Signal-Transduction Pathways toward the Regulation of Brassinosteroid Biosynthesis

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Brassinosteroids (BRs) collectively refer to the steroidal plant hormones that stimulate dramatic growth by cells and organs. Because of their essential roles, mutants that are defective in either the biosynthetic or signaling pathway exhibit severe dwarfism. Numerous reports from biochemical, genetic, and physiological studies agree that *DWARF4* mediates the flux-determining step in the biosynthetic pathway. Rather than exercising allosteric feed-back regulation of the enzyme by the end product brassinolide, transcriptional control of the *DWF4* gene is considered the most important for precise modulation of the pool size for bioactive BRs. *DWF4* expression is localized to actively growing tissues, including shoot and root tips, and the junction tissues between inflorescences and roots. This expression is significantly down-regulated by exogenous or endogenous applications of BRs, but increased by auxin treatment. Direct measurement of the endogenous BRs in different tissues of *Arabidopsis* has demonstrated that the accumulated BRs are mainly attributable to *DWF4* expression. Regarding their interaction with other hormones, BRs induce many auxin-associated genes, e.g., Aux/IAA, GH3, and SAUR. Therefore, it is likely that the brassinosteroids synthesized by a limited number of tightly controlled cells in actively growing tissues are responsible for the demands by those tissues. Future research should elucidate which portion of the BRs is transported via which mechanisms, as well as reveal the roles of auxin-associated genes expressed in a BR-dependent manner.

Keywords: *Arabidopsis*, brassinolide, cytochrome P450, homeostasis, rice

PHYSIOLOGICAL EFFECTS OF BRASSINOSTEROIDS

Brassinosteroids (BRs) are plant growth-promoting hormones whose chemical structures are based on polyhydroxylated steroids (Choe, 2004). Numerous physiological processes across a life cycle are controlled by BRs. The existence of phenotypes for mutants that are defective either in BR biosynthesis or signal-transduction pathways suggest that these compounds are primarily involved in the directional growth of cells that leads to photomorphogenesis, skotomorphogenesis, vascular system differentiation, cell elongation, and overall morphogenesis of the leaves and stems. Furthermore, physiological analyses have revealed that BRs confer tolerance to heat, salt, and pathogen stresses via molecular mechanisms that are still largely unknown (Krishna, 2003; Nakashita et al., 2003).

In their interactions with other plant hormones, BRs possess growth-promoting attributes similar to the auxins. Physiological studies have found that when bean seedlings are treated with auxins following BR exposure, such applications result in synergistic effects on ethylene production that are proportional to the effects of a single-hormone treatment (Arteca et al., 1988). Interestingly, when plants are instead treated first with auxins and then with a brassinosteroid, only additive effects are noted. Likewise, BRs are known to induce many auxin-associated genes, including AuxIAA, GH3, and SAUR-AC (Goda et al., 2002, 2004). Thus, it is probable that BRs act through their own signaling pathways as well as partly by modifying the signaling or biosynthesis of other plant hormones, as is the case in *Arabidopsis* apical hook formation (de Grauwe et al., 2005). It would be interesting to find the molecular mechanisms that fine-tune plant growth via the concerted actions of these different hormones: BRs, auxins, and ethylene.

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HOMEOSTASIS OF BIOACTIVE BR LEVELS

BRs are primarily biosynthesized by using campesterol as a pathway precursor (Fujioka and Yokota, 2003). Bioconversion analysis using a brassinolide (BL)-overproducing cell line derived from periwinkle (*Catharanthus roseus*) has facilitated the establishment of the entire pathway from campesterol to the end product BL. Currently, BL is known as the most active among more than 45 compounds. Further elucidation has come by studying the mutants that are defective in genes encoding enzymes for those pathways. *Arabidopsis* BR biosynthetic mutants include, from the earlier steps to the later, *dwarf5* (*dwf5*) (Choe et al., 2000), *dwf7* (Choe et al., 1999b), *dwf1* (Choe et al., 1999a), *det2/dwf6* (Li et al., 1996), *dwf4* (Choe et al., 1998), and *cpd/dwf3* (Szekeres et al., 1996). Their shared phenotypes are manifested as short hypocotyls both under light and darkness, greatly decreased overall heights in adult plants (approx. 20 to 40% of that measured in the wild type), mechanical sterility due to insufficient growth of stamens, short round leaves, and reduced sizes for petioles, pedicels, and siliques (Choe, 2004). Interestingly, *hydra* mutants that are defective in the two steps before *dwf5* display different phenotypes than the other conventional BR dwarf mutants described above; these are not rescued by exogenous applications (Souter et al., 2002). This suggests that DWF5 should be considered an enzyme for the first committed step in the BR-biosynthetic pathways. Similar to the monogenic dwarf mutants, a double mutant for the two cytochrome P450 genes, *cyp85a1/cyp85a2*, also displays a characteristic dwarf phenotype, whereas the monogenic mutant does not exhibit these phenotypes due

to functional redundancy of those two genes (Kwon et al., 2005).

Many active research programs using rice mutants have revealed additional genes that participate in BR biosynthesis. The amino acid sequence for rice CYP724B1 shares 41% identity with that of *Arabidopsis* DWF4 (CYP90B1), and the former has the same function as the steroid C-22 hydroxylation of DWF4 (Tanabe et al., 2005; Sakamoto et al., 2006). Therefore, due to the redundancy that is contributed by a functional CYP724B1, the monogenic OsDWF4 mutant does not display a severe dwarf phenotype. However, it does exhibit a slightly stunted and erect leaf phenotype that results in greater rice yields. This is similar to a green revolution mutant that is altered in its gibberellin biosynthesis or signal-transduction pathways (Peng et al., 1999; Sasaki et al., 2002).

Interestingly, all the biosynthetic enzymes from the DWF4-mediated steps and downstream belong to a group of cytochrome P450 proteins (Choe, 2006; Nomura and Bishop, 2006). Thus, in a likely scenario, BRs evolved as plant hormones at a similar evolutionary time as when the P450 genes burst into multiplication, even before divergence occurred for flowering plants into monocots and dicots.

At least two genes are now known to be involved in BR deactivation in *Arabidopsis*. These include CYP734A1 (At2g26710) (Neff et al., 1999; Turk et al., 2003, 2005) and CYP72C1 (At1g17060) (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). The first mediates a C-26 hydroxylation reaction, whereas a biochemical role for the second remains to be resolved. Recently, Park et al. (2006) have found that a loss-of-function mutation for the rice CYP734A6 (LOC_Os01g29150) gene shows an exaggerated lamina-bending phenotype, possibly due to accumulated BRs that are supposedly degraded by the functional CYP734A6 enzyme. This suggests that rice CYP734A6 acts as a BR-catabolic enzyme, similar to the activity of *Arabidopsis* CYP734A1 and tomato CYP734A7 (Ohnishi et al., 2006).

Conjugation of phytohormones with sugar, lipids, or amino acids often results in a decrease in the pools of bioactive hormones. In the case of BRs, UGT-glycosyltransferase deactivates them by attaching a glucose molecule to such bioactive BRs such as BL and castasterone (CS) (Poppenberger et al., 2005). The sequenced genomes of *Arabidopsis* and rice contain tens of genes that belong to the CYP734 and CYP72 groups; it will be interesting to determine whether all of these are involved in BR deactivation as directed by certain environmental or developmental cues.

DWF4 TRANSCRIPT LEVEL-DEPENDENT EFFECTS

The amount of *DWF4* transcript in *Arabidopsis* is crucial to the maintenance of an appropriate pool size for bioactive BRs and, subsequently, the overall morphology (Choe et al., 1998, 2001). For example, *dwf4* mutants display severe dwarfism due to a disruption in the steroid C-22 hydroxylase, which leads to significantly decreased levels of biosynthetic activity toward BL. In the reverse, when DWF4 is ectopically over-expressed using a constitutive promoter from cauliflower mosaic virus (CaMV) 35S, a completely opposite phenotype is produced, i.e., enlarged shapes, especially along the long axes of the organs (Choe et al., 2001). Unlike with *DWF4*, overexpression of other biosynthetic genes, e.g., *DWF5*, *DWF7*, and *CPD*, does not result in bigger plants, suggesting that the steady-state level of *DWF4* is important in establishing the biosynthetic activity for entire BR pathways. As previously suggested, one may infer that *DWF4* mediates the rate-determining step in the BR pathway, partly because of tight control over *DWF4* transcription.

In contrast, simple overexpression or a gain-of-function mutation of BR-signaling genes, such as *BRI1*, *BAK1*, *BES1*, and *BZR1*, result in a phenotype similar to that from *DWF4*-overexpression lines (Fig. 1; Vert et al., 2005). This suggests that increasing the sensitivity of BR-signaling serves as an alternative means for regulating plant growth.

In addition to its transcriptional control, regulation of DWF4 enzyme activity is critical to determining BR biosynthetic activity. When *Arabidopsis* seedlings are treated with the biosynthetic inhibitor brassinazole (Brz), they display a characteristic dwarf phenotype of short hypocotyls under either illumination or darkness (Asami et al., 2001). Brz is known to specifically depress DWF4 activity, in contrast to other CYP450 proteins including CPD. Therefore, it might act as an artificial competitive inhibitor of DWF4.

Biochemical functioning of DWF4 has been elucidated via enzymatic reconstitution experiments (Fujita et al., 2006). Surprisingly, it has been found that a cholesterol substrate is preferred over previously used campesterol (CR). In *Arabidopsis*, the endogenous concentration of cholesterol is significantly lower relative to CR and stigmasterol, which constitute the major bulk sterols. This may be the reason that BL replaces *nor*-brassinolide, which can be biosynthesized by using cholesterol as a pathway precursor in *Arabidopsis*. Moreover, these reconstitution experiments have shown that BL does not allosterically inhibit DWF4 enzyme activity. When enzyme essays are challenged in the presence of both the substrate and the end product BL, activity is not meaningfully decreased. This suggests that the DWF4-mediated reaction is the rate-limiting step, perhaps not due to allosteric inhibition by the end product but because of transcriptional control of the DWF4 gene.

REGULATION OF *DWF4* EXPRESSION: DEVELOPMENTAL AND SPATIAL PATTERNS

Obviously, regulation of *DWF4* expression is critical in determining the flux toward BL biosynthesis. Kim et al. (2006) have employed a *DWF4p-GUS* reporter system to examine spatial and temporal patterns in *Arabidopsis*. There, *DWF4p-GUS* expression is limited to actively dividing and expanding cells, including the marginal meristem of embryonic cotyledons and the junction tissues of hypocotyls and roots during seedling establishment. In adult plants, the hydathode tissues of developing leaves, shoot apical meristems, root tips, and junction tissues for inflorescences and roots are distinctly GUS-positive. In addition, when seedlings that are grown for 3 d under different lighting regimens are compared, *DWF4* expression is differentially regulated in

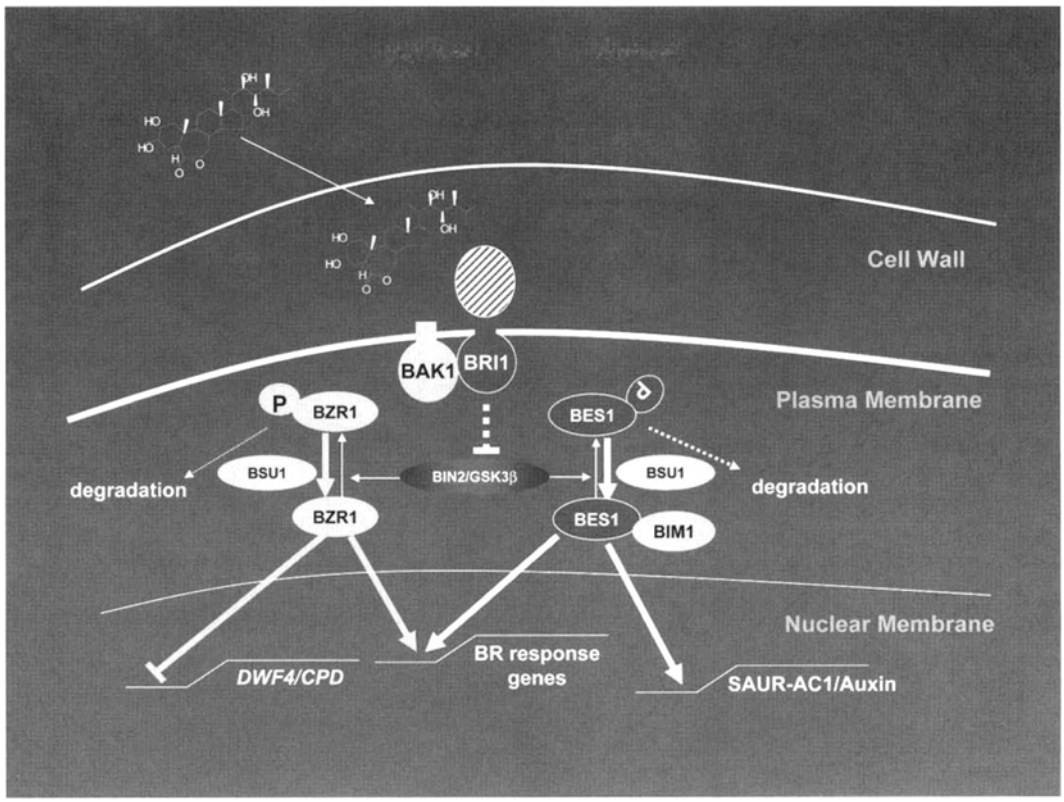

Figure 1. Brassinosteroid-signaling pathways toward regulation of BR-biosynthetic genes *DWF4* and *CPD*. BRs produced by neighboring cells move to target cell that harbors functional receptors BRI1 and BAK1. BR-activated receptors repress activity of BIN2, a glycogen synthase 3-like kinase. BIN2 phosphorylates BR-specific transcriptional factors, e.g., BZR1 and BES1, to inactivate nuclear functioning of these factors. Otherwise, de-phosphorylated functional BZR1 enters into nuclear membrane and binds to cognate sequences of biosynthetic genes *DWF4* and *CPD*. Binding of promoters by BZR1 represses transcriptional activity of RNA polymerase II via a mechanism still to be resolved. Broken lines indicate signaling pathways not yet clarified at molecular level yet.

the cotyledons; whereas the entire blade region from dark-grown seedlings shows expression, only the hydathodes in light-grown seedlings are GUS-positive. This demonstrates the contribution of light to spatially different expression patterns. Future studies should reveal how light-dependent transcriptional factors, e.g., PIF3, transmit their signals to regulate the DWF4 promoter.

The spatial and temporal expression patterns of *DWF4p-GUS* are greatly amplified in the *cpd-388*, *bri1-5*, and *bin2-2* mutant backgrounds (Kim et al., 2006; Fig. 1). However, apart from *cpd-388* and *bri1-5*, the expression pattern in *bin2-2* is quite different, especially in the root. There, the tips and emerging laterals are GUS-positive in the *DWF4p-GUS, cpd-388,* and *bri1-5* mutants, while long-stretched vascular tissues throughout the root system also express the *DWF4p-GUS* transgene in the *bin2-2* mutant background. This suggests that proper regulation of *DWF4* expression requires functional proteins that are involved in both the biosynthesis and signal-transduction of BRs.

Not surprisingly, *DWF4p-GUS* expression is down-regulated by exogenous treatment with BL. A signaling pathway that is triggered by exogenous BL may down-regulate *DWF4* expression via a feedback mode. However, this is not observed when the *DWF4p-GUS* is placed in either the *bri1-5* or *bin2-2* mutant background, implying that the signal-transduction pathways that include these two components are necessary to perform such feedback down-regulation. However, *DWF4p-GUS* activity is significantly down-regulated in the *bzr1-1D* mutant background. BZR is considered a transcriptional repressor (He et al., 2005) (Fig. 1). Compared with the wild type, mutants of *bzr1-D* that are grown in the light show a relatively stunted semi-dwarf phenotype. This suggests that the dominant gain-of-function mutation of BZR1 down-regulates *DWF4* expression in that mutant, consequently resulting in semi-dwarfism due to a reduced amount of bioactive BRs. Likewise, endogenous levels of BRs in *bzr1-D* mutants are lower than in the wild-type control (Wang et al., 2002), and semi-dwarfism is rescued to the WT phenotype when plants are supplemented with BL.

The pool size for endogenous BRs in one cell depends on at least four biochemical events: *de novo* biosynthesis, catabolic deactivation, influx from neighboring cells, and release from intracellular organelles, such as the vacuole and endoplasmic reticulum. However, no unequivocal data are currently available about the transportation of BRs either from nearby cells or long distance. Future studies should reveal whether BRs are transported as a prerequisite for completing our understanding of their mode-of-action. Regardless of the biochemical processes, it is important to know which tissues in *Arabidopsis* have the greater steady-state level of bioactive BRs. Those concentrations have already been measured directly in different organs from that genus (Shimada et al.,

2001; Kim et al., 2006). In young seedlings, BR contents are enriched four-fold in the root tissues compared with the shoots (Kim et al., 2006), while levels are highest in the junction tissues from mature plants. These two organ types are the primary tissues that significantly express the *DWF4p-GUS* transgene. Therefore, it is likely that *DWF4*-expressing tissues produce BRs and retain most of them in those tissues to support local BR-dependent activities. However, even organs in which *DWF4p-GUS* expression is not detectable also contain non-negligible amounts of BRs. Thus, we cannot rule out the possibility that the BRs produced in *DWF4*-expressing tissues are moved into nearby stems and rosette leaves to accommodate those potential demands.

CONCLUDING REMARKS

The contributions gained via molecular genetics analysis of *Arabidopsis* BR dwarf mutants has enabled the scientific community to acknowledge that brassinosteroid hormones are essential to the proper growth and development of plants. It is now imperative that we further examine the molecular mechanisms by which those signals are made and transduced to give rise to a physiological outcome such as cell elongation. BRs have been shown to be enriched in specific tissues within *Arabidopsis*, but we must obtain even more precise identifications of those biosynthetic cells. Such critical questions that remain focus on whether permanently brassinosteroidogenic cells exist and whether BRs are synthesized by many competent cells after induction signals are received.

Furthermore, there is a wide gap in our knowledge of processes ranging from environmental signals to BR biosynthesis. For example, although it is accepted that brassinosteroids confer stress tolerance and respond to light signals, it remains largely unknown which environmental signal-transduction components directly activate or repress biosynthesis. In addition, BRs have been shown to induce many auxin-associated genes, leading us to conclude that BRs and auxins are seemingly engaged in close crosstalk. Nevertheless, we must further clarify the molecular mechanisms for that communication.

Cell elongation is an outcome of multiple processes, including the biosynthesis of cell wall components and the coordinated action of numerous gene products. We must still elucidate the biochemical events that occur downstream of BR-specific transcription factors toward actual cell growth. It is challenging work to examine entire pathways, starting from environmental signals to the physiological effects that are modulated by BRs. However, holistic approaches, such as integrative evaluation of the data obtained from transcriptome analysis, metabolomics, and proteomics, might soon shed greater light on these complicated physiological processes.

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