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A Double Mutant for the *CYP85A1* and *CYP85A2* Genes of *Arabidopsis* Exhibits a Brassinosteroid Dwarf Phenotype

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Brassinosteroid (BR)-6-oxidases mediate the bridge reactions that connect the late and early C-6 oxidation pathways by converting 6-deoxoBR to 6-oxoBRs. Two similar genes of *Arabidopsis*, *CYP85A1* (At5g38970) and *CYP85A2* (At3g30180), are proposed to encode BR-6-oxidases based on findings that heterologously expressed genes mediate BR-6-oxidation reactions in yeast. However, genetic evidence that both genes are critically involved in the BR-6-oxidation step in *Arabidopsis* has been limited. Here, we show that a double mutant for the two genes displays dwarfism similar to that of typical BR biosynthesis-deficient mutants, suggesting that they are the major BR-6-oxidases in *Arabidopsis*. Examination of endogenous BR levels and metabolism monitoring tests using this double mutant revealed a great reduction in the levels of 6-oxoBRs, e.g., TY and CS, due to a lack in the conversion reactions from 6-deoxoCS to CS, and from 6-deoxoTY to TY. Surprisingly, the double mutant accumulated a significant amount of 6-oxocampestanol, suggesting that the upstream C-6 oxidation of campestanol to 6-oxocampestanol is not catalyzed by the two BR-6-oxidases in *Arabidopsis*, rather, by another enzyme yet to be discovered.

Keywords: brassinosteroid, BR-6-oxidase, C-6 oxidation, cell elongation, CYP85, cytochrome P450

Brassinosteroids (BRs) are a class of poly-hydroxylated plant steroidal hormones that influence diverse physiological processes, e.g., embryogenesis, cell elongation, vascular differentiation, fertility, and senescence (Kwon and Choe, 2005). Brassinolide (BL) and castasterone (CS) are synthesized from campesterol (CR) via a series of enzymatic steps, including reduction, hydroxylation, epimerization, and oxidation. The conversion of CR to campestanol (CN) occurs through the early and late C-22 oxidation pathways, and further downstream events from CN or 6-oxocampestanol (6-oxoCN) to BL take place in two parallel early- and late-C-6 oxidation pathways (Choe, 2005). These alternative C-6 oxidation pathways seem to be conserved in many plant species (Fujioka et al., 2000; Noguchi et al., 2000; Nomura et al., 2001).

The discovery of *Arabidopsis* BR-deficient mutants, such as *de-etiolated-2* (*det2*), *dwarf4* (*dwf4*), *constitutive photomorphogenesis and dwarfism* (*cpd/dwf3*), *ste1/dwf7*, *dwf1*, and *dwf5*, has facilitated elucidation of the BR biosynthetic pathway (reviewed in Choe, 2005; Kwon and Choe, 2005). However, several steps, i.e., CN to 6-oxoCN, 6-deoxoteasterone (6-deoxoTE) to 3-dehydro-6-deoxoteasterone (6-deoxo3DT), 6-deoxo3DT to 6-deoxotyphasterol (6-deoxoTY), and 6-deoxoTY to 6-deoxoCS, are still not characterized at the molecular level in *Arabidopsis*. In other plants, several cytochrome P450 enzymes have been proposed to be involved in these steps, such as C-2 hydroxylation by DDWRF in pea (Kang et al., 2001), or the conversion of the 6-deoxoTE (TE) to 6-deoxo-3DT (3DT) by CYP90D2 in rice (Hong et al., 2003).

CYP85/BR-6-oxidase was first identified via isolation of tomato loss-of-function dwarf mutant (d^x) and is characterized as a C-6 oxidase that converts 6-deoxoBRs to 6-oxoBRs via two consecutive steps: hydroxylation of the C-6 position and further dehydrogenation (Bishop et al., 1996). In addition, yeast functional assay of the heterologously expressed recombinant DWARF has further demonstrated that BR-6-oxidases have broad substrate specificity not only for 6-deoxoTY and 6-deoxoCS but also for 6-deoxoTE and 6-deoxo3DT (Bishop et al., 1999; Hong et al., 2002). Thus, BR-6-oxidases are thought to be involved in the multiple oxidation steps that connect the late and early C-6 oxidation pathways in plants.

Arabidopsis BR-6-oxidases are encoded by two

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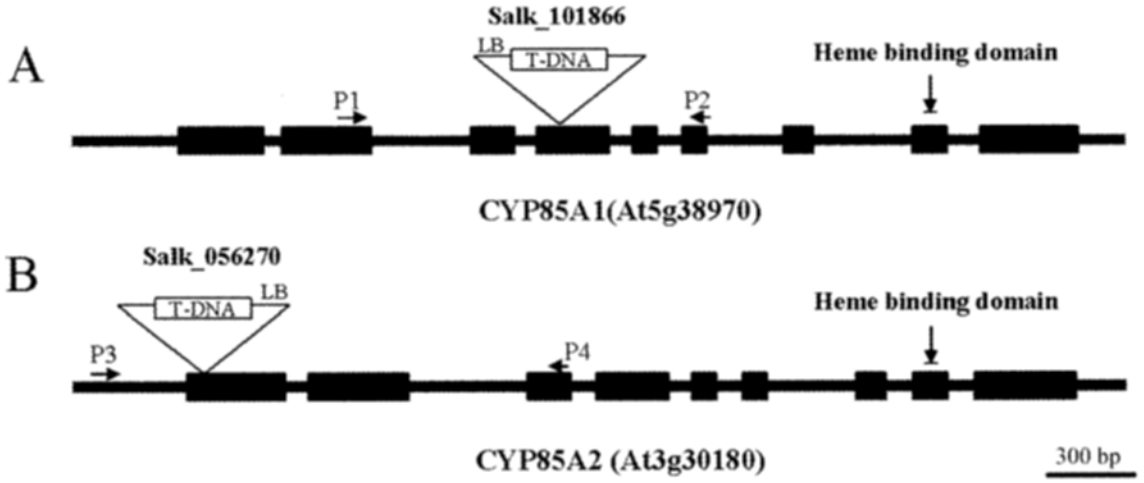

Figure 1. Schematics of *Arabidopsis CYP85A1* and *CYP85A2* with T-DNA insertion site for Salk T-DNA insertion mutant lines. Both genes have nine exons whose exon/intron boundaries are completely conserved between the two. SALK_101866 has a T-DNA inserted in the 4th exon of the At5g38970 gene while SALK_056270 has a T-DNA insertion at approximately 130 b downstream from a translational start codon. P1, P2, P3, and P4 refer to positions for oligonucleotides used for genotyping.

highly homologous *CYP85A* classes of cytochrome P450s, AtBR6ox1/At5g38970/*CYP85A1* and AtBR6ox2/At3g30180/*CYP85A2*, thereby sharing a well-conserved gene structure (Shimada et al., 2003). The functions of *Arabidopsis* CYP85A1 and CYP85A2 as BR-6-oxidases have been studied in yeast after heterologous expression of those genes. Nomura et al. (2005) have reported that *Arabidopsis CYP85A2* is the ortholog of tomato *CYP85A3*, and that it plays an additional role as a BL synthase in addition to its BR6-oxidase activity. In our current study, we generated a double mutant for *CYP85A1* and *CYP85A2*, and determined the genetic basis by which those two genes are critically involved in the BR-6-oxidation step in *Arabidopsis*. Metabolic feeding tests were used as well as an examination of the endogenous levels of BR intermediates to determine whether the dwarf phenotype of the double mutant was due to a disruption in BR-6-oxidation activities.

MATERIALS AND METHODS

Plant Materials

Arabidopsis SALK_101866 and SALK_056270 T_3 seeds from the ABRC stock center (Alonso et al., 2003) were used to generate double-mutant plants defective in both genes that encode BR-6-oxidases. All seeds were sterilized according to the method of Kwon et al. (2005). Plants and seedlings were grown under the long day condition.

Genetic Crosses and Identification of Double Homozygote of *cyp85a1/cyp85a2-1*

To isolate a homozygote for T-DNA insertional mutant lines, primers were designed for the flanking sequences (Fig. 1) and genotyped for the T-DNA insertion. The oligonucleotide sequences were 5′-atcttgatcagtggaatgagcttgagg-3′ (P1), 5′-taggaccccattaacgatcgttgcc-3′ (P2), 5′-gggcataatgatgatgattttgggtc-3′ (P3), 5′-tccactgcg gtaattcgttcccg-3′ (P4), and 5′-gcgtggaccgcttgctgcaact-3′ (LB). Individual homozygote lines for SALK_101866 and SALK_056270 were crossed with each other, and the resulting F_1 plants were allowed to self for production of F_2 seeds. Double mutants were confirmed by PCR using primers for the genotyping as well as scoring a segregation ratio at the F_3 generation. All progeny of the F_2 double mutant stably inherited dwarfism in the F_3 generation without any segregating wild types.

Response to CS, BL, and Brassinazole (Brz)

Biochemical complementation of *cyp85a1/cyp85a2-1* was performed with 10^{-9}~10^{-6} M CS or BL-supplemented solid media. We also studied the effects of different doses (10^{-9} M, 10^{-8} M, 10^{-7} M, or 10^{-6} M) of brassinazole, a BR biosynthetic inhibitor, on seedlings of that double mutant. After sterilization, subsequent stratification and 5 h of light induction, we grew the seedlings for 7 d on a half-strength Murashige and Skoog medium containing 0.8% (w/v) plant agar and 1% sucrose, plus various concentrations of CS, BL, or Brz.

Endogenous BR Analysis and Metabolism Tests

Wild-type 'Columbia' and *cyp85a1/cyp85a2-1* seeds were planted in soil and grown for 5 weeks in the light (long day condition). Aerial portions of plants were harvested when the first 2 or 3 siliques were fully matured. After tissues were freeze-dried, endogenous levels of the BR intermediates were determined via gas chromatography-mass spectrometry (GC-MS) (Noguchi et al., 1999). We also conducted a feeding test using deuterium-labeled 6-deoxoTY and 6-deoxoCS (Choe et al., 1999).

RESULTS AND DISCUSSION

cyp85a1/cyp85a2-1 Displays Weak Dwarfism with a Typical BR Biosynthetic Mutant Phenotype

To further investigate functioning in the *Arabidopsis* cytochrome P450 85A family, we have obtained two independent mutants, in which T-DNA inserted in the coding regions of At5g38970 (*CYP85A1*) and At3g30180 (*CYP85A2*). Genotyping with gene-specific and T-DNA left border-specific primers revealed that T-DNA was inserted in the 4th exon of At5g38970 (SALK_101866), and approximately 130 b downstream of the translation start codon of At3g30180 (SALK_056270; Fig. 1). In order to determine whether transcription of At5g38970 and At3g30180 was completely blocked, we performed RT-PCR using total RNA isolated from *cyp85a1* and *cyp85a2-1*. *CYP85A2* mRNA was not detectable in *cyp85a2-1* (data not shown), suggesting that *cyp85a2-1* is a null allele.

To generate a double mutant and determine the effect of defects in *CYP85A1* and *CYP85A2*, we crossed a homozygous mutant for each locus, *cyp85a1* and *cyp85a2-1*. The double mutant (*cyp85a1/cyp85a2-*

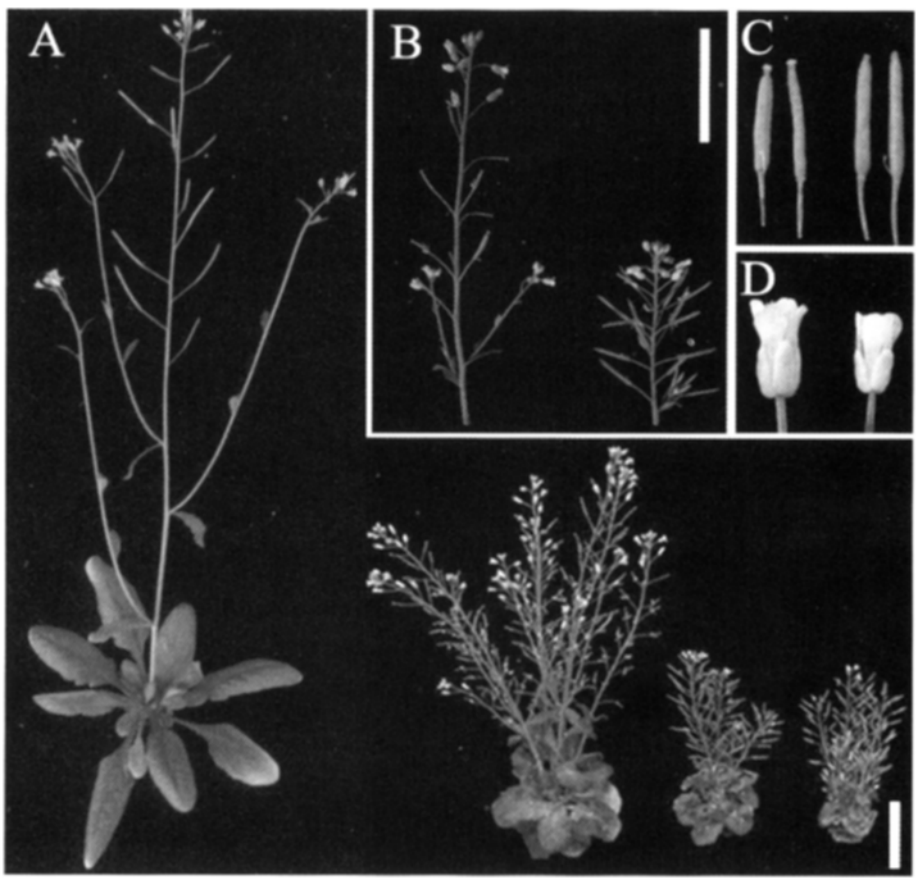

Figure 2. Morphology, from left to right, of 6-week-old *Arabidopsis* wild-type ('Columbia'), *cyp85a1/cyp85a2-1*, *dwf7-1*, and *cpd-388* plants (**A**). Inflorescences (**B**), siliques (**C**), and flowers (**D**) of *cyp85a1/cyp85a2-1* (left) and *dwf7-1* (right). *cyp85a1/cyp85a2-1* shows dwarfism, short robust inflorescences, round leaves, short pedicels, and siliques with significantly reduced fertility. Scale bar = 2 cm (**A** and **B**).

Table 1. Morphometric comparison of *cyp85a1/cyp85a2-1*, *dwf7-1*, and *cpd-388* in 6 week-old *Arabidopsis* plants. Inflorescence from double mutant was approximately twice as tall as from *dwf7-1* and *cpd-388*, indicative of a relatively weak dwarf phenotype Data represnt the mean±S.E. of 20 individual plants.

Organ sizes[a] (n=20)	*cyp85a1/cyp85a2-1*	*dwf7-1*	*cpd-388*
Inflorescence height	109.10±5.24	43.8±1.08	47.7±1.90
Length of silique	6.01±0.16	5.42±0.10	5.32±0.09
Length of pedicle	3.94±0.10	3.38±0.09	3.28±0.08
Rosettes	18.79±0.46	13.56±0.34	10.92±0.34
Leaf blade length[b]	13.75±0.41	9.72±0.24	10.38±0.32

[a]Length in mm.
[b]Second pair of rosette leaves.

1) was then isolated based on PCR-assisted genotyping as well as scoring a segregation ratio for dwarfism (data not shown). As predicted, *cyp85a1/cyp85a2-1* showed dwarfism with a typical BR-deficient mutant phenotype (Fig. 2A). Compared with 6-week-old wild-type plants, the double mutant, when grown for 6 weeks in the light, had short, robust inflorescences, round leaves, reduced fertility, and short pedicels and siliques (Fig. 2A). Interestingly, dwarfism of *cyp85a1/cyp85a2-1* was relatively weaker than what was observed with the *cpd-388* and *dwf7-1* mutants (Fig. 2A). The latter is known as weak among those BR-deficient mutants already discovered (Choe, 2005). Inflorescences from the double mutant were twice as tall as those from either *dwf7-1* or *cpd-388*. Moreover, its leaf blades were significantly longer (Table 1). Another characteristic feature of the *cyp85a1/cyp85a2-1* was a significant reduction in fertility; only one or two siliques per plant produced fertile seed-filled siliques at 6 weeks after germination in contrast to all siliques of *dwf7-1* bearing viable seeds (Fig. 2B and C). However, floral development was apparently normal, with sepals and petals being longer in the flowers of *cyp85a1/cyp85a2-1* than in *dwf7-1* (Fig. 2D). This suggests that the biochemical activities missing from the double mutant are necessary to support ordinary seed development in the silique.

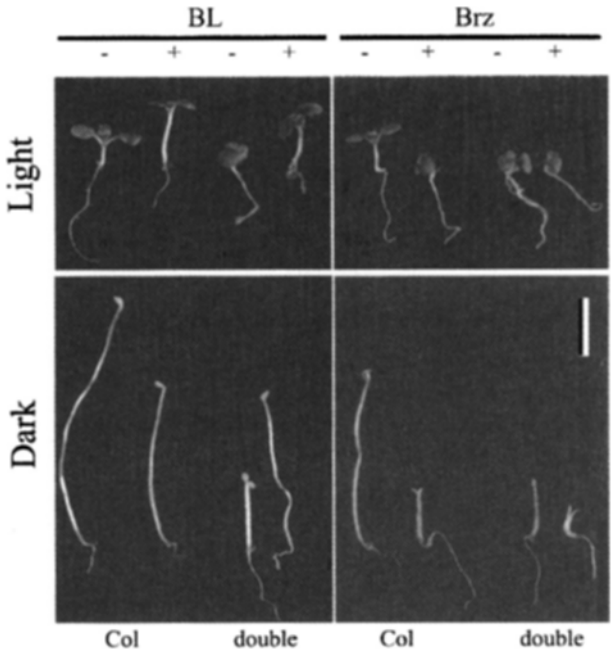

Figure 3. Response by 7-d-old 'Columbia' wild-type and *cyp85a1/cyp85a2-1* seedlings to BL and Brz in light and dark. Double mutant seedlings reacted to BL and Brz with increased or decreased hypocotyl lengths, respectively, regardless of lighting conditions. Scale bar =0.5 cm.

Both CS and BL Rescue Dwarf Phenotypes

The developmental defects of BR biosynthetic mutants are rescued by exogenous application of BL, the final product of the BR biosynthetic pathway (Kwon and Choe, 2005). For biochemical complementation, we grew *cyp85a1/cyp85a2-1* seedlings either in the light or under darkness for 7 d on media supplemented with 10^{-6} M BL. Exogenous supply completely rescued the short hypocotyl phenotype regardless of lighting conditions (Fig. 3), a response similar to that found with most BR-deficient mutants, e.g., *dwf7-1* and *cpd-388*. Unlike wild-type seedlings, which produced shorter hypocotyls when treated with BL under the darkness, dark-grown *cyp85a1/cyp85a2-1* seedlings responded to BL with lengthened hypocotyls (Fig. 3). Such an inhibitory effect by exogenous BL on dark-grown wild-type seedlings has been previously reported (Choe et al., 2001). In addition, the double-mutant seedlings exhibited even shorter hypocotyls when treated with brassinazole (Brz), in both the light and the dark (Fig. 3). This inhibition of growth by Brz suggests that the double

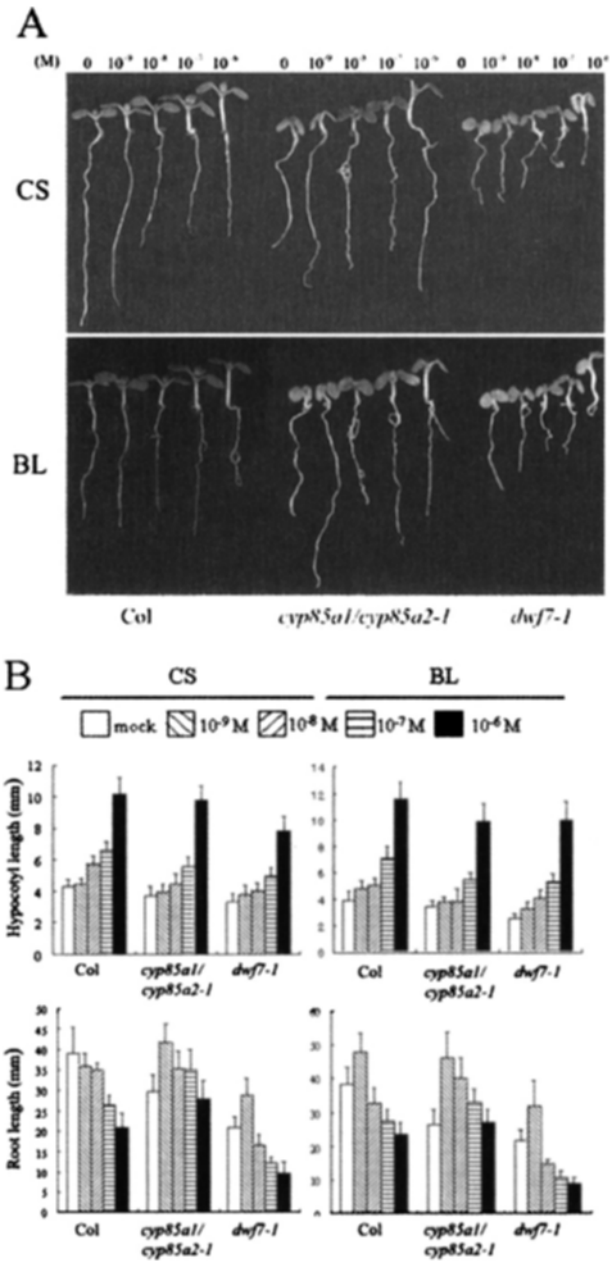

Figure 4. Responses of hypocotyls and roots at 7 d after germination to growth media supplemented with brassinosteroids. Both CS and BL induced hypocotyl growth. In contrast, roots of double mutants displayed reduced sensitivity to higher concentrations (10^{-6} M) of both CS and BL. Inhibition of root growth by 1 μM CS or BL was obvious in wild type and *dwf7-1*, but not in double mutant. Shown are representative seedlings treated with different concentrations of CS and BL (**A**), and mean values of hypocotyl and root lengths (n>15) (**B**) with standard deviation on each bar.

mutant still possessed some of the endogenous pool of bioactive BRs, despite the disruption to the *CYP85A1* and *CYP85A2* genes. Besides blocking BR-6-oxidation, Brz further reduced the metabolic flux that acts on DWF4, perhaps retarding the growth of the hypocotyls from those Brz-treated double-mutant seedlings (Fig. 3; Asami et al., 2001).

Nomura et al. (2005) have demonstrated that *CYP85A2* as a BL synthase as well as a BR-6-oxidase in tomato, and have proposed distinct functions for CS and BL, the former being the major brassinosteroid hormone essential to normal vegetative growth, the latter having an organ-specific role in fruit development (Nomura et al., 2005). Thus, we would expect a double mutant for two BR-6-oxidase genes to be defective in both CS- and BL-biosynthetic steps. To determine whether treatments with CS and BL induced different sensitivities by the double mutant and *dwf7-1*, we applied various concentrations of those two brassinosteroids. Overall, both CS and BL effectively promoted hypocotyl elongation in the wild type, *dwf7-1*, and *cyp85a1/cyp85a2-1* (Fig. 4). CS seemed to be a nascently-bioactive compound that promoted this lengthening, because the CS to BL conversion reaction is supposedly to be defective in *cyp85a1/cyp85a2-1*. In contrast to the hypocotyls, the roots of the double mutants and the *dwf7-1* seedlings responded to BRs differently than the wild types. Unlike wild type, root lengths increased at lower concentrations of CS and BL (10^{-9} M), but then showed a decline in growth as the concentrations rose above 10^{-8} M. Furthermore, the lengths of wild type and *dwf7-1* roots, when treated with 1 mM of CS or BL, were almost half that measured for the control. However, the lengths of the double-mutant roots at 1 μM did not differ significantly from those that resulted from the control dose (Fig. 4B).

Double Mutant is Defective in Multiple BR-6-Oxidation Steps in the Conversion of 6-Deoxoty and 6-DeoxoCS to 6-OxoBRs but Not CN to 6-OxoCN

We used two approaches to determine the biochemical basis for growth-retarding phenotypes in the double mutant: 1) examination of endogenous BR levels and 2) use of a deuterium-labeled intermediate. First, when BR biosynthetic intermediates in *cyp85a1/cyp85a2-1* were analyzed by GC-MS, the levels of CN and 6-deoxoCT did not significantly differ from those of the wild type (Fig. 5). However, many of those intermediates in the late C-6 oxidation pathway, such as 6-deoxoTE, 6-deoxo3DT, 6-deoxoTY, and 6-deoxoCS, accumulated more in *cyp85a1/cyp85a2-1* than in the wild types (Fig. 5). In particular, the amount of 6-deoxoCS was quadrupled

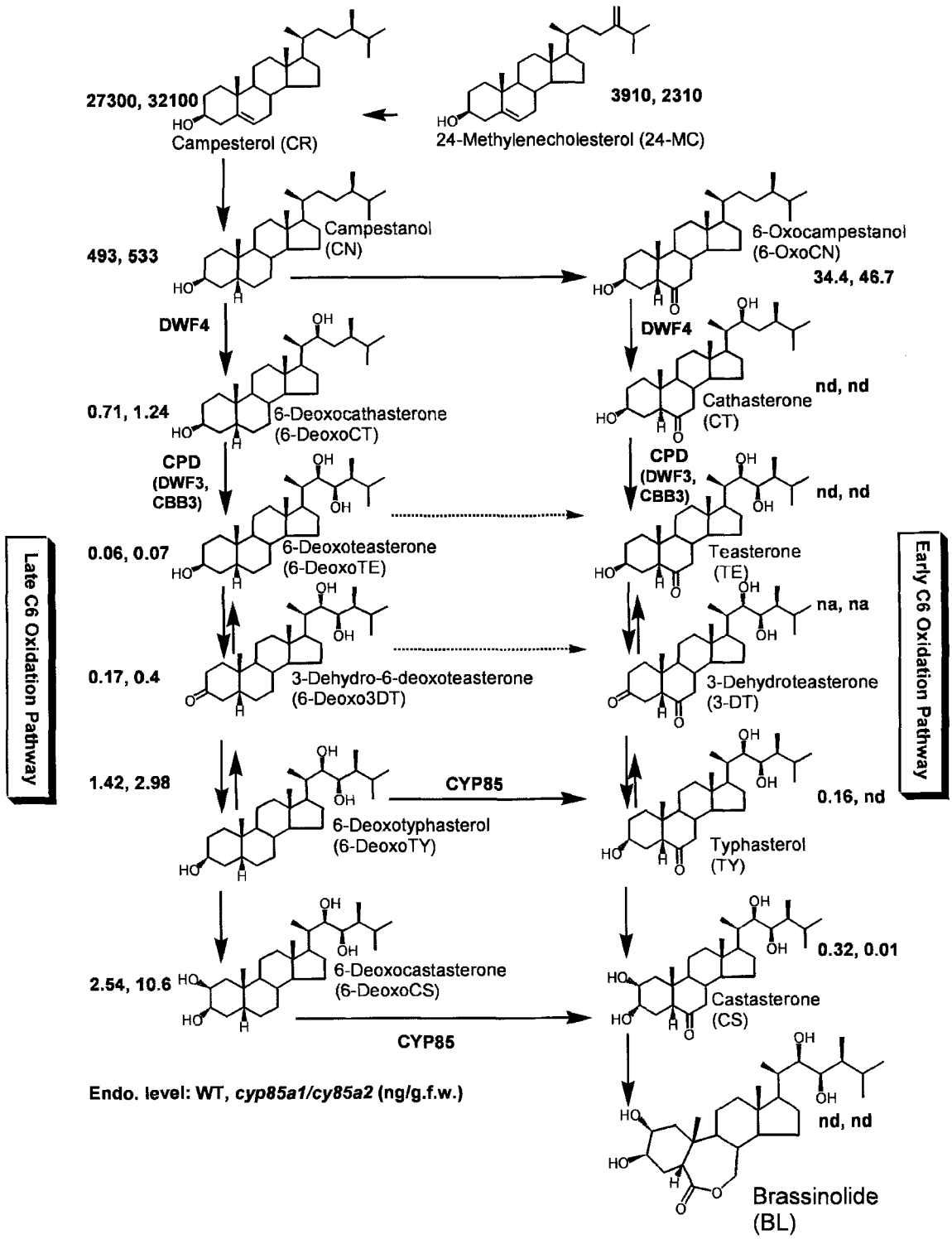

Figure 5. Endogenous levels of BR biosynthetic intermediates in wild type and double mutant. The 6-deoxoBRs in late C-6 oxidation pathway were converted to 6-oxoBRs via actions of BR-6-oxidases in wild type. In *cyp85a1/cyp85a2-1*, 6-deoxoTE, 6-deoxo-3DT, 6-deoxoTY, and 6-deoxoCS were accumulated, compared with wild type. In particular, the level of 6-deoxoCS was almost quadrupled in double mutant while CS levels decreased approximately 30-fold in double mutant, suggesting critical impairment in C-6 oxidation of 6-deoxoCS to CS. Each biosynthetic intermediate is labeled with 2 numbers to indicate levels, as shown in lower portion.

Table 2. Metabolic experiments of [^{2}H]6-DeoxoCS and [^{2}H]6-DeoxoTY in the wild type and the *cyp85a1/cyp85a2-1* double mutant. When one flask containing seedlings of either genotype was fed with 10 μg of deuterium-labeled 6-deoxoTY, its metabolites were detected as 6-deoxoTE, 6-deoxo3DT, and 6-deoxoCS, suggesting that 3-epimerization reactions are reversible in *Arabidopsis*. In addition, lower-than-detectable levels of TY and CS in the double mutant confirmed that its C-6 oxidation is deficient.

Substrate	Metabolite	
	Wild Type (Columbia)	*cyp85a1/cyp85a2-1*
[$^2H_6$]6-DeoxoCS	[$^2H_6$]CS (3)	[$^2H_6$]CS (nd)
[$^2H_6$]6-DeoxoTY	[$^2H_6$]6-DeoxoTE (46)	[$^2H_6$]6-DeoxoTE (38)
	[$^2H_6$]6-Deoxo3DT (36)	[$^2H_6$]6-Deoxo3DT (17)
	[$^2H_6$]6-DeoxoCS (1)	[$^2H_6$]6-DeoxoCS (3)
	[$^2H_6$]TY (1)	[$^2H_6$]TY (nd)
	[$^2H_6$]CS (1)	[$^2H_6$]CS (nd)

nd: not detected (below detection limit)
Values in parentheses indicate detected amount (ng) of each metabolite.

in the double mutant, suggesting that BR-6-oxidation of 6-deoxoCS is critically impaired in this double mutant. Shimada et al. (2001, 2003) have heterologously expressed *CYP85A1* and *CYP85A2* in yeast, and have shown that both genes mediate the conversion reactions of 6-deoxoTE, 6-deoxo3DT, 6-deoxoTY, and 6-deoxoCS to their corresponding 6-oxo-BRs. In contrast, the conversion of 6-deoxoTE to TE and 6-deoxo3DT to 3DT was not detected in the *in vivo* metabolic feeding tests with wild-type *Arabidopsis* seedlings (Noguchi et al., 2000). Therefore, the slight increase in the level of 6-deoxoTE and 6-deoxo3DT noted in our study may have been due to both the blockage of C-6 conversion to TE and 3DT as well as to inhibition of the downstream conversion of 6-deoxoTY and 6-deoxoCS to TY and CS, respectively.

In contrast to the great reduction in endogenous levels of TY and CS in our double mutant, 6-oxoCN levels increased approximately 50% as compared with that of the wild type. Thus, it is obvious that C-6 oxidation of CN to 6-oxoCN operates normally or is even more activated in the *cyp85a1/cyp85a2-1* (Fig. 5). Normal conversion of CN to 6-oxoCN has also been reported in rice *brd1 (brassinosteroid deficient 1)* (Hong et al., 2002). Therefore, it is likely that conversion of CN to 6-oxoCN is catalyzed by other enzymes besides *CYP85A1* or *CYP85A2* in *Arabidopsis*.

To further confirm the metabolic flux of 6-deoxoTY, we fed wild type and double-mutant seedlings with deuterium-labeled 6-deoxoCS and deuterium-labeled 6-deoxoTY, then monitored their metabolic fates (Table 2). Consistent with the level of endogenous BRs in the double mutant, 6-deoxoCS was not metabolized to CS in those plants. When seedlings were fed with deuterium-labeled 6-deoxoTY, it was metabolized toward both forward and reverse reactions. The 6-deoxoTY metabolism went backward than forward reaction which leads to 6-deoxoCS in wild type, based on the finding that the level of 6-deoxoTE ranked highest (46 ng per flask) among all metabolites tested. In comparison, levels of 6-deoxoCS, TY, and CS were as low as 1 ng per flask. We observed a similar backward metabolism pattern in the double mutant. However, the amount of 6-deoxoCS (3 ng per flask) in the double mutant was triple that of the wild-type level (1 ng per flask), thereby confirming that the 6-oxidation of 6-deoxoCS was blocked in the *cyp85a1/cyp85a2-1*. This also demonstrated that the 3-epimerization reaction was reversible *in vivo*.

In conclusion, we have found that *CYP85A1* and *CYP85A2* in *Arabidopsis* encode functionally redundant BR-6-oxidases that convert 6-deoxoTY and 6-deoxoCS to TY and CS, respectively, but not CN to 6-oxoCN. Therefore, tissue-specific and/or developmental regulation of *CYP85A1* and *CYP85A2* expression might impart overlapping but distinct roles for CS and BL. In future research, clues to the possible roles of these functionally redundant enzymes may be obtained through studies of ectopic over-expression of each *CYP85* gene, promoter analysis of *CYP85A1* and *CYP85A2* under different light regimes, and the examination of endogenous BR levels in *cyp85a2* single mutants.

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