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# The Influence of Chemical Genetics on Plant Science: Shedding Light on Functions and Mechanism of Action of Brassinosteroids Using Biosynthesis Inhibitors

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## Abstract

When exogenous chemicals allow rapid, conditional, reversible, selective, and dose-dependent control of biological functions, they act like conditional mutations, either inducing or suppressing the formation of a specific phenotype of interest. Exploration of the small molecules that induce the brassinosteroid (BR) deficient-like phenotype in *Arabidopsis* led us to identify brassinazole as the first candidate for a BR biosynthesis inhibitor. Brassinazole treatment reduced BR content in plant cells. Investigation of target site(s) of brassinazole revealed that the compound directly binds to the DWF4 protein, a cytochrome P450 monooxygenase that catalyzes 22-hydroxylation of the side chain of BRs. These results suggest that brassinazole is a BR biosynthesis inhibitor. There are currently at least two BR biosynthesis inhibitors that act like conditional mutations in BR biosynthesis. They allow the investigation of the functions of BRs in a variety of plant species. Application of BR biosynthesis inhibitors to a standard genetic screen to identify mutants that confer resistance to these inhibitors allowed the identification of new components working in BR signal transduction. This method has advantages over mutant screens using BR-deficient mutants as a background. Development of chemicals that induce phenotypes of interest is now emerging as a useful way to study biological systems in plants and this would be a complement to classical biochemical and genetic methods.

## Introduction

Combining knowledge of organic chemistry and modern aspects of plant research is very useful for investigating the interaction between chemicals and enzyme(s), or chemicals and receptor(s). For

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example, finding suicide substrates of the abscisic acid 8′-hydroxylase was a great help in identifying this enzyme (Cutler and others 2000). The use of biotinylated abscisic acid derivatives demonstrated that there are proteinaceous ABA perception sites on the plasma membrane of *Vicia faba* guard cells, and direct visualization and quantitative analyses of the ABA (abscisic acid) perception sites was possible (Yamazaki and others 2003). Photoaffinity labeling of membrane proteins with a photoactivatable phytosulfokine (PSK) analog characterized the PSK binding proteins, which were then purified with affinity chromatography using immobilized PSK (Matsubayashi and others 2002). These are straightforward biochemical approaches. There are alternative ways to determine the function of proteins and genes using chemicals.

Genetics has been a powerful tool for biologists. A classical forward genetic analysis starts with an outward physical characteristic (called a phenotype) of interest and ends with the identification of the gene or genes that are responsible for it. In classical reverse genetics, scientists start with a gene of interest and try to find what it does by looking at the phenotype when the gene is mutated. Recently, ''chemical genetics'' has been used as a new tool for dissecting and understanding biological systems (Schreiber 1998). This term impresses on us the importance of biologically active small molecules in biology. In chemical genetics small molecules are used as a switch to turn on or turn off the biological event by affecting protein functions rather than genes. In a forward chemical-genetic screen, instead of mutating genes at random, scientists generate many small molecules and then systematically introduce them into living organisms to determine their effects. Small molecules that create a change in the phenotype of interest are selected for further study. Because these small molecules probably change the phenotype by binding to proteins inside cells, thus changing the way these proteins work, there is great interest in finding the protein targets of these small molecules. In a sense, the small molecules that bind to proteins and affect their activities mimic the random mutations used in classical genetic screens. However, there are important differences. In a genetic screen the activity of a protein is altered indirectly—by mutating its gene —but in chemical genetics this change is direct and occurs in real time (when the molecule is added). Another difference between the two approaches is that the effect of the ''mutation'' caused by a small molecule is reversed when the small molecule is removed. In contrast, the effect of mutating a gene is, in most cases, permanent. Therefore, chemical-genetic approaches may be more useful when scientists want to study genes that are essential to an organism's survival. A small molecule can be administered to cells or organisms for a very short time to study the function of the target protein (Tan 2002).

One of the most difficult aspects of forward chemical genetics is identification of the target(s) of the small molecule, especially if the target is novel. This difficulty could be overcome by utilizing a standard genetic screen to identify mutants that confer resistance to a small molecule. That is, by using a small molecule as a specific mutagen, the combination of chemical and classical genetics can be a powerful tool for new discoveries in biology (Specht and Shokat 2002).

In a reverse chemical-genetic screen, a protein is purified and then tested against a large number of small molecules. The candidates are retested several times under different conditions, and only those that ''pass'' subsequent tests are used to determine the biological consequences of altering the target protein's function in a cell or whole organism. Thus, using this strategy of chemical genetics, it is possible to identify new reagents that act like conditional mutations, either inducing or suppressing the formation of a specific phenotype of interest. In this context, we look back over brassinosteroid (BR) biosynthesis inhibitors.

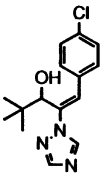
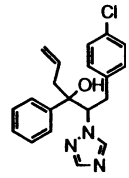
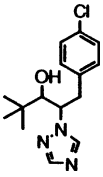
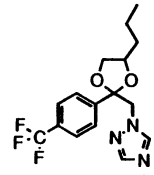
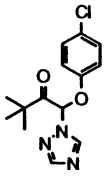
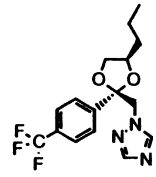
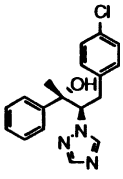
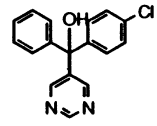
Five years have passed since the discovery of the first BR biosynthesis inhibitor (Min and others 1999). One of the scientific goals of working with BR biosynthesis inhibitors is to find new functions of BRs and to identify novel components involved in BR biosynthesis and signal transduction. From the point of view of specific approaches to achieve this goal, we discuss the following topics in this review: (1) development of BR biosynthesis inhibitors, (2) functions of BRs in plant development unveiled by BR biosynthesis inhibitors, (3) disease resistance induced by BRs, (4) use of BR biosynthesis inhibitors to study BR-regulated gene expression, and (5) BR biosynthesis inhibitors as a useful screening tool for BR signaling mutants. In Table 1, BR biosynthesis inhibitors reported up to now are listed. Brz22012 is the most potent and specific inhibitor among them.

## Development of BR Biosynthesis Inhibitors

### Development of Small Molecules That Induce BR-Deficient-like Phenotype in Plants

Progress in understanding the detailed mechanism of BR biosynthesis and signal transduction has been quite rapid recently, and two main chemical ap-

**Table 1.** Cytochrome P450 Inhibitors and BR Biosynthesis Inhibitors

| Chemicals | References | Chemicals | References |
|---|---|---|---|
|  | Uniconazole (Iwasaki and Shibaoka 1991) |  | Brz2001 (Sekimata and others 2001) |
|  | Paclobutrazol |  | Brz220 (Sekimata and others 2002a) |
|  | Triadimefone (Asami and others 2003) |  | Brz22012 (Sekimata and others 2002b) |
|  | Brassinazole (Brz) (Min and others 1999) (Asami and Yoshida 1999) (Asami and others 2000, 2001) |  | DPPM4 (Wang and others 2001) |

proaches have been devised to antagonize the action of BRs. One is a search for a chemical that acts directly at the BR receptor as an antagonist, and the other is a chemical that blocks BR biosynthesis by inhibiting a key enzyme of the process. With respect to the latter strategy, we developed a BR biosynthesis inhibitor because BR biosynthesis mutants had already been identified (Li and others 1996; Szekeres and others 1996). These mutants display strong dwarfism with curly, dark-green leaves in the light, and a de-etiolated phenotype with short hypocotyls and open cotyledons in the dark. The characterization of BR-deficient mutants by biochemical studies and molecular genetic analysis has established the biosynthetic pathway for brassinolide (BL), the most biochemically active BR (Bishop and Yokota 2001). These mutants are the standard by which to predict the physiological effects of BR biosynthetic inhibitors. BL is synthesized from campesterol via either early or late C-6 oxidation pathways that include cytochrome P450 monooxygeneses. These steps include the production of 6α-hydroxycampestanol from campestanol, cathasterone from 6-oxo-campestanol (Choe and other 1998), teasterone from cathasterone (Szekeres and others 1996), castasterone from typhasterol, and BL from castasterone (Yokota 1997). Thus, the biosynthetic pathway of BRs includes several potential active sites for cytochrome P450 inhibitors. Uniconazole, a gibberellin (GA) biosynthesis inhibitor, has been reported to inhibit BR biosynthesis, even though its main target is GA biosynthesis rather than BR biosynthesis (Yokota and others 1991). Various triazole compounds, including uniconazole and other GA biosynthesis inhibitors, have been shown to inhibit many types of cytochrome P450s (Raymond and others 1989). From studies of these cytochrome P450 inhibitors, the azole moiety of the inhibitors is believed to act as a ligand binding to the iron atom of the heme prosthetic group of the cytochrome P450 enzyme, forming a coordinated complex. Chemical structure other than a triazole moiety is considered to be the important factor, which results in the selective nature of the interaction. In an effort to illustrate azole-binding sites in BR biosynthesis and to identify essential structural features among azole compounds, the structure–activity relationship of uniconazole has been studied for BR biosynthesis inhibition.

## Assay Methods for BR Biosynthesis Inhibitors

Because a good biological system for identifying BR biosynthesis inhibitors had not yet been found, we

combined some biological assays. First, chemicals were assayed using a rice-stem elongation test to identify and eliminate GA biosynthesis inhibitors because rice is very sensitive to GA deficiency and therefore a good plant for this purpose. Some of the synthesized chemicals retarded rice-stem elongation, and such retardation was reversed by treatment with GA. The second screening for BR biosynthesis inhibitors was performed to find chemicals that induce dwarfism in *Arabisopsis* and which resemble BR biosynthesis mutants and can be rescued by the addition of BL. BL has been shown to be effective in rescuing the *Arabidopsis* BR-deficient mutants, but they cannot be rescued by other plant hormones, such as auxins and GAs. Finally, selected compounds were assayed using a cress hypocotyl elongation test. Cress is very sensitive to an internal deficiency of BRs and is therefore a useful species for evaluating BR biosynthesis inhibitors (Min and others 1999; Sekimata and others 2001).

## Structure–Activity Relationship Study

The presence of a *tert*-butyl group at C-2 of uniconazole and paclobutrazol could be essential for the inhibitory activity of GA biosynthesis. The chemical structure of paclobutrazol is closely related to that of uniconazole but it has no double bond. A substitution of a *tert*-butyl group of these compounds with a phenyl group caused a drastic loss of inhibition of rice-stem elongation, whereas it caused strong inhibition of *Arabidopsis* and cress hypocotyl elongation (Min and others 1999). This retardation was recovered by the coapplication of BL but not of GA. These studies revealed that the phenyl moiety at C-2 of uniconazole and paclobutrazol is essential for the selectivity of BR biosynthesis inhibition. In addition to the substitution, an introduction of an alkyl or aryl group at C-2 of paclobutrazol caused more potent BR biosynthesis inhibition and reduced the effect on GA biosynthesis (Min and others 1999; Sekimata and others 2001). As a result the structural difference between paclobutrazol and brassinazole derivatives is only the existence of an alkyl or aryl group and a phenyl group attached to the carbinol carbon. These groups drastically change the character of triazole derivatives from GA biosynthesis inhibitors to BR biosynthesis inhibitors.

## Target Sites(s) of BR Biosynthesis Inhibitor

To investigate the biosynthetic steps affected by brassinazole, we examined the effect of biosynthetic intermediates downstream from cathasterone on hypocotyl elongation of brassinazole-treated *Arabidopsis* (Asami and others 2000). The feeding experiment suggests that the target(s) of brassinazole could be the two-step conversion of 6-oxocampestanol to teasterone via cathasterone, catalyzed by DWF4 and CPD, which are *Arabidopsis* cytochrome P450s isolated as putative steroid 22- and 23-hydroxylases, respectively. In addition, we analyzed endogenous BRs in brassinazole-treated and-nontreated *Catharanthus roseus* cells (Asami and others 2001). In brassinazole-treated plant cells, the levels of campestanol and 6-oxo-campestanol levels were increased, and levels of BR intermediates with hydroxy groups on the side chains were reduced, suggesting that brassinazole treatment reduced BR levels by inhibiting the hydroxylation of the 22-position catalyzed by DWF4. Thus, DWF4 was expressed in *Escherichia coli*, and the binding affinity to brassinazole and its derivatives to the recombinant DWF4 was analyzed (Asami and others 2001). Among several triazole derivatives, brassinazole had both the highest binding affinity to DWF4 and the highest growth inhibitory activity. The binding affinity and activity for inhibiting hypocotyl growth were well correlated among the derivatives. On the other hand, brassinazole did not bind to the recombinant CPD proteins (Mizutani, personal communication), which suggested that CPD was not the target site of brassinazole. In brassinazole-treated *Arabidopsis*, the CPD gene was induced within 3 h, most likely because of feedback activation caused by the reduced levels of active BRs. These results indicate that brassinazole inhibits the hydroxylation of the 22-position of the side chain in BRs by direct binding to DWF4 and that DWF4 catalyzes this hydroxylation reaction. Because the involvement of DWF4 protein in the BR biosynthesis pathway was suggested only by comparing the phenotypes of *dwf4* mutants to that of other BR-deficient mutants and feeding biosynthesis intermediates, the combination of the chemical analysis of internal BRs in brassinazole-treated plant cells and the binding assay of brassinazole to DWF4 should have been an alternative way to investigate the role of the DWF4 in BR biosynthetic pathway.

## Searching for Novel BR Biosynthesis Inhibitors

To develop more specific and potent BR biosynthesis inhibitors, we screened for various triazole derivatives with the cress hypocotyl elongation test. Through this screening experiment, fenarimol, triadimefon, and propiconazole were selected as likely inhibitors of BR biosynthesis. Fenarimol is a pyrimidine derivative and an inhibitor targeting

a wide variety of cytochrome P450 monooxygenases. Chemical modification of fenarimol led us to discover a new BR biosynthesis inhibitor, DPPM4, which is specific for BR biosynthesis but not as potent as brassinazole ( Wang and others 2001a). Triadimefon affects GA biosynthesis and 14α-demethylase in ergosterol biosynthesis. In our experiment, triadimefon shows good affinity to expressed DWF4 proteins and induces the BR-deficiency phenotype in plants (Asami and others 2003). These results indicate that triadimefon inhibits BR biosynthesis. Propiconazole is a fungicide that targets lanosterol 14α-demethylase in the ergosterol biosynthesis pathway. Moreover, the triazole is reported to show plant growth regulator activity by the inhibition of obtusifoliol 14α-demethylase (Raymond and others 1989). Propiconazole-treated cress showed dwarfism that could be rescued considerably by BL treatment. This implies that the morphological alteration of cress seedlings treated with propiconazole should be partly due to the deficiency of BL (Sekimata and others 2002a). Because propiconazole showed considerable inhibitory activity in the cress hypocotyl elongation test, the synthesis of propiconazole derivatives with optimized activity and selectivity was started. Intensive study of structure–activity relationships of propiconazole led to the discovery of a more potent and specific inhibitor, Brz220 (Sekimata and others 2002a). Because it contains two stereogenic carbon atoms, there are four epimeric stereoisomers of Brz220. Since the stereoisomers of azole compounds often have different biological activities, we examined the relationship between the stereochemical structure and biological activity of Brz220. The configuration of enantiomers of Brz220 was determined by a combination of asymmetric syntheses (Sekimata and others 2002b). Finally, Brz22012, one of the stereoisomers of Brz220, was found to be the most potent BR biosynthesis inhibitor (the structure of Brz22012 is shown in Table 1). In inhibiting BR biosynthesis, the (*S*)-configuration of Brz220 at C-2 predicts whether a stereoisomer can bind to its receptor site on a cytochrome P450 in the BR biosynthesis pathway, as occurs with brassinazole. Further study to reveal the site of action of Brz220, both *in vivo* and *in vitro*, could be accomplished.

## Functions of BRs in Plant Development Unveiled by BR Biosynthesis Inhibitors

In general, plant hormone inhibitors are useful tools, as demonstrated in the study of GAs (Yokota and others 1991). Therefore, brassinazole should be helpful in clarifying the function of BRs in plants, as a complement to studies of BR-deficient mutants. Mutant or inhibitor studies have already demonstrated quite well that BRs are essential for normal plant growth (Asami and Yoshida 1999). Therefore, as a next step to understand novel functions of BR in plants, brassinazole was applied to investigate the functions of BRs in photomorphogenesis in the dark and in xylem development. To be able to use brassinazole as a tool, it is necessary to confirm in detail that various morphological and cytological changes in brassinazole-treated plants are due to inhibition of BR biosynthesis and not to side effects of the inhibitor. In the experiments in photomorphogenesis, the brassinazole-induced phenotype was carefully compared with that of BR-deficient mutant (Nagata and others 2000).

### Plastid Differentiation

*De-etiolated2* (*det2*), which was the first BR biosynthesis-deficient mutant identified, was originally found to be defective in light-regulated growth. When grown in the dark, plants exhibited the phenotype of light-grown plants (Chory and others 1991). Furthermore, they accumulated high levels of light-regulated RNA and photosynthetic proteins in the absence of light. Subsequently, an increase in the expression of light-regulated genes was also confirmed in seedlings of the constitutive *photomorphogenesis and dwarfism* (*cpd*) mutant grown in the dark. This mutant is defective in a cytochrome P450 (CYP90), which plays an essential role in the biosynthesis of BRs (Szekeres and others 1996). In this context, BRs seem to affect light-dependent signaling pathways. In some BR-biosynthesis-deficient mutants, such as *det2* and *dwarf4* (*dwf4*), electron microscopy has shown that etioplasts fail to differentiate to chloroplasts, suggesting that the mutation uncouples the plastid differentiation pathway (Chory and others 1991; Azpiroz and others 1998).

When a BR biosynthesis inhibitor, brassinazole, was applied at concentrations ranging from 0.1 to 2 μM, *Arabidopsis thaliana* (L.) Heynh seedlings grown in the dark exhibited morphological features of light-grown plants, that is, short hypocotyls, expanded cotyledons, and true leaves, in a dose-dependent manner (Nagata and others 2000). Control (non-brassinazole-treated) seedlings grown in the dark for 40 days did not develop leaf primordia, whereas treatment with the lowest concentration of brassinazole induced the development of leaf buds, although it hardly induced any short hypocotyls,

and treatment with the highest concentration of brassinazole induced both short hypocotyls and leaves. Labeling experiments with the thymidine analogue 5-bromo-2′-deoxyuridine revealed that amplification of cell nuclei and organellar nucleoids is activated in the shoot apical meristems of dark-grown brassinazole-treated seedlings. These results suggest that brassinazole treatment induces development of true leaves. Furthermore, condensation and scattering of plastid nucleoids, which are known to occur during the differentiation of etioplasts into chloroplasts, were observed in the plastids of dark-grown brassinazole-treated cotyledons. In addition, high levels of ribulose-1,5-bisphosphate carboxylase-oxygenase proteins accumulated in the plastids of the cotyledons. Electron microscopy showed that the plastids were etioplasts with a prolamellar body and few thylakoid membranes. These results suggest that brassinazole treatment in the dark induces the initial steps of plastid differentiation, which occur prior to the development of thylakoid membranes. This is a novel presumed function of BRs. These cytological changes seen in brassinazole-treated *Arabidopsis* were exactly the same as those seen in a BR-biosynthesis-deficient mutant, *det2*, supporting the hypothesis that brassinazole has no side effects except inhibiting BR biosynthesis and should prove a useful tool in clarifying the role of BRs.

To summarize, various changes in brassinazole-treated *Arabidopsis* are perfectly coincident with those in a BR-biosynthesis-deficient mutant, *det2*, suggesting that brassinazole has no side effects except inhibition of BR biosynthesis. Second, it was revealed that brassinazole treatment (inhibition of BR biosynthesis) induces the development of true leaves and short hypocotyls independently in the dark. Third, through the study of the cytological changes in brassinazole-treated *Arabidopsis* from a different perspective than that of previous studies of BR-deficient mutants, novel characteristics were found, suggesting that brassinazole treatment induces a very early step in plastid differentiation.

## Vascular Differentiation

BRs have been shown to play a role during differentiation of xylem in *in vitro* experiments (Clouse and Zurek 1991; Iwasaki and Shibaoka 1991; Yamamoto and others 1997). A plant-growth retardant, uniconazole, inhibits GA biosynthesis and partially inhibits BR biosynthesis. Iwasaki and Shibaoka (1991) reported that inhibition of tracheary element differentiation by uniconazole depends upon inhibition of BR synthesis in cultured cells of *Zinnia elegans*. Tracheary element formation of cultured *Zinnia* cells has been divided into three stages: during stage I, cells dedifferentiate; stage II involves the differentiation to precursor cells of tracheary elements; stage III is the final stage and involves secondary wall formation and cell death (Fukuda 1997). Yamamoto and others (1997) revealed that BRs induce stage III in cultured *Zinnia* cells. BRs have also been isolated from the cambial region in Scotch pine (Kim and others 1990). These *in vivo* results support the notion that BRs function during vascular differentiation.

In a *cpd* mutant of *Arabidopsis* defective in cytochrome P450, which is involved in BR biosynthesis, a slight predominance of phloem at the expense of xylem was observed (Szekeres and others 1996). The sterol- and BR-deficient mutant *dwf7* also exhibits an increase in phloem vs. xylem cells, and the number of vascular bundles is reduced from eight in the wild type to six in the mutant, with irregular spacing between vascular bundles (Choe and others 1999). However, some of BR-biosynthesis-deficient mutants contain normal vascular tissues. Thus, previous reports on *Arabidopsis* mutants were insufficient to support the notion that BRs promote vascular differentiation *in vivo*.

In this context cress plants were treated with brassinazole to investigate the involvement of BRs in vascular differentiation *in vivo* (Nagata and others 2001). Figure 1 shows the morphological phenotype of cress plants treated with brassinazole and/or BL for 40 days. Brassinazole-treated cress exhibited a severe dwarf phenotype, with dark-green curled leaves. Morphological changes were similar to those seen in other plants treated with brassinazole, and much like the phenotype of BR-deficient mutants (Asami and Yoshida 1999). Normal growth was restored by application of BL, but not GA, to brassinazole-treated cress. The function of BRs in vascular differentiation of cress plants was also investigated using microscopic analysis.

In hypocotyls of cress grown for 5 days, development of the primary phloem and primary xylem of brassinazole-treated hypocotyls seemed to be normal or promoted. In 12-day-old cress, control plants exhibited secondary xylem formation from vascular cambium, but secondary phloem was not yet formed. In contrast, 12-day-old brassinazole-treated cress contained secondary phloem before there was marked secondary xylem formation. The secondary xylem region of the hypocotyl of 40-day-old brassinazole-treated cress was much smaller than that of control cress. In the brassinazole-treated cress, it seemed that formation of the sec-

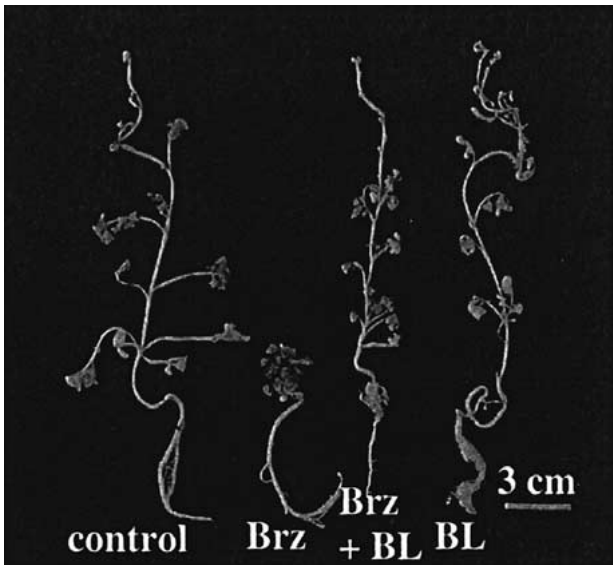



**Figure 1.** Morphological effects of Brz and/or BL on cress plants. Cress plants were grown for 40 days in continuous light.

ondary phloem ring was promoted at the expense of formation of the secondary xylem.

In stems at the second internode of cress grown for 40 days, control plants exhibited a continuous ring of secondary xylem formed from vascular cambium, although a ring of secondary phloem had not yet formed. In contrast, the brassinazole-treated cress grown for 40 days exhibited no rings of either secondary xylem or secondary phloem in the stem. Thus, the results clearly indicate that brassinazole treatment inhibits secondary xylem development. Normal xylem development in brassinazole-treated cress was restored by application of BL, but not GA. Brassinazole treatment in cress plants seems to specifically inhibit BR biosynthesis. Although uniconazole treatment induced severe dwarfism, few changes in the vascular system of uniconazole-treated cress were observed. Although uniconazole is known to inhibit the biosynthesis of BL in addition to GA in cultured *Zinnia* cells, it may be a specific inhibitor of GA in cress plants. This result indicates that the abnormal vascular formation induced by brassinazole-treatment is not a secondary effect of dwarfism. That is, BRs are essential for the development of the vascular system in cress plants. It might be that brassinazole treatment affects secondary wall formation, such as lignification of either the xylem or phloem. This point is the subject for a future study. Figure 2 shows a schematic illustration of the development of the vascular system in brassinazole-treated cress as predicted from observation of both hypocotyls and stems.

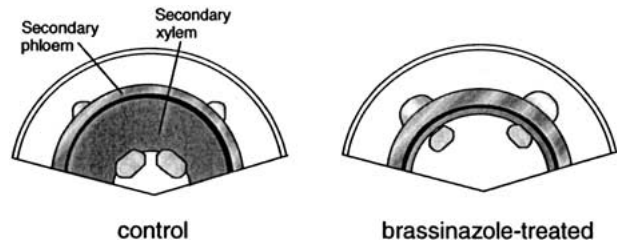



**Figure 2.** Schematic illustration of the changes during development of the vascular system in Brz-treated cress.

### Other Functions

Spraying morning glory (*Pharbitis nil*) with BR biosynthesis inhibitors retarded plant growth, especially internode elongation and leaf development. The diameter of the flowers of inhibitor-treated plants was clearly smaller than that of nontreated plants. These results suggest that BRs should have an essential role for the plant growth and the development of both leaves and flowers (unpublished data).

Recently, Bajguz and Asami reported the effect of a BR biosynthesis inhibitor on *Chlorella vulgaris* cells (Bajguz and Asami, unpublished data). Treatment of cultured *C. vulgaris* cells with Brz2001 inhibited their growth in the light. This inhibition was prevented by the co-application of BR. This result suggests that the presence of endogenous BRs during the initial steps of the *C. vulgaris* cell cycle is indispensable to their normal growth in the light.

## Disease Resistance Induced by BRs

Plants have evolved a unique self-protection system in addition to morphological adaptations because diseases caused by microorganisms are inevitable and constitute a serious stress for plants. The primary response in this system involves the specific recognition of the pathogens and a rapid induction of localized host cell death (Ross 1961). The secondary response is to develop an induced resistance to protect the plant from further attacks by the pathogens (Kuc 1982; Mclntyre and others 1981). These responses are governed by hormonal regulation, in which salicylic acid (SA), jasmonic acid (JA), or ethylene participate. The most well-characterized induced resistance is systemic acquired resistance (SAR) that is activated through SA biosynthesis after infection by a necrotizing pathogen and confers resistance against a broad spectrum of pathogens in other uninfected plant parts (Chester 1933; Durner and others 1997).

There have been several reports describing the relationship between BRs and plant stress responses such as activation of cold resistance (Hotta and others 1998) and induction of ethylene biosynthesis (Yi and others 1999), suggesting that BRs may play a role in stress-responding systems. Protective activity of BRs against plant diseases has been indicated based on evaluations from field trials (Khripach and others 2000), but its mechanism at the molecular level remains to be clarified. Recent studies revealed the roles of some other phytohormones in disease resistance, as mentioned above, using model plant–microbe interaction systems. BRs' function in defense response against pathogens was also demonstrated using model plant systems (Nakashita and others 2003).

Wild-type tobacco treated with 20 μM BL exhibited enhanced resistance to the viral pathogen tobacco mosaic virus (TMV). Treatment with BL also reduced the disease symptoms of tobacco wildfire disease, caused by the bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (*Pst*), and of tobacco powdery mildew disease, caused by the fungal pathogen *Oidium sp*. BL did not have any antimicrobial activity in liquid culture at tested concentrations. Thus, the results of these infection tests indicated that BL induced a disease resistance in tobacco to a broad spectrum of pathogens, which bears similarities to SAR.

During the development of known systemically induced resistances, the accumulation of specific hormones in tissues is observed. For example, the infection of necrotizing bacteria induces SA accumulation resulting in the development of SAR. Furthermore, the requirement of SA for SAR development was confirmed by using NahG transgenic tobacco plants, which are unable to accumulate SA due to the expression of salicylate hydroxylase, an SA-degrading enzyme (Delaney and others 1994; Gaffney and others 1993). In either TMV-inoculated or mock-inoculated tobacco leaf tissue, the BL level was less than the detection limit but some biosynthetic precursors were detected. The levels of castasterone and 6-deoxocastasterone in TMV-inoculated leaves of wild-type plants were 0.27 and 2.88 ng/gfw, respectively, whereas those of mock-inoculated leaves were 0.16 and 1.52 ng/gfw, respectively, indicating that *N*-gene-mediated defense response against TMV resulted in increased levels of BRs in leaf tissues. These slight differences do not cause drastic morphological changes. In addition, the requirement of BR biosynthesis for the plant defense response was demonstrated by experiments using Brz2001 (Sekimata and others 2001), an inhibitor for BL biosynthesis. The Brz2001-treated wild-type plants exhibited a similar level of resistance against TMV to that of the water-treated control plant. However, the Brz2001-treated NahG transgenic plants exhibited reduced resistance against TMV compared with the water-treated control plants. Brz2001 concentration up to 150 μM exhibited the suppressive effect on disease resistance in a dose-dependent manner, with a slight effect on growth. Thus, in wild-type plants, it is speculated that the more intense effect of SA in the defense response obscures the effect of Brz2001, which becomes visible when the effects of SA are excluded. Another possible explanation is that BL functions in situations in which the induction of SA biosynthesis is not involved. Therefore, these experiments demonstrated that brassinosteroid-mediated disease resistance (BDR) takes part in the innate immunity system in tobacco, independent from the SA-mediated defense response.

Characterization of BDR revealed that it is effective against a variety of pathogens by a mechanism different from that of known induced disease resistance responses in tobacco. BDR development does not accompany SA accumulation in the wild-type plant and can be induced in NahG transgenic tobacco plants, indicating that BDR does not require SA biosynthesis for its development. Furthermore, BDR development does not accompany the expression of defense-related genes, which are SA- or JA-inducible in SAR development or wound responses, respectively.

Various cross-talks among phytohormone-mediated signaling in stress responses have been reported. SA-mediated signaling for SAR development and JA-mediated wound response are reported to interfere with each other (Niki and others 1998). However, simultaneous activation of SAR and BDR results in an enhanced level of protection against TMV and *Pst*. This additive effect likely depends on basic mechanisms of these resistances and is caused by a parallel activation of defense responses.

As opposed to dicotyledonous species, in monocotyledonous species, especially rice, the functions of SA and JA in the defense response have not been clarified yet. BL treatment resulted in a moderate level of resistance induction, suggesting that BDR functions also in rice plants, although the requirement of BR biosynthesis in this plant has not been determined.

Conclusively, BL functions in innate immunity systems of higher plants, including dicotyledonous and monocotyledonous plant species, where the resistance mechanism mediated by BL is distinct from other currently identified ones. Because BRs are essential phytohormones for the growth and

development of plants, they may function in the defense response by activating the fundamental biological systems that function not only in stress responses but also in other aspects of cellular regulation.

## Use of BR Biosynthesis Inhibitors to Study BR-Regulated Gene Expression

### Use of BR Biosynthesis Inhibitors to Study Regulation of BR Biosynthesis

Exogenous treatment of *Arabidopsis* with specific BR intermediates or end products, such as BL or CS, downregulates gene expression of *CPD* (Mathur and others 1998) and other BR-biosynthetic and putative BR-biosynthetic genes (Goda and others 2002). By contrast, Noguchi and others reported the upregulation of the *DWF4* gene in the BR-deficient mutants *dwf1-1* and *cpd3939* or in the BR-insensitive mutant *bril-5*. These results suggested that transcript abundance of BR-biosynthetic genes is regulated in a feedback regulatory manner through *BR11*. These findings were supported by using triazole-type BR-biosynthetic inhibitors, brassinazole (Asami and others 2001) and triadimefon (Asami and others 2003). In particular, the *CPD* gene expression was upregulated after 3-h treatment with brassinazole, indicating that the endogenous BR intermediates are metabolized within the 3-h brassinazole treatment and BR biosynthesis is activated at the level of transcription, which is regulated by decreased endogenous active BRs. This result suggests rapid depletion of the BR precursor pool within the 3-h brassinazole treatment, that is, the BR precursors are rapidly metabolized to BL and/or degraded to the inactive form during the 3-h treatment with brassinazole.

### Use of BR Biosynthesis Inhibitors to Extract Specific Responsive Genes in a Microarray Study

Recent progress in DNA microarray technology enabled comprehensive studies of hormone-regulated genes. However, it is not easy to identify hormone-regulated genes, specifically in a population of false-positively responding (noisy) genes. Because these studies handle huge numbers of genes, extraction of hormone-responsive genes by a statistical analysis could result in inclusion of false-positive genes. For example, extracting 1000 genes at a significance level of $p < 0.05$ by statistical analysis gives 50 false-positive genes. Therefore, it is reasonable to confirm specificity of the extracted gene list by alternative sets of experiments. Goda and others (2002) analyzed BR-regulated genes and confirmed the BR-responsive genes in *Arabidopsis* by using the newly developed BR biosynthesis inhibitor Brz220 (Sekimata and others 2002a), which has the strongest and the most specific effects on *Arabidopsis* among the BR biosynthesis inhibitors (Sekimata and others 2002b). Wild-type seedlings were exposed to either $3 \times 10^{-6}$M Brz220 or a mock treatment for 3 h and the abundance of transcripts was compared using GeneChip oligonucleotide microarrays. The Signal Log Ratio values from the Brz experiments were plotted against that from the BL experiments for all genes on the GeneChip (Figure 3A), for BL-responsive genes in a single GeneChip experiment (Figure 3B), for genes responding to BL reproducibly in two independent GeneChip experiments (Figure 3C), or for genes responding to BL reproducibly in three independent GeneChip experiments (Figure 3D). The correlation coefficient for the Brz and BL experiments was −0.004 for all genes ($n > 8000$), indicating that there was no significant correlation between the BL and Brz treatments for global expression comparison. On the other hand, a correlation coefficient of −0.793 was calculated for the genes in Figure 3D, suggesting a strong inverse correlation between the BL and Brz220 treatments for extracted genes. These observations indicated that a number of false-positive genes are included in a BL-responding gene list based on a single GeneChip experiment and that BR-regulated genes were successfully extracted in three independent GeneChip experiments. In addition, Brz220 efficiently inhibited BR biosynthesis in *Arabidopsis*.

## BR Biosynthesis Inhibitors as a Useful Screening Tool for BR Signaling Mutants

In the past decade, the identification and characterization of *Arabidopsis* BR biosynthetic mutants such as *det2* (Li and others 1996) and *dwf4* (Choe and others 1998) has revealed the importance of BRs in plant growth regulation. These BR-deficient mutants have a pleiotropic dwarf phenotype that can be reverted to a wild-type-like phenotype by feeding with BL. The *Arabidopsis bri1* mutant was identified by its ability to elongate roots in the presence of high concentrations of BR and it also exhibits a dwarf phenotype (Clouse and others 1996; Li and Chory 1997). Study of *bri1* revealed that *BRI1* is a critical component in BR signaling and

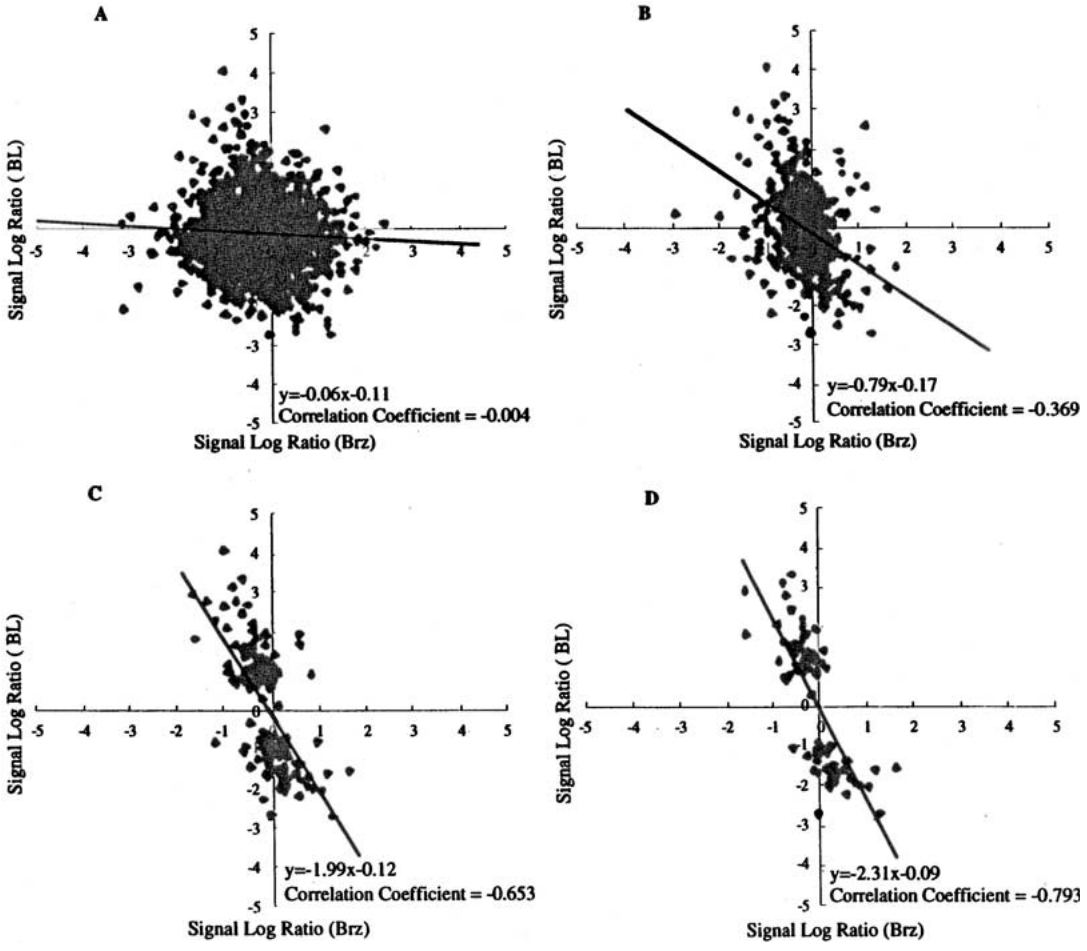



**Figure 3.** Comparison of BL and Brz treatment with the use of GeneChip experiment. The distribution of Signal Log Ratio values for treatments with BL (*y*-axis) and Brz (*x*-axis) are shown. (**A**) All of the genes (more than 8000) on the GeneChip are plotted. (**B**) Genes that are induced or reduced more than two-fold in a single GeneChip experiment are plotted. (**C**) Genes that are induced or reduced more than two-fold in two of the three GeneChip experiments are plotted. (**D**) Genes that are induced or reduced more than two-fold in three GeneChip experiments are plotted. The Signal Log Ratio represents the ratios of hybridization signals using a log (base 2) scale. A Signal Log Ratio of 1 represents a gene whose expression is increased two-fold by treatment with either BL or Brz, and a Signal Log Ratio of −1 represents a gene whose expression is reduced two-fold by treatment with either BL or Brz. (This figure is reproduced from Goda and others 2002).

that mutation in its *BRI1* gene causes an increase in BR levels. *BRI1* is a member of the leucine-rich repeat (LRR) receptor kinase family, and BL binds strongly to a plasma membrane fraction purified using an anti-*BRI1* antibody (Wang and others 2001b). In animal cells, steroid hormones are perceived through nuclear-localized steroid-binding proteins, but plants can perceive steroid hormones at the cell surface (Schumacher and others 2000). How this signal is transduced to regulate plant nuclear gene expression is unknown.

Many studies of the molecular mechanisms of plant growth have been performed using genetic methods in *Arabidopsis*. As initial steps in the study of the molecular genetics of plant hormone action, screens are conducted to identify phytohormone-deficient and phytohormone-insensitive mutants. These trials can identify a number of genes, but these genes are likely not all of the players in the regulation of plant growth by the phytohormone (Kende and Zeevaart 1997). The next step to consider is a screen to identify suppressor mutants that repress phytohormone deficiency or signaling mutants, because these repressors may be permanently activated in phytohormone signaling. In the GA research field, the *spy* mutant was identified on the basis of its resistance to the GA biosynthesis inhibitor paclobutrazol, and the gene was found to encode a homolog of *N*-acetylglucosamine transferase (Jacobsen and others 1996). Furthermore, *rga* was

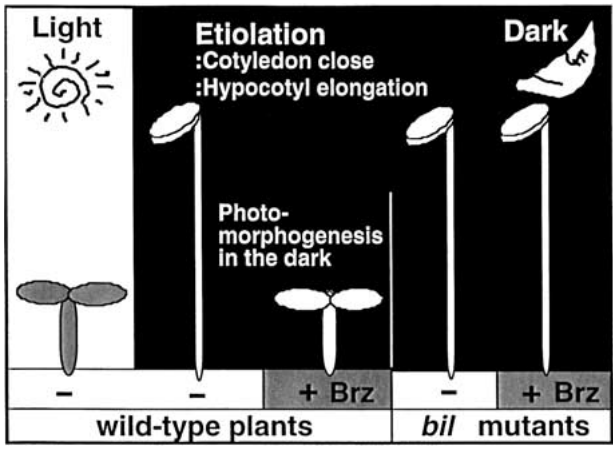



**Figure 4.** Screening strategy for *bil* (Brz-insensitive-long hypocotyl) mutants.

identified as a suppressor mutant of the *ga1-3* GA biosynthesis mutant, and the mutated gene was found to belong to the *VHIID* family of transcription factors (Silverstone and others 2001). Research in the BR signaling field is now proceeding to the second strategy for *Arabidopsis* mutant screening, using BR-deficient and signaling mutants.

To analyze in detail the mechanisms of BR biosynthesis and signal transduction, we performed a screen for mutants with altered responses to Brz220 treatment in darkness in the germination stage. A screen of 140,000 *Arabidopsis* seeds that had been subjected to EMS and fast neutron mutagenesis revealed several mutants that had significantly longer hypocotyls than the wild type when grown in the dark and treated with Brz220 (Figure 4). These plants were designated *bil* mutants (Brz-insensitive-long hypocotyl). Initially, we identified a dominant mutant, *bil1-1D*, from the EMS-treated lines. When grown in medium containing 3 μM Brz220, wild-type plants had quite short hypocotyls, but *bil* mutants had hypocotyls as long as those of wild-type plants grown on unsupplemented medium. The *bil* mutants did not display a dwarf phenotype like that of *bri1*. In parallel, *bzr1-1D* and *bes1-1D* were identified as Brz-resistant and *bri1*-suppressor mutants, respectively. Gene sequencing revealed that the *bzr1-1D* gene is the same gene as *bil1-1D*, even containing the same mutation (Wang and others 2002). These genes are 88% identical to *BES1*, and the *bes1* mutant has the same nucleotide substitution (Yin and others 2002a). The plant-specific gene family encompassing *BZR1*, *BES1*, and *BIL1* encodes novel phosphoproteins containing a putative nuclear localization signal. The BZR1:CFP fusion protein localizes mainly to the cytoplasm and also to the nucleus at low levels, but treatment with BRs results in a significant increase of BZR1:CFP levels in the nucleus within 30 min. In contrast, a BZR1:CFP protein containing the mutation localizes continuously to the nucleus (Wang and others 2002). These results suggest that *BZR1/BIL1* and *BES1* are key components in BR signaling from the cell surface to the nucleus.

The phenotypes of the *bzr1/bil1* and *bes1* Brz220-resistance and *bri1*-suppression mutants, respectively, are very strong, with the resistant mutant just like wild-type plants in appearance, even though it is severely deficient in BRs. These mutants, the mutant alleles of which are both dominant, result from the substitution of just one amino acid as compared to the wild type. Overexpression of the *BZR1* or *BES1* wild-type genes via the CaMV 35S promoter resulted in only weak resistance against BR deficiency (Wang and others 2002; Yin and others 2002b). These results suggest that *bzr1/bil1* and *bes1* mutants would be difficult to identify from activation-tagged pools of plants in the background of a mutant deficient in BRs, such as *det2* or *dwf4*. An alternative method is to induce point mutations by chemical treatment of BR-deficient mutants. The most widely used method to identify point mutations is genomic walking, performed using backcrosses with another ecotype. The screening is best performed on recombinant F2 plants to allow identification of the BR-deficiency mutation as a homozygous *det2/det2* plant and not as *det2/DET2* or *DET2/DET2* plant. However, distinguishing between putative BR signaling mutants with a *det2/det2* homozygous background and *det2/DET2* or *DET2/DET2* plants with no mutation is very difficult, because they would all have a phenotype of resistance against BR deficiency. Therefore, identifying a suppressor mutant resulting from a point mutation might also be challenging. These predictions suggest that the combination of BR biosynthesis inhibitors and a simple point mutation in the wild-type Columbia ecotype can allow rapid identification of crucial signaling proteins. This role for BR biosynthesis inhibitors will be a great contribution to plant science.

*brs1* and *bak1* were identified as *bri1-5* dwarf suppressor mutants by activation tagging. *BRS1* encodes a carboxypeptidase and its role in BR signaling has not been defined (Li and others 200la). *BAK1*, however, encodes a leucine-rich-repeat type receptor-like kinase that could interact directly with BRI1 (Li and others 2002; Nam and others 2002). On the basis of their phenotypes, *bak1-1D* and *brs1-1D* mutants could potentially be Brz-insensitive mutants. Several other BR-insensitive dwarf mutants, *bin2* and *bin3/bin5*, have also

been identified. *BIN2* encodes a cytosolic GSK kinase (Li and Nam 2002), whereas *BIN3* and *BIN5* encode proteins of the topoisomerase family (Yin and others 2000 ). The idea that these proteins could be related to BR signaling was investigated by examining the phenotypes of transformed plants in which expression of these genes has been modified, such as by overexpression, and by monitoring the plants for a Brz-insensitive phenotype (Li and Nam 2002; He and others 2002).

Rop2 is a type of GTPase, and transformants in which this protein is constitutively active show hypersensitivity to BRs (Li and others 2001b). Because dominant negative *Rop2* transformant does not display BR insensitivity, the actual relationship of this gene with BRs is not yet clear. The *det3* mutant, with a lesion in a gene encoding a vacuole-localized ATPase, is less sensitive to BRs (Schumacher and others 1999). Future studies using this mutant should help reveal the currently unknown role of the vacuole in BR signaling. These two genes have possible roles in BR signal transduction, and transformed plants with altered expression of these genes also could be Brz220-insensitive. The combined analysis of the above mutants, gene-modified plants, and Brz should give further insights into BR signaling.

In another approach toward the understanding of BR signaling, Drs. Joanne Chory and Detlef Weigel and their colleagues have mapped the quantitative trait loci (QTL) responsible for natural variations in hormone and light responses. They first collected 141 *Arabidopsis thaliana* accessions from the Northern hemisphere and analyzed the lengths of their hypocotyls in different hormone and light conditions (Maloof and others 2001). From these accessions, an *Arabidopsis* recombinant inbred line (RIL) resulting from a cross of the Cape Verde Islands (Cvi) and Landsberg erecta (Ler) accessions was chosen for detailed analysis with Brz treatment (Borevitz and others 2002). The resulting QTL map predicted at least three strong loci that confer BR biosynthesis inhibitor insensitivity and long hypocotyls in darkness, and five weaker loci were also identified. As these strong Brz-insensitivity loci do not map near the already confirmed or potential BR biosynthesis inhibitor-insensitivity genes, a more detailed QTL analysis and more genetic screening for BR signaling mutants will be needed to clarify the mechanisms of plant growth regulation by BRs.

Recently, gene chip methods have been used to predict genes induced or reduced by BRs or brassinazole (Müssig and others 2002; Goda and others 2002). However, it is difficult to determine which genes are actually involved in BR signaling in plants from the data obtained by gene chip analyses, but reverse genetics approaches will be a great help for identifying components of BR signaling. That is, when a transgenic plant in which BRs or Brz-regulating genes are overexpressed or suppressed shows insensitivity against treatment with BRs or a BR biosynthesis inhibitor, then it is possible that the product of such genes would be involved in BR signaling.

## Concluding Remarks

Now there are at least two characterized BR biosynthesis inhibitors and they act like conditional mutations in BR biosynthesis. They allow the investigation of the functions of BRs in a variety of plant species. Applications of BR biosynthesis inhibitors to a standard genetic screen to identify mutants that confer resistance to BR biosynthesis inhibitors allow us to identify new components of the BR signal transduction pathway. This method has advantages over mutant screening using a BR-deficient mutant as background. Thus, development of chemicals that induce phenotypes of interest is now emerging as a useful and supplementary way to study biological systems of plants, enhancing classical biochemical and genetic methods.

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