4 cm, while the

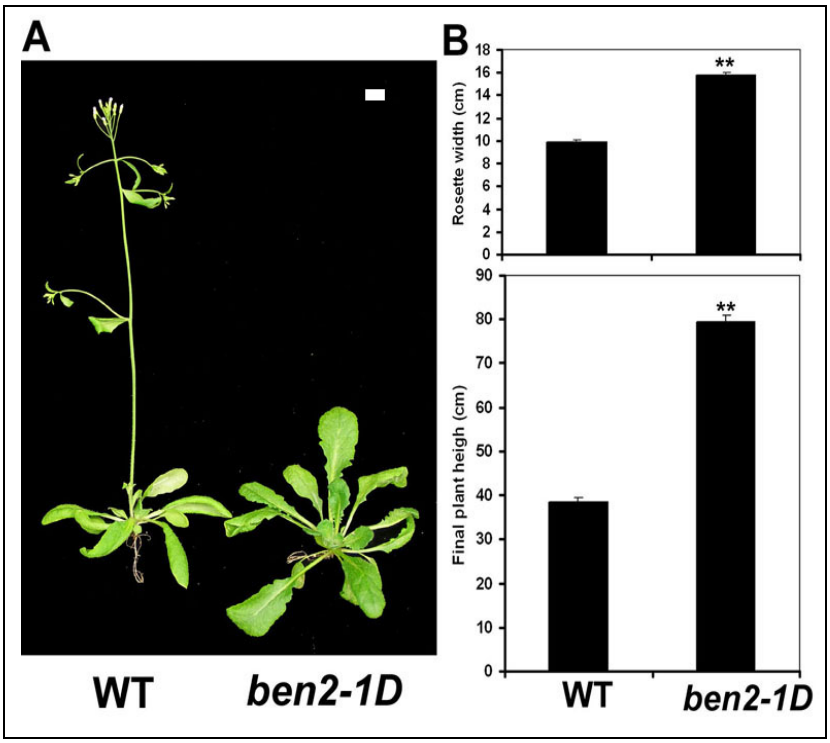

Figure 11. Phenotype of *ben2-1D* single mutant. **A.** *ben2-1D* single mutant dramatically enlarges the plant size and delays flowering in wild-type plants. Scale bar = 1 cm. **B.** *ben2-1D* gain-of-function mutation caused the elongated inflorescence length and enlarged rosette width. For rosette width, measurements from 5-week-old plants are shown; and for inflorescence length, the measurements from 12 week-week-old plants are shown. The experiments were repeated three times and the data from one representative experiment are presented as means ± standard errors (n = 40). ** Significant differences determined by Student's unpaired *t*-tests, two-tailed ($P < 0.001$).

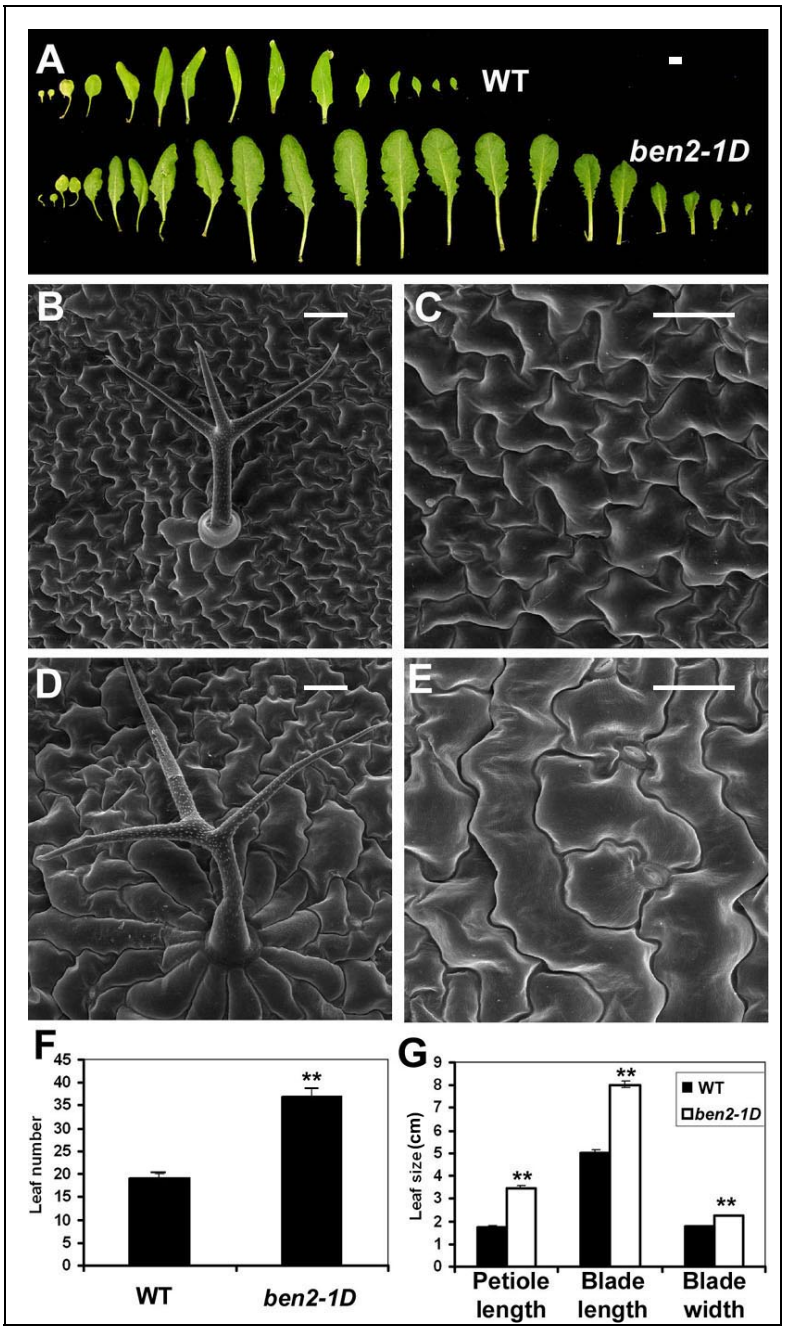

Figure 12. Leaf phenotypes of *ben2-1D* single mutant plants. **A.** 4-week-old *ben2-1D* plants show larger leaf size and greater leaf numbers. Scale bar = 1 cm. **B to E.** Scanning electron micrographs of epidermal cells from the third pair of leaves of 4-week-old WS2 (B, C) and *ben2-1D* (D, E) plants, showing *ben2-1D* plants have more expanded cells. Scale bar = 40 μm. **F. G.** Leaf number (F) and leaf size (G) of 5-week-old wild-type (WS2) and *ben2-1D* plants. The data are presented as means ± standard errors (n = 40). Student's *t*-tests indicated that all measurements from *ben2-1D* are statistically different (**) from those of WS2 ($P < 0.01$).

average height of wild-type plants is 38.6 cm. Thus, the mutant plants are about twice as tall as the wild-type plants.

Homozygous *ben2-1D* plants also produce larger leaves and longer petioles (Figure 12A). The petioles of *ben2-1D* mutants are twice as long as that of wild-type plants, and leaf blades in *ben2-1D* are 58.9% larger and 24.7% wider than that of wild-types (Figure 12G). Student's *t*-tests showed that the differences between mutant plants and wild-type plants were all significant ($P < 0.001$). It appears that the lager leaf size is mainly caused by greater cell expansion (Figure 12B to 12E). These results suggest that *BEN2* function is partially dependent on a functional BR signaling pathway.

BEN2 regulates hypocotyl elongation of Arabidopsis seedlings in the light; regulation is dependent on the function of BRI1

The *bri1-5 ben2-1D* mutant plants show enhanced defective phenotypes of *bri1-5* plants in the light, whereas *ben2-1D* plants exhibit enlarged organs and epidermal cells compared to its wild-type background, suggesting that the regulation of *BEN2* in plant growth requires a functional *BRI1*. To further test this supposition, the seedlings of *bri1-5 ben2-1D (-/-)* and *ben2-1D (-/-)* were carefully compared (Figure 13). Similar to what was observed by Park et al. (2003); the 8-day-old gain-of-function mutant seedlings in wild-type background are taller than wild-type seedlings, reaching almost twice the height of wild-type seedlings. Cotyledon petiole length is 61.5% longer, but the root length is 32.6% shorter than that of wild-type (Figure 13A, 13B). Student's *t*-tests indicated that the differences between the *ben2-1D* and wild-type seedlings were all

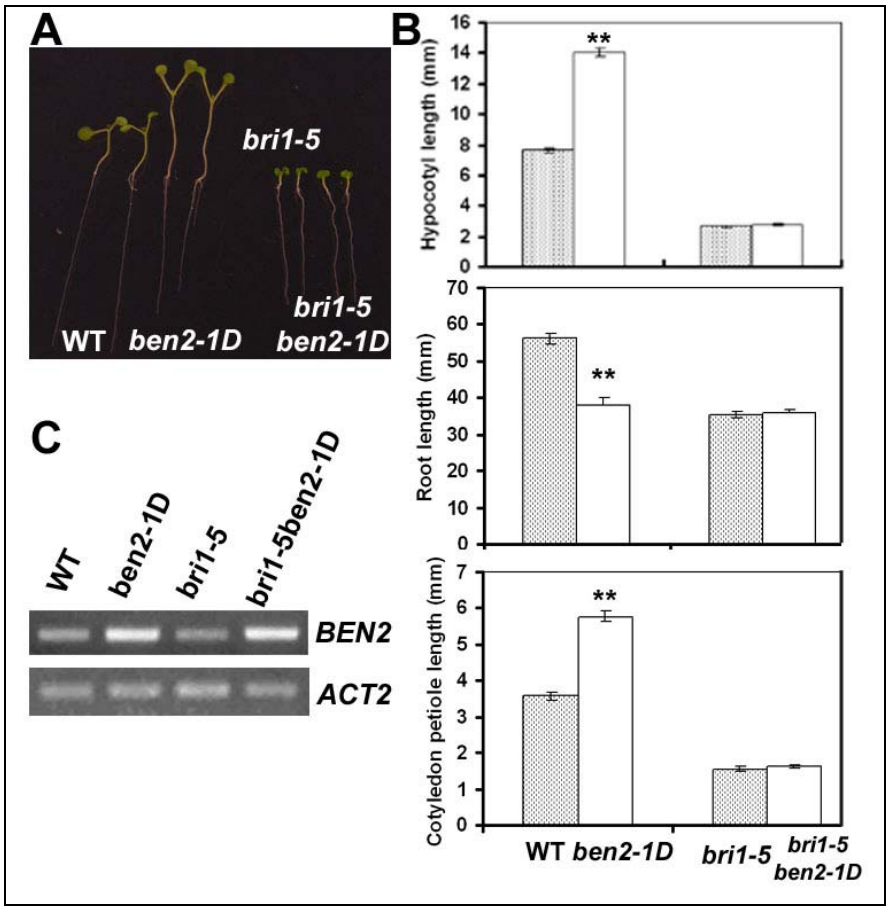

Figure 13. Regulation of *BEN2* on the elongation of *Arabidopsis* seedlings is dependent on a functional *BRI1*. **A.** Phenotypes of WT (WS2), *ben2-1D* (in WS2 background), *bri1-5* and *bri1-5 ben2-1D* seedlings grown in ½ MS medium supplemented with 1% sucrose. **B.** Measurements of the seedlings shown in (A). Measurements were taken 6 to 8 days after germination and presented as means ± standard errors (n=60 to 90). Experiments were repeated three times and the data from one representative experiment are shown. ** Significantly difference between the *ben2-1D* and the WS2, based on Student's unpaired *t*-tests, two-tailed ($P < 0.001$). **C.** RT-PCR analysis of the seedlings shown in (A), shows *BEN2* transcript was overexpressed in *ben2-1D* and in *bri1-5 ben2-1D* plants. *ACT2* was used to confirm equal amount of mRNA used for RT-PCR. The PCR cycles for *BEN2* and *ACT2* were 26 and 18, respectively.

statistically significant ($P < 0.001$). On the contrary, when *BEN2* was overexpressed in *bri1-5* background, the differences between *bri1-5 ben2-1D* and *bri1-5* were indistinguishable. Hypocotyl, cotyledon petiole and root length of the 8-day-old *bri1-5 ben2-1D* double mutant seedlings were not statistically different from those of the same age *bri1-5* seedlings (Figure 13A, 13B). RT-PCR was performed to confirm *BEN2* expression levels in both wild-type and *bri1-5* backgrounds (Figure 13). This result clearly indicates that *BRI1* activity is required for *BEN2*-mediated hypocotyl elongation.

Regulation of BEN2 on seedling elongation is dependent on endogenous levels of BR

bri1-5 is a weak allele of *bri1* that harbors a point mutation of C69Y at the N-terminus of the BRI1 receptor (Noguchi et al., 1999). As a result, the BR signaling pathway is partially blocked and BRs cannot effectively regulate normal plant growth and development. In order to further test if *ben2-1D* phenotypes require BRs, we directly reduced the endogenous BR levels by applying exogenous brassinazole (BRZ) during seeding growth. BRZ is a specific BR biosynthesis inhibitor that blocks BR biosynthesis at the C-22 hydroxylation step, leading to the dramatic reduction of the endogenous BR levels (Asami et al., 2000). As expected, the *ben2-1D* plants treated with 4μM BRZ showed phenotypes similar to the *bri1-5 ben2-1D* plants (Figure 14). These results clearly indicated that normal BR signaling pathway is essential to the function of BEN2 in regulating seedling growth.

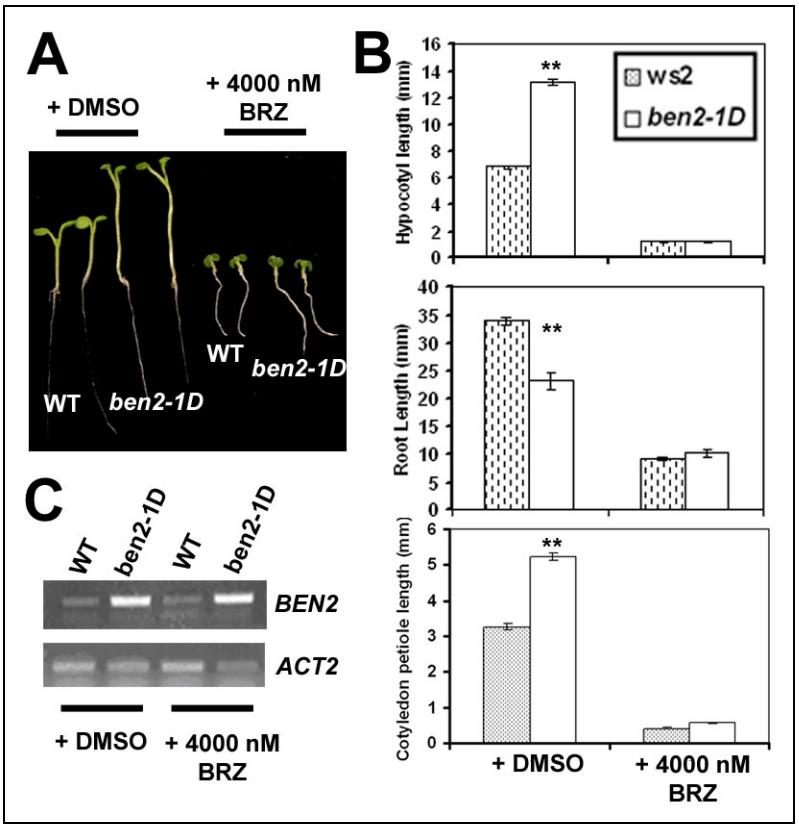

Figure 14. The elongated hypocotyl phenotype of *ben2-1D* can be diminished by the supplementation of exogenous BRZ. **A.** Phenotypes of WT (ecotype: WS2) or *ben2-1D* (in WS2 background) seedlings grown in ½ MS medium supplemented with 1% sucrose, and either DMSO (as control) or 4000 nM BRZ (dissolved in DMSO). **B.** Hypocotyl, root and cotyledon petiole lengths in BRZ treated and control seedlings. Measurements were taken 6 days after germination and presented as means ± standard errors (n = 60 to 90). The experiments were repeated three times and the data from one representative experiment are shown. ** Significant differences based on Student's unpaired *t*-tests, two-tailed ($P < 0.001$). **C.** RT-PCR analysis of *BEN2* in BRZ treated and control seedlings, showing that the *BEN2* overexpression status was not altered in mutant *ben2-1D* after the BRZ treatments. *ACT2* was used to show same amount of mRNA used for RT-PCR analysis. The PCR cycles for *BEN2* and *ACT2* were 26 and 18, respectively.

Response of ben2-1D to exogenous BL and BRZ differs from wild type

The *ben2-1D* single mutant seedlings have longer hypocotyls but shorter primary roots than those of wild-type when grown in light (Figure 13A, Figure 14A), similar to the phenotypes of wild-type plants grown in media containing BL (Li et al., 2002). It was reported that root growth of wild-type plants is inhibited by 66% after 1 nM BL treatment (Li et al., 2002). The phenotype of *ben2-1D* plants suggests that the endogenous BR levels of *ben2-1D* mutant may be increased. Therefore, we examined whether BL or BRZ can affect the growth of *ben2-1D* mutant seedlings. The *ben2-1D* single mutant plants showed reduced sensitivity to BL treatment (Figure 15). For example, when grown in 1 nM and 1000 nM of BL, wild-type hypocotyls were increased by 48.4% and 67.7% respectively; whereas the *ben2-1D* hypocotyls were only increased by 1.3% and 5.2%, respectively. Root growth and cotyledon expansion in response to BL treatment also reduced sensitivity to exogenous BL. For example, in the medium containing 1 nM or 1000 nM, wild-type root growth decreased by 2.6-fold or by 16-fold, respectively; *ben2-1D* root growth, on the other hand, reduced only by 1.7-fold and 5-fold , respectively. These data suggest that *ben2-1D* plants show reduced sensitivity to BL treatment. The observation can also be explained by high endogenous levels of BR in the single mutant which cause exogenous BL has less effect on their growth. To test this hypothesis, BR biosynthesis inhibitor, BRZ, was used to reduce endogenous BR levels. As expected, *ben2-1D* seedlings were more sensitive to the BRZ treatment (Figure 16). The root growth of *ben2-1D* can be completely restored to that of wild-type seedlings when 0.1 μM BRZ was applied to the growth media. Similar results were obtained measuring hypocotyl and cotyledon growth after BRZ treatment. For example, wild-type hypocotyl

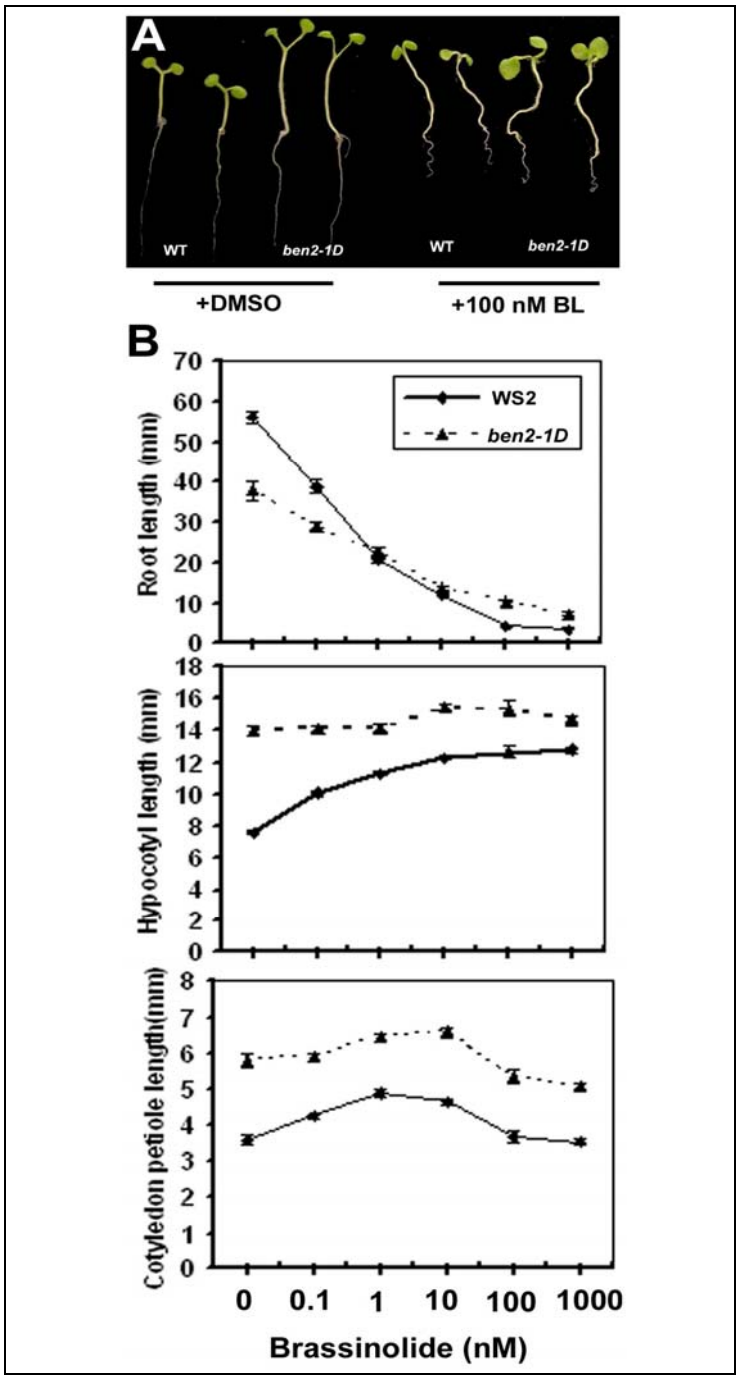

Figure 15. Addition of exogenous BL on *ben2-1D* growth, showing *ben2-1D* reduced sensitivity to BL treatment. **A.** Phenotypes of WT (ecotype: WS2) and *ben2-1D* (in WS2 background) seedlings grown in ½ MS medium supplemented with 1% sucrose and either DMSO or 100 nM 24-epiBL (dissolved in DMSO). **B.** Root (upper panel), hypocotyl (middle panel) and cotyledon petiole (lower panel) lengths of 8-day-old seedlings on ½ MS medium agar plates containing various concentrations of 24-epiBL. Each point represents the means ± standard errors (n = 60 to 90). The experiments were repeated three times and the data from one representative experiment are shown.

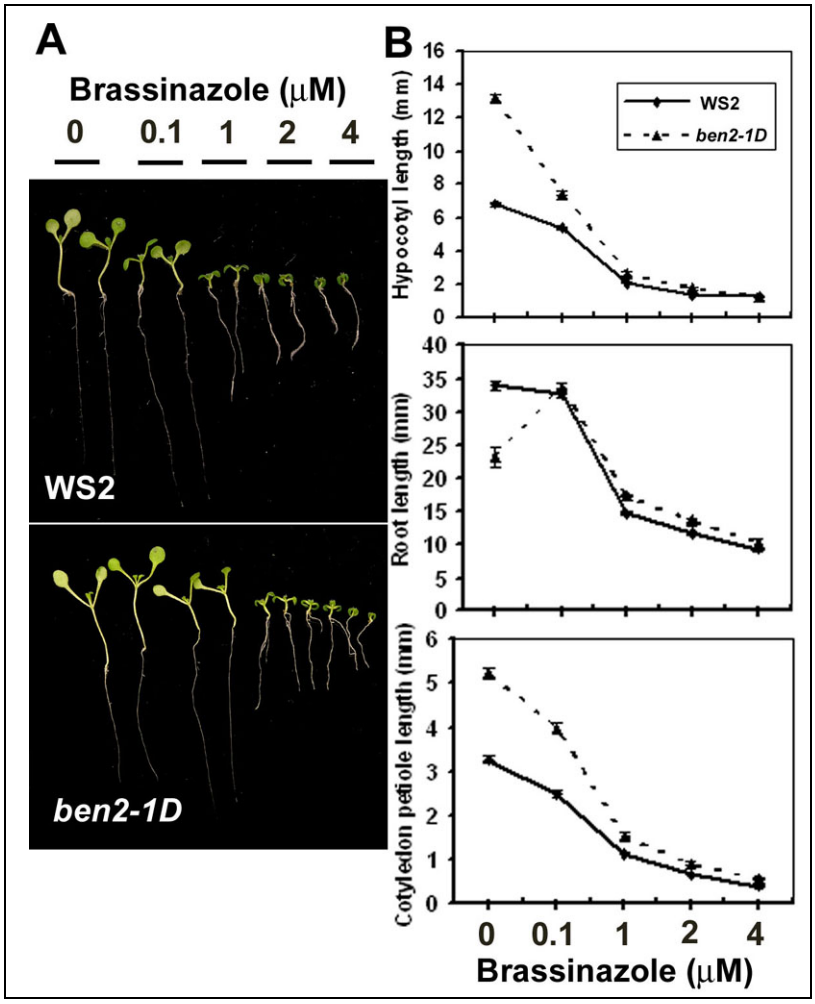

Figure 16. Addition of exogenous BRZ on *ben2-1D* growth, showing *ben2-1D* seedling is more sensitive to exogenous BRZ. **A.** Phenotypes of 6-day-old WT (ecotype: WS2) or *ben2-1D* (in WS2 background) seedlings grown in ½ MS medium supplemented with 1% sucrose, and various concentrations of BRZ. **B.** Measurements of the seedlings shown in (A). Measurements were taken 6 days after germination and each point presented as means ± standard errors (n = 60 to 90). The experiments were repeated three times and the data from one representative experiment are shown.

length was reduced by 3.3-fold after 1 μM BRZ treatment and by 5.5-fold after 4 μM BRZ treatment. *ben2-1D* hypocotyl length was reduced by 5-fold after 1 μM BRZ treatment and by 10.7-fold after 4 μM BRZ treatment. In fact, in the presence of 4 μM BRZ, the phenotype of *ben2-1D* mutants was similar to that of the WT (Figure 14, Figure 16). Although differences in the response to exogenous application of BL and BRZ between mutant and wild-type could be interpreted that the endogenous BR levels are altered (Neff et al., 1999; Fujioka and Yokota, 2003), we are unable to discern the mechanism of this effect. Additional work is required to clarify whether it is caused by the alteration of the BL biosynthesis or signal transduction.

Gene expression analysis of key flowering time genes

Besides the altered growth phenotype seen in the mutant, the gain-of-function *ben2-1D* mutant also show delayed flowering time phenotype. Flowering time is controlled by four genetic pathways: photoperiod pathway (day length), vernalization pathway (low temperatures), autonomous pathway (plant age) and gibberellic acid pathway (Mouradov, et al., 2002; Boss, et al., 2004). It has been reported that the photoperiod pathway promotes flowering through *CONSTANS* (*CO*) to up-regulate *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) gene expression. On the other hand, the vernalization and the autonomous pathways promoted plant flowering through inhibition of flowering repressor *FLOWERING LOCUS C* (*FLC)* to up-regulate the expression of *SOC1*. It seemed as if all these different pathways converge to regulate some common genes, like *SOC1*, to regulate flowering time (reviewed in Mouradov et al., 2002; Putterill et al., 2004; Amasino et al., 2005). Through examining the expression of these key

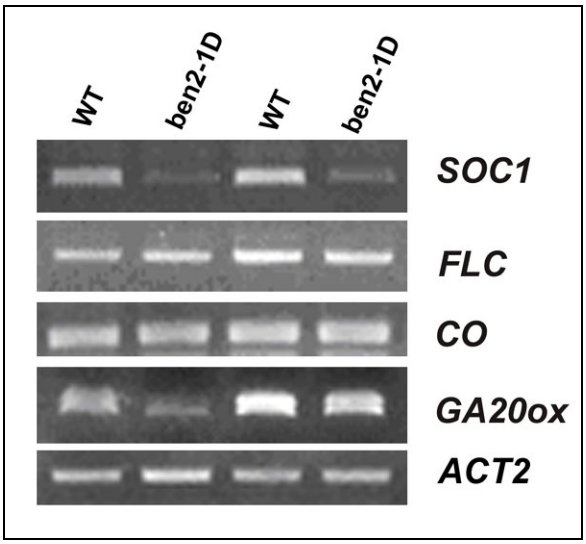

Figure 17. RT-PCR analysis of expression levels of flowering time response genes: *SOC1, FLC*, *CO* and *GA20ox.* Paired lanes represent 3-week-old WT and *ben2-1D* plants (left) and 8-day-old WT and *ben2-1D* seedlings (right). The PCR cycles for *SOC1*, *FLC*, *CO, GA20ox* and *ACT2* were 26, 23, 23, 26 and 18, respectively. *ACT2* was used as a loading control.

flowering time genes, we may obtain some information about how *BEN2* regulates flowering. We used the total RNA from 3-week-old plants (just before wild-type bolting) and from 8-day-old seedlings to perform RT-PCR analysis of several key flowering time genes (Figure 17). Because *SOC1* regulation is a common phenomenon observed in a flowering plant, we first examined the expression levels of *SOC1*. The result showed that overexpression of *BEN2* downregulates *SOC1*, suggesting that *BEN2* plays a role in regulating flowering. We subsequently compared the expression of several key flowering time genes in regulating photoperiod, vernalization and autonomous pathways, such as *CO* and *FLC*. None of these genes showed notable altered expression levels in the mutant, compared to wild-type plants. Further we examined the expression of GA response gene GA20ox to determine whether the endogenous GA level was changed. Overexpression of *BEN2* can dramatically reduce the expression of GA20ox, a key gene in the GA biosynthesis pathway. This result suggests that the endogenous GA level has been changed in the mutants. This preliminary data suggests that *BEN2* may be involved in delaying flowering time by regulating endogenous GA levels instead of other flowering pathways. More studies need to be carried out in the future to test this hypothesis.

Discussion

BR is perceived by two distinct leucine-rich repeat receptor-like protein kinases, BRI1 and BAK1. Although many critical regulatory components have been identified in the BR signaling transduction cascade, important regulators connecting these proteins are still missing. We have been using a gain-of-function genetic screen to identify novel proteins

in this pathway (Li et al., 2001a; Li et al., 2002; Zhou et al., 2004; Yuan et al., 2007). Previous studies demonstrated that activation tagging is indeed an excellent genetic strategy for identifying a variety of signaling molecules directly or indirectly involved in this pathway. For example, BRS1 was identified as a suppressor of a weak *bri1* mutant, *bri1-5*. Overexpression of *BRS1* selectively suppressed *bri1* mutants but failed to suppress other LRR-RLK mutants such as *clv1-1* (Li et al., 2001b). BRS1 encodes a secreted carboxypeptidase (Zhou and Li, 2005). Recent studies suggested that BRS1 may be involved in a regulatory process at an early step of BR signal transduction. BAK1 was identified simultaneously by a yeast two-hybrid analysis for specific BRI1 interacting proteins and by our activation tagging (Li et al., 2002; Nam and Li, 2002). It has become clear that the interaction between BAK1 and BRI1 is ligand (BR)-dependent (Wang et al., 2005). Upon the binding of BR with its receptor, BRI1, BRI1 can be autophosphorylated by itself and a negative regulator named BKI1, normally associated with the inactive form of BRI1, detaches from BRI1, and the BR-BRI1 complex is then able to interact with BAK1 (Wang and Chory, 2006). BAK1 can therefore be activated by protein phosphorylation. The activated BRI1 and BAK1 can then further transduce the signal to undefined downstream components. Another BR regulator identified by activation tagging is called BRL1. Because *BRL1* can suppress both weak and null alleles of *bri1* (Zhou et al., 2004), it is likely that BRL1 plays a redundant role with BRI1 in mediating the BR signal transduction. Using the same activation tagging approach, we have also identified a number of *bri1-5* genetic enhancers. For instance, *bri1-5 ben1-1D* double mutant plants are more severe than *bri1-5* single mutant, indicating that BEN1 may play a negative role in regulating the BR signal pathway. Gene cloning, however, revealed that

BEN1 encodes a protein homologous to a well defined reductase named dihydroflavonol 4-reductase (DFR; Yuan et al., 2007). It is therefore hypothesized that BEN1 may indeed act as a novel steroid reductase which may be responsible for the inactivation of a number of plant steroids including bioactive catastrone and brassinolide. *ben2-1D* and *ben2-2D*, two gain-of-function discussed in this chapter, are alleles of another protein which may play a negative role in either BR signal transduction or BR biosynthetic pathways.

The mutants were named as *bri1-5 enhanced 2-1 dominant* (*ben2-1D)* and *bri1-5 enhanced 2-2 dominant* (*ben2-2D)* because the they show the enhanced defective phenotypes of *bri1-5* including more compact rosette leaves and significantly delayed flowering time. BEN2 was previously named AtDof1.5/COG1. Earlier studies of transgenic plants overexpressing *AtDof1.5* in wild-type background showed elongated hypocotyls and petioles, but reduced leaf blades in the later stage of development (Park et al., 2003). Consistent with the results from Park et al. (2003), we observed that the *ben2-1D* single mutant showed enhanced hypocotyls growth by 83.5% compared to wild-type plants. We also observed some phenotypes in *ben2-1D* plants which are not consistent with the results of *cog1*. For example, *ben2-1D* plants had significantly enlarged leaf blades and elongated petioles (1.6 fold bigger and 2 fold longer than wild-type). In addition, overexpression of *BEN2* in *bri1-5* or in wild-type background can significantly delay the plants'In addition, overexpression of *BEN2* in *bri1-5* or in wild type background can significantly delay the plants' by up to six weeks (Table 2). The leaf phenotype difference between *ben2-1D* and *cog1* may be caused by the growth

conditions; plants grown on medium or in soil show different leaf phenotypes (unpublished results). The delayed flowering phenotype was not previously reported.

Although RT-PCR revealed that *BEN2* transcription product could be detected in all tissues examined, our GUS stain showed that BEN1 may only be expressed in restricted tissues. The discrepancy may be caused by several reasons: 1) RT-PCR is a more sensitive approach for detecting the gene expression. Weak expression may be revealed by RT-PCR but not by GUS staining; 2) collection conditions of the samples were not consistent. It is reported that the expression level of this gene (*COG1)* is induced in response to far-red and red light (Park et al., 2003); Yin et al. (2002) also found the transcription factor BES1 existed in the nucleus at very low levels but accumulated at high levels in response to BL treatment. In the same way, expression of *BEN2* perhaps also depends on environmental cues, such as light and hormones. That could explain why the same T2 lines of GUS transgenic plants sometimes showed the GUS staining signals but sometimes not. 3) Another possibility is that the expression of this gene is controlled by a biological clock. Consistent with this explanation, we recently searched the published microarray data from the internet, the expression of this genes show a typical circadian rhythm. We collected the samples at different times of the day, which could have affected our results.

In 1985, Klug first found a projection in the structure of the general transcription factor TFIIIA from *Xenopus*. This protein has 9 repeat regions, each with two closely spaced cysteines and two histidines that fold around a central $Zn2^{+}$ ion; this forms a finger-shaped domain, which is termed a Zinc finger motif (Evans, 1988; Evans and

Hollenbery, 1998). According to the number and position of the cysteine and histidine residues available for coordination with the zinc ion, zinc finger motifs can be divided into several classes, including C2-C2 Zn fingers, C2-H2 Zn fingers, C6 Zn fingers, and LIM domains (Evans, 1988). The most common Zn finger motif, C2-H2 Zn fingers, contain a pair of cysteine residues in β-sheets and two histidine residues in an α-helix which coordinated with a zinc ion. Usually, a transcription factor comprises multiple copies of a Zn finger motif. Dof domains, in contract, contain only one Zn finger motif. The Dof domain contains a CX2CX21CX2C (where X is any amino acid) motif, and therefore belongs to the C2-C2 Zn finger group (Umemura et al., 2004; Yanagisawa, 2004).

The first C2-C2 Zn finger was identified as receptors for steroid hormones in animals. Because similar intracellular receptors for non-steroid hormones were subsequently found, C2-C2 Zn finger transcription factors are now called nuclear receptors. The widely accepted mechanism for mammalian steroid hormone signal transduction includes the binding of the steroid to an intracellular receptor, followed by dimerization, and ligand-dependent transcriptional activation of gene expression (Mangelsdorf et al., 1995). Unlike animals, plants have different mechanisms for steroid hormone signal transduction. Plant steroid hormones are recognized at the cell surface by BRI1, a leucine-rich repeat receptor-like kinase (Li et al, 1996). Previous studies, however, indicated that some of the BR responsive genes can respond BR in a *bri1* null mutant background, suggesting that there might be other BR signaling pathway regulated by different BR receptors. BEN2 is a C2-C2 Zn finger that beard significant structural homology to animal nuclear steroid receptor, thus potentially representing a novel class

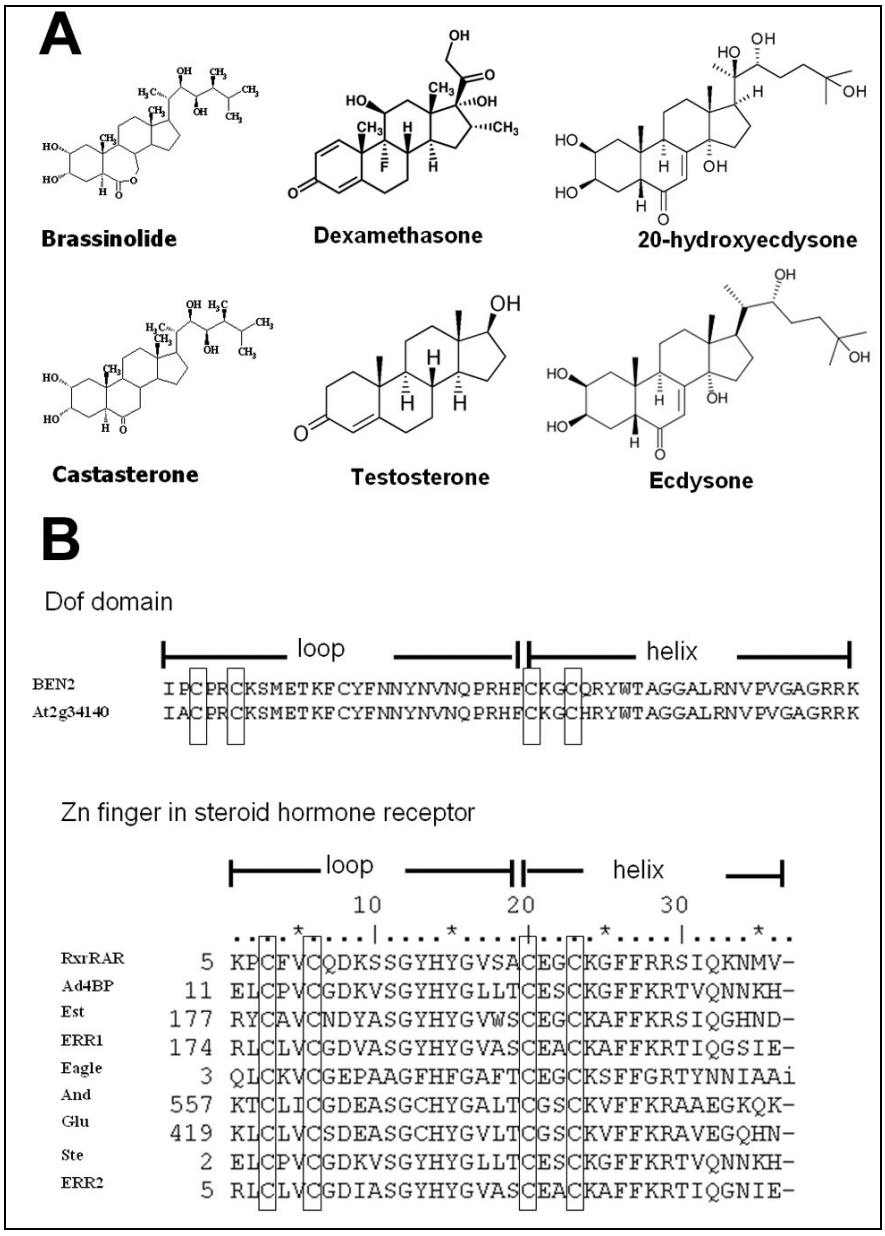

Figure 18. The structure of BEN2 Zn finger motif is similar to that of steroid hormone receptor. **A.** Comparison of the structures of BRs and some animal steroid hormones. **B.** Comparison of the amino acid sequences of BEN2 as well as its close homolog, At2g34140, with the steroid hormone receptors. The DNA-binding domains of the steroid hormone receptor have two zinc-binding units, U1 and U2. The U1 units of Retinoid X receptor (RxrRAR; 1DSZ_A), Steroid hormone receptor Ad4BP (Ad4BP; Q04752), estrogen receptor (Est; P06212), Estrogen-related receptor-α (ERR1; P11474), Protein embryonic gonad receptor (Eagle; P15370), Androgen receptor--Dihydrotestosterone receptor (And, P10275), Glucocorticoid receptor (GR, P04150), Steroidogenic factor receptor (Ste, 2FF0_A) and Estrogen-related receptor 2 (ERR2; 1LO1_A) are compared. The four Cys conserved residues are boxed.

of steroid nuclear receptor in plants. This is supported by the observations that BRs have a molecular frame similar to animal steroid hormones such as ecdysone, progesterone and glucocorticoid (Figure 18A), and the position of the four conserved cysteine residues in BEN2 is analogous to those of steroid hormone receptors (Figure 18B). Similar to the BRI1 extracellular domain functions in protein-protein interactions, and the zinc finger motif can also function in protein–protein interaction (Mackay and Crossley, 1998). The Dof domain has been proven as a bifunctional domain for DNA-binding and protein–protein interactions. One example is the *Arabidopsis* Dof domain protein OBP1, which interacts with a basic domain-leucine zipper proteins (bZIP protein) OBF4, to modulate stress responses (Zhang et al., 1995). Similarly, maize Dof domain protein PBF can interact with Opaque2, a maize bZIP protein. The Dof domain could also interact with non-transcription factors, such as nuclear high-mobility group proteins (Yanagisawa, 1997; Krohn et al., 2002). Elucidating the molecular mechanisms of BEN2 in the BR related signal transduction pathway would provide significant insight into our understanding the effect of plant steroid hormones on plant growth and development.

Overexpression of BEN2 can dramatically delay plant flowering time, indicating that BEN2 might be a negative regulator flowering timing control. It has been reported that *bri1* could delay flowering. The BR signaling regulates flowering time through elevating expression of the potent floral repressor FLC (Domagalska et al., 2007). RT-PCR results showed that overexpression of *BEN2* did not affect *FLC* expression and the regulation was independent of *bri1-5*. Preliminary RT-PCR results indicated that the late flowering of *ben2-1D* plants may be caused by the deficiency of GA (Figure 17). We also applied exogenous GA to *ben2-1D* and *bri1-5 ben2-1D*, and the result showed that

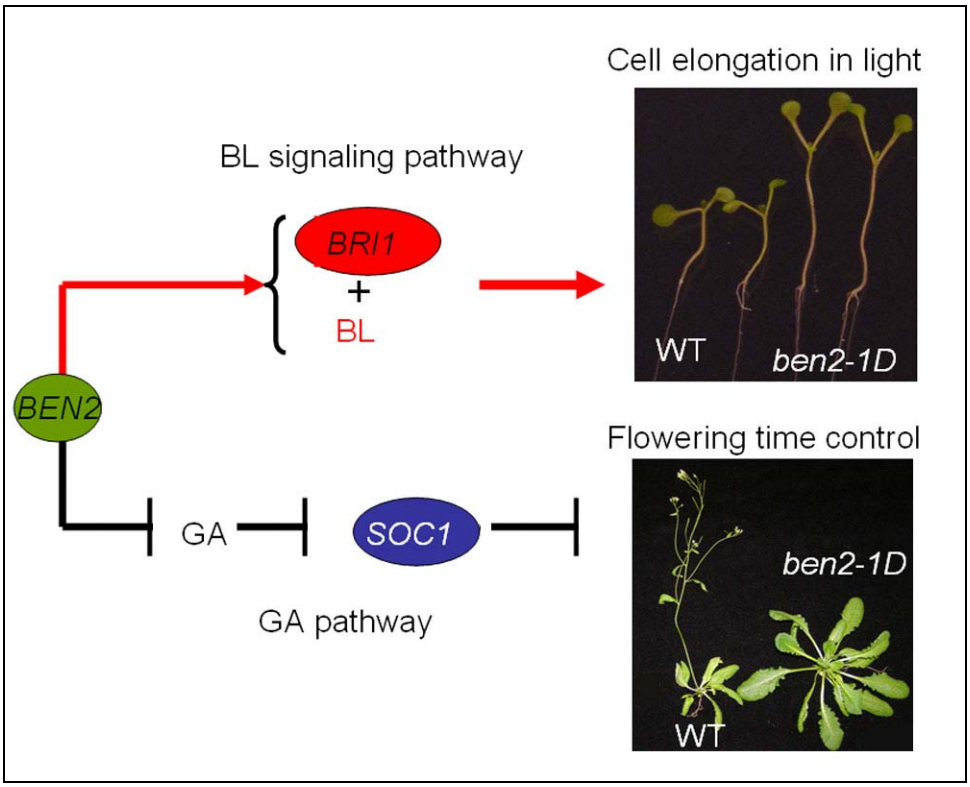

Figure 19. The possible model of BEN2 in connecting the BR biosynthesis/signal transduction and GA biosynthesis/signal transduction pathways.

exogenous GA can complement the late flowering phenotype seen in the mutant plants (results not shown). We therefore hypothesize that the hypocotyl elongation phenotype of *ben2-1D* is controlled by a *BRI1*-mediated BR signaling pathway; whereas the late flowering phenotype is likely regulated by the GA-related signaling pathway (Figure 19). Previous studies have suggested that At Dof1.5/COG1 is involved in the phytochrome signaling pathway (Park et al., 2003). Our results indicate that this gene may encode an important protein regulating BR and GA signaling pathways. Understanding the crosstalk among signaling pathways may provide future better manipulation of crops for the best traits using genetic engineering.

Since current roles of BEN2 are revealed by the gain-of-function genetic analysis, it is also important to consider loss-of-function genetic mutants. Genome analysis in *Arabidopsis* suggests that there is at least one gene (*At2g34140*) that shows high identity with *BEN2*. To further understand physiological role of BEN2, it would be necessary to make double knock outs of these two genes. The detailed molecular mechanisms regulating the crosstalk among BR, GA and phytochrome will also be studied.

Experimental procedures

Activation-tagging mutant screening

Arabidopsis thaliana bri1-5 mutants (in *Arabidopsis* ecotype WS2 background) were transformed with the activation-tagging construct, pBASTA-AT-2 (Yuan et al., 2007).

ben2-1D and *ben2-2D* were obtained by screening extragenic modifiers of the *bri1-5* mutant as described previously (Li et al., 2001a; Li et al., 2002; Zhou et al., 2004; Yuan et al., 2007). T2 and T3 seeds from self-fertilized *bri1-5 ben2-1D* were carefully checked to confirm that the mutant phenotype was linked to the transgene and that only one locus contained the activation T-DNA.

All plants were grown in long day growth conditions with 16 h light and 8 h dark at 20°C.

Cloning of the BEN2 gene

The location of gene *BEN2* T-DNA was determined by amplifying the flanking genomic DNA using TAIL-PCR (Liu and Whittier, 1995; Terauchi and Kahl, 2000). Sequence analysis indicated that the insertion site of the T-DNA is located in chromosome I, between *At1g29160* and *At1g429170*. The activation-tagged gene was determined by RT-PCR.

Recapitulation of the bri1-5 ben2-1D mutant phenotype

To generate *bri1-5 ben2-1D* recapitulation construct, *BEN2* cDNA was amplified by RT-PCR from total RNA of *Arabidopsis* Columbia (Col-0) using the following primers: BEN2- BP1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGACCCA AGATTCTCAA-3') and BEN2-BP2 (5'-GGGGACCACTTTGTACAAGAAAGCTG G GTCACAAGATTGACCATCGGTGTAACA-3'). The PCR product was cloned into destination vector pBIB-BASTA-35S-GFP-GWR via Gateway® BP and LR recombination reactions. The *bri1-5* was transformed with this construct via *A.*

tumefaciens–mediated transformation (Clough and Bent, 1998). The T_1 transgenic plants were screened for resistance to BASTA and analyzed for a *bri1-5* enhanced phenotype.

RT-PCR analysis

For tissue specificity of *BEN2* expression analysis, total RNA was extracted from 8-day-old seedlings and multiple organs, including roots, stems, cauline leaves and rosette leaves, flowers, and siliques using the RNeasy plant mini kit (Qiagen; http://www.qiagen.com/). For wild-type and mutant expression in different background or treatment analysis, total RNA was isolated from 8-day-old seedlings of each sample with the same method. For flowering time gene expression analysis, the entire plants of 8-day-old seedling grown in petri dishes or leaf tissue of 3-week-old soil-grown plants were harvested after 8 hours growth in light. Total RNA (5 µg) was used as template for first strand synthesis in a 50 µl volume using the SuperScript III first-strand synthesis system (Invitrogen; http://www.invitrogen.com/). The RT product was diluted to 200 µl volume, and a 5 µl volume of cDNA was used as PCR template. Preliminary experiments were performed to ensure RT-PCR in a linear range of accuracy for each individual gene. All final RT-PCR experiments were performed at least 4 times.

BEN2 gene was amplified using primers *BEN2*- fw (5'-TTAACAAGATTGACC ATCGGTGTA -3') and *BEN2*-rv (5'-ATGGCGACCCAAGATTCTCAA-3'); *GA20ox* was amplified using primers GA20-fw (5'-CAAGAGTTTGTATGGCCTGACCA-3') and GA20 -rv (5'-TCAGAAACTTCCTTTGTTCTTGAGCCA-3'); *SOC1* was amplified using primers *SOC1*-fw (5'-CTGAGGCATACTAAGGATCG-3') and *SOC1*-rv (5'-GAACAAGGTAACCCAATGAA-3'); *FLC* was amplified using primers *FLC* -fw (5'-

CCTTGATCGATATGGGAAACAG-3') and *FLC* -rv (5'-CTAGTCACGGAGAGGGC AGTC-3'); *CO* was amplified using primers *CO* -fw (5'-AGGAGGTTGCTTCGTGGCT GTTC-3') and *CO*-rv (5'-CTTTGGGCGTTCTTGGGTGTGA-3'); *ACTIN2* was amplified using primers specific *ACTIN2*-fw (5'-AGCGCTGAGGCTGATGATATT CAAC-3') and *ACTIN2*-rv (5'-TCTAGAAACATTTTCTGTGAACGATTC-3').

The parameters used for PCR thermal cycling were conducted as follows: 95°C denaturing for 3 min; 95°C for 15 sec, 56°C for 30 sec, and 72°C for 2 min, 21 to 30 cycles; 72°C extension for 7 min.

Construction of the 35S::BEN2-GFP fusion and confocal microscopy analysis

The full-length cDNA of *BEN2* without a stop codon was amplified by RT-PCR from total RNA of the *bri1-5 ben2-1D* mutant with the following primers: *BEN2*- BP1 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGACCCAAGATTCTCA A-3') and *BEN2*-BP2 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCACAAGA TTGACCATCGGTGTAACA-3'). After purification, the PCR product was product was cloned into destination vector pBIB-BASTA-35S-GFP-GWR via Gateway® BP and LR recombination reactions. After BP reaction, the plasmid was sequenced to confirm that there were no errors affecting the open reading frame. The resulting construct was transformed into Col-0 wild-type plants (Clough and Bent, 1998) and transgenic plants were selected. T_2 seeds were grown vertically on semisolid ½ MS medium containing 1% sucrose and 0.8% agar in continuous light. To view the GFP signal, root tips of 5-day-old seedlings were chosen and viewed by confocal microscopy using an Olympus FluoView 500 laser-scanning microscope as previously described (Zhou and Li, 2005).

More than 20 independent transgenic lines were observed, and all showed the same localization patterns.

Construction of the promoter BEN2::BEN2-GUS fusion construct and GUS analysis

The 2100 bp *BEN2* promoter region with *BEN2* gene (except stop codon) was PCR-amplified using the primers: *BEN2*- BP1 (5'- GGGGACAAGTTTGTACAAAAAAGCA GGCTTCCTCTCCATCAATCAGATGTTTCAT-3') and *BEN2*-BP2 (5'-GGGGACCA CTTTGTACAAGAAAGCTGGGTCACAAGATTGACCATCGGTGTAACA-3'), and then cloned into pBIB-BASTA-GUS-GWR vector via Gateway® BP and LR recombination reactions. The plasmid from BP reaction was sequenced to ensure that there were no errors. The resulting construct was transformed into *Arabidopsis* ecotype Col-0 by the floral dipping method (Clough and Bent, 1998). Homozygous lines were identified by conducting several serial screening of BASTA-resistant plants. GUS analysis was performed using 6-day-old seedlings and different development stage tissues using vacuum-infiltration in GUS staining solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl ammonium salt, 50 mM $NaHPO_4$, pH 7.2, and 0.05% [v/v] Triton X-100; Robatzek and Somssich, 2001) for 4 hours and incubation at 37°C overnight, followed by destaining with 70% ethanol. The stained material was examined with a dissecting microscope.

Scanning electron microscopy (SEM)

The middle part of the third pair leaves from 4-week-old plants, including WS2, *bri1-5*, *bri1-5 ben2-1D (+/-), bri1-5 ben2-1D (-/-),* and *ben2-1D (-/-),* were cut into 2 mm X 2

mm pieces and fixed in 3% glutaraldehyde with 0.1 M cacodylate-0.1 M Sucrose buffer (pH 7.4) for 4 h at room temperature. Vacuum was used to remove air from the intercellular spaces during fixation. After three changes of chilled buffer, the samples were postfixed in chilled 2% osmium tetroxide for 2 hours on ice. The samples were subsequently dehydrated using a cold graded ethanol series up to 100%. Following two more changes of cold 100% ethanol (30 minutes per change), the samples were dried by critical-point dryer and coated with carbon by vacuum evaporator. Specimens were observed using a JEOL JSM-880 high resolution scanning electron microscope.

Seedling measurements

Seeds were surface sterilized with 100% ethanol and twice with 70% ethanol (30 minutes per change) and planted in ½ MS medium supplemented with 1% sucrose, 0.8% agar and different concentrations of 24-epiBL or BRZ. The plates were kept at 4°C for 2 days, and then grown vertically in continuous white light at 22°C. After 6 to 8 days, the seedlings were transferred onto agar plates and scanned using a scanner at a resolution of 600 dpi. Roots, hypocotyls and cotyledons were measured using ImageJ 1.29J (National Institute of Health imaging software; http://rsb.info.nih.gov/ij/java1.3.1). All measurements were repeated three times independently and each time 60 to 90 seedlings were measured.

Flowering time measurements

Seeds of *bri1-5*, *bri1-5 ben2-1D (+/-), bri1-5 ben2-1D (-/-),* WS2 and *ben2-1D (-/-)* were germinated in the same flat containing well-watered potting mix. After 2 days of cold treatmentat 4°C, plants were grown under long day condition (16-h/8-h dark/light) until flowering. Flowering time was scored by the time (days) when the bolt was approximately 0.5 cm high and by the rosette leaf numbers when the first flower opened. A total of 30 to 40 plants were analyzed for each genotype. Data is shown as mean ± standard error. Student's unpaired two-tailed *t*-tests were used for the statistical analysis.

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