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. 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 widespread 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-oxalactonatization 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. 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 synthesis as well as for BR 6-oxidase.

As shown in Fig. 1, accumulating evidence suggests that plants operate multiple biosynthetic pathways to synthesize diverse naturally-occurring BRs such as 28-norBRs (C20-BRs), 24-methylene-BRs (C21-BRs), 24-methyl-BRs (C22-BRs) and 24-ethyl-BRs (C23-BRs). Although carbon skeletons, especially the alkyl groups at C-24, are different in C20-, C21- and C22-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. 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. Galactose-induced cells were diluted in 20 mL of YPL to an OD630 of 0.4 to 0.6 and about 5 μg 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 C18, 10 x 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, 28-4BL: 16 min; CS: 20 min) were collected and analyzed by GC-MS after suitable derivatizations.

First, successful expressions of CYP85A1 and A2 in yeast strains (CYP85A1/V60/WAT21 and CYP85A2/V60/WAT21) were examined. When 6-deoxoCS was added as a substrate, as summarized Table 1, the expressed CYP85A1 and CYP85A2 catalyzed the conversion of 6-deoxoCS to CS. When CS was used as a substrate, the expressed CYP85A2 produced CS. However, 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 24-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-oxalactonation 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.

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Substrate</th>
<th>Product</th>
<th>Rt* on GC</th>
<th>Prominent ions (m/z: relative intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP85A1/V60/WAT21</td>
<td>6-DeoxoCS</td>
<td>CS</td>
<td>29.20</td>
<td>512(M+, 84), 497(94), 411(16), 399(21), 358(24), 257(12), 287(40), 155(100)</td>
</tr>
<tr>
<td></td>
<td>6-Deoxo-28-norCS</td>
<td>28-NorCS</td>
<td>28.16</td>
<td>498(M+, 100), 483(8), 399(3), 358(11), 328(8), 287(35), 141(54)</td>
</tr>
<tr>
<td></td>
<td>6-DeoxoDS</td>
<td>none</td>
<td>(27.81)</td>
<td>(540(M+, 16), 378(10), 237(82), 158(70), 124(100))</td>
</tr>
<tr>
<td>CYP85A2/V60/WAT21</td>
<td>6-DeoxoCS</td>
<td>CS</td>
<td>29.20</td>
<td>512(M+, 84), 497(94), 411(16), 399(21), 358(24), 257(12), 287(40), 155(100)</td>
</tr>
<tr>
<td></td>
<td>6-Deoxo-28-norCS</td>
<td>28-NorCS</td>
<td>28.16</td>
<td>498(M+, 100), 483(8), 399(3), 358(11), 328(8), 287(35), 141(54)</td>
</tr>
<tr>
<td></td>
<td>CS</td>
<td>BL</td>
<td>32.07</td>
<td>528(M+, 16), 374(39), 344(22), 322(40), 177(61), 163(26), 155(100)</td>
</tr>
<tr>
<td></td>
<td>28-NorCS</td>
<td>none</td>
<td>(25.28)</td>
<td>(496(M+, 10), 481(5), 411(8), 356(7), 327(100), 287(10), 159(70))</td>
</tr>
<tr>
<td></td>
<td>DS</td>
<td>none</td>
<td>(38.20)</td>
<td>(526(M+, 6), 404(42), 385(50), 343(100), 153(80), 124(92))</td>
</tr>
<tr>
<td></td>
<td>25-HomoCS</td>
<td>none</td>
<td>(35.58)</td>
<td>(542(M+, 8), 475(20), 397(21), 374(70), 345(42), 169(100))</td>
</tr>
</tbody>
</table>

The data in parenthesis are for only authentic BRs. The samples are analyzed as a derivative of bismethaneboronate. "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 CYP85A1, no BL 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. 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, 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. 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-28-norCS (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-oxa-lactonase, CYP85A2 can catalyze 7-oxa-lactonation of CS (C24-methyl), yielding BL, but can not drive 7-oxa-lactonation of 28-norCS (C24-nor), DS (C24-methylene) and 28-homoCS (C24-ethyl) to 28-norBL, DS 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-oxa-lactonase; 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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Reference