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Tomato cytochrome P450 CYP734A7 functions in brassinosteroid catabolism

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Abstract

Several cytochrome P450 monooxygenases (P450s) catalyze essential oxidative reactions in brassinosteroid (BR) biosynthesis as well as in BR catabolism; however, only limited information exists on the P450s involved in the BR catabolic pathway. Here, we report the characterization of two P450 mRNAs, CYP734A7 and CYP734A8, from *Lycopersicon esculentum*. These P450s show high homology with *Arabidopsis* CYP734A1/BAS1 (formerly CYP72B1), which inactivates BRs via C-26 hydroxylation. Transgenic tobacco plants that constitutively overexpressed CYP734A7 showed an extreme dwarf phenotype similar to BR deficiency. Quantitative gas chromatography–mass spectrometry analysis of endogenous BRs in the transgenic plants showed that the levels of castasterone and 6-deoxocastasterone significantly decreased in comparison with those in wild-type plants. By measuring the Type I substrate-binding spectra using recombinant CYP734A7, the dissociation constants for castasterone, brassinolide, and 6-deoxocastasterone were determined to be 6.7, 12, and 12 μM, respectively. In an *in vitro* assay, CYP734A7 was confirmed to metabolize castasterone to 26-hydroxycastasterone. In addition, 28-norcastasterone and brassinolide were converted to the hydroxylated products. The expression of *CYP734A7* and *CYP734A8* genes in tomato seedlings was upregulated by exogenous application of bioactive BRs. These results indicated that CYP734A7 is a C-26 hydroxylase of BRs and is likely involved in BR catabolism in tomato. The presence of the CYP734A subfamily in various plant species suggests that oxidative inactivation of BRs by these proteins is a widespread phenomenon in plants.

Keywords: *Lycopersicon esculentum*; Solanaceae; Brassinosteroid; Brassinolide; Castasterone; Brassinosteroid catabolism; Cytochrome P450; CYP734A7; CYP734A8

1. Introduction

Brassinosteroids (BR) are steroidal phytohormones that are ubiquitously distributed throughout the plant kingdom (Bishop and Yokota, 2001). BRs regulate many important physiological and developmental processes, such as cell elongation and division, pollen fertility, photomorphogenesis, differentiation of vascular elements, and stress resistance (Mandava, 1988; Clouse and Sasse, 1998). Recent molecular genetic studies for BR-deficient mutants of *Arabidopsis thaliana* L., rice (*Oryza sativa* L.), tomato (*Lycopersicon esculentum* L.), and garden pea (*Pisum sativum* L.) have identified several genes in the pathway of BR biosynthesis from campesterol to brassinolide (BL) (Fujioka and Yokota, 2003). Cytochrome P450 monooxygenases (P450s) play crucial roles in BR biosynthesis as many oxygenations

Abbreviations: BL, brassinolide; BR, brassinosteroid; CS, castasterone; P450, cytochrome P450 monooxygenase.

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at the steroidal skeleton and side-chain structure can occur. To date, several P450 genes have been identified (Bishop and Yokota, 2001; Fujioka and Yokota, 2003).

Fine-tuning of the rates of de novo biosynthesis and catabolism controls the endogenous bioactive BR pool size in order to regulate BR action in plants. BR catabolism involves the conversion of bioactive BRs to inactive forms by enzymatic reactions such as hydroxylations and conjugations. Although published results indicate that BRs exogenously applied to plants are rapidly metabolized (Yokota, 1999), very little is known about the enzymes involved in the catabolism of endogenous BRs. Exogenous applications of radiolabelled 24-*epi*-castasterone (24-*epi*-CS) and 24-*epi*-brassinolide (24-*epi*-BL) to cell suspension cultures of *Ornithopus sativus* (Kolbe et al., 1996) and tomato (Schneider et al., 1994; Hai et al., 1995; Winter et al., 1997; Kolbe et al., 1998) revealed that a variety of modifications of the BR molecules occur (Fig. 1). Epimerization of the 3α-hydroxy group has been observed in tomato and *Ornithopus*, and the resulting 3β-hydroxy group forms conjugates with glucose and fatty acids, respectively. After 3-epimerization in *Ornithopus* suspension cells, hydroxylation at C-20 and successive cleavage of the side chain between C-20 and C-22 were also observed (Kolbe et al., 1996). In cultured tomato cells, hydroxylation at C-25 and C-26 and subsequent glucosylation were observed, suggesting that the plants possess endogenous enzymes responsible for these reactions.

In mammals and insects, P450s play important roles in biosynthesis and catabolism of steroidal hormones. Mammalian CYP11A catalyzes the hydroxylation of C-20 and C-22 and the successive side chain cleavage of cholesterol to produce pregnenolone. Vitamin D3 is derived from 7-dehydrocholesterol and is metabolized by three human P450s, CYP24A1, CYP27A1, and CYP27B1 (Sakaki et al., 2005). In *Drosophila*, ecdysteroids, which are insect molting steroids, are synthesized from cholesterol by successive hydroxylation of C-25, C-22, C-2, and C-20. These reactions are catalyzed by CYP306A1, CYP302A1, CYP315A1, and CYP314A1, respectively (Niwa et al., 2004). Hydroxylation of C-26 and further oxidation to the 26-oic acids by P450 inactivate ecdysone (Williams et al., 2000). Similarly, several P450s are involved in the hydroxylation reactions in the BR catabolic pathways in plants (Fig. 1). In particular, hydroxylations at C-25 and C-26 in tomato suspension cells are likely catalyzed by P450 enzymes, since several P450 inhibitors, such as carbon monoxide, cytochrome *c*, clotrimazole, and ketoconazole, have been found to affect these hydroxylation reactions (Hai et al., 1995; Winter et al., 1997).

In the model plant *Arabidopsis*, an activation-tagging screen for photomorphogenesis mutants identified the *bas1-D* mutant (Neff et al., 1999). The *BAS1* gene encodes the P450 CYP734A1 (formerly CYP72B1). Enhanced expression of CYP734A1 in *Arabidopsis* shows a dwarfed phenotype and accumulates 26-hydroxybrassinolide **1** in feeding experiments (see Fig. 1 for structures). In addition, CYP734A1 catalyzes C-26 hydroxylation of BL **3** and castasterone (CS) **1** in a yeast functional assay (Turk et al., 2003). Recently, a second activation-tagging mutant with a BR-deficient phenotype was identified and designated *chi2*/*sob7-D*/*shk1-D* (Nakamura et al., 2005; Takahashi et al., 2005; Turk et al., 2005). This mutant exhibited the dwarfed phenotype due to the enhanced expression of

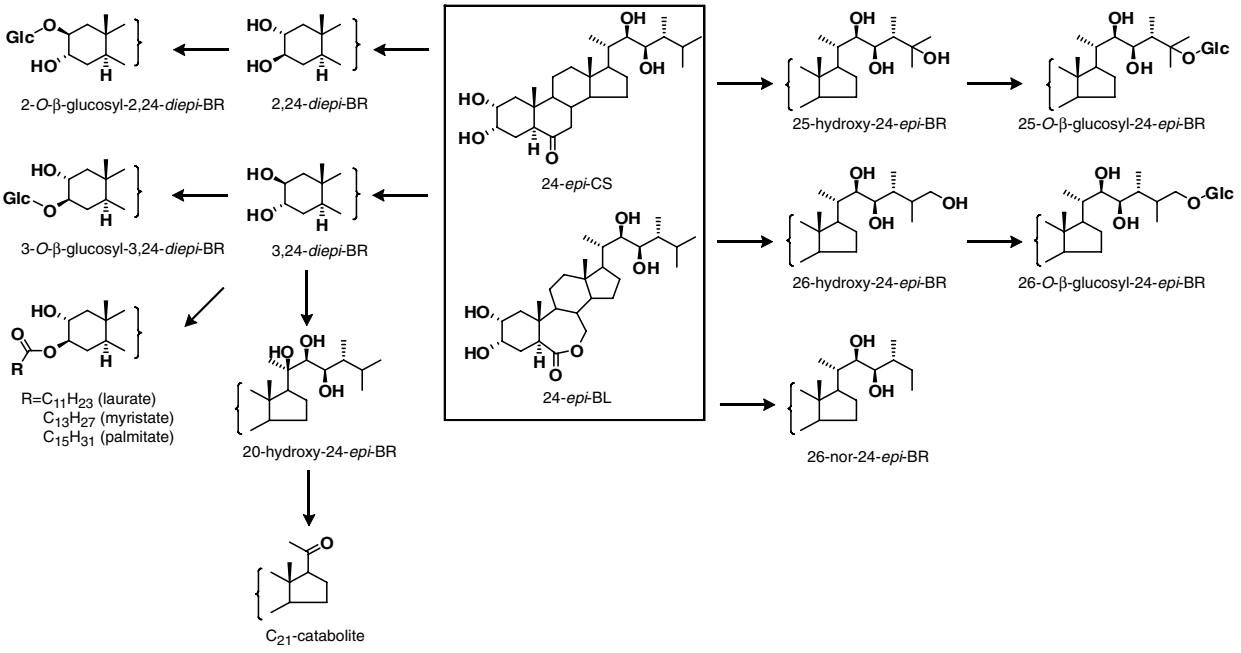

Fig. 1. The BR catabolic pathway in cultured cells of *L. esculentum* and *O. sativus*. Epimerization of the 3α-hydroxy group and the 3β-hydroxy group forms conjugates with glucose and fatty acids in tomato and *Ornithopus*, respectively. The hydroxylation at C-20 and the successive cleavage of the side chain between C-20 and C-22 are also observed in *Ornithopus* suspension cells. The hydroxylations at C-25 and C-26 and subsequent glucosylation are observed in tomato cultured cells.

CYP72C1, suggesting that this gene product decreases endogenous active BR levels through inactivation of BRs. Although CYP734A1 and CYP72C1 appear to possess unique biochemical mechanisms for BR inactivation, the mechanism of catalysis of CYP72C1 remained undefined. While these two P450s have been isolated based on a BR-inactivating phenotype from *Arabidopsis*, homologues of CYP734A1 and CYP72C1 have not been identified in other plant species.

Tomato is an extremely important crop and thus, several deficiencies in BR biosynthesis, including *dwarf* (Bishop et al., 1999) and *dumpy* (Koka et al., 2000), and in BR signaling, such as *curl3* and *abs1* (Koka et al., 2000; Montoya et al., 2002), have been identified. No genes involved in BR catabolism, however, have been isolated. In this study, we isolated two P450s, CYP734A7 and CYP734A8 from tomato. Each P450 in the P450 subfamily has a unique catalytic activity. For example, CYP71Cs functions in cyclic hydroxamic acid biosynthesis (Frey et al., 1997; Nomura et al., 2002), CYP71Ds functions in terpenoid metabolism (Haudenschild et al., 2000), and CYP725As functions in taxol biosynthesis (Jennewein et al., 2001; Schoendorf et al., 2001). Prediction of the physiological and catalytic functions of plant P450s by their primary amino acid sequences proves difficult, and this difficulty prevents the assumption that the CYP734A subfamily is involved in the oxidative inactivation of BRs in plants. Here, we report the characterization of tomato CYP734As and show that CYP734A7 is a C-26 BR hydroxylase. This work provides a molecular mechanism in support of the earlier work on BR metabolism in tomato suspension cultures (Hai et al., 1995; Winter et al., 1997). Moreover, these results implicate the CYP734A subfamily as BR catabolic P450s in higher plants.

2. Results

2.1. Isolation of *CYP734A7* and *CYP734A8* cDNAs from *L. esculentum*

Since *Arabidopsis* CYP734A1 (BAS1) inactivates BRs through hydroxylation at C-26, other CYP734A subfamily members are expected to be involved in BR catabolism. Previous feeding experiments with 24-*epi*-BL **4** suggested the presence of P450s catalyzing hydroxylation at either C-25 or C-26 in tomato suspension cells (Schneider et al., 1994; Hai et al., 1995; Winter et al., 1997). Scanning the tomato EST database (http://tigrblast.tigr.org/tgi/) resulted in the identification of several CYP734A clones. Two of them, cTOD5K13 and cTOA30H6, seemed to contain the entire coding region. These EST clones were obtained from the Clemson University Genomics Institute, and their cDNA inserts were completely sequenced. The cDNA insert in cTOD5K13 contained an intron insertion, which was located at the position corresponding to the second intron of the *BAS1* gene. The full-length cDNA of the cTOD5K13 clone was isolated by RT-PCR. The nucleotide sequence of the cTOA30H6 cDNA was significantly different from that of the sequence of cTOD5K13. The open reading frames from the cDNAs of cTOD5K13 and cTOA30H6 consisted of 1548 bp and 1668 bp and encoded 515 and 555 amino acid polypeptides, respectively. The deduced amino acid sequences showed more than 55% identity with *Arabidopsis* CYP734A1. As a result, the P450s encoded by cTOD5K13 and cTOA30H6 cDNAs were designated as CYP734A7 (Accession No. AB223041) and CYP734A8 (AB223042), respectively.[3] Tomato clearly has at least two CYP734As while *Arabidopsis* has only one, CYP734A1. Fig. 2 shows the alignment of the amino acid sequences of *Arabidopsis* CYP734A1 and tomato CYP734A7, and CYP734A8. CYP734A7 and CYP734A8 share 59% identity, and their amino acid sequences show 60% and 69% similarity, respectively, to *Arabidopsis* CYP734A1. These similarities suggest that CYP734A7 and CYP734A8 are involved in BR catabolism like CYP734A1 in *Arabidopsis*.

CYP734A8 has a 30 amino acid insert compared to CYP734A7, that is rich in His and Asn residues but lacks a stop codon. This insert may indicate a splicing mutation in the *CYP734A8* gene; however, the predicted protein sequence for rice CYP734A2 also contains an insertion of 18 amino acids at the same position (data not shown). The CYP734A8 transcript that was isolated by RT-PCR was identical to the cTOA30H6 clone sequence, indicating that the CYP734A8 mRNA is naturally transcribed in plants. The CYP734A8 protein with the insertion is likely expressed *in vivo*, although it is not evident that CYP734A8 is a functional protein. Therefore, we focused on the characterization of the catalytic function of CYP734A7.

2.2. Overexpression of *CYP734A7* cDNA in tobacco

We generated transgenic tobacco plants that constitutively overexpressed the CYP734A7 cDNA under the control of the CaMV35S promoter (*35S:CYP734A7*) (Fig. 3). The phenotype of 90-day-old transgenic plants exhibited a dramatic dwarfed phenotype with curled and shrunken leaves. At termination of flowering, the average height of the *35S:CYP374A7* lines was only about one-fifth that of the wild-type plants. The dwarf phenotype of the *35S:CYP374A7* plants is quite similar to that of tobacco plants that constitutively overexpress *Arabidopsis* CYP734A1 (BAS1) (Neff et al., 1999). Furthermore, this phenotype is characteristic of BR-deficient mutants, strongly suggesting that the bioactive BR levels in the *35S:CYP734A7* plants are decreased.

To confirm this speculation, the endogenous levels of BRs were analyzed by gas chromatography-selected ion monitoring (GC-SIM) (Table 1). The levels of castasterone

[3] The nucleotide sequences reported will appear in the GenBank Nucleotide Sequence Database under the Accession Nos. AB223041 for CYP734A7 and AB223042 for CYP734A8.

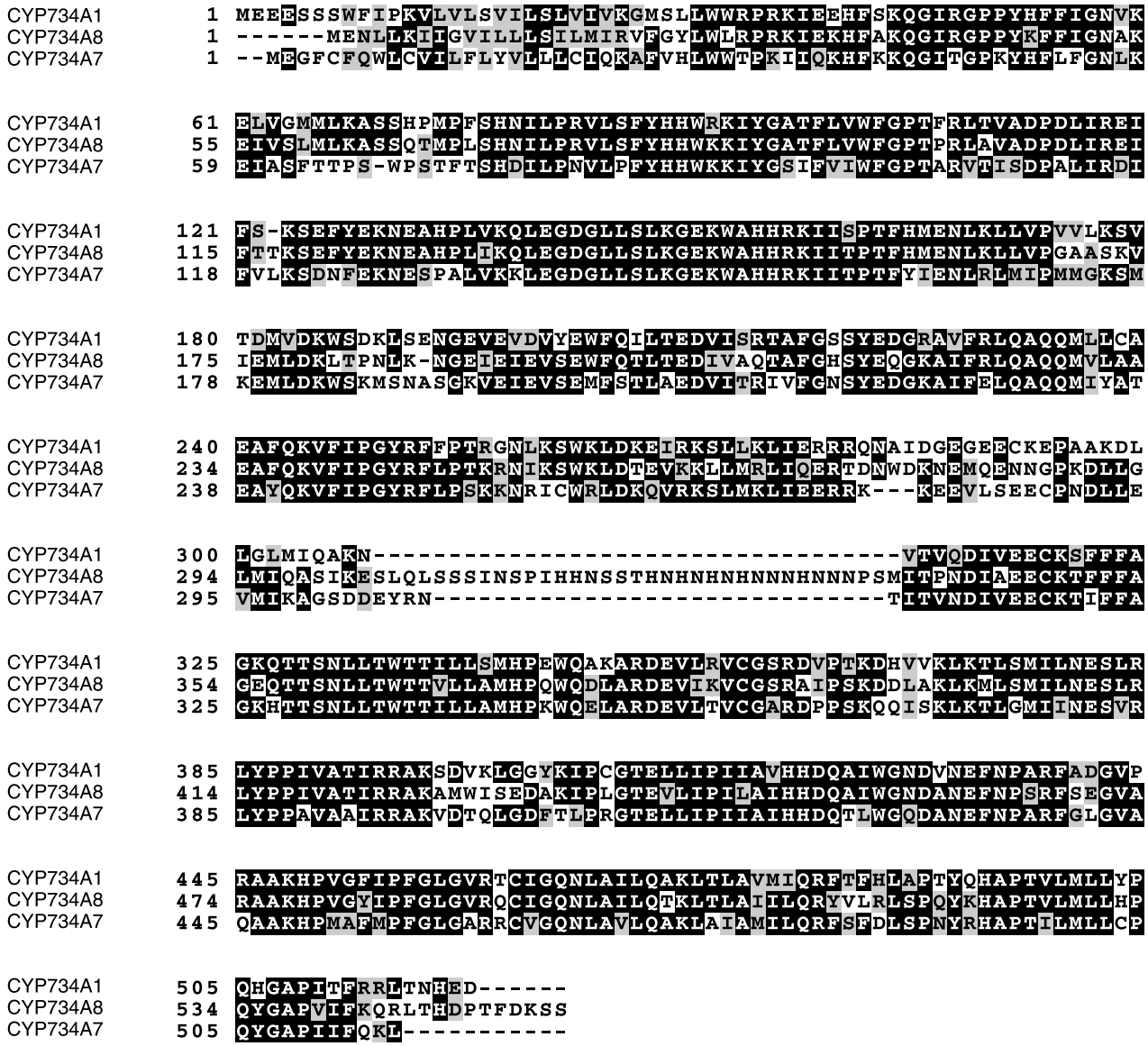

Fig. 2. Alignment of the deduced amino acid sequences of the CYP734A family from *Arabidopsis* and *L. esculentum*. The multiple sequence alignment was performed using the CLUSTALW analysis tool. Identical and similar amino acid residues are shaded in black and gray, respectively.

(CS) **1** and 6-deoxocastasterone (6-deoxoCS) (structure not shown) in the *35S:CYP734A7* plants were reduced by 34% and 72%, respectively, compared to wild-type plants. BL **3** was not detected in the shoots of either the *35S:CYP734A7* or the wild-type plants. On the other hand, a slight increase in amounts of 6-deoxotyphasterol (6-deoxoTY), 3-dehydro-6-deoxoteasterone (6-deoxo3DT), 6-deoxoteasterone (6-deoxoTE), and 6-deoxocathasterone (6-deoxoCT) (structures not shown) was observed in the *35S:CYP734A7* plants, probably due to upregulation of BR biosynthesis genes by the BR deficiency. These results suggest that endogenous active BRs are inactivated by CYP734A7.

2.3. Heterologous expression in insect cells

To characterize the biochemical properties of CYP734A7, recombinant CYP734A7 was expressed in insect cells using a baculovirus expression system. SDS–PAGE analysis showed that a 58 kDa band appeared in the microsomal fractions of the insect cells infected with the recombinant viruses of CYP734A7 (date not shown). The reduced-CO difference spectrum of the solubilized fractions of the CYP734A7 microsomes showed a clear absorption peak at 450 nm while the mock-infected cells did not (data not shown). Thus, the recombinant CYP734A7 protein was expressed in an active form in insect cells.

Next, the CYP734A7 fraction solubilized with 1% sodium cholate was used for substrate binding experiments with BRs (Fig. 4). The addition of CS **1** showed a typical type I difference spectra with a peak at 390 nm and a trough at 420 nm. The K_s value for CS **1** was determined to be 6.7 μM. CS **1** exhibited a higher affinity for CYP734A7 than BL **3** ($K_s = 12$ μM) or 6-deoxoCS ($K_s = 12$ μM). The other

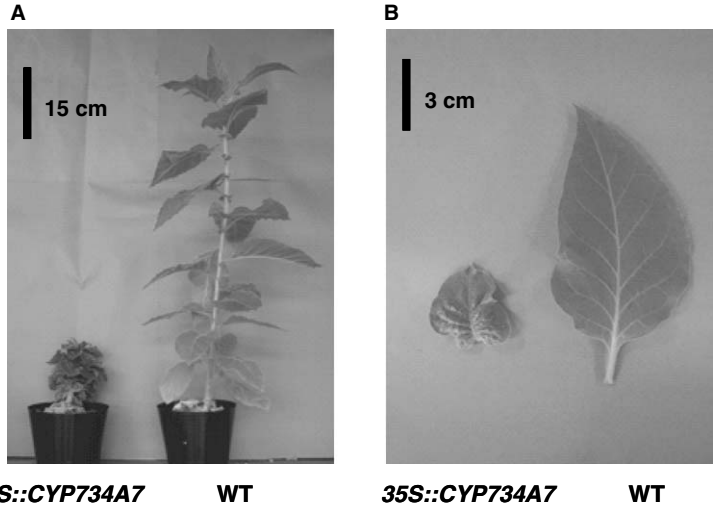

Fig. 3. Effect of *35S:CYP734A7* on the phenotype of tobacco plants. (A) 90-day-old *35S:CYP734A7* (left) and wild-type (right) plants grown in soil at 25 °C. Bar: 15 cm. (B) Leaves from *35S:CYP734A7* (left) and wild-type (right) plants. Bar: 3 cm.

Table 1
Endogenous levels of BR in wild-type and the *35S:CYP734A7* (ng kg^{-1} fresh weight)

BRs	*Arabidopsis* line	
	Wild-type	*35S:CYP734A7*
Brassinolide	ND[a]	ND
Castasterone	58	20
6-Deoxocastasterone	2554	1843
6-Deoxotyphasterol	197	244
3-Dehydro-6-deoxoteasterone	51	64
6-Deoxoteasterone	189	233
6-Deoxocathasterone	1250	1580
Typhasterol	ND	ND
3-Dehydroteasterone	ND	ND
Teasterone	9	7
Cathasterone	ND	ND

[a] Not detectable.

BR intermediate compounds induced no spectral shift (data not shown).

2.4. Functional assay of CYP734A7

To confirm that CYP734A7 is involved in BR catabolism, an *in vitro* biochemical assay was employed. Recombinant CYP734A7 activity was analyzed using the insect cell microsomal fractions in a reconstitution assay with *Arabidopsis* NADPH-cytochrome P450 reductase (Mizutani and Ohta, 1998) and NADPH. The reaction products were converted to methaneboronate-trimethylsilyl derivatives and analyzed by GC–MS. In the assay with CS **1**, a new product was detected at 18.61 min (Fig. 5A). The EI mass spectrum of the product derivative yielded prominent

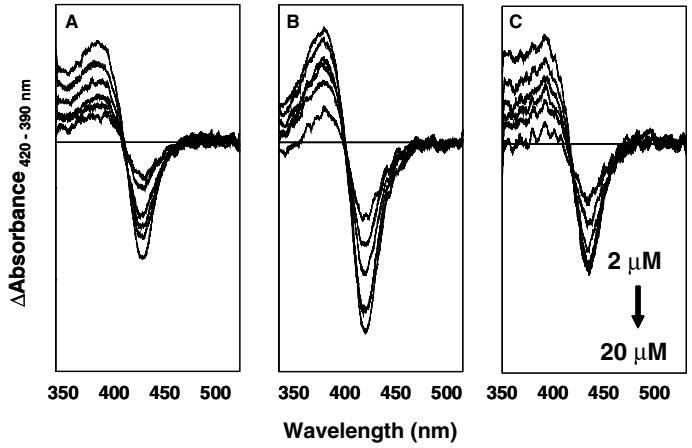

Fig. 4. Substrate binding spectra of the CYP734A7 protein. The recombinant CYP734A7 microsomes were solubilized with 1.0% (w/v) sodium cholate. Solubilized CYP734A7 was obtained by centrifugation at 100,000 × *g*. Type I difference spectra of CYP734A7 with 6-deoxocastasterone (A), castasterone (B), and brassinolide (C). Each BR was added to the solubilized CYP734A7 at a final concentration of 20 μM. CYP734A7 was titrated spectrophotometrically with BRs and then was added to the solubilized CYP734A7 at concentrations ranging from 2 to 20 μM, and difference spectra were recorded between 500 and 350 nm. The spectral dissociation constant (K_s) was calculated from a double reciprocal plot of the absorbance difference $\Delta A_{(390–420\,nm)}$ versus the substrate concentration.

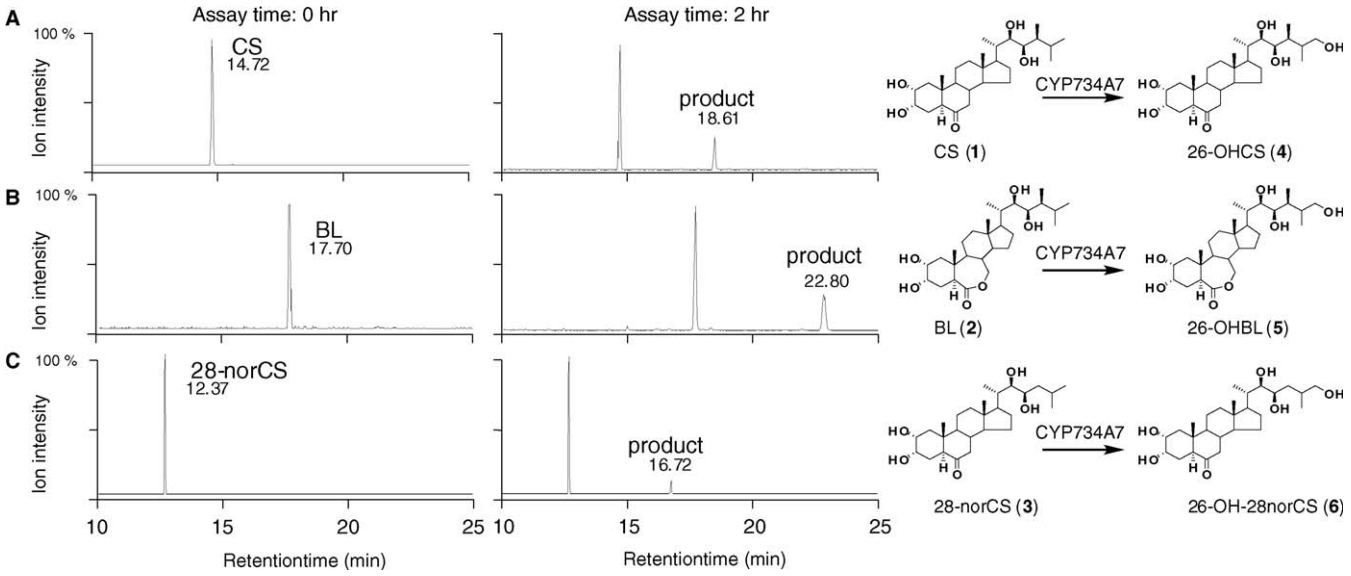

Fig. 5. GC–MS analysis of BR metabolites produced by recombinant CYP734A7 microsomes. Selected ion chromatograms for reaction products generated by CYP734A7 with BL (A), CS (B), and 28-norCS (C) are shown.

peaks at *m/z* 720 (M^+), 705 ($M - CH_3$), 603 ($M - 117$), and *m/z* 514 ($M - 206$) (Table 2). The C_{20}–C_{22} fission fragment ion of the CS derivative was *m/z* 155, whereas that of the product derivative was *m/z* 243. This result indicates that the difference (*m/z* 88) results from the presence of one trimethylsilyloxy group in the side chain of the product, suggesting that CYP734A7 catalyzes the hydroxylation of the side chain of CS.

To determine the specific position of the hydroxyl group introduced by CYP734A7, we chemically synthesized a candidate product 26-hydroxycastasterone (26-OHCS). This product was identical to the authentic 26-OHCS in GC retention time and in mass spectrum. In addition, BL **3** was metabolized to a new product that appeared at 22.80 min (Fig. 5B). Similarly, the side chain of BL **3** was hydroxylated by CYP734A7; however, the position of the hydroxyl group was not defined without the authentic standard. The mass spectrum (prominent peaks at *m/z* 736, 721, 619, 577, 564, 299, and 156) of the product derivative was identical to that of the derivative of the 26-hydroxybrassinolide **1** (26-OHBL) standard (Table 2) (Turk et al., 2003), strongly suggesting that the product was 26-OHBL **1**. In contrast, no product was detected when 6-deoxoCS was used, although 6-deoxoCS can bind to CYP734A7. Overall, these results indicate that CYP734A7 catalyzes hydroxylation at C-26 on CS **1** and BL **3** but not on 6-deoxoCS. Although the biological activity of 26-OHCS has not been determined, 26-OHBL **1** is an inactive form (Seto et al., 1999; Pharis et al., 2001; Turk et al., 2003), indicating that hydroxylation at the C-26 position is a key reaction for inactivation of BRs.

C_{27} BRs such as 28-norcastasterone (28-norCS) occur in tomato as well as in other lower and higher plant species (Nomura et al., 2001): however, the metabolic pathway of C_{27} BR has yet to be determined. Upon examination of the metabolism of 28-norCS by the CYP734A7 microsomes, a new product peak was detected (Fig. 5C). The mass spectra of the derivative of the 28-norCS metabolite showed prominent peaks at *m/z* 706, 691, 589, and 500, which were 14 mass units smaller than those of the 26-OHCS derivative (*m/z* 720, 705, 603, and 514). This result strongly suggests that the 28-norCS metabolite is 26-OH-28-norCS. Thus, CYP734A7 is a C-26 hydroxylase that inactivates both C_{28} and C_{27} BRs.

2.5. CYP734A7 and CYP734A8 transcripts in tomato

Expression profiles of the *CYP734A7* and *CYP734A8* genes were determined in various tissues from tomato plants (Fig. 6A). The transcripts of both of these genes

Table 2
GC–MS data of products in the reconstitution assay of CYP734A7

Substrate	Product identified[a]	Retention time (min)	Characteristic ions (*m/z*, relative intensity, %)
BL **3**	26-OHBL[b]	22.80	736 [M^+] (2), 721 (2), 619 (26), 577 (22), 564 (10), 299 (40), 156 (100)
CS **1**	26-OHCS	18.61	720 [M^+] (1), 705 (2), 603 (100), 514 (42)
	Authentic	18.60	720 [M^+] (1), 705 (4), 603 (100), 514 (44)
28-norCS	28-nor-26-OHCS[b]	16.72	706 [M^+] (1), 691 (2), 589 (100), 500 (14)
6-deoxoCS	ND[c]	–	–

[a] Analyzed by the methaneboronate-trimethylsilyl ether.
[b] Characterization of these products was based solely on fragmentation patterns indicated because of unavailability of standards.
[c] Not detected.

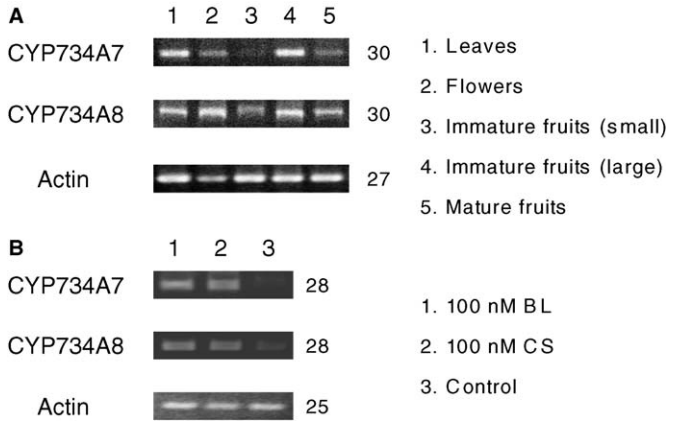

Fig. 6. RT-PCR analysis of tomato *CYP734A7* and *CYP734A8*. (A) Tissue specific expression of tomato *CYP734A7* and *CYP734A8*. Various organs, leaves, flowers, immature fruits (small: diameter = 1–2 cm, large: diameter = 5–6 cm), were harvested from tomato plants grown in soil for 90 days. *Actin* RT-PCR was included as a constitutive control. The number of PCR cycles after RT-PCR for *CYP734A7*, *CYP734A8* and *Actin* were 30, 30, and 27, respectively. (B) Expression patterns of *CYP734A7* and *CYP734A8* in response to BRs. Tomato plants (2 weeks old) that were cultured in GM liquid medium were treated with or without 100 nM of brassinolide (BL) or castasterone (CS). *Actin* RT-PCR was included as a constitutive control.

were ubiquitously detected in all organs but at different levels of expression. The transcript levels of *CYP734A7* in leaves and flowers were greater than those in the other tissues, while mature fruits showed low expression of *CYP734A7*.

Expression of *Arabidopsis CYP734A1* (*BAS1*) is upregulated by exogenous BL **3** application (Choe et al., 2001; Goda et al., 2002; Tanaka et al., 2005). Therefore, we investigated whether the gene expression of *CYP734A7* and *CYP734A8* was affected by exogenous application of BL **3** and CS **1**. Following application of BL **3** and CS **1**, the transcript levels of *CYP734A7* and *CYP734A8* were analyzed by semi-quantitative RT-PCR (Fig. 6B). The mRNA levels of *CYP734A7* and *CYP734A8* genes increased dramatically following application of 100 nM of either BL **3** or CS **1** compared with those of the untreated plants. Hence, the increased gene expression of *CYP734A7* and *CYP734A8* in response to exogenous BRs further implicates both CYP734A genes in BR catabolism in tomato.

3. Discussion

3.1. BR metabolism in tomato

Previous metabolic experiments with 24-*epi*-BL **4** showed that cultured tomato suspension cells converted 24-*epi*-BL **4** to 25-*O*-β-glucosyl-24-*epi*-BL and 26-*O*-β-glucosyl-24-*epi*-BL (Fig. 1). These results indicate that enzymes in tomato cells hydroxylate 24-*epi*-BL **4** at positions C-25 and C-26 and then glucosylate the introduced hydroxy groups (Hai et al., 1995). These studies also suggested the involvement of two different enzymes in each hydroxylation reaction based on the observation that several P450 inhibitors differently affected the C-25 and C-26 hydroxylations (Winter et al., 1997). Recently, Kim et al. (2004) reported that a cell-free enzyme solution prepared from tomato seedlings converted CS **1** to 26-norCS, demonstrating that C-26 demethylation also occurs in tomato. Thus, metabolic feeding experiments indicate the potential metabolic abilities of plants, but the observed pathways do not always reflect the naturally-occurring BR metabolism in tomato plants.

In this study, we isolated two P450 cDNAs, CYP734A7 and CYP734A8, from tomato. Biochemical assays with recombinant CYP734A7 expressed in insect cells showed that CS **1**, BL **3**, and 6-deoxoCS bind to CYP734A7. In addition, these studies demonstrated that the dissociation constant for CS **1** is lower than those for BL **3** and 6-deoxoCS, suggesting that CS **1** is a preferable substrate of CYP734A7. The functional assay revealed that CYP734A7 catalyzes C-26 hydroxylation of CS **1** and BL **3**. Although we could not quantitate the relative activities for CS **1** and BL **3** because of the unavailability of an authentic 26-OHBL **1**, CYP734A7 appeared to catalyze the C-26 hydroxylation of CS **1** better than BL **3** (data not shown). No metabolite of 6-deoxoCS was detected under our assay conditions; however, the value of the dissociation constant for 6-deoxoCS was 12 μM, which was approximately as same as that for BL **3**. These findings indicate that 6-deoxoCS may competitively inhibit the enzymatic activity of CYP734A7. The endogenous level of 6-deoxoCS is greater than that of CS **1** in some plants including *Arabidopsis*, tomato, and pea (Fujioka et al., 1996; Yokota et al., 1997; Nomura et al., 1997). 6-deoxoCS may be important for the regulation of the endogenous BR levels through inhibition of the inactivation process by CYP734A7. The amount of 6-deoxoCS in transgenic tobacco plants, which constitutively overexpress CY734A7, significantly decreased in comparison with that in the wild-type plants. The ratios of the endogenous content of 6-deoxoCS relative to CS **1** are 20:1 in tomato (Nomura et al., 2001) and 44:1 in tobacco (Table 1). Therefore, the endogenous 6-deoxoCS is much higher than that of CS **1**. Conversely, CS **1** and BL **3** were not converted to the C-25 hydroxylated metabolites, indicating that CYP734A7 is not a C-25 hydroxylase. We have not, however, examined the metabolism of 24-*epi*-BL **4** by the recombinant CYP734A7.

In addition, the tomato plant accumulates high levels of C_{27} BRs with no alkyl group at the C-24 position in the 5α-cholestane skeleton. The endogenous content of 28-norCS is comparable to that of CS **1** in 60-day-old tomato shoots (Nomura et al., 2001). Examination of the metabolism of 28-norCS by the CYP734A7 microsomes revealed that 28-norCS is converted to the hydroxylated product, presumably 26-OH-28-norCS. This finding indicates that CYP734A7 is also a C-26 hydroxylase of C_{27} and C_{28} BRs (Fig. 6A–C).

3.2. Inactivation of BRs by CYP734A7

Chemical synthesis and biological evaluation revealed the apparently weak bioactivity of 25-OHBL **5** and 26-OHBL **1** relative to BL **3**. Therefore, hydroxylations at C-25 and C-26 are the inactivation steps of BL **3** (Pharis et al., 2001). In this study, we showed that CYP734A7 catalyzed hydroxylation of CS **1**, BL **3**, and 28-norCS at the C-26 position. In addition, tobacco plants that overexpressed CYP734A7 showed a dwarf phenotype, which is typical for a BR deficiency, and contained reduced levels of CS **1** and 6-deoxoCS compared with wild-type plants (Fig. 3). These results indicate that CYP734A7 functions in inactivation of the endogenous BRs *in planta*. CS **1** is the major bioactive brassinosteroid during vegetative growth in tomato (Bishop et al., 1999; Nomura et al., 2001; Montoya et al., 2005), and therefore, the endogenous level of CS **1** must be controlled through the balance of biosynthesis and catabolism. The facts that the CYP734A7 transcript was abundantly detected in tomato leaves (Fig. 6A) and that CS **1** is a preferable substrate of CYP734A7 (Fig. 6B) strongly suggest that CS **1** is inactivated by CYP734A7 in tomato shoots. On the other hand, BL **3** accumulates in developing fruits (Montoya et al., 2005; Nomura et al., 2005). The CYP734A7 mRNA was also detected in immature fruits (Fig. 6A), implicating CYP734A7 in the inactivation of BL **3** in tomato fruits.

In *Arabidopsis*, the exogenous application of BL **3** downregulates the transcription of genes involved in the BR biosynthesis pathway including *DWF4*/*CYP90B1*, *CPD*/*CYP90A1*, *BR6ox*/*CYP85A*, *ROT3*/*CYP90C1*, and *CYP90D1* (Mathur et al., 1998; Choe et al., 2001; Goda et al., 2002; Tanaka et al., 2005) and upregulates the expression of the BR inactivation gene *BAS1*/*CYP734A1* (Choe et al., 2001; Goda et al., 2002; Tanaka et al., 2005). These results indicate that BR homeostasis is controlled by elaborate feedback and feedforward mechanisms of gene activation and inactivation. Pretreatment of tomato suspension cells with BL **3** and 24-*epi*-BL **4** induced increased conversion of 24-*epi*-BL **4** to 25-*O*-β-glucosyl-24-*epi*-BL and 26-*O*-β-glucosyl-24-*epi*-BL, suggesting a feedforward activation of C-25 and C-26 hydroxylase activities in tomato (Winter et al., 1997). In this study, we demonstrated that the expression of tomato *CYP734A7* is upregulated in response to the exogenous application of BL **3** and CS **1**. This result suggests that *CYP734A7* is indeed involved in the regulation of BR homeostasis through catabolism of bioactive BRs in tomato.

3.3. P450s involved in BR catabolism

All of the P450 genes responsible for BR biosynthesis are classified in the CYP85, CYP90, and CYP724 families, which have been placed in the CYP85 clade by phylogenetic analyses of plant P450s (Nelson et al., 2004). Thus, P450s involved in BR biosynthesis appear to be evolutionarily related. In contrast, the P450 genes (CYP734As and CYP72C1), which encode proteins involved in BR catabolism, are distant from the CYP85 clade, indicating that the P450s in BR biosynthesis and in BR catabolism evolved independently although they share a common substrate structure.

Phylogenetic analysis indicates that the CYP72, CYP721, and CYP734 families are relatively close to CYP734A7 (Fig. 7). The CYP734A subfamily consists of seven known P450 genes: *CYP734A1* in *Arabidopsis*, *CYP734A2/4/5/6* in rice, and *CYP734A7/8* in tomato. Moreover, a Blast search of the plant EST database (The Institute for Genomic Research, TIGR, http://www.tigr.org/tdb/tgi/plant.shtml) identified EST clones with homology to CYP734A in various plants such as *Aquilegia*, *Lotus japonicus*, *Zea mays* (maize), *Petunia hybrida*, *Solanum tuberosum* (potato), *Sorghum bicolor*, *Glycine max* (soybean), and *Saccharum officinarum* (sugarcane). These findings indicate that the CYP734A family is widely conserved throughout plant species. Tomato CYP734A7 and *Arabidopsis* CYP734A1 function in inactivation of BRs by C-26 hydroxylation, strongly suggesting that oxidative inactivation of BRs by the CYP734A subfamily is widespread in plants. On the other hand, homologues for CYP72C1 have not been found in other plant species such as rice and tomato, suggesting that inactivation by CYP72C1 may be unique to *Arabidopsis*. In contrast, the CYP72A subfamily is widely distributed from gymnosperms to monocots and dicots (Nelson et al., 2004). CYP72A1 from *Catharunthus roseus* catalyzes the conversion of loganin to secologanin in indole alkaloid biosynthesis (Irmler et al., 2000). Thus, the CYP72A subfamily is involved in secondary metabolism rather than BR catabolism. Interestingly, the CYP721 family is evolutionarily very close to the CYP734 family while CYP72C1 splits from the ancestor of the CYP721 and CYP734 families. Thus, CYP721 may be involved in steroid metabolism. There are several oxidative reactions in the BR metabolic pathways in tomato (Fig. 1), and oxidations such as C-25 hydroxylation and successive C-26 oxidation/demethylation also seem to be catalyzed by P450s. CYP734A8 may be responsible for catalyzing BR oxidation at either C-25 or C-26 position. Further biochemical and genetic characterization of the CYP734 and CYP721 families from various plants will increase our understanding of BR catabolism in plants.

4. Materials and methods

4.1. Chemicals

Brassinolide (BL) **3** and castasterone (CS) **1** were purchased from Fuji Chemical Industries Ltd. (Toyama, Japan). 26-Hydroxycastasterone (26-OHCS) was chemically synthesized (Watanabe, manuscript in preparation). Other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

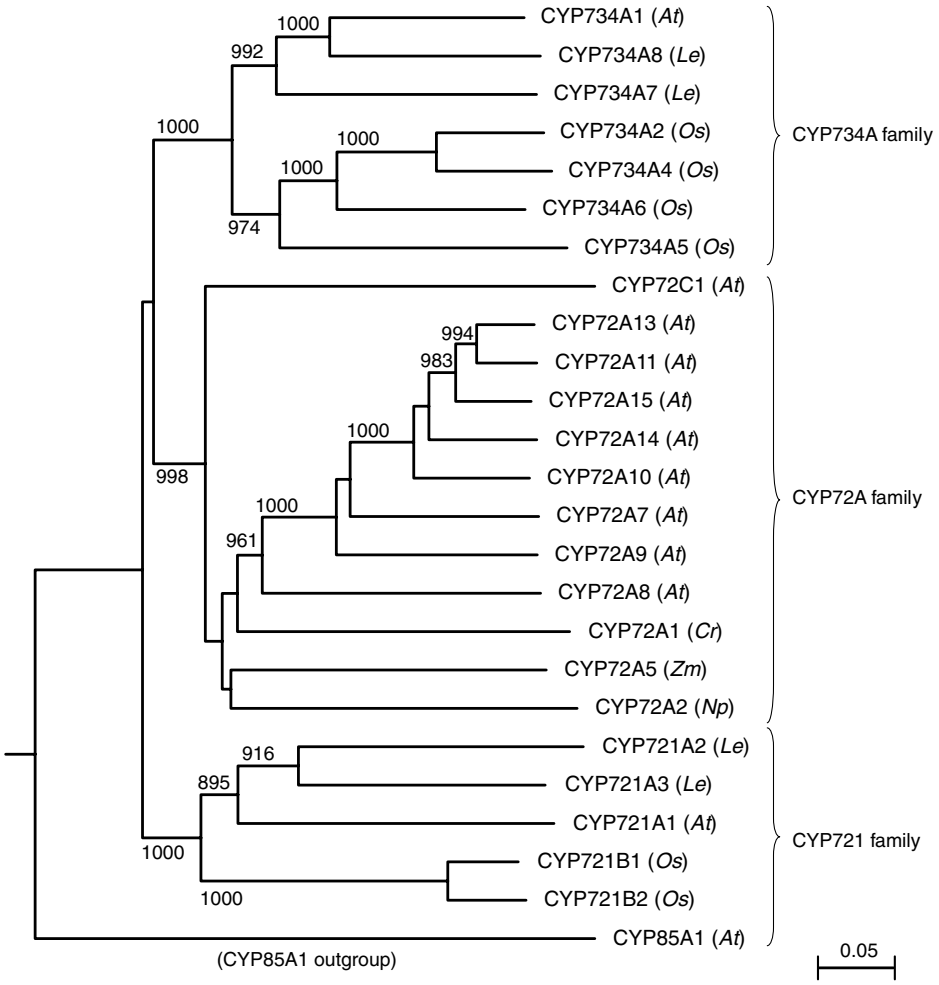

Fig. 7. Phylogenetic tree of the CYP734, 72, and 721 families. A multiple alignment of the CYP734, 72, and 721 family members from various plants was constructed with ClustalW 1.81. Abbreviations are as follows: *At*, *Arabidopsis*; *Cr*, *Catharunthus roseus*; *Le*, *Lycopersicon esculentum* (tomato); *Np*, *Nicotiana plumbaginifolia*; *Os*, *Oryza sativa* (rice); *Zm*, *Zea mays* (maize). *Arabidopsis* CYP85A1 was included in the alignment as an outgroup. The statistical significance of the NJ tree topology was evaluated by bootstrap analysis with 1000 iterative tree constructions. Scales indicate the evolutionary distances of the base substitution per site, estimated by the Kimura's two-parameter method.

4.2. Plant materials

Tobacco (*Nicotiana tabacum* cv. SR1) and tomato (*L. esculentum* cv. Momotarou) were used as the wild-type plants. For examination of the growth of seedlings of SR1 and *35S:CYP734A7* plants, and for harvest of various organs of tomato seedlings for expression studies, non-sterilized plants were grown in soil under natural photoperiods. To analyze endogenous BRs of SR1 and *35S:CYP734A7* plants, seeds were sterilized by treatment with EtOH–H_2O (7:3, v/v) for 10 s, followed by 1% (w/v) SDS-5% (v/v) bleach solution, and finally by rinsing three times with sterile H_2O. These seeds were grown for 1 month under sterile conditions on 0.8% (w/v) agar plates containing GM medium supplemented with 1× Murashige Skoog (MS) salt (Murashige and Skoog, 1962) and 1% (w/v) sucrose. To isolate total RNA from tomato plants, seedlings were grown for 2 weeks under the sterile conditions described above. For phytohormone treatment, the 2-week-old seedlings were transferred to GM liquid medium supplemented with 100 nM BL or CS and incubated in a rotary shaker at 110 rpm for 12 h.

4.3. Cloning of CYP734A7 and CYP734A8 from tomato

From an extensive BLAST searching of the tomato EST database of the Institute for Genomic Research (TIGR, http://www.tigr.org/tdb/at/at.html), two tomato EST clones, cTOD5K13 (Accession No. AW738072) and cTOA30H6 (Accession No. BI926826), were identified. Both of these clones contained an entire open reading frame homologous to the *Arabidopsis* CYP734A1. These tomato EST clones were obtained from the Clemson University Genomics Institute. The cDNA inserts were completely sequenced. The cDNA insert in cTOD5K13 contained an intron insertion, and therefore, the corresponding full-length cDNA was isolated from total RNA obtained from *L. esculentum* cv. Momotarou by RT-PCR.

Nucleotide sequences of gene-specific primers for cTOD5K13 were as follows: MM63, 5′-*ggatcc*ATGGAAGGATTTTGCTTTCAATGGTTA-3′ (a *Bam*HI site was added as indicated in italics and the start codon is underlined) and MM64, 5′-*ctcgag*CTACAACTTTTGGAATATAATAGGAGC-3′ (an *Xho*I site is indicated in italics and the stop codon is underlined). First-strand cDNA was synthesized in a 10-μl reaction mixture containing 1.0 μg of total RNA with an oligo dT primer and Rever Tra Ace reverse transcriptase (Toyobo, Osaka, Japan). The reverse-transcription reactions were carried out at 42 °C for 30 min, then 99 °C for 5 min, and finally chilled to 5 °C for 5 min. Five microliters of the reaction product was used as a template for PCR. The PCR was carried out in a volume of 50-μl containing 2.5 mM $MgCl_2$, 10 mM Tris–HCl, 50 mM KCl, 1 unit of KOD plus Taq DNA polymerase (Toyobo), 2 mM of each deoxynucleotide triphosphate, and 0.1 μM of each gene-specific primer as shown above. Addition of 3′A-overhangs was performed by incubation of the PCR with 1 unit of Taq DNA polymerase at 72 °C for 10 min. The PCR product was gel-purified, cloned into the TA cloning vector pCR2.1 (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA), and the insert was sequenced completely. Sequencing reactions were carried out using a BigDye terminator cycle sequencing kit (PE Applied Biosystems, WI, USA). A DNA sequencer Model 377 (PE Applied Biosystems) was used for DNA sequencing. Nucleotide sequences were analyzed using the DNASIS software system (Hitachi, Tokyo, Japan).

4.4. Generation of transgenic plants

To construct the vector for constitutive expression of CYP734A7 cDNA under control of the CaMV35S promoter, the β-glucuronidase coding region in the pBI121 vector (Clontech, Palo Alto, CA, USA) was replaced by the entire coding region of CYP734A7 cDNA. *Agrobacterium tumefaciens* strain LBA4404 was transformed with this construct and was used for plant transformation of tobacco (*N. tabacum* cv. SR1) by the leaf disk method. Several *35S:CYP734A7* plants showed an extreme dwarf phenotype, and seeds from these plants were harvested. T_1 seeds were germinated and selected on MS medium supplemented with 50 μg ml^{-1} kanamycin, 1% (w/v) sucrose, and 0.8% (w/v) agar. T_1 plants were grown to collect T_2 seeds. For analysis of *35S:CYP734A7* plant phenotype, *35S:CYP734A7* T_2 plant seeds were grown in a growth chamber at 25 °C for 90 days under continuous light.

4.5. Analysis of the endogenous levels of BRs

BRs were extracted and purified from twenty plants of SR1 seedlings ($n = 20$, 85.86 g) and *35S:CYP734A7* seedlings ($n = 80$, 72.39 g) as described by Nomura et al. (2001). To quantify endogenous BRs using GC–MS, the BRs were derivatized by conversion to methaneboronates, methaneboronate-trimethylsilyl ethers, or trimethylsilyl ethers (Nomura et al., 2001). Identification of BRs was verified when the relative intensities of the M^+ ion and the two daughter ions for each molecule were consistent with those of the corresponding deuterated internal standards. The endogenous levels of BRs were calculated from the peak area ratios of $^2H_6$ and $^2H_0M^+$ ions.

4.6. Heterologous expression of the CYP734A7 protein in insect cells

The entire coding region of the CYP734A7 cDNA in pCR2.1 plasmid vector was excised with the restriction enzymes *Bam*HI and *Xho*I and was purified by 1% (w/v) agarose gel electrophoresis. The cDNA was then ligated into the pFastBac1 vector (Life technologies, Grand Island, NY, USA). The pFastBac1-CYP734A7 construct was then used for the preparation of the corresponding recombinant bacmid DNA by transformation of *Escherichia coli* strain DH10Bac (Life technologies). Preparation of the recombinant baculovirus DNA containing the CYP734A7 cDNA and transfection of *Sf*9 (*Spodoptera frugiperda* 9) cells were carried out according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). For expression of the recombinant P450 proteins, *Sf*9 cells were propagated in Grace's Insect Cell Culture Medium at 27 °C (Life Technologies) supplemented with 10% (v/v) fetal bovine serum, 200 μM 5-aminolevulinic acid, and 200 μM ferrous citrate to compensate for the low heme synthetic capacity of the insect cells. Microsomal fractions of CYP734A7 were obtained using previously described methods for P450 (Fujita et al., 2006). The concentration of CYP734A7 protein was estimated from the CO-difference spectrum using the extinction coefficient ($\Sigma =$ 91 mM^{-1} cm^{-1}) (Omura and Sato, 1964).

4.7. Measurements of CYP734A7 activities

CYP734A7 activity was reconstituted by mixing CYP734A7 microsomes with NADPH-cytochrome P450 reductase. The reaction mixture consisted of 50 pmol recombinant CYP734A7 microsomes, 0.1 unit NADPH-cytochrome P450 reductase, 100 mM potassium phosphate (pH 7.25), 1 mM NADPH, and 40 μM BRs. Reactions were incubated at 30 °C for 30 min. The reaction products were extracted and then treated with 2 mg/ml methaneboronic acid in pyridine at 80 °C for 30 min. These reactions were then incubated with 10 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 80 °C for 30 min. The derivatized products were analyzed by GC–MS. GC–MS was performed using the same conditions as described by Fujita et al. (2006).

4.8. Expression analysis by RT-PCR

All tissues were frozen in liquid nitrogen and stored at −80 °C until use. Total RNA from tomato plants was isolated with a MagExtractor-RNA (Toyobo). RT-PCR was

performed using a ReverTra Dash RT-PCR kit (Toyobo) according to the manufacturer's instructions. The cDNA was synthesized in a 10-μl reaction mixture containing 1 μg of total RNA, an oligo dT primer, and ReverTra Ace reverse transcriptase (Toyobo). The RT reactions were performed at 42 °C for 30 min, followed by incubation at 99 °C for 5 min and then 4 °C for 5 min. The 10 μl PCR mixtures contained 1 μl RT product, 2.5 mM $MgCl_2$, 10 mM Tris–HCl, 50 mM KCl, 0.25 unit of KOD dash Taq DNA polymerase (Toyobo), 2 mM of each deoxynucleotide triphosphate, and 0.5 μM of each gene-specific primer as shown below. Nucleotide sequences of gene-specific primers were as follows: for CYP734A7, 734A7-F 5′-AGGAATGCTAGGCTAAGG-3′, 734A7-R, 5′-GATATTGATATGGTAGGGC-3′; for CYP734A8, 734A8-F 5′-GGCTGATCCTGATCTTATTAGAGA-3′, 734A8-R 5′-GATCGCTAGAATCGGAATCAACAC-3′; for actin, act2-F 5′-CGTCACACTGGTGTGATGGTT-3′, act2-R 5′-ACCAGGGAACATTGTGGTACC-3′.

The PCRs were denatured at 94 °C for 2 min and then amplified under the following conditions: 30 cycles of 94 °C for 30 s, 60 °C for 2 s, 74 °C for 45 s in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems). The PCR products were analyzed on a 1% (w/v) agarose gel containing 20 ng/ml of ethidium bromide.

4.9. Phylogenetic analysis

Amino acid sequences were aligned at the Bioinformatics Center, Institute for Chemical Research, Kyoto University (http://www.genome.jp/) using the ClustalW program. Phylogenetic trees were determined using the neighbor-joining method with bootstrap analysis (1000 replicates) and Kimura's correction for protein distances. The tree was visualized using TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The accession numbers in GenBank/EMBL/DDBJ database are as follows: the CYP72 family, CYP72A1 (Accession No. L19074), CYP72A2 (U35226), CYP72A5 (AY072300), CYP72A7 (AY072303), CYP72A8 (AY052208), CYP72A9 (NM_112324), CYP72A10 (NM_112325), CYP72A11 (NM_112326), CYP72A13 (BT002745), CYP72A14 (BT001008), CYP72A15 (AY096748), CYP72C1 (NM_101566); the CYP721 family, CYP721A1 (NM_106169), CYP721B1 (AC104708), CYP721B2 (AC104708); the CYP734A family, CYP734A1 (BT010564), CYP734A2 (AP005008), CYP734A4 (AP003612), CYP734A5 (AP003822), CYP734A6 (AP006237), CYP734A7 (AB223041), CYP734A8 (AB223042), CYP85A1 (NM_203133).

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