Journal of Plant Biology, March 2005, 48(1) : 1-15 **REVIEW**

Brassinosteroid Biosynthesis and *dwarf* Mutants

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Plants enjoy their entire life exactly where they were initially rooted. Because of this fixed life pattern, plants have to devise a different type of strategy than animals to survive the numerous biotic and abiotic challenges. Many different plant hormones that act alone or in concert underpin these mechanisms. Brassinosteroids (BRs) collectively refer to plant-originated 5α-cholestane steroids that elicit growth stimulation in nano- or micromolar concentrations. BRs that are biosynthesized using sterols as precursors are structurally similar to the cholesterol derived, mammalian steroid hormones, insect molting hormones and ecdysteroids. BRs have been known for decades to be effective in plant growth promotion. However, definitive evidence for their roles in growth and development remained unclear until the recent characterization of BR *dwarf* mutants isolated from *Arabidopsis* and other plants. This review aims to provide a cohesive summary of information obtained from the molecular genetic characterization of mutants that are defective in sterol and BR biosynthetic pathways.

Keywords: Arabidopsis, brassinolide, cytochrome P450, dwarf, hormone

BRIEF HISTORY AND NATURAL OCCURRENCE OF BRASSINOSTEROIDS

A variety of plant growth regulators are involved in the intricate processes of reproduction. Thus, plant scientists recognized that pollen could be a rich source of phytohormones. A search for novel plant hormones from pollen was begun in the 1960s by a United States Department of Agriculture (USDA) group (Yokota, 1999). This led to the discovery of a substance, named Brassin, from rape (*Brassica napus*) pollen that stimulated growth in a bean second internode bioassay (Yokota, 1999). The first characterized BR, brassinolide (BL), was discovered from bee-collected rape pollen at a concentration of 200 parts per billion (Grove et al., 1979). The BL structure elucidated by X-ray diffraction technology was (22*R*,23*R*, 24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa -5α-cholestan-6-one (Fig. 1).

In addition to BL, at least 50 additional BR structures have been identified from various species in the plant kingdom examined to date (Fujioka et al., 2002). These include one species of algae (*Hydrodictyon reticulatum*), a pteridophyte (*Equisetum arvense*), 5 species of gymnosperms, and 37 different species of angiosperms (Fujioka et al., 1997). Thus, it is conceivable that BRs are ubiquitous in the plant kingdom (Fujioka and Sakurai, 1997).

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CHECIAL STRUCTURES OF BRASSINOSTEROIDS

BRs are primarily classified into seven categories depending on the side chain structures that are derived from different parental sterols (Fig. 1). In addition to the side chain variation, two additional structural variations occur: 1) differing numbers of hydroxyl groups with varying configurations at the C-2 and C-3 positions; and 2) either a ketone or a lactone functional group at C-6 and C-7. Among the 50 different BRs, BL was shown to possess the greatest growth-promoting activity (Thompson et al., 1982). As inferred from the chemical structure of BL, it was hypothesized that active BRs should possess the following structural requirements. First, the A and B rings must be in the *trans* configuration, which is determined by an α hydrogen at C-5. Second, the B ring should contain a 6-oxo or a 6-oxo-7-oxa group. Third, the hydroxyl groups at C-2 and C-3 in ring A should be *cis* -oriented. Fourth, the *cis* α-oriented hydroxyl groups at the C-22, C-23, and the C-24 positions should be occupied either by α-oriented methyl or ethyl groups (Mandava, 1988; Arteca, 1996). As a rule, the more these requirements are met, the more active the compound.

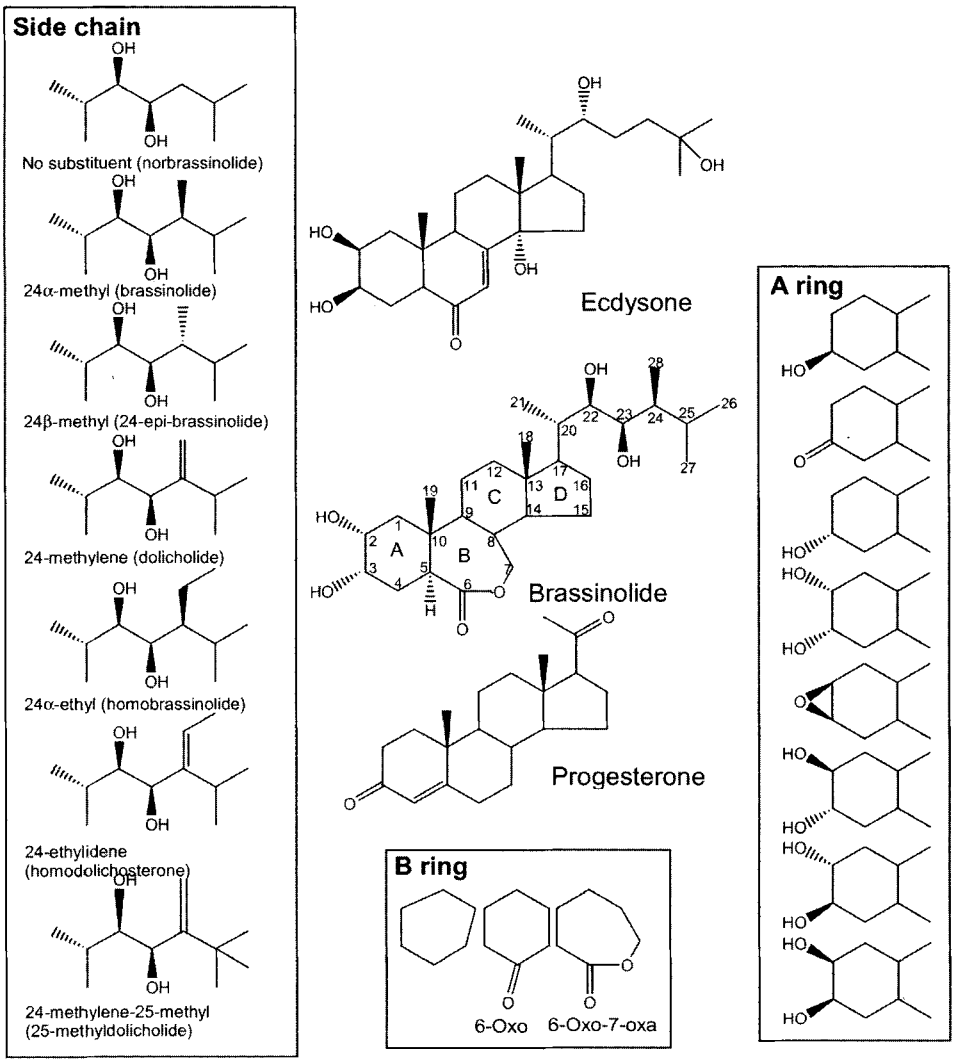

Figure 1. Structural variation in brassinosteroids. Brassinosteroids share structural similarities with the human steroid hormone progesterone and the insect molting hormone ecdysone in that they all have a 4-membered steroid ring backbone and multiple oxidations. Structural variations are primarily based on the different status of oxidation at ring A, B, and the side chain. Different combinations of each element in the three variable regions are reflected in the approximately 50 BRs identified to date. The BR names in the side chain box are applicable when the BL side chain is replaced with one of the structures in the box.

DWARF MUTANTS AND BRASSINOSTEROID BIOSYNTHETIC PATHWAYS

Isolation and characterization of the dwarf mutants that are defective in BR biosynthesis has been instrumental in understanding the brassinosteroid biosynthetic pathways. Although genetic defects in other plant hormones such as auxin and gibberellin result in dwarfism, phenotypes of the BR dwarf mutants differ from those of GA and auxin dwarfs in that the former tend to possess more severe alteration in leaf and inflorescence development than GA and auxin dwarfs do. In addition, many of the GA dwarfs fail to germinate in the absence of exogenously added GA, whereas BR dwarfs generally do (Clouse and Sasse, 1998), and only BR mutants are recovered to wild-type phenotype by exogenous application of BRs. *Arabidopsis* BR dwarfs are characterized by the phenotypes of short robust stature, reduced fertility, prolonged life span, and leaves with dark-green, round, and curled shape. In addition, when grown in the dark, severe BR dwarfs often display abnormal etiolation patterns, including reduced hypocotyls length, open cotyledons, and an absence of apical hooks (Fig. 3). Alleles of BR dwarf mutants that were isolated and studied by different research groups are com-

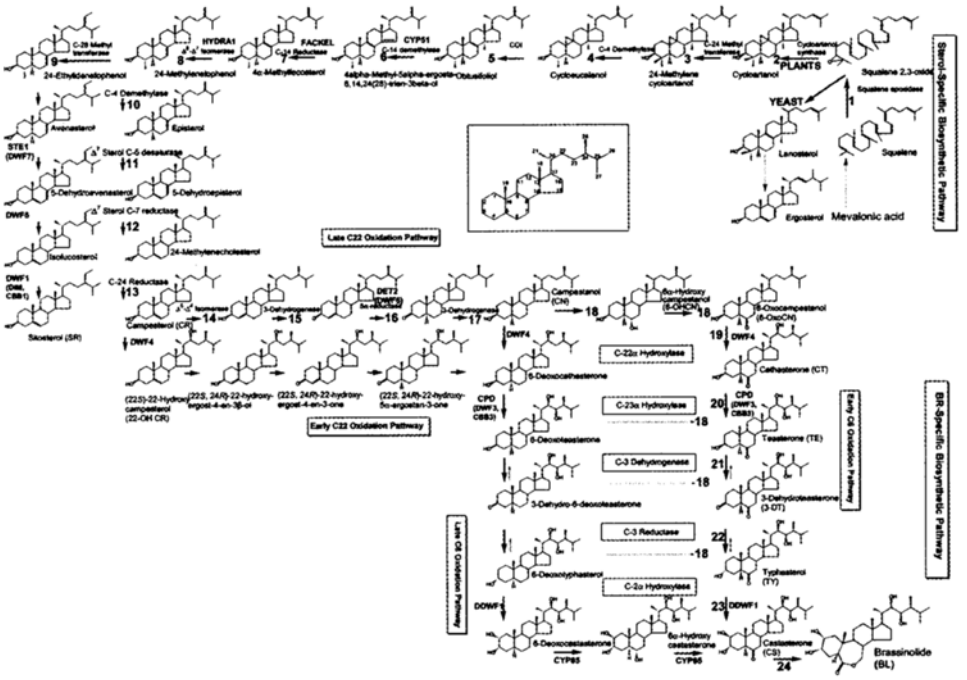

Figure 2. The brassinolide biosynthetic pathway. The biosynthetic pathway is divided into multiple subunits. The sterol-specific pathway refers to the steps from Squalene to CR (or STR). The sterols are modified by the BR-specific pathway to produce BL. Depending on C22 hydroxylation at campesterol, the BR pathway is further divided into the early and late C22 oxidation pathways. Similarly, the C6 position can be oxidized at campestanol or later at 6-deoxocathasterone stage, and thus these are called the early and late C6 oxidation pathways, respectively. Each enzymatic step is numbered and referred in the text, and steps identified with mutants are marked with bold-faced letters. Acronyms used in the text are written in parentheses. In the inset, the carbon atoms of the sterol core rings and side chain are numbered.

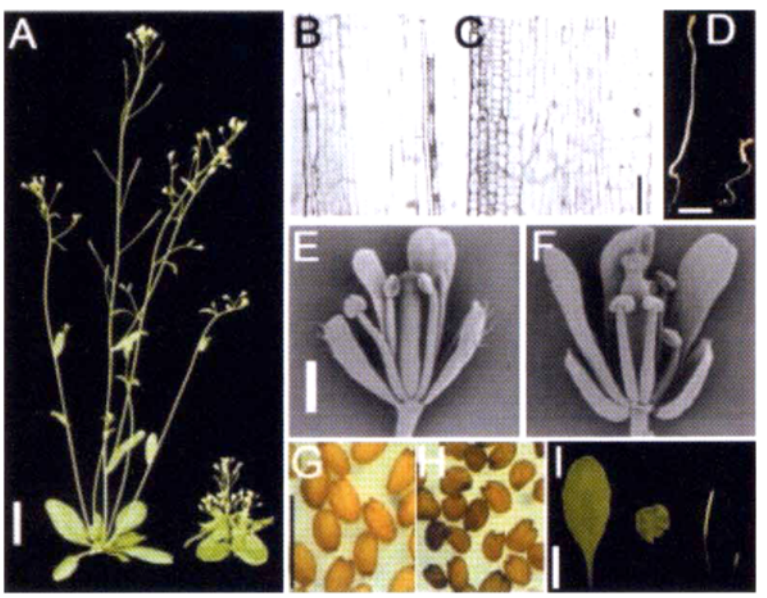

Figure 3. Morphological characteristics of the BR *dwarf* mutants. BR dwarf mutants display greatly reduced stature **(A)**, the cell's size especially in the epidermal layer decreased to only 20% that of a wild type **(B** and **C)**, dark-grown seedlings exhibit de-etiolation phenotypes **(D)**, dwarf flowers are generally sterile due to shorter length of the filaments **(E** and **F)**, seeds are rounder and dark brown **(G** and **H)**, a rosette leaf looks rounder, and siliques are short due to the defects in elongation growth **(I)**. In each panel, phenotypes of wild type and *dwarf* mutant are placed at the left and right, respectively. Unit bars in A = 2 cm, C = 0.1 mm, D = 1 cm, E and G = 1 mm, and I = 1 cm.

Table 1. Genes involved in brassinosteroid biosynthesis. Genes referred to as in the text are summarized with their alleles, other names, mutagen used to generate the specific mutant allele, their ecotypic background, and stock no that refers to the stock ID number for Arabidopsis biological resource center (ABRC).

- Genetic locus - Chromosome Locus - Function - Yeast locus	Allele	Other name	Mutagen	Ecotype	Stock No
- *COTYLEDON VASCULAR PATTERN1 (CVP1)* - At1g20330 - Sterol C-24[1] methyltransferases	*cvp1-1*	-	EMS	Columbia	-
	cvp1-2	-	EMS	Columbia	-
	cvp1-3	-	EMS	Columbia	-
	cvp1-4	-	EMS	Columbia	-
- *FACKEL (FK)* - At3g52940 - Sterol C-14 reductase - *erg24*	*fk-x224*	fk	X-rays	Ler	CS6149
	fk-2	ell	T-DNA	Ws-2	-
	fk-3	hydra2	T-DNA	Ws-2	-
- *HYDRA1 (hyd1)* - At1g20050 - Sterol Δ^8-Δ^7 isomerase - *erg2*	*hyd1*	-	T-DNA	Ws-2	-
- DWARF7 (*DWF7*) - At3g02580 - Steroid C-5 desaturase - *erg3*	*ste1-1*	-	EMS	Columbia	-
	dwf7-1/ste1-2	1845	T-DNA*S	Ws-2	-
	dwf7-2/ste1-3	WM5-3	EMS	Ws-2	-
- *DWARF5 (DWF5)* - At1g50430 - $\Delta^{5,7}$-sterol-Δ^7-reductase	*dwf5-1*	2	T-DNA*	Ws-2	-
	dwf5-2	398	EMS	En-2	CS398
	dwf5-3	402	EMS	En-2	CS402
	dwf5-4	le-1	EMS	Estland	CS72
	dwf5-5	WM9-4	EMS	Ws-2	-
	dwf5-6	maria1	EMS	Ws-2	-
- DWARF1 (DWF1) - At3g19820 - Δ^5-sterol-Δ^{24}-reductase - *erg5*	*dwf1-1*	T-31-90	T-DNA	Ws-2	-
	dwf1-2	diminuto	T-DNA	C24	CS6100
	dwf1-3	318	EMS	En-2	CS318
	dwf1-4	355	EMS	En-2	CS355
	dwf1-5	356	EMS	En-2	CS356
	dwf1-6	cabbage1	Ac/Ds	Columbia	CS291
	dwf1-7	WM1-7	EMS	Ws-2	-
	dwf1-8	WM3-1	EMS	Ws-2	-
	dwf1-9	WM5-5	EMS	Ws-2	-
	dwf1-10	WM9-3	EMS	Ws-2	-
	dwf1-11	WM12-1	EMS	Ws-2	-
- *DE-ETIOLATED2 (DET2)* - At2g38050 - Steroid $\Delta^{5,6}$ reductase	*det2-1~det2-8*	-	EMS	Columbia	CS6159
	det2-9	28	T-DNA*	Ws-2	-
	det2-10	87	T-DNA*	Ws-2	-
	det2-11	3648	T-DNA*	Ws-2	-
	det2-12	303	EMS	En-2	CS303
	det2-13	319	EMS	En-2	CS319
	det2-14	352	EMS	En-2	CS352

Table 1. continued.

- Genetic locus - Chromosome Locus - Function - Yeast locus	Allele	Other name	Mutagen	Ecotype	Stock No
- DWARF4 (DWF4) - At3g50660 - Steroid C-22α hydroxylase	*dwf4-1*	22	T-DNA	Ws-2	-
	dwf4-2	211	T-DNA*	Ws-2	-
	dwf4-3	365	EMS	En-2	CS365
	dwf4-4	374	EMS	En-2	CS374
	dwf4-5	409	EMS	En-2	CS409
- CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD) - At5g05690 - Steroid C-23α hydroxylase	*cpd-1*	-	T-DNA	Columbia	-
	cpd-2	cabbage3	Ac/Ds	Columbia	-
	cpd-3	*dwf3-3939*	T-DNA	Ws-2	-
	cpd-4	*dwf3-317*	EMS	En-2	CS317
	cpd-5	*dwf3-388*	EMS	En-2	CS388
	cpd-6	*dwf3-417*	EMS	En-2	CS417

*Untagged mutant isolated from T-DNA insertional mutant population. The full names of the ecotypes Ws-2 and En-2 are Wassilewskija -2 and Enkheim-2, respectively.

piled in Table 1. BR biosynthetic dwarf mutants are, by definition, those that are rescued by exogenous application of BRs. These BR biosynthetic dwarf mutants include *Arabidopsis dwf1, cpd/dwf3, dwf4, dwf5, det2/dwf6, ste1/dwf7*, pea *lka* and *lkb*, and tomato *dwarf* mutants. The main feature of the characteristic phenotype of a BR dwarf results from, by-and-large, a unidirectional reduction in organ size. The length of pedicels, siliques, roots, and leaf blades are all reduced. The width of leaf blades is, however, not reduced nearly as much as their length. This differential reduction in leaf size gives rise to leaves with a rounder appearance. This is in contrast to the leaf phenotypes of GA biosynthetic and signaling mutants that display shorter and narrower leaves relative to a wild type (Fig. 3). BR *dwf* mutants have a smaller cell volume, but contain the same number of chloroplasts as wild type, which causes the darker green appearance (Azpiroz et al., 1998).

BR *dwf* mutants display varying degrees of fertility. Generally, the reduced fertility of dwarf plants is ascribed to differential reduction in cell elongation of specific floral organs. Elongation is more severely affected in the filament than the gynoecium, resulting in pollen being shed on the walls of the gynoecium instead of being shed directly on the papillae. When BR dwarf flowers are hand-pollinated, they show increased fertility, suggesting that the sterility is at least partially due to lack of access of pollen. As the siliques are shorter, even when flowers are hand-pollinated, the seeds are smaller than wild type (Choe et al., 2000).

Mutants defective in light-signal transduction pathways such as *constitutive photomorphogenesis (cop)/de-etiolated (det)/fusca (fus)* mutants display dwarfism, and seedlings of these mutants grown in the dark exhibit differentiated plastids and the gene expression pattern of light-grown wild-type plants (McNellis and Deng, 1995). Dark-grown hypocotyls of these mutants were shown to respond to the exogenous application of BRs (Szekeres et al., 1996); however BRs do not rescue their phenotype. Similar to these light signaling mutants, several of the BR dwarfs possess part of the *de-etiolation (det)* phenotype in the dark. These include short hypocotyls, expanded cotyledons, absence of an apical hook, and initiation of leaf development in the dark (Fig. 3). Dwarf mutants exhibit shorter hypocotyls in the dark than those of wild type, but the hypocotyls are still capable of responding to darkness with more elongation than light-grown plants (Azpiroz et al., 1998). This response to darkness suggests that dwarf plants can distinguish light from dark, and that other hormones are also responsible for hypocotyl growth. Presumably, the de-etiolated phenotype of dark-grown dwarfs is primarily due to a generalized defect in cell elongation.

Delayed senescence has been observed in BR dwarf mutants. Dwarf plants stay green and produce flowers for a longer period of time than wild type plants. In the dwarf mutants, the length of time for important developmental events such as bolting, flower opening and senescence are noticeably delayed (Choe et al., 1999a, b). The prolonged life span is cor-

related with the degree of reduced fertility in *dwf* mutants. Relatively fertile dwarfs such as *dwf5* have a shorter life span than dwarfs that set few seeds (Choe et al., 2000).

It has been reported that the primary cause of the reduced stature of dwarf plants is not fewer number of cells but reduced cell elongation (Husselstein et al., 1996; Choe et al., 1999b). Longitudinal sections of *dwf* stems display a dramatic reduction in cell length to approximately 20% of that of wild type, whereas cell width is equal or even greater than wild type. The wider cell files partly contribute to the thicker stems seen in *dwf7* plants (Choe et al., 1999b).

BR dwarfs retain sensitivity to other plant growth-promoting hormones (Kauschmann et al., 1996; Azpiroz et al., 1998). Although BR dwarfs respond to auxins and GAs, only BRs rescue the dwarf phenotype. Exogenous application of BRs causes elongated internodes, petioles, pedicels, and roots (Choe et al., 1998). However, the sterility of dwarfs is not completely rescued by exogenous application of BR. Precise spatial and temporal regulation of BR action seems to be important in rescuing all the defects of dwarf mutants.

Mutants that have lesions in the sterol-specific pathways can be divided into two groups based on their response to exogenous application of BRs. One group, which includes *dwf1*, *dwf5*, and *dwf7*, have mutations in the genes regulating the later steps in the sterol-specific pathway; these are successfully rescued by BRs. The members of the other group, including *fackel* and *hydra1*, are defective in the earlier steps in the pathway and display multiple growth defects that are not corrected by exogenously-supplied sterols and BRs.

BIOSYNTHESIS OF BRASSINOSTEROIDS

BRs belong to triterpenoids that are consisted of 30 carbon atoms. Because BRs are said to be a group of modified sterols, the BL biosynthetic pathways can be divided into two major parts: sterol-specific and BR-specific pathways, squalene to campesterol (CR) and CR to BL, respectively (Fig. 2). Mevalonic acid (MVA), which serves as the starting molecule of the terpenoid pathway, is condensed and cyclized to produce 2,3-oxidosqualene. 2,3-oxidosqualene is further modified to form the major plant sterols such as sitosterol (SR) and CR. All of these parent sterols then serve as precursors of BL isologs such as homo- or nor-BL (Fig. 1). To become a bioactive BR, sterols must be processed by the BR specific pathway. In short, sterols are modified to have the following functional groups: 1) saturation of a double bond at Δ^5, 2) formation of a 6-oxo-group, 3) addition of α-oriented vicinal hydroxyl groups at C-22 and C-23, 4) epimerization of a 3β-hydroxyl group to the 3α configuration, 5) addition of a 2α hydroxyl group, and finally 6) a Baeyer-Villiger type oxidation in the B ring (Fig. 2).

Individual biosynthetic steps have been elucidated by metabolic tests using BR overproducing cell lines of the periwinkle *Catharanthus roseus* (Sakurai and Fujioka, 1996). These BL-overproducing lines were developed to overcome low biosynthetic activities in regular plant tissues or cell lines, which technically limits extraction and detection of BRs. BR biosynthesis proceeds through multiple branched pathways. The first branch occurs at CR and the second at campestanol (CN). CR can be either C-22 hydroxylated or C-5 reduced in bifurcated pathways that are termed the early and the late C-22 oxidation pathways, respectively (Choe et al., 2001) (Fig. 2). In addition, CN proceeds to one of two alternative pathways, the early and the late C-6 oxidation pathways. The early C-6 oxidation pathway undergoes a two-step oxidation of the C-6 position at the CN stage (Fujioka et al., 2002). In the late C-6 oxidation pathway, C-6 is oxidized at the second to last step. The order of chemical substitutions other than the branching steps is conserved between the parallel pathways, such that these reactions are performed by single enzyme acting on both the early and late intermediates (Choe et al., 1998). The BR biosynthetic pathway that was established in the periwinkle feeding experiments has served as a framework for further validation and modification by results from analyses of dwarf mutants that are defective in the BR biosynthesis and signaling pathways.

STEPS IN THE BRASSINOSTEROID BIOSYNTHETIC PATHWAYS

Sterols are ubiquitous in eukaryotic organisms. Two major roles played by sterols in biological systems are as precursors of steroid hormones and as bulk components of biological membranes. Thus, genetic defects in sterol biosynthetic pathways cause a broad spectrum of phenotypes attributable to both deficiency of steroid hormones as well as loss of membrane integrity. A comprehensive description of each step in sterol biosynthesis is well reviewed by Benveniste (2002). Each step in the sterol biosynthetic pathway, where a mutant and its gene is known will

be highlighted here.

STEROL SPECIFIC BIOSYNTHETIC PATHWAYS

S-adenosylmethionine-sterol-C-methyltransferases1 (SMT1): C-24 methylation (Reaction 3)

The presence or absence of an alkyl group at the C-24 position of sterols distinguishes plant and fungal sterols from those of animals. Major vertebrate sterols are devoid of an alkyl group at C-24, whereas fungi have a methyl, and plants possess either a methyl or an ethyl group. In plants, the alkylation at C-24 occurs via two non-consecutive steps. Methylation at C-24 of cycloartenol forms C28 sterols such as CR. Additional methylation at C28 of 24-methylenelophenol results in C29 sterols such as stigmasterol and SR (Fig. 2). The sterol S-adenosylmethionine-sterol-C-methyltransferases transfer a methyl group from S-adenosylmethionine (SAM) to C-24 of cycloartenol to give rise to 24-methylenecycloartenol.

A mutant for this step (*smt1*) has been recovered from screening for mutants with a developmentally altered root system (Clouse and Sasse, 1998). *smt1* plants are characterized by short malformed roots, greatly reduced fertility, and abnormal embryogenesis. The swelling root phenotype is due to increased sensitivity to exogenous calcium ions in certain types of agar-solidified growth medium. Interestingly, the sterility results from a maternal defect: no seeds are produced when *smt1/smt1* stigma are fertilized with wild type pollen, whereas the reciprocal cross is fertile. In addition, developing *smt1/smt1* embryos display irregular patterns of morphogenesis in that the different stages of embryogenesis are not distinguishable, and the germinating seedlings also show varying cotyledon number and shape. Biochemical analysis of *smt1* plants reveals that the endogenous level of cholesterol (C27) increases while SR (C29) decreases, an indication of suppressed methylation at C-24. Interestingly, the endogenous level of CR (C28) which is used as a precursor of BR biosynthesis was similar to wild type level, suggesting that a functionally redundant gene may exist in the mutant. Indeed, the *Arabidopsis* genome contains three *SMT* genes: *SMT1* (At5g 13710), *SMT2* (At1g20330), and *SMT3* (At1g76090) (Benveniste, 2002). All three SMT genes can complement the yeast *erg6* mutant that is defective in C-24 methylation, indicating that all three *Arabidopsis* SMTs are functional homologs of yeast ERG6 (Carland et al., 2002). Of the three genes, SMT1 is the most similar to ERG6. Decreased C24-methylation activity in *smt1* mutants also suggests that SMT1 is most likely involved in the first methylation reaction in the plant sterol biosynthetic pathway (Clouse and Sasse, 1998).

In relation to the BR dwarf phenotype, the overall growth of *smt1* plants is not significantly reduced as compared to typical BR mutants such as *dwf1*, *dwf5*, or *dwf7*. Thus it is plausible that the unique phenotypes of *smt1* may be due to a deficiency in specific sterols that are required at certain levels in particular cell types and tissues.

SMT2 and SMT3 are likely to mediate the second methylation reaction that converts 24-methylenelophenol to 24-ethylidenelophenol. The *Arabidopsis cotyledon vascular pattern1* (*cvp1*) mutant is defective in the SMT2 gene. As the mutant name suggests, *cvp1* displays abnormal phenotypes in vascular cell arrangement which results in abnormal leaf vascular patterning (Carland et al., 2002). Biochemically, the *cvp1* mutant accumulates C28 sterols such as 24-methylelenelophenol, whereas C29 sterols including 24 methylidenelophenol and its downstream compounds are greatly decreased (Carland et al., 2002). Consequently, the ratio of CR to SR is shifted from approximately 0.2 in wild type to 1.9 in *cvp1*. These data clearly indicate that SMT2 (CVP1) preferentially mediates the second methylation step in the sterol pathway (Fig. 2). In addition, evidence that *Arabidopsis* SMT3 is a functional homolog of SMT2 comes from the results of *SMT3* overexpression using CaMV 35S promoter to complement the *cvp1* mutant. The two genes also show partially overlapping gene expression patterns, indicative of the same function in different tissues.

Conversely, overexpression of the SMT2 gene using the 35S promoter in *Arabidopsis* resulted in increased metabolic flux toward C29 sterol pathways and increased SR level at the expense of CR (Schaeffer et al., 2001). Furthermore, the reduced CR levels led to decreased BL biosynthesis that resulted in dwarfed plants that could be rescued by exogenous BL treatment (Schaeffer et al., 2001).

Fackel/extra long lifespan/hydra2: C-14 reduction (Reaction 7)

The FACKEL-mediated step converts 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol to 4α-methylfecosterol using NADPH as a hydrogenation source (Fig. 2) (Benveniste, 2002; Fujioka and Yokota, 2003). Three independent groups isolated mutants that are allelic with each other: *fackel* (*fk*) and *hydra2* (*hyd2*) were

recovered from screening for mutants possessing abnormal embryonic patterning, and *extra long lifespan* (*ell*) mutants for constitutive cytokinin responses such as longevity. Examination of endogenous sterol and BR content showed that *fk* accumulates $\Delta^{8,14}$ sterols, and thus BR levels are greatly reduced (Fig. 2) (Jang et al., 2000; Schrick et al., 2000). Introduction of the *Arabidopsis FK* gene into the yeast *erg24* mutant, defective in the C-14 reduction step, successfully rescued *erg24*, suggesting that *FK* is an ortholog of yeast ERG24. *Arabidopsis* FK (At3g52940) displays sequence identity to a sterol reductase domain of the human Lamin B receptor and signature sequence of sterol reductases "LLXSGYWGXXRH" (Benveniste, 2002). Unlike *smt1* mutants, *fk* mutants display severe growth retardation possibly due to a relatively low level of CR, and accordingly a decreased level of bioactive BRs. However, despite the reduced BR levels in the *fk* mutant, exogenous application of BRs did not rescue the *fk* phenotype, suggesting that the abnormal development in these mutants is partly attributable to unique roles played by sterols. In support of this, *fk* mutants show different gene expression patterns from BR biosynthetic mutants including *dwf4* in that the *TOUCH4* gene, which is normally induced by BL, and repressed in BR mutants is increased (Grove et al., 1979). This suggests that sterols play an important role as signaling molecules whose molecular mechanism is yet to be discerned.

HYDRA1: Δ^8- Δ^7 isomerization (Reaction 8)

A sterol Δ^8-Δ^7 isomerase converts 4-methylfecosterol to 24-methylenelophenol. This enzymatic step is conserved among vertebrates, fungi, and plants; thus it was possible to isolate an *Arabidopsis* cDNA clone for this gene by functional complementation of a yeast *erg2* mutant that is defective in this step (Fujioka and Yokota, 2003). A loss-of-function mutation for this gene, *hydra1* (*hyd1*), as recovered from screening for mutants that show altered embryonic and seedling cell patterning morphology in *Arabidopsis* (Souter et al., 2002). A genomic DNA fragment containing the 2 kb promoter region and the coding sequence of *Arabidopsis* sterol Δ^8-Δ^7 isomerase (At1g20050) successfully complemented the *hyd1* mutants. Similar to *fk*, *hyd1* displays pleiotropic developmental alterations such as the formation of multiple leaf-like cotyledons. In addition, similar to *cvp1*, *hyd1* shows aberrant vascular patterning. Examination of the sterol profiles in the *hyd1* mutant revealed that CR and SR are merely 12% and 2% that of wild type, respectively, suggesting that molecular lesions exist in the sterol isomerase gene. Although the reduced CR level led to a decreased BL level, exogenous treatment with BL did not rescue the phenotypes of the *hyd1* mutants. However, it is noteworthy that the *dwf7* mutants, which block two steps downstream of this sterol Δ^8-Δ^7 isomerase reaction, display dwarfism and are successfully rescued by exogenous application of bioactive BRs. Thus it is likely that the functional roles of sterols as steroid hormone precursors and as bulk component of membranes could be conferred by the enzymatic modifications catalyzed by sterol Δ^8-Δ^7 isomerase or one step before this.

DWARF7/STE1: C-5 desaturation (Reaction 11)

DWARF7 (DWF7) converts episterol (avenasterol) into 5-dehydroepisterol (5-dehydroavenasterol) by a stereo-specific removal of the two protons at C-5 (Bishop et al., 1999). Cytochrome b5, NADH, and a molecular oxygen are involved in this oxidation step (Benveniste, 2002). A mutant that accumulates sterols without C-5 desaturation was isolated from an *Arabidopsis* EMS mutant population and named *ste1* (He et al., 2003). The gene for this enzymatic function was identified by isolation of a cDNA clone that functionally complemented the yeast *erg3* mutant. The *erg3*-rescuing clone was shown to encode a protein of 281 amino acids with characteristic His-rich motifs whose molecular role is yet to be discovered (Choe et al., 1999b). When the *35S::STE1* clone was re-introduced into the *ste1* mutant, the genetic defect in C-5 desaturation was completely rescued. Sequencing the C-5 desaturase gene in *ste1* revealed that the threonine at position 114 was replaced by isoleucine, and this resulted in a slight change in the function of the native protein. This can be inferred from the *ste1* mutant phenotype as it has no obvious growth retardation except for the slight reduction in C5 saturation activity. Severe mutants for this gene were independently isolated and characterized by analysis of *Arabidopsis* as BR dwarf mutants.

Arabidopsis dwf7 mutants display typical BR dwarf phenotypes, and are successfully rescued by exogenous application of BRs (Choe et al., 1999b). Examination of the endogenous sterol levels showed that intermediates, such as 24-methylenecholesterol and downstream compounds, are greatly diminished as compared to wild type. In addition, metabolite conversion tests performed by feeding ^{13}C-labeled MVA into *dwf7* seedlings suggested that *dwf7* is not able to produce C-5 desaturated sterols. Sequencing of the

C-5 desaturase gene (At3g02580) in *dwf7-1* and *dwf7-2* mutants revealed that mutations caused premature stop codons at position 230 and 60, respectively, and are predicted to be nulls (Choe et al., 1999b).

The *DWF7* gene (At3g02580) has a contiguous homolog (At3g02590) in the *Arabidopsis* genome. When the homologous gene to *DWF7* (*HDF7*) was overexpressed in the *dwf7* mutant, the mutant phenotype was complemented (Choe and Tanaka, unpublished data), suggesting that the two genes were duplicated in recent evolutionary time. The loss-of-function phenotype of *dwf7*, in spite of presence of a functionally equivalent gene, suggests that the two genes may be expressed in different cell types in *Arabidopsis* or different affinities for substrates. Although a null mutant of *dwf7* exhibits severe dwarfism and reduced fertility, *dwf7* does not display severe defects in embryo development as are commonly found in upstream sterol mutants such as *smt1*, *fk*, and *hyd1* mutants. This suggests that essential roles played by CR and SR may have been replaced by surrogate sterols in the *dwf7* mutants, accordingly *dwf7* displays more of the phenotype attributable to BR deficiency only.

DWARF5: C-7 reduction (Reaction 12)

$\Delta^{5,7}$-sterol-Δ^{7}-reductase (S7R) hydrogenates the double bond at the Δ^{7} position. An *Arabidopsis* S7R gene (At1g50430) has been identified by selecting yeast strains that are resistant to Nystatin after being transformed with an *Arabidopsis* cDNA expression library (Lecain et al., 1996). Nystatin is toxic to wild-type yeast because it acts on sterols with a double bond at the C-7 position. A yeast strain harboring a functional S7R may have saturated the double bond, and therefore show resistance to this fungicide. Sequencing of the gene selected from the Nystatin-resistant clone revealed that the protein possess the characteristic sterol reductase signature sequence also found in HYDRA2 (LLXSGWWGXXRH). It has long been known that the human sterol metabolic disease Smith-Lemli-Opitz syndrome (SLOS) is due to a genetic defect in the S7R step. Cloning of the *Arabidopsis* S7R gene accelerated the isolation of a corresponding human gene and subsequent molecular characterization of this devastating genetic disease.

Arabidopsis mutants for the S7R gene were identified from a population of canonical BR dwarf mutants. Unlike the typical sterol mutants *smt1*, *fk*, *cvp1*, and *hyd1*, but similar to *dwf7*, *dwf5* mutants display characteristic BR dwarf phenotype (Choe et al., 2000). Examination of endogenous sterol and BR levels revealed that intermediates after the S7R step are greatly diminished in *dwf5* mutants. Many of the growth defects in *dwf5* are rescued by exogenous application of BL and its early precursors including 22-hydroxycampesterol (22-OHCR) (Fig. 2). In addition, metabolites from ^{13}C-labeled MVA metabolism tests revealed that C-7 reduced compounds are not detectable in the *dwf5* mutant. Instead, *dwf5* skips the step and forms novel compounds such as 7-dehydroCR and 7-dehydroCN. One characteristic phenotype specific to *dwf5* includes a greatly increased fertility relative to other dwarfs, however, their seed size is small and color is dark-brown (Fig. 3). Sequencing the S7R gene in *dwf5* showed that mutations are located in splice donor or acceptor sites as well as substitution mutations mostly in the 3′ half of the gene. Localization of *dwf5* mutations in the 3′ half of the gene suggests that some yet to be identified important domains reside in the C-terminal region of the protein.

DWARF1: C-24 reduction (Reaction 13)

A Δ^{5}-sterol-Δ^{24}-reductase converts the double bond at $\Delta^{24(28)}$ into a saturated single bond. It has been suggested that the reduction step occurs via two consecutive reactions: isomerization of the $\Delta^{24(28)}$ double bond from 24-methylenecholesterol to 24-methyldesmosterol ($\Delta^{24(25)}$) then saturation of the double bond into CR. *Arabidopsis dwf1* is known to be defective in this step. The *DWF1* (At3g19820) gene was cloned long before a precise biochemical role was elucidated by BR intermediate feeding tests and examining the endogenous BR levels because the sequence did not initially indicate the function of the protein (Klahre et al., 1998; Choe et al., 1999a). Feeding tests showed that altered developmental defects in *dwf1* mutants are rescued by exogenous application of 22-OHCR, which suggests that the biosynthetic defect resides prior to CR. In addition, measurement of endogenous sterol levels in the *dwf1* mutants demonstrated that 24-methylelenecholesterol accumulates to 12 times the level of wild type, whereas the CR level stayed at 0.3% that of wild type (Choe et al., 1999a). These data clearly suggest that *dwf1* is blocked in a step converting 24-methylenecholesterol to CR. The *lkb* mutant of garden pea has a mutation in an orthologous gene of *DWF1* and is defective in the two consecutive steps mediated by the single enzyme DWF1 (Nomura et al., 1999). The amino acid sequence of

DWF1 contains a domain identifiable as a flavin adenine dinucleotide (FAD)-binding motif. At least 7 of 10 *dwf1* mutations were mapped to conserve amino acid residues of this domain, a strong indicator of the importance of the FAD-binding domain in proper functioning of this enzyme. Although fungi have the sterol C-24 reduction step (*erg5*), the protein sequences are divergent from those of the plant. However, human and other eukaryotic organisms besides fungi were found to possess sequences as similar as 40% to DWF1. The human DWF1-homologous gene is called Seladin-1 (GenBank Acc. # Q15392) and is responsible for conferring resistance to Alzheimers disease-related neurodegeneration as well as oxidative stress (Grebenok et al., 1998). Future research will elucidate whether Seladin-1 could mediate a C-24 reduction in the plant sterol biosynthetic pathway.

BRASSINOSTEROID SPECIFIC BIOSYNTHETIC PATHWAYS

DE-ETIOILATED2: C-5 reduction (Reaction 16)

Different sterols such as CR, SR, or cholesterol can be subjected to specific BR biosynthetic pathways resulting in BL, homo-BL, and nor-BL, respectively. These sterol modification steps are collectively referred to as the BR-specific biosynthetic pathway, and consist of reduction, oxidation, and isomerization reactions (Fig. 2). The first enzymatic step that has been identified with mutants in *Arabidopsis* is mediated by a sterol Δ^5 reductase. In humans, before a reduction occurs, a double bond Δ^5 is isomerized to Δ^4 by the action of a multifunctional enzyme 3-hydroxysteroid dehydrogenase/$\Delta^{5\text{-}4}$-isomerase (Lorence et al., 1990). A reductase then hydrogenates the Δ^4 double bond in the presence of NADPH.

Arabidopsis de-etiolated2 (*det2*) mutants are defective in this reduction step, and were isolated by screening for mutants that display a light-grown phenotype in the dark (Chory et al., 1991). *det2* mutants exhibit a typical BR dwarf phenotype including abnormal skotomorphogenic patterns, such as short hypocotyls, open cotyledons, hook opening, and expression of light dependent genes (Li et al., 1996). *DET2* (At2g38050) is homologous to human steroid 5α-reductase, and when introduced into human cell lines, it converts progesterone (3-oxo Δ^4) to 4,5-dihydroprogesterone. The reverse is also true; the human steroid 5α-reductase gene functionally complements *det2* mutants (Li et al., 1996). The precise biochemical defect in *det2* was resolved by feeding $^2H_6$-labeled CR to *det2* seedlings and subsequently examining the metabolites using gas chromatography-mass spectrometry (GC-MS) (Fujioka et al., 1995). Precursors having a 3-oxo Δ^4 structure accumulated 3-fold more in the *det2* mutant than in wild type, whereas CN level is less than 10% that of wild type. Detection of CN in *det2* indicates that either the *det2* mutation may not be null, or another functional homolog may be present in *Arabidopsis*.

DWARF4: C-22 hydroxylation (Reaction 19)

The C-22α hydroxylation step is considered a rate-determining step in the BR biosynthetic pathways based on the findings that the endogenous level of 6-oxoCN was 500 times greater than that of cathasterone (CT). However the bioactivity of CT is as much as 500 times greater than that of its 6-oxoCN in a rice lamina bending assay (Diener et al., 2000). Recent biochemical evidence has revealed that *Arabidopsis* C-22α hydroxylase uses various steroids as substrates. These include CR, (24*R*)-ergost-4-en-3-one, (24*R*)-5α-ergostan-3-one, CN, 6-OxoCN, and possibly many other C-24 reduced sterols (Fig. 2) (Choe et al., 1998; Fujioka et al., 2002).

Arabidopsis dwf4 mutants have mutations in this enzyme, and display typical BR deficient dwarf phenotypes. Feeding tests with biosynthetic intermediates showed that only steroids that were C-22 hydroxylated rescued the dwarfism (Choe et al., 1998). In addition, examination of the endogenous levels of BR biosynthetic intermediates in *dwf4* revealed that substrates such as 6-oxoCN and CN accumulate, whereas the C-22 hydroxylated products are present only in trace amounts (Choe et al., 1998). Sequence analysis showed that DWF4 (At3g50660) belongs to the cytochrome P450 (CYP90B1) superfamily and shares great similarity with previously identified BR biosynthetic enzymes such as *Arabidopsis* CONSTITUTIVE PHOTOMOTPHOGENESIS AND DWARFISM (CPD: CYP90A1) and tomato DWARF (CYP85) (Choe et al., 1998). The *Arabidopsis* genome has 4 members in the CYP90 family: CYP90A1, CYP90B1, CYP90C1, and CYP90D1. Of these, CYP90A1 is another name for CPD that mediates the step after DWF4, but the precise functions of the other two enzymes CYP90C1 and CYP90D1 in BR biosynthesis remain to be elucidated. RNA gel blot analysis revealed that *DWF4* is not highly expressed, suggesting that a lower gene expression level is a part of a mechanism to keep DWF4 enzymatic activity low, resulting in a rate

determining step in the pathway. In accordance with this, overexpression of the *DWF4* gene using the CaMV 35S promoter gave rise to constitutive BL responses including elongated inflorescences, long petioles, elongated leaf blades, increased number of siliques and consequently elevated seed productions (Choe et al., 2001). Completion of the rice genome sequencing revealed that rice has a single *DWF4* homolog. In light of elevated seed production by *DWF4* overexpression in *Arabidopsis*, characterization and application of the rice *DWF4* gene in rice may result in elevated seed yield from this important crop.

CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM: C-23 hydroxylation (Reaction 20)

Steroid C-23-hydroxylase is a cytochrome P450 monooxygenase that adds a hydroxyl group to the C-23 position. The *Arabidopsis cpd* mutant for this enzymatic step has been identified from a T-DNA-tagged mutant population (Szekeres et al., 1996). Feeding studies using biosynthetic intermediates indicated that only C-23 hydroxylated steroids such as teasterone (TE) and its downstream compounds could rescue the extreme dwarfism of *cpd*, suggesting that the C-23 hydroxylation step is defective in this mutant. Cross sections of stems from *cpd* mutant plants showed that vascular system differentiation is altered, resulting in extranumerary phloem cells and fewer xylem cells, possibly due to unequal cell division activity in the cambium (Szekeres et al., 1996). Examination of gene expression in *cpd*, wild type, and *CPD*-overexpression lines revealed that transcripts of stress-related genes, such as alcohol dehydrogenase, are significantly increased, whereas pathogenesis-related (PR) genes, including *PR1*, *PR2*, and *PR5*, are decreased in *cpd*, suggesting that BRs are involved in conferring resistance to biotic attacks. The CPD cytochrome P450 protein (At5g05690) is classified as CYP90A1. A genetic defect similar to *cpd* has also been found in the tomato *dumpy* (*dpy*) mutant, where dwarfism is rescued only by C-23 hydroxylated BRs (Koka et al., 2000).

In addition to CYP90A1 (CPD) and CYP90B1 (DWF4), the *Arabidopsis rotundafolia3* (*rot3*) mutant for CYP90C1 has been isolated. Unlike the severe growth retardation seen in *cpd* and *dwf4*, the *rot3* loss-of-function mutant displays normal stature but the leaf shape is most obviously altered, being more round than the wild type (Kim et al., 1998). Interestingly, the width of the *rot3* leaves is not affected as much, thus it is thought that CPY90C1 is involved in metabolic pathways that produce molecules that control cell elongation especially in the leaf. Despite significant sequence similarity of ROT3 with CPD and DWF4, the metabolic step that ROT3 mediates has not been clearly defined.

Tomato DWARF: C-6 oxidation (Reaction 18)

The gene that is responsible for the C-6 oxidation was first identified from tomato. Transposon-based insertional mutagenesis of tomato resulted in an extreme *dwarf* mutant (Bishop et al., 1996). It has been found that the transposon disrupted a gene called *Dwarf* (*D*), and the tomato *D* gene encodes an enzyme catalyzing the C-6 oxidation step. A C-6 oxidase converts 6-deoxoBRs to 6-oxoBRs via two consecutive steps: hydroxylation at the C-6 position first, then further dehydrogenation to a ketone group (Bishop et al., 1999). The D protein belongs to a cytochrome P450 (CYP85) superfamily. This gene was functionally expressed in yeast, and shown to mediate two consecutive steps of C-6 oxidation (Bishop et al., 1999). Examination of endogenous BR biosynthetic intermediates in wild type, the d^x mutant, and a *D*-overexpression line (*35S::D*) revealed that 6-deoxoCS accumulates about 4-fold in the d^x mutant, whereas the content of 6-deoxoCS in the *35S::D* line decreased to 1/50 that of wild type (Bishop et al., 1999). This confirms that the tomato *D* gene encodes a C-6 oxidase enzyme in tomato plants.

Similarly to the tomato CYP85, rice genome has a single copy of functional homolog to this gene. Rice *brd1* (*brassinosteroid deficient 1*) mutant is deficient in the C-6 oxidation step, and this mutant shows severe growth retardation (Hong et al., 2002).

Interestingly, although tomato and rice results in dwarf phenotype by a monogenic recessive mutation in a single gene, *Arabidopsis* does not show any phenotype when a putative CYP85 homolog was disrupted (Shimada et al., 2003). The *Arabidopsis* genome has two copies of the tomato D homologs: At5g38970 (CYP85A1) and At3g30180 (CYP85A2). It could be due to overlapping functions of the two CYP85 genes in *Arabidopsis*. When these two genes were functionally expressed in yeast, both of them converted not only 6-deoxocastasterone (6-deoxoCS), but also 6-deoxoTE, 6-deoxo3DT, and 6-deoxotyphasterol (6-deoxoTY) to their 6-oxidized forms: CS, TE, 3DT, and TY, respectively (Shimada et al., 2003). Real time RT-PCR analysis of the two genes revealed that both of the two genes are highly expressed in apical shoots with higher transcript level of CYP85A2. Although,

CYP85A1 expression was extremely low compare to that of CYP85A2, CYP85A1 expression was relatively high in silique and root, where CYP85A2 expression level was low. Thus it is likely that these two functionally redundant genes possess different levels of activity in spatially separate tissues.

Pea DDWF1: C-2 hydroxylation

The conversion of 6-deoxotyphasterol and typhasterol to 6-deoxocastasterone and castasterone occur via C-2 hydroxylation reaction as shown in *Arabidopsis* using deuterium-labeled BR intermediate (Noguchi et al., 2000). DDWF1 (Dark-induced-DWF like protein1) encoding cytochrome P450 (CYP92A6) has C2-hydroxylation activity and has been first identified from yeast-two-hybrid screening as Pra2 interacting protein in pea (Kang et al., 2001). Since *Arabidopsis* genome does not seem to encode any member of CYP92 family, C-2 hydroxylation reaction converting (6-deoxo) typhasterol to (6-deoxo)castasterone probably performed by a different cytochrome P450 enzymes in *Arabidopsis*.

BR BIOSYNTHETIC INHIBITORS

Although the BR dwarf mutant served as a strong tool to dissect the biosynthetic pathway, only a few plant species are known for BR dwarf mutants. Furthermore, information on other possible roles of brassinosteroids is limited because the mutant analysis has been limited to a relatively small number of plant species (Asami and Yoshida, 1999). In these cases, exogenously supplied BR biosynthetic inhibitors can be used to determine the physiological functions of the brassinosteroids and to screen the mutant defective in BR signaling pathway (Wang et al., 2002).

As described previously, brassinosteroids are synthesized from the phytosterols and its biosynthetic pathway is characterized by plant-specific steps such as the cyclization of squalene oxide to cycloartenol, by which sterols are synthesized through a series of reactions including single or double methylation. Since the reduction of the normal phytosterols caused by inhibition of obtusifoliol 14α-demethylase can lead to phytoxicity, the step downstream of sterol biosynthesis has been preferable for the target of the specific inhibitors of brassinosteroid biosynthesis (Asami and Yoshida, 1999).

Many steps of brassinosteroid biosynthesis after episterol are catalyzed by the cytochrome P450

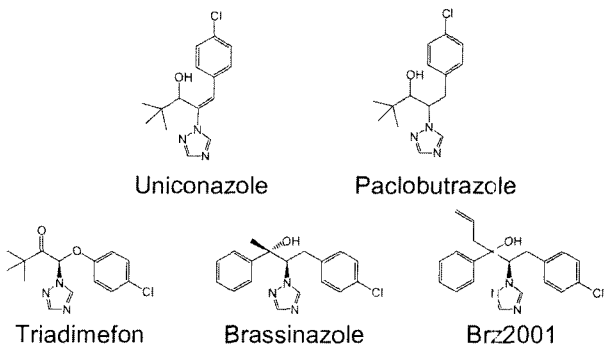

Figure 4. Structure of the biosynthetic inhibitors, uniconazole, paclobutrazole, triadimefon, brassinazole and brz2001.

enzymes as indicated by the identification of DWF4 (Choe et al., 1998) and CPD (Szekeres et al., 1996) from *Arabidopsis*, DWARF from tomato (Bishop et al., 1999) and DDWF1 from pea (Kang et al., 2001). Triazole compounds are known to inhibit cytochrome P450s since its hydrophobic moiety interacts with the site normally occupied by the substrate, thus preventing the binding of the oxygen molecules that would normally be activated and transferred to the substrate. Work with triazoles has provided an insight into the affinity of these inhibitors for the different forms of cytochrome P450 which catalyze the various steps during the sterol and brassinosteroid biosynthesis in fungi and plants. Uniconazole and paclobutrazol are triazole derivatives that inhibit cytochrome P450s involved in the GA biosynthesis (Fig. 4). They also show slight inhibition of the BR biosynthesis as shown in pea shoot and cultured cells of *Zinnia elegans* (Iwasaki and Shibaoka, 1991; Crozier et al., 2000). Triadimefon is a widely used traizole-type fungiside and interferes not only with oxidative demethylation reaction in 14á-demethylase in ergosterol (BR) biosynthesis pathway in fungi, but also blocks gibberellin biosynthesis (Asami et al., 2003).

Although cytochrome P450 is ubiquitous and involved in various steps in brassinosteroid biosynthesis, inhibition of enzymes can be strictly controlled by specific inhibitors since every enzyme has its own characteristic structure of three dimensional inhibitor binding site. Recent works showed the possibility of developing selective inhibitors which can function at specific biosynthetic conversion among broad range of the cytochrome P450 enzymes catalyzing steps in BR biosynthesis (Asami and Yoshida, 1999; Asami et al., 2000).

Brassinazole, which is a triazole derivative in which tertbutyl group of uniconazole was replaced by a phenyl group, has produced strong inhibitor of BR

biosynthesis among triazole derived inhibitors (Asami and Yoshida, 1999; Asami et al., 2000). Unlike Triadimefon, brassinazole seems to be specific brassinosteroid biosynthetic inhibitors that might function at oxidation steps catalyzing by the cytochrome P450 (Asami et al., 2000). Direct analysis of the interaction between DWF4 protein expressed in *Escheichia coli* and brassinazole and its derivatives has revealed that brassinazole targets C22--hydroxylation catalysed by DWF4 to induce BR deficiency in plants (Asami et al., 2001). Chemically synthesized Brz2001 is a more specific BR-biosynthesis inhibitor than brassinazole, and this allyl-type compound with a modified methyl on the carbon of brassinazole with a hydroxyl group does not affect the physiological function of GA (Sekimata et al., 2001).

CONCLUDING REMARKS

Brassinosteroid *dwarf* mutants of *Arabidopsis* greatly contributed to our understanding of brassinosteroid biosynthesis. However, many of the BR biosynthetic steps including reactions 14, 15, 17, 21, 22, 23 and 24 await for identification of enzymes and corresponding genes in *Arabidopsis*. In mammalian system, the steps 14, 15 and 17 are mediated by a multifunctional 3β-hydroxysteroid dehydrogenase/isomerase enzyme. *Arabidopsis* genome possesses multiple copies of the genes showing limited homology to the human enzymes. These include At1g47290, At2g26260, At2g33630, and At2g43420. Further biochemical analysis with these genes would reveal if they indeed mediate steroid 3-epimerization step in *Arabidopsis*. Recently, Hong et al. (2003) reported that rice CYP90D2 mutant *d2* is defective in the 3-dehydration step, and they could not find any homologs from rice (Hong et al., 2003). This suggests that animals and rice adopted a different enzymatic system in 3-dehydrogenase reaction. Interestingly, many of the BR biosynthetic steps are shown to be mediated by cytochrome P450 proteins. In pea, the enzyme that mediates the penultimate step, C-2 hydroxylation, also belongs to cytochrome P450 (Kang et al., 2001). Although sequence similarity search does not seem to show a corresponding gene from *Arabidopsis*, one of the P450 enzymes that has significant identity with known BR- biosynthetic P450s, such as CYP90s and CYP85s, may mediate the 2-hydroxylation step as well as the final oxidation step. Functional expression and subsequent biochemical analysis of the P450s may clarify currently unknown genes in BR biosynthesis.

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