Substrate Specificity for Cytochrome P450 85A1 and 85A2 in Brassinosteroids Biosynthesis

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There are over forty members of the class of compounds known as plant steroidal hormones.[1-2] They are collectively named brassinosteroids (BRs). Among these, castasterone (CS) and brassinolide (BL) are considered the most important BRs because of their strong biological activities and wide-distribution in the plant kingdom. CS is biosynthesized from 24-methylcholesterol via two parallel pathways, namely, the early and late C-6 oxidation pathways, and then CS is further oxidized by 7-oxalactonation to produce BL. In the late C-6 oxidation pathway (Fig. 1), conversion of 6-deoxoCS to CS is catalyzed by two BR 6-oxidases which are cytochrome P450s designated as CYP85A1 and A2.[3] Recently we found that CYP85A2, has additional effects on BL synthase-it mediates the conversion of CS to BL, indicating that CYP85A2 is a bi-functional enzyme for BL synthase as well as for BR 6-oxidase.[4]

As shown in Fig. 1, accumulating evidence suggests that plants operate multiple biosynthetic pathways to synthesize diverse naturally-occurring BRs such as 28-norBRs (C_{27}-BRs), 24-methylene-BRs (C_{28}-BRs), 24-methyl-BRs (C_{28}-BRs) and 24-ethyl-BRs (C_{29}-BRs).[5] Although carbon skeletons, especially

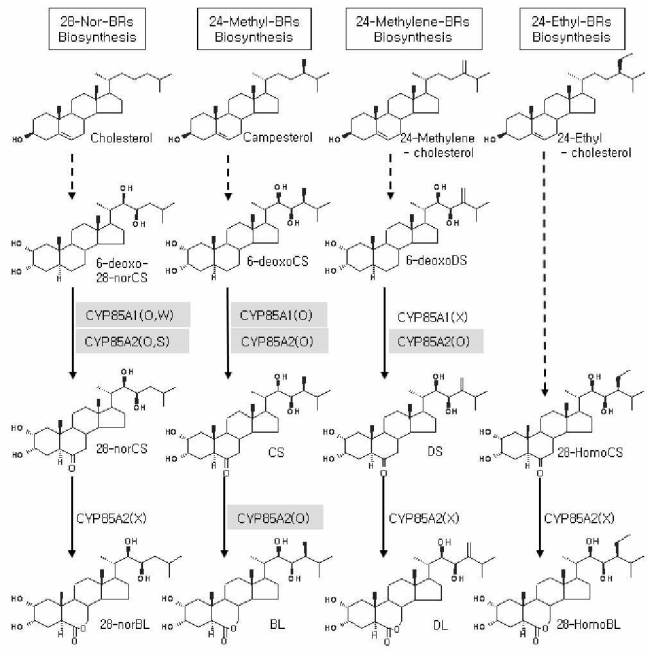

Figure 1. Diverse biosynthetic pathways for BRs in plant. The shadow box indicates catalyzing activity of CYP85A1, and/or CYP85A2. O: active, X: inactive, W: weak, S: strong

the alkyl groups at C-24, are different in C_{27}-, C_{28}- and C_{29}-BR biosynthesis, biosynthetic reactions involved in the multiple BR biosyntheses are thought to be basically identical, suggesting that the same genes or proteins characterized in 24-methyl-BR biosynthesis are likely to catalyze the corresponding biosynthetic reactions for the other BR biosyntheses.[3] To test the possibility, involvement of CYP85A1 and A2, which are potentially useful proteins for enhancing BRs activity to develop commercially valuable plants, in 28-nor-, 24-methylene- and 24-ethyl-BRs biosynthesis was investigated in this study.

The preparation of Cyt P450 overexpressing yeast strains (CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21) and galactose induction were done as previously described.[6] Galactose-induced cells were diluted in 20 mL of YPL to an OD_{550} of 0.4 to 0.6 and about 5 ug of suitable substrates were added to the cells. After 6 hrs, metabolites were extracted using 20 mL of ethyl acetate and then subjected to reversed-phase HPLC (RP-HPLC) (SenshuPak C_{18}, 10 × 150 mm) with a flow rate of 2.5 mL/min and 45% acetonitrile as the elution. Fractions were collected every minute. Fractions corresponding to the retention times of authentic BRs for expected products (28-norBL, DL: 10 min; 28-norCS, DS, BL: 14 min; 28-HBL: 16 min; CS: 20 min) were collected and analyzed by GC-MS after suitable derivatizations.[4]

First, successful expressions of CYP85A1 and A2 in yeast strains (CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21) were examined. When 6-deoxo-CS was added as a substrate, as summarized Table 1, the expressed CYP85A1 and CYP85A2 catalyzed the conversion of 6-deoxo-CS to CS. When CS was used as a substrate, the expressed CYP85A2 induced production of BL, but CYP85A1 did not. Therefore, it is confirmed that the function of the expressed CYP85A1 and A2 is correct.

Second, a possible function of CYP85A1 and CYP85A2 in 28-nor-, 24-methylene- and 28-ethyl-BRs biosynthesis was examined. When 6-deoxo-28-norCS was added as a substrate, both CYP85A1 and CYP85A2 produced 28-norCS by 6-oxidation of 6-deoxo-28-norCS. The conversion yield by CYP85A2 was higher than that by CYP85A1 (Fig. 2b), suggesting that CYP85A2 is a more powerful BR 6-oxidase in 28-norBR biosynthesis. When 28-norCS was used as a substrate for CYP85A2, the expected product, 28-norBL, was not detected even in GC-SIM analysis, indicating that CYP85A2 is not functional in the 7-oxalactionation of 28-norCS to 28-norBL in 24-methylene-BR biosynthesis. When 6-deoxoDS was added as a substrate for both CYP85A1 and CYP85A2, CYP85A2 catalyzed conversion of 6-deoxoDS to DS, but

Table 1. GC-MS data for authentic BRs and the products catalyzed by CYP85A1 and CYP85A2.

Yeast strain	Substrate	Product	Rt[a] on GC	Prominent ions (*m z*, relative intensity)
CYP85A1/V60/WAT21	6-DeoxoCS	CS	29.20	512 (M^+,84), 497 (9), 441 (16), 399 (21), 358 (24), 327 (12), 287 (40), 155 (100)
	6-Deoxo-28-norCS	28-NorCS	28.16	498 (M^+,100), 483 (8), 399 (3), 358 (11), 328 (8), 287 (35), 141 (54)
	6-DeoxoDS	none	(27.81)	(510(M^+16), 387(10), 327(82), 158(70), 124(100))
CYP85A2/V60/WAT21	6-DeoxoCS	CS	29.20	512 (M^+,84), 497 (9), 441 (16), 399 (21), 358 (24), 327 (12), 287 (40), 155 (100)
	6-Deoxo-28-norCS	28-NorCS	28.16	498 (M^+,100), 483 (8), 399 (3), 358 (11), 328 (8), 287 (35), 141 (54)
	CS	BL	32.07	528 (M^+,6), 374 (39), 344 (22), 332 (40), 177 (61), 163 (26), 155 (100)
	28-NorCS	none	(25.28)	(496(M^+,10), 481(5), 411(8), 356(7), 327(100), 287(10), 139(70))
	DS	none	(38.20)	(526(M^+6), 403(42), 385(30), 343(100), 153(80), 124(92))
	28-HomoCS	none	(35.58)	(542(M^+8), 457(20), 397(21), 374(70), 345(42), 169(100))

The data in parenthesis are for only authentic BRs. The samples are analyzed as a derivative of bismethaneboronate. [a]Retention time

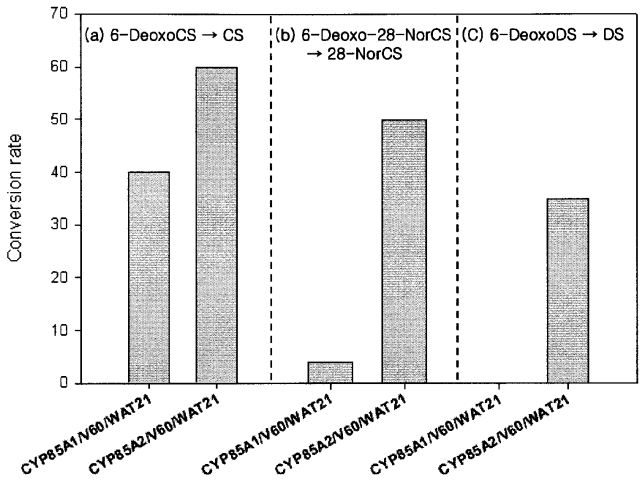

Figure 2. Conversion rate of CYP85A1 and CYP85A2 expressed in Yeast strain(V60/WAT21).

CYP85A1 did not (Table 1 and Fig. 2c), showing that only CYP85A2 has BR 6-oxidase activity in 24-methylene-BR biosynthesis. When DS was used as a substrate for CYP85A2, no DL was identified as a product of CYP85A2, indicating that CYP85A2 has only BR 6-oxidase activity in 24-methylene-BR biosynthesis. When 28-homoCS was added as a substrate for CYP85A2, CYP85A2 did not generate 28-homoBL, implying that CYP85A2 has no 28-homoBL synthase activity in 24-ethyl-BR biosynthesis.

In the rice lamina inclination test, 6-deoxo-BRs such as 6-deoxo-28-norCS, 6-deoxoDS and 6-deoxoCS showed no biological activity.[3-7] 6-Keto-BRs, 28-norCS, DS, CS and 28-homoCS, exhibited quite strong activity, indicating that 6-oxidation is important for acquiring BR activity. 28-norBL, DL, BL and 28-homoBL show approximately 5 to 10 times higher activity than 28-norCS. DS, CS and 28-homoCS, respectively. These findings show that enzymes catalyzing 6-oxidation and 7-oxa-lactonation are important candidates for increasing BR activity for the development of commercially useful plants.

Both CYP85A1 and CYP85A2 consist of 465 amino acids.[3] They share 83% identity and 92% similarity. In spite of this, CYP85A1 and CYP85A2 possess different substrate specificities for BRs. CYP85A1 can catalyze 6-oxidation of 6-deoxo-28-norCS (C24-nor) and 6-deoxoCS (C24-methyl) but not 6-deoxoDS (C24-methylene), indicating that CYP85A1 recognizes alkyl groups at the C-24 position of BRs as its substrates. In contrast, CYP85A2 can mediate 6-oxidation of 6-deoxo-28norCS (C24-nor), 6-deoxoDS (C24-methylene) and 6-deoxoCS (C24-methyl). Further, the conversion rate of 6-oxidation by CYP85A2 is much higher than that by CYP85A1 (Fig. 2). These findings suggest that CYP85A2 is a universal and more powerful enzyme for driving BR 6-oxidation than CYP85A1 in BR biosyntheses.

As a 7-oxalactonase, CYP85A2 can catalyze 7-oxalactonation of CS (C24-methyl), yielding BL, but can not drive 7-oxalactonation of 28-norCS (C24-nor), DS (C24-methylene) and 28-homoCS (C24-ethyl) to 28-norBL, DL and 28-homoBL, respectively, which implies that CYP85A2 recognizes alkyl groups on BR side chains, especially at C-24. Therefore, CYP85A2 is not a universal BR 7-oxalactonase: it is only a BL synthase that mediates conversion of CS to BL, the last biosynthetic reaction in 24-methyl-BRs biosynthesis.

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