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Cytochrome P450-catalyzed brassinosteroid pathway activation through synthesis of castasterone and brassinolide in *Phaseolus vulgaris*

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Abstract

The last reaction in the biosynthesis of brassinolide has been examined enzymatically. A microsomal enzyme preparation from cultured cells of *Phaseolus vulgaris* catalyzed a conversion from castasterone to brassinolide, indicating that castasterone 6-oxidase (brassinolide synthase) is membrane associated. This enzyme preparation also catalyzed the conversions of 6-deoxocastasterone and typhasterol to castasterone which have been reported to be catalyzed by cytochrome P450s, CYP85A1 of tomato and CYP92A6 of pea, respectively. The activities of these enzymes require molecular oxygen as well as NADPH as a cofactor. The enzyme activities were strongly inhibited by carbon monoxide, an inhibitor of cytochrome P450, and this inhibition was recovered by blue light irradiation in the presence of oxygen. Commercial cytochrome P450 inhibitors including cytochrome c, SKF 525A, 1-aminobenzotriazole and ketoconazole also inhibited the enzyme activities. The present work presents unanimous enzymological evidence that cytochrome P450s are responsible for the synthesis of brassinolide from castasterone as well as of castasterone from typhasterol and 6-deoxocastasterone, which have been deemed activation steps of BRs.

Keywords: *Phaseolus vulgaris*; Brassinosteroids; Cytochrome P450 monooxygenase

1. Introduction

Brassinosteroids (BRs) represent a large group of plant steroids which include more than 40 congeners distributed from lower to higher plants. Physiological functions proposed for BRs include cell elongation, cell division, leaf bending, vascular differentiation, proton pump-mediated membrane polarization, sink/source regulation, gravitropic responses, and modulation of stress responses (Clouse, 1996; Clouse and Sasse, 1998; Fujioka, 1999; Kim, 1991; Kim et al., 2000; Sasse, 1999; Yokota, 1997). Recent molecular genetic and biochemical analyses of BR-deficient mutants of *Arabidopsis*, garden pea, and tomato revealed that BR-deficiency causes abnormal development of plants such as reduced stem elongation (dwarfism), reduced fertility, altered photomorphogenesis and vasculature, and delayed senescence. Application of BRs alone can rescue these pleiotropic abnormalities, indicating that BRs are essential for normal growth and development of plants (Altmann, 1999; Bishop and Yokota, 2001; Bishop et al., 1999; Choe et al., 1998; Clouse and Feldmann, 1999; Fujioka et al., 1997; Kauschmann et al., 1996; Klahre et al., 1998; Koka et al., 2000; Mathur et al., 1998; Noguchi et al., 1999; Nomura et al., 1997, 1999; Szekeres et al., 1996; Yokota, 1997).

Since the first plant cytochrome P450 monooxygenase (Cyt P450) CYP71A1 was characterized by analyzing its cDNA (Bozak et al., 1990), many plant Cyt P450 genes

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have been isolated using nucleotide sequence information, especially a highly conserved fingerprint region (FxGxxxCxG) near the C-terminus, which contains the heme-binding cystein (Meijer et al., 1993; Frank et al., 1996; Holton and Lester, 1996). To date, more than 500 plant Cyt P450 sequences comprising 53 families have been deposited in the Cyt P450 database (http://drnelson.utmem.edu/CytochromeP450.html). Cyt P450s are typically NADPH-dependent monooxygenases requiring molecular oxygen. Cyt P450-mediated reactions include hydroxylation, dealkylation of alkylated N, O and S, deamination, sulfoxide formation, dehalogenation and C–C bond cleavage (Durst, 1991; Guengerich, 1993, 1996; Omura, 1999; Ortiz de Montellano, 1995; Schuler, 1996; Wachenfeldt and Johnson, 1995). Like animal Cyt P450s, plant Cyt P450s are involved in oxidative metabolism of endogenous compounds such as sterols, terpenes, flavonoids, fatty acids, alkaloids, phenylpropanoids and cyanogenic glucosides and also participated in detoxification of xenobiotics such as herbicides (Bolwell et al., 1994; Chapple, 1998; Durst, 1991; Schuler, 1996; West, 1980). Furthermore, Cyt P450s are responsible for kaurene oxidation and kaurenoic acid 7α-hydroxylation in gibberellin biosynthesis as well as for the oxidative catabolism of abscisic acid (Hedden and Kamiya, 1997; Helliwell et al., 1998; Krochko et al., 1998; Winkler and Helentjaris, 1995; Yamaguchi and Kamiya, 2000).

Cyt P450s have been known to play crucial roles in the biosynthesis of brassinolide **14** (BL) which starts from campesterol **1** (CR, 24α-methylcholesterol) either through the early or late C-6 oxidation pathways (Fig. 1) (Choi et al., 1996, 1997; Müssig and Altmann, 1999; Sakurai, 1999; Suzuki et al., 1994, 1995; Yokota, 1997). The hydroxylations at C-22 and C-23 have been demonstrated to be catalyzed by Cyt P450s, DWF4 and CPD, respectively, by biochemical and molecular genetic analyses of the *Arabidopsis* mutants *dwf4* and *cpd* and these proteins had significant homology to the mammalian steroid hydroxylases (Choe et al., 1998; Szekeres et al., 1996). C-6 oxidation of 6-deoxocastasterone **7** (6-deoxoCS) to castasterone **13** (CS) has been demonstrated to be mediated by Cyt P450 by analyzing the tomato *Dwarf (D)* gene which also had high homology to the mammalian steroid hydroxylase genes (Bishop et al., 1999). Furthermore, a *D* ortholog termed *AtBR6ox*, has been isolated from *Arabidopsis* (Shimada et al., 2001). These proteins, CPD, DWF4 and D/AtBR6*ox*, were new members of Cyt P450s and classified into CYP90A1, CYP90B1 and CYP85A1, respectively. Recently, it was reported that a pea gene *DDWF1* (Dark induced DWF-like protein1, CYP92A6) encodes a Cyt P450 that might catalyze C-2 hydroxylation of typhasterol **12** (TY) to CS **13** and of 6-deoxoTY **6** to 6-deoxoCS **7** through a small GTP-binding protein, Pra2 (Kang et al., 2001).

The oxidative conversion of CS **13** to BL **14** has been deemed as an important activation step because BL **14** is the most biologically active BR. However, nothing has been characterized about this enzyme, termed CS **13** 6-oxidase. Furthermore, CS **13** has also been considered to have a bioactivity because CS **13** is not convertible to BL **14** in many biological systems in which CS **13** is biologically active (Yokota, 1997; Yokota et al., 2001). We have already demonstrated that various BRs including TY **12**, CS **13**, 6-deoxoCS **7** and BL **14** are endogenous in the *Phaseolus vulgaris* seed (Kim, 1991; Kim et al., 2000; Yokota et al., 1987) and also CS **13** and BL **14** are major BRs in *P. vulgaris* shoots (Yokota et al., 1990). This situation prompted us to characterize these enzymes through in vitro enzymatic system of *P. vulgaris* cells. In this paper we propose evidence that CS 6-oxidase is indeed a Cyt P450. Furthermore, we demonstrated enzymatical evidence for the Cyt P450-catalyzed synthesis of CS **13** from TY **12** and 6-deoxoCS **7**, which are direct precursors of CS in the early and late C-6 oxidation pathway of the BR biosynthesis, respectively.

2. Results and discussion

2.1. Microsomal enzymes catalyze conversions of TY 12 to CS 13, 6-deoxoCS 7 to CS 13 and CS 13 to BL 14

Suspension cultured cells of *P. vulgaris* were homogenized prior to ultracentrifugal separation into cytosolic and microsomal fractions. Authenticity of the fractionation was confirmed by measuring the enzyme activities of NADPH-Cyt c reductase and alcohol dehydrogenase which are markers for microsomal and cytosolic enzymes, respectively (Table 1). After confirming that no detectable amount of BRs was contained in the fractions (data not shown), both the fractions were examined the abilities to convert TY **12** to CS **13**, 6-deoxoCS **7** to CS **13** and CS **13** to BL **14**. Enzyme assays were initiated by adding the substrates and NADPH in an aerobic atmosphere. After completion of reactions, deuterium labelled CS **13** or BL **14** was added to the assay mixture as an internal standard to quantitatively measure the amounts of expected products, CS **13** and BL **14**. After ethyl acetate extraction followed by reversed phase HPLC, the extracts were subjected to methaneboronation and analyzed by GC-MS and/or GC-SIM.

Analysis of the metabolite of TY **12** in the microsomal fraction gave rise to *m*/*z* 512 [M^+], 441, 358, 327, 287 and 155 ions characteristic of CS bismethaneboronate at the appropriate GC retention time of 16.40 min (Table 1), indicating the presence of TY 2α-hydroxylase in the *P. vulgaris* cells. The specific activity for TY 2α-hydroxylase in the microsomal enzyme solution was 2.66 ng mg protein^{-1} min^{-1} (Fig. 2A and Table 2). The

cytosolic fraction contained only 6-fold lower activity than the microsomal fraction (Table 2).

Based on the same mass spectrum and GC retention time of bismethaneboronate of 6-deoxoCS metabolite as those of authentic CS bismethaneboronate, the metabolite of 6-deoxoCS **7** in the microsomal fraction was identified as CS **13** (Table 1), indicating the presence of 6-deoxoCS 6-oxidase in *P. vulgaris* cells. The specific activity of this enzyme was 0.76 ng mg protein^{-1} min^{-1}, much weaker than that of TY 2α-hydroxylase (Fig. 2B and Table 2). The cytosolic fraction contained only marginal activity of 6-deoxoCS oxidase.

GC-SIM analysis of the metabolite of CS **13** gave rise to *m*/*z* 528 (M^+) and 155 (base peak) ions characteristic of BL **14** bismethaneboronate at the appropriate GC retention time of 20.12 min (Fig. 2C), indicating the presence of CS 6-oxidase (BL synthase) in *P. vulgaris* cells. The activity for CS 6-oxidase was detected only in the microsomal solution, being 0.48 ng mg protein^{-1} min^{-1} (Table 2).

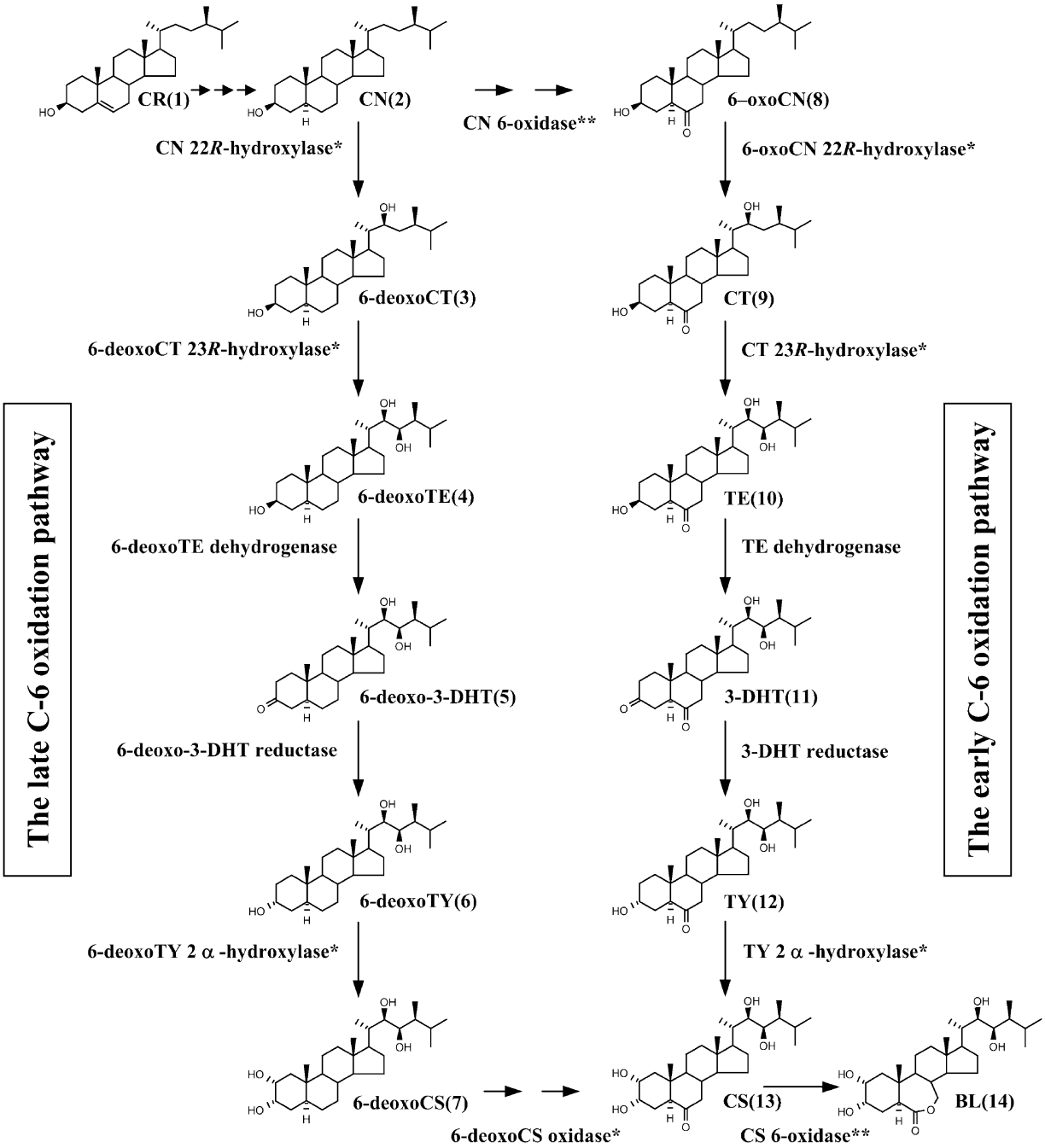

Fig. 1. Tentative names of enzymes involved in the early and late C-6-oxidation pathway. Asterisk (*) and double-asterisk (**) denote a Cyt P450 and Cyt P450-like enzyme, respectively.

Table 1
GC-MS/SIM analysis for the products of TY 2α-hydroxylase, 6-deoxoCS oxidase and CS 6-oxidase in *P. vulgaris* cells

Compound[a]	R_t (min)	Prominent ions (*m*/*z*, relative intensity %)
Product of TY 2α-hydroxylase[b]	16.40	512(M^+, 72), 441(11), 358(36), 327(12), 287(31), 155(100)
Product of 6-deoxoCS oxidase[b]	16.40	512(M^+, 74), 441(10), 358(36), 327(11), 287(33), 155(100)
Product of CS 6-oxidase[c]	20.12	528(M^+, 4), 374(47), 344(28), 332(45), 177 (78), 163(33), 155 (100)
Authentic CS[b]	16.40	512(M^+, 75), 441(11), 358(38), 327(13), 287(34), 155(100)
Authentic BL[c]	20.12	528(M^+, 4), 374(48), 344(28), 332(46), 177(80), 163(34), 155 (100)

[a] The sample was analyzed as its bismethaneboronate derivative.
[b] The sample was analyzed directly by GC-MS.
[c] The sample was analyzed directly by GC-SIM.

Table 2
Activity for enzymes in subcellular fraction prepared from cultured cells of *P. vulgaris*

Subcellular fraction	TY 2α-hydroxylase[a]	6-deoxoCS oxidase[a]	CS 6-oxidase[a]	Alcohol dehydrogenase[b]	NADPH-Cyt c reductase[b]
Microsomal	2.66	0.76	0.48	ND[c]	23.9
Cytosolic	0.46	0.09	ND	0.72	0.45

[a] The enzyme activities were expressed as ng of product mg protein^{-1} min^{-1}.
[b] The marker enzyme activities were expressed as μmol of product mg protein^{-1} min^{-1}.
[c] ND, Not detected.

The data obtained clearly indicate localization of TY 2α-hydroxylase, 6-deoxoCS oxidase and CS 6-oxidase (BL synthase) in the microsomes.

The present work first demonstrated enzymatical evidence, using *P. vulgaris* cell-free system, that Cyt P450s synthesize BL **14** from CS **13** and also CS **13** from either TY **12** or 6-deoxoCS **7** in microsomes. Animal P450s are known to be bound to the membrane of endoplasmic reticulum (ER) or mitochondria. However, all plant Cyt P450s known so far were demonstrated to be bound to ER except CYP86B1 with chloroplast transit peptide and allene oxide synthase (CYP74A) which has several unusual characteristics (Bolwell et al., 1994; Froehlich et al., 2001; Schuler, 1996; Watson et al., 2001). Therefore, it is most probable that CS 6-oxidase, TY 2α-hydroxylase, 6-deoxoCS oxidase in *P. vulgaris* cells are associated to endoplasmic reticulum.

2.2. Molecular oxygen and NADPH are required to elicit activities of TY 2α-hydroxylase, 6-deoxoCS oxidase and CS 6-oxidase

Enzyme analysis is a prerequisite measure to characterize Cyt P450s in a crude enzyme system, especially when no relevant mutant for the enzyme in question is available. Cyt P450 enzymes must be furnished with several, if not all, of the following criteria: (a) a requirement for molecular oxygen, (b) a requirement of NADPH and a reductase, (c) inhibition by CO that forms an adduct with Cyt P450, (d) reversal of the CO inhibition by blue light at 450 nm that dissociates CO from the enzyme, (e) membrane association, (f) 1:1:1 stoichiometry between the amount of O_2 and NADPH used and product formed, (g) the presence in the reduced enzyme preparation of a CO-binding pigment with a maximum absorption at 450 nm, (h) a substrate-dependent type I-binding spectrum, and (i) incorporation of an oxygen atom from O_2 into the product (Krochko et al., 1998; West, 1980).

In the reactions catalyzed by TY 2α-hydroxylase, 6-deoxoCS oxidase or CS 6-oxidase, single oxygen was incorporated into substrates in the presence of NADPH. Requirement of molecular oxygen and NADPH by this enzyme system was examined by removing one of them from the standard assay mixtures. When oxygen was depleted from the assay mixtures by flushing with nitrogen gas, activities of TY 2α-hydroxylase, 6-deoxoCS oxidase and CS 6-oxidase were reduced to as low as 5, 6 and 6%, respectively. When NADPH was removed from the assay mixtures, activities of TY 2α-hydroxylase and 6-deoxoCS oxidase were reduced to 5 and 6%, respectively, while no CS 6-oxidase activity was detected (Table 3). When the *P. vulgaris* enzyme system was operated in the atmosphere of CO, activities of TY 2α-hydroxylase, 6-deoxoCS oxidase and CS 6-oxidase were largely reduced (Table 4). This inhibition was partially recovered by introducing O_2 to the CO-saturated assay mixtures. Further illumi-

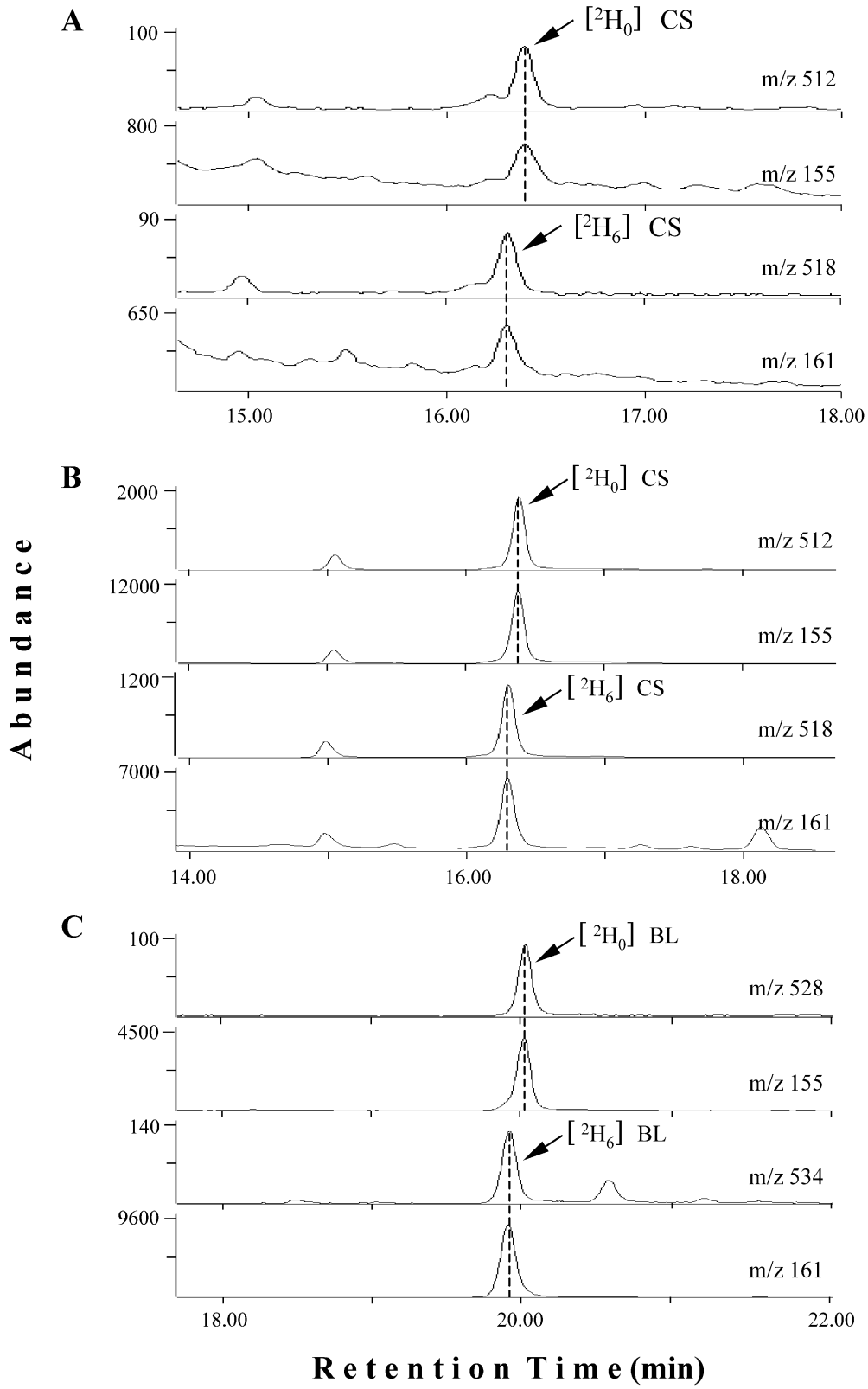

Fig. 2. Quantitative GC-SIM analysis for activity of TY 2α-hydroxylase (A), 6-deoxoCS oxidase (B) or CS 6-oxidase (C) in the microsomal enzyme solution prepared from cultured cells of *P. vulgaris*.

Table 3
Effect of NADPH, O_2, and Cyt P450s inhibitors on the enzyme activities

Treatment	TY 2α-hydroxylase (% activity)	6-deoxoCS oxidase (% activity)	CS 6-oxidase (% activity)
Control[a]	100	100	100
− NADPH	6	6	ND[b]
− O_2 (anaerobic by N_2)	5	6	6
+ Cyt c (50 μM)	23	13	19
+ SKF 525A (1 mM)	21	4	41
+ Aminobenzotriazole (100 μM)	58	22	59
+ Ketoconazole (100 μM)	64	34	61

[a] Control means enzyme activity in standard assay mixture containing NADPH under aerobic conditions.
[b] ND, Not detected.

Table 4
Inhibition of enzyme activities by CO and its reversal by blue light

Treatment	TY 2α-hydroxylase (% activity)	6-deoxoCS oxidase (% activity)	CS 6-oxidase (% activity)
Control[a]	100	100	100
+ CO	7	29	28
+ CO/O_2, dark	42	38	67
+ CO/O_2, blue light	65	57	104

[a] Control means enzyme activity in standard assay mixture containing NADPH under aerobic conditions.

nation of blue light significantly enhanced the recovery rates (Table 4). All together these findings furnish evidence for these enzymes being Cyt P450s.

2.3. Cyt P450 inhibiting chemicals suppress activities of TY 2α-hydroxylase, 6-deoxoCS oxidase and CS 6-oxidase at varying degrees

Activities of the three enzymes were measured in the presence of Cyt P450 inhibitors including cytochrome c (Cyt c), SKF 525A, aminobenzotriazole or ketoconazole. Cyt c consumes electrons that are to be utilized by NADPH-Cyt P450 reductase, thereby inhibiting Cyt P450 activities. SKF 525A is N-de-ethylated by the Cyt P450s to yield an N-oxide, which then inhibits the enzyme activity via heme ligation. Aminobenzotriazole is oxidized by Cyt P450 to release benzyne, which in turn causes phenylation of the prosthetic heme group of Cyt P450s to produce an abnormal porphyrin with irreversible loss of enzyme activity. Ketoconazole not only acts as a heme ligand of P450s, but also interacts with certain amino acid residues in the Cyt P450s active site, resulting in inhibition of enzyme activity (Colby et al., 1995; Karp et al., 1990; Lewis, 1996; Miranda et al., 1998; Nagai et al., 1986; Ortiz de Montellano and Correia, 1995; Reichhart et al., 1982; Thies et al., 1996).

Among these inhibitors tested, Cyt c was found to be equally effective on the inhibition of all of the three enzymes. SKF 525A was an effective inhibitor against TY 2α-hydroxylase and 6-deoxoCS oxidase, but much less inhibitory against CS 6-oxidase. Aminobenzotriazole and ketoconazole were the weakest inhibitors although some activities were observed against 6-deoxoCS oxidase (Table 3).

In the reaction that converts CS **13** to BL **14**, an oxygen atom is inserted into a C–C bond. Such chemical reactions have been classed into the Baeyer-Villiger reaction. Bacteria such as *Acinetobacter* NCIB9871 (Donoghue et al., 1976; Walton and Stewart, 2002) and *Nocardia globerula* CL1 (Donoghue et al., 1976; Norris and Trudgill, 1976) produce cyclohexanone 1,2-monooxygenases that convert cyclohexane to ϵ-caprolactone. A bacterium *Pseudomonas* NCIB9872 also produces cyclopentanone 1,2-oxygenase (Griffin and Trudgill, 1976), while *Pseudomonas putida* produces camphor 1,2-monooxygenase that converts its cyclopentanone ring into a six-membered lactone (Ougham et al., 1983). Androst-4-ene-2,17-dione-16,17-monooxygenase, a product of *Penicillium lilacinum*, modifies the steroidal D ring (cyclopentanone) into a six-membered lactone ring (Prairie and Talalay, 1963). These enzymes responsible for the biological Baeyer-Villiger reaction are all monooxygenases that belong to a group of flavoproteins with FAD as prosthetic group, requiring NADPH as electron donor. Recently a Baeyer-Villiger monooxygenase-identifying sequence motif has been uncovered (Fraaije et al., 2002). Flavin-containing monooxygenases (FMO) and Cyt P450s are located in the same subcellular

compartment, require NADPH and oxygen as cofactors, and have overlapping substrates (Ziegler, 1988). However, any evidence for inhibition by carbon monoxide on FMOs has not been established yet. In fact, an enzyme responsible for the formation of aldicarb sulfoxide in Japanese medaka (*Oryzias latipes*) was characterized as a FMO because the enzyme activity was not inhibited by carbon monoxide (El-Alfy and Schlenk, 1998). As far as the biological Baeyer-Villiger oxidation, plants seem to be different from bacteria and fungi because the present work revealed that lactonization from CS **13** to BL **14** *in planta* is elaborated by a P450(s) rather than a flavoprotein.

Conversion of CS **13** to BL **14**, an activation step that increases the biological activity 4- or 5-fold, was first found in cultured cells of *Catharanthus roseus* (Suzuki et al., 1993; Yokota et al., 1990) and later in its seedlings (Suzuki et al., 1995a). However, this conversion has not been found in the seedlings of tobacco and rice (Suzuki et al., 1995a) nor in mung bean explants (Suzuki et al., 1993). Furthermore, no indication has been reported that such a reaction occurred when 24-epiCS was administered to cultured cells of tomato and serradella (*Ornithopus sativus*), although extensive analysis has been done on the metabolites (Hai et al., 1996; Kolbe et al., 1996). There is the possibility that these are inactive tissues or cells which do not contain CS 6-oxidase. However, it is also probable that these tissues or cells have a regulatory mechanism that CS **13** is decomposed before it contacts CS 6-oxidase, as suggested in etiolated rice lamina explants (Yokota et al., 1992). Our successful characterization of CS 6-oxidase as a Cyt P450 in *P. vulgaris* cell-free system may be due to the fact that the system is free from membrane permeability as well as such metabolism that might have happened during transport.

Peculiarly, no mutant with respect to CS 6-oxidase has been found from *Arabidopsis*, tomato, pea and rice from which a variety of BR biosynthesis mutants were isolated. One reason for this could be that plants have more than one Cyt P450 enzyme that catalyze oxidation of CS **13** to BL **14**. An alternative reason may be that CS **13** is biologically active by its own, as described above, so that any mutant in CS 6-oxidase does not show significantly altered phenotype. This idea seems to be in accord with the recent finding that CS **13** interacts with BRI1 receptor protein competitively with BL **14** (Wang et al., 2001).

In this context, 2α-hydroxylation of TY **12** to CS **13** could be deemed an activation step. However, there are no known mutants defective in this conversion, so that very poor knowledge is available on this enzyme. Recently, Kang et al. (2001) reported that the pea gene *DDWF1* encodes a Cyt P450 enzyme that functions as 2α-hydroxylase of TY **12** and 6-deoxoTY **7**. However, the product obtained in the reaction of typhasterol **12** with the enzyme was not analyzed by GC-MS, leaving an ambiguity for the enzyme function. Nonetheless the present work was in accord with their finding that 2α-hydroxylation is conducted by a Cyt P450. The expression of this pea gene is upregulated in the dark while suppressed in the light. As far as the BR levels of *Arabidopsis* reported by Choe et al. (2001) are concerned, the ratio of the CS **13** content to the TY **12** content was indeed increased in the dark, but the ratio of the 6-deoxoCS **7** content to the 6-deoxoTY **6** content was decreased in the dark. These observations may suggest that two or more 2α-hydroxylation enzymes are present in plants and could be regulated by light or dark in different fashions. In support of this idea, Fujioka et al. (1997) earlier demonstrated that the late C-6 oxidation pathway is dominant in the light while the early C-6 oxidation pathway plays an important role in the dark.

BR 6-oxidases (CYP85A1) of tomato and *Arabidopsis* were shown to catalyze not only conversion from 6-deoxoCS **7** to CS **13** but also conversions from 6-deoxoTY **6** to TY **12**, 6-deoxo-3-dehydroteasterone to 3-dehydroteasterone and 6-deoxoteasterone to teasterone (Shimada at al., 2001). However, these enzymes cannot catalyze the conversion of campestanol **2** (CN) and cathasterone to their 6-oxo counterparts. Our preliminary experiment using *P. vulgaris* microsomal enzyme showed that conversion of CN **3** to 6-oxoCN **8** occurred when NADPH was added under aerobic condition (data not shown). Therefore, CN 6-oxidase may be different from CYP85A1 identified so far. Now characterization of CN 6-oxidase is underway using the same enzyme system.

3. Experimental

3.1. Plant materials and chemicals

Cell-suspension cultures of *Phaseolus vulgaris* were prepared according to John and Lorin (1995) and maintained at 25 °C in a shaking incubator at 120 rpm in the dark. The cells, 2 weeks after subculturins, were harvested by vacuum filtration and stored at −70 °C until required.

All chemicals used in this study including NADPH and inhibitors, Cyt c, SKF 525A, 1-aminobenzotriazole, ketoconazole were purchased from Sigma Chemical (St. Louis).

3.2. Enzyme preparation

During all enzyme preparation steps, the temperature was maintained at 0–4 °C. The harvested cells (50 g) were ground in a prechilled mortar and pestle in 80 ml of 0.1 M sodium phosphate (pH 7.4) containing 250

mM sucrose, 15 mM 2-mercaptoethanol, 1 mM EDTA, 40 mM ascorbate, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 15% (v/v) glycerol and 1% (w/v) insoluble polyvinylpyrrolidone. The homogenate was filtered and centrifuged at 15,000×*g* for 30 min. The resulting supernatant was re-centrifuged at 190,000×*g* for 120 min (Kontron Centrikon T-1180). The obtained pellet was re-suspended in 4 ml of 0.1 M sodium phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% (v/v) glycerol and used as a microsomal enzyme solution. Cytosolic enzymes were precipitated from the supernatant by addition of cold acetone to 40% (v/v) final concentration. The supernatant-acetone mixture was kept at −25 °C for 20 min and centrifuged at 13,000×*g* for 10 min. Resulting precipitate was dissolved in 8 ml of 0.1 M sodium phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% (v/v) glycerol and used as a cytosolic enzyme solution. Protein concentrations of the microsomal and cytosolic solution were estimated with a microassay from Bio-Rad using BSA as a standard (Bradford, 1976).

3.3. Enzyme assays

The enzyme assays were performed in duplicate and the standard assay mixture consisted of 2–5 mg protein ml^{-1} from either cytosolic or microsomal enzymes in the re-suspension buffer, 0.8 mM NADPH and 5 μg substrate (TY **12**, 6-deoxoCS **7** or CS **13**) in a total volume of 1.2 ml. The reaction was initiated by addition of NADPH. The reaction mixture was thoroughly mixed and incubated at 37 °C for 30 min with shaking. The reaction was terminated by addition of 1.2 ml of ethyl acetate and [C26, 28-$^2H_6$]-labelled BR (CS **13** or BL **14**) 50 ng was added to the reaction mixtures as an internal standard for quantitative analysis. The reaction mixture was then centrifuged at 2000 rpm for 10 min and ethyl acetate soluble fraction was obtained (three times in 1.2 ml). The ethyl acetate soluble fraction was concentrated in vacuo, dissolved in MeOH-H_2O (1:1, 5 ml), and charged on an octadecylsilane cartridge column (Sep-Pak Plus C18, Waters). The fraction eluted with 100% methanol (5 ml) was concentrated in vacuo and subjected to a reversed phase HPLC (Novapak C18, 8 × 100 mm) at a flow rate of 1 ml min^{-1} with 45 and 40% acetonitrile for elution of the product for TY 2α-hydroxylase or 6-deoxoCS oxidase and CS 6-oxidase, respectively. The fractions corresponding to retention time of authentic CS **13** (12.6 min) and BL **14** (14.8 min) were collected and analyzed by GC-MS and/or GC-SIM after bismethaneboronation. To detect and calculate the amount of the product for TY 2α-hydroxylase and 6-deoxoCS oxidase, ions at *m/z* 512/518 (M^+) and 155/161 (base peak) characteristic for bismethaneboronate of [$^2H_0$]/[$^2H_6$]-CS were monitored. For CS 6-oxidase, ions at *m/z* 528/534 (M^+) and 155/161 (base peak) for [$^2H_0$]/[$^2H_6$]-BL bismethaneboronate were monitored.

NADPH-Cyt c reductase, an ER marker enzyme, was assayed using oxidized Cyt c as described previously (Park et al., 1999). Alcohol dehydrogenase, a cytosolic marker enzyme, was assayed using a Spectra Max 340 spectrometer (Molecular device com.). The assay mixture contained 1 to 2 mg protein ml^{-1} in 50 mM Tris/HCl (pH 9.0) containing 1 mM NAD^+ and 20% (v/v) ethanol in a total volume of 1 ml. The enzyme reaction was initiated by addition of ethanol to 20% (v/v) of final concentration in the reaction mixture at room temperature, and formation of NADH was measured spectrophotometrically at 340 nm. The extinction coefficient used to calculate NADH concentration was 6.22 mM^{-1} cm^{-1}.

3.4. Inhibition test

Inhibition by CO and reversal of the CO inhibition by blue light were assayed in the following manner. Assays were run in septum-capped tubes. The microsomal enzyme solution was thoroughly saturated with CO gas for 2 min and sealed with a septum. Then, a substrate and a cofactor were introduced by a syringe needle to the assay mixture, and the mixture was incubated at 37 °C for 30 min. To test light reversal of CO inhibition, the microsomal solutions was thoroughly saturated with CO for 2 min followed by saturation with O_2 for 30 s. After capping with a septum, a substrate and a cofactor were introduced to the assay mixture by a syringe needle, and the mixture was incubated at 37 °C for 30 min under blue light or in the dark. The exclusion of O_2 was accomplished by flushing the microsomal solution with N_2 gas. After sealing the tube, then a substrate and a cofactor were introduced by syringe needle. Inhibition test for SKF 525A (1 mM), aminobenzotriazole (100 μM), ketoconazole (100 μM) and Cyt c (50 μM) were carried out by addition of the inhibitors to the standard assay. When SKF 525A and aminobenzotriazole were used, the microsomal enzyme solution was preincubated for 15 min with them before the addition of a substrate. After finishing all assays, the enzyme products were purified and analyzed by GC-SIM by the same methods described above.

3.5. GC-MS and GC-SIM analysis

GC-MS and GC-SIM analyses for the enzyme products were carried out with a Hewlett-Packard 5973 mass spectrometer (Electron impact ionization, 70 electron voltage) connected with 6890 gas chromatography fitted with a fused silica capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness). GC oven temperature was maintained at 175 °C for 2 min, elevated to 280 °C at a rate of 40 °C min^{-1} and then maintained at

280 °C. The carrier gas was He at a flow rate of 1 ml min^{-1}, and the samples were introduced by an on-column injector. Prior to injection, the samples were treated with methaneboronic acid in pyridine (2 mg ml^{-1}) at 70 °C for 30 min to produce a bismethaneboronate.

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