Cytochrome P450 *CYP710A* Encodes the Sterol C-22 Desaturase in *Arabidopsis* and Tomato [W][OA]

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Δ22-Unsaturated sterols, containing a double bond at the C-22 position in the side chain, occur specifically in fungi and plants. Here, we describe the identification and characterization of cytochrome P450s belonging to the CYP710A family as the plant C-22 desaturase. Recombinant proteins of CYP710A1 and CYP710A2 from *Arabidopsis thaliana* and CYP710A11 from tomato (*Lycopersicon esculentum*) were expressed using a baculovirus/insect system. The *Arabidopsis* CYP710A1 and tomato CYP710A11 proteins exhibited C-22 desaturase activity with β-sitosterol to produce stigmasterol (CYP710A1, K_m = 1.0 μM and kinetic constant [k_{cat}] = 0.53 min^{-1}; CYP710A11, K_m = 3.7 μM and k_{cat} = 10 min^{-1}). In *Arabidopsis* transgenic lines with *CYP710A1* and *CYP710A11* overexpression, stigmasterol levels increased by 6- to 32-fold. *Arabidopsis* CYP710A2 was able to produce brassicasterol and stigmasterol from 24-*epi*-campesterol and β-sitosterol, respectively. Sterol profiling analyses for *CYP710A2* overexpression and a T-DNA insertion event into *CYP710A2* clearly demonstrated in planta that *CYP710A2* was responsible for both brassicasterol and stigmasterol production. Semiquantitative PCR analyses and promoter:β-glucuronidase transgenic approaches indicated strict tissue/organ-specific regulation for each *CYP710A* gene, implicating differential tissue distributions of the Δ^{22}-unsaturated sterols in *Arabidopsis*. Our results support the possibility that the CYP710 family may encode P450s of sterol C-22 desaturases in different organisms.

INTRODUCTION

Sterols, isoprenoid-derived lipids produced via the mevalonate pathway, are involved in various cellular functions in eukaryotes, such as maintenance of membrane integrity and biosynthetic precursors of steroid hormones. One of the most remarkable differences in sterol composition among biological kingdoms is the specific occurrence of Δ^{22}-sterols (Figure 1) containing a double bond at C-22 in the sterol side chain in fungi (ergosterol) and plants (stigmasterol). The plant C-22 desaturase has yet to be clarified.

In plants, a vast majority of sterols are present in membranes as a mixture of several major molecular species, including β-sitosterol, stigmasterol, campesterol, and cholesterol (Schaller, 2003; Benveniste, 2004). These bulk sterols modulate membrane permeability and fluidity. Furthermore, recent studies (Mongrand et al., 2004; Borner et al., 2005) have suggested that phytosterols, as well as cholesterol in mammals and ergosterol in yeast, are

involved in the formation of membrane microdomains that serve as a platform for crucial physiological processes, such as signal transduction for cellular proliferation and differentiation, vesicular trafficking, and cytoskeleton organization. Sterols also serve as precursors for the biosynthesis of steroid hormones involved in many different cellular processes (Schaller, 2003). In plants, brassinosteroids (BRs) are the only class of steroidal phytohormones, functioning in a variety of postembryonic events, including cell division and expansion, responses to light and dark, morphogenesis, apical dominance, and gene expression (Clouse, 2002; Nemhauser and Chory, 2004).

Recent findings with the aid of molecular genetics approaches have suggested possible involvement of phytosterols in BR-independent processes of embryonic and postembryonic development (Clouse, 2000; Lindsey et al., 2003). Thus, *Arabidopsis thaliana* mutants in the sterol biosynthetic pathway (Figure 1) display severe impairment in embryogenesis and development at specific stages, which cannot be rescued by the application of exogenous BRs. *Arabidopsis* mutants, *sterolmethyltransferase1* (*smt1/orc*), are defective in cycloartenol C-24 methyltransferase (Diener et al., 2000; Willemsen et al., 2003), and *fackel* (*fk*) phenotype is ascribed to the mutation of the C-14 reductase (Jang et al., 2000; Schrick et al., 2000). The *hydra1* (*hyd1*) mutants carry the mutation in the C8-C7 isomerase gene (Souter et al., 2002). Other embryonic mutants of *cephalopod* (*cph*) and *hyd2* have been found to be allelic to *smt1* and *fk*, respectively (Schrick et al., 2002; Souter et al., 2002). The *smt1/orc/cph* mutants exhibit developmental abnormalities with altered sterol
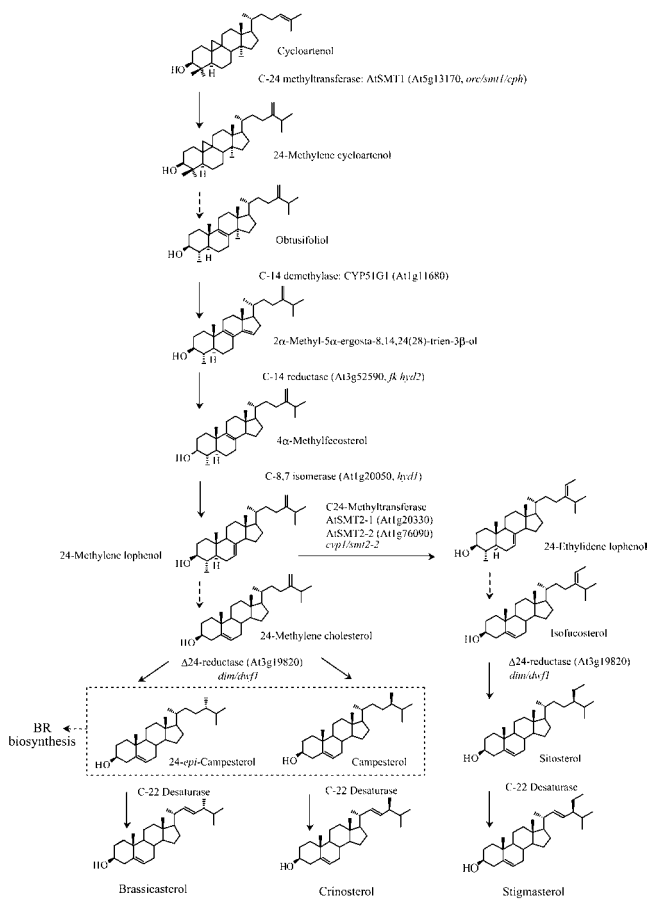
Figure 1. Sterol Biosynthesis in *Arabidopsis.*

The pathway from cycloartenol to the Δ^{22}-sterols is shown. 24-Methyl sterols are a mixture of (24*R*)- and (24*S*)-epimers of campesterol (Schaller, 2003). The Δ^{22}-desaturases catalyze the C22-desaturation reaction to yield stigmasterol, crinosterol, and brassicasterol from their immediate precursors. Dashed arrows indicate several enzymatic steps not shown here.

compositions (Lindsey et al., 2003). It has also been reported that the *smt1/orc/cph* mutation influenced pattern formation mostly by interfering with the polar localization of PIN proteins (Willemsen et al., 2003), while PIN protein trafficking was not affected in either the *hyd1* or *hyd2/fk* mutant (Souter et al., 2002), implicating differential developmental roles of membrane sterols. It has been reported that unusual sterols accumulated in *fk* mutant plants, while it is not clear whether or not such sterols have specific roles in BR-independent developmental processes (He et al., 2003; Schrick et al., 2004). On the other hand, *smt2* and *cotyledon vascular pattern1* (*cvp1*) are due to mutations in the methyltransferase gene *SMT2* encoding the C-24 methyltransferase. They produce 24-ethylidene lophenol from 24-methyl lophenol (Schaeffer et al., 2001; Carland et al., 2002). *cvp1* plants displayed postembryonic vascular patterning defects (Carland et al., 2002), and *SMT2* cosuppression lines contained higher levels of campesterol and lower levels of sitosterol, exhibiting developmental abnormalities such as reduced apical dominance and reduced fertility that cannot be restored by exogenous

BRs (Schaeffer et al., 2001). Furthermore, it has recently been reported that a T-DNA insertion mutagenesis within the obtusifoliol 14α-demethylase gene *CYP51G1* caused defects in membrane integrity and hypocotyl elongation, leading to post-embryonic seedling lethality (Kim et al., 2005). These results indicate that altered sterol profiles due to the mutations both upstream and downstream of the *SMT2* step affect membrane properties, thereby influencing signaling cascades involved in normal plant growth, such as cell polarity, auxin efflux, and ethylene signaling. However, no molecular basis for the phytosterol-dependent developmental processes has been established. Thus, complete elucidation of the sterol biosynthetic pathway is a prerequisite to understanding the mechanisms for maintaining correct membrane sterol compositions required for normal plant growth.

Specifically, biosynthesis and physiology of the Δ^{22}-sterols are not clearly understood. In higher plants, membrane sterols are present as a complex mixture of 24-ethyl sterols and 24-methyl sterols with cholesterol as a minor component (Schaller, 2003; Benveniste, 2004). The ratio of 24-ethyl sterols (up to 60% of total sterol) and 24-methyl sterols (<40% of total sterol) is determined by the two sterol methyltransferase activities (Schaller, 2003; Benveniste, 2004). The configuration at C-24 is 100% *R* in β-sitosterol, while 24-methyl sterols are a mixture of campesterol (campest-5-en-3β-ol) and its C-24 methyl epimer, 24-*epi*-campesterol (ergost-5-en-3β-ol). Plants in the family *Brassicaceae*, including *Arabidopsis*, contain a 24-ethyl-Δ^{22}-sterol, stigmasterol, and 24-methyl-Δ^{22}-sterols, a mixture of brassicasterol [(22*E*)-ergost-5,22-dien-3β-ol] and crinosterol [(22*E*)-campest-5,22-dien-3β-ol] (Matsumoto et al., 1983; Benveniste, 2004). Brassicasterol (24*R*-epimer) and crinosterol (24*S*-epimer) are thought to be derived from 24-*epi*-campesterol and campesterol, respectively (Figure 1). However, no sequence has thus far been reported for any higher plant enzyme catalyzing the C-22 desaturase reaction (Benveniste, 2004).

A fungal cytochrome P450 monooxygenase, CYP61, is known as the sterol C-22 desaturase to produce ergosta-5,7,22,24(28)-tetraenol from the immediate precursor, ergosta-5,7,24(28)-trienol, at the penultimate step in the ergosterol biosynthetic pathway (Skaggs et al., 1996; Kelly et al., 1997). A sequence comparison (Figure 2) revealed striking sequence conservation as [FLFA(A/S)QDAS(T/S)S] between plant CYP710A proteins and fungal CYP61 proteins, while the overall sequence similarity is only ~30%. The conserved sequence is located at one of the putative substrate recognition sites of P450s (Gotoh, 1992) on the I helix (SRS4) that is positioned on the distal side of the heme group. Within this I helix, two Ala residues, corresponding to Ala-295 and Ala-299 in *Arabidopsis* CYP710A1 (Figure 2, At 710A1), are absolutely conserved between the CYP710 and CYP61 family proteins. By contrast, most P450s have a conserved Thr residue at the second of these two positions that is known to participate in the oxygen activation and proton delivery for hydroxylation reactions catalyzed by P450s (Meunier et al., 2004). These observations suggested the possibility of CYP710A family proteins as the C-22 desaturases in plants (Benveniste, 2004). On the basis of this sequence information, we initiated an investigation to clarify whether plant CYP710A proteins were the functional homologs of fungal CYP61 proteins.
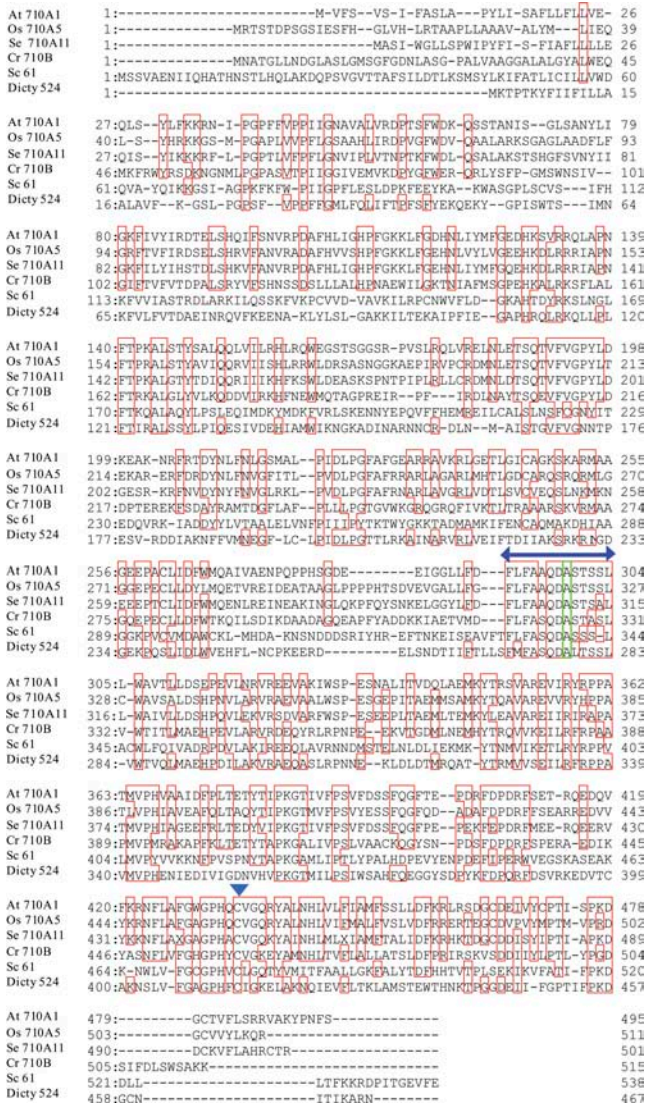
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At 710A1    1:------------------------M-VFS--VS-I-FASLA---PYLI-SAFLLFLLVE- 26
Os 710A5    1:-------------MRTSTDPSGSIESFH--GLVH-LRTAAPLLAAAV-ALYM---LIEQ 39
Se 710A11   1:-------------MASI-WGLLSPWIPYFI-S-FIAFLLLLE 41
Cr 710B     1:------------MNATGLLNDGLASLGMSGFGDNLASG-PALVAAGGALALGYALWEQ 45
Sc 61       1:MSSVAENIIQHATHNSTLHQLAKDQPSVGVTTAFSILDTLKSMSYLKIFATLICILLVWD 60
Dicty 524   1:-------------------------------MKTPTKYFIIFILLA 15

At 710A1    27:QLS--YLFKKRN-I-PGPFFVPPIIGNAVALVRDPTSFWDK-QSSTANIS--GLSANYLI 79
Os 710A5    40:L-S--YHRKKGS-M-PGAPLMVPFLGSAAHLIRDPVGFWDV-QAALARKSGAGLAADFLF 93
Se 710A11   27:QIS--YIKKKRF-L-PGPTLMFFFLGNVIPLVTNPTKFWDL-QSALAKSTSHGFSVNYII 81
Cr 710B     46:MKFRWYRSDKNGNMLPGPASVTPIIGGIVEMVKDEYGFWER-QRLYSFP-GMSWNSIV-- 101
Sc 61       61:QVA-YQIKNGSI-AGPKFKFW-PIIGPFLESLDPKFEEYKA--KWASGPLSCVS----IFH 112
Dicty 524   16:ALAVF--K-GSL-PGFSF--VPPFFGMLFQLIFTFFSFYEKQEKY--GPISWTS---IMN 64

At 710A1    80:GKFIVYIRDTELSHQIFSNVRPDAFHLIGHPFGKKLEGDHNLIYMFGEDHKSVRRQLAFN 139
Os 710A5    94:GRFTVFIRDSELSHRVFANVRPDAFHLIGHPFGKKLFGEHNLVYLVGEEHKDLRRRIAFN 153
Se 710A11   82:GKFILYIHSTDLSHKVFANVRPDAFHLIGHPFGKKLFGEHNLIYMFGGEHKDLRRRIAFN 141
Cr 710B     102:GIFTRVFVTDPALSRYVFSHNSSDSLLLALHPNAEWIIGKTNIAFMSGPEHKALRKSFIAL 161
Sc 61       113:KFVVIASTRDLARKILQSSKFVKPCVVD-VAVKILRPCNWVFLD--GKAHTDYRKSLNGL 169
Dicty 524   65:KFVLFVTDAEINRQVFKEENA-KLYLSL-GAKKILTEKAIPFIE--GAPHRQLKQLIFL 120

At 710A1    140:FTPKALSTYSALQQLVILRHLRQWEGSTSGGSR-PVSLRQLVREINLETSQTVFVGPYLD 198
Os 710A5    154:FTPRALSTYAVIQQRVIIISHLRRWLDRSASNGGKAEPIRVPCRDMNLETSQTVFVGPYLT 213
Se 710A11   142:FTPKALGTYTDIQQRIIIKHFKSWLDEASKSPNTPIPLELLCRDMNLDTSQTVFVGPYLD 201
Cr 710B     162:FTRKALGLYVLKQDDVIRKHFNEMMQTAGPREIR--PF---IRDLNAYTSQEVFVGPYLD 218
Sc 61       170:FTKQALAQYLPSLEQIMDKYMDKEVRLSKENNYEPQVFFHEMREILCALSLNSFGNYIIT 229
Dicty 524   121:FTIRALSSYLPIQESIVDEHIAMVIKNGKADINARNNCFR-DLN--M-AISTGVFVGNNTP 176

At 710A1    199:KEAK-NRFRTDYNLFNLGSMAL--PIDLPGFAFGEARFAVKRLGETLGICAGKSKARMAA 255
Os 710A5    214:EKAR-ERFDRDYNLFNVGFITL--PVDLPGFAFRNRALGAARLMHTLGDCARGRQRMLG 270
Se 710A11   202:GESR-KRFNVDYNYFNVGLRKL--PVDLPGFAFRNARLAVGRLVDTLSVCVEQSLNKMKN 258
Cr 710B     217:DPTEREKFSDAYRAMTDGFLAF--PLLLPGTGVWKGRQGRQFIVKTLITRAAARSKVRMAA 274
Sc 61       230:EDQVRK-IADDYLVTAALELVNEFIIPYTKTWYGKFTADMAMKIFENCAQMAKDHIAA 288
Dicty 524   177:ESV-RDDIAKNFFVMNEGF-LC-LPIDLPGTTLRKAINARVRLVEIFTDDIIAKSRKRNGD 233

At 710A1    256:GEEPACILDFWMQAIVAENPQPPHSGDE---------EIGGLLFD---FLFAAQDASTSSL 304
Os 710A5    271:GEEPECILDYLMQETVREIDEATAAGLPPPPHTSDVEVGALLFG---FLFAAQDPSTSSL 327
Se 710A11   259:EEEPTCILDFWMQENLREINEAKINGLQKPFQYSNKELGGYLFD---FLFAAQDTSTSAL 315
Cr 710B     275:GQEPECILDFWTKQILSDIKDAADAGQEAPFYADDKKIAETVMD---FLFAAQDATTASL 331
Sc 61       289:GGKFVCVMDAWCKL-MHDA-KNSNDDDSRIYHR-EFTNKEISEAVFTFLFASQDSSTSSL 344
Dicty 524   234:GEKEQSLIDLMVEHFL-NCPKEERD---------ELSNDTIIETLLSEMFASQDUTSSL 283

At 710A1    305:L-WAVTLLDSEPEVLNRVREEVAKIWSP-ESNALITVDQLAEMKYTRSVAREVIRYRPPA 362
Os 710A5    328:C-WAVSALLDSHPNVLARVRAEVWLASP-ESGEPITAEMVSAMKYTQVAREVVRVRHPA 385
Se 710A11   316:L-WAIVLLDSHPQVLEKVRSDVARFWSP-ESEEPITAEMLTEMKYLEAVAREIIRIRAPA 373
Cr 710B     332:V-WTITLMAEHPEVLARVRDEQYRLRPNPEL--EKVIGDMLNEMHYTRQVVKEILRIRPPA 388
Sc 61       345:ACWLFQIVAADRPDVLAKIREEQLAVRRNNDMSTELNLDLIEKMK-YTNMVIKETTLRYRPPV 403
Dicty 524   284:-VWTVQLLMAEHDILAKVRAEQASLRPNNE--KLDLDTMRQAT-YIRMVVSEILSFRPPA 339

At 710A1    363:TMVPHVAAIDFHLTETYTIPKGTIVFPSVFDSSFQGFTE--PDRFDPDRFSET-RQEDQV 419
Os 710A5    386:TLVPHIAVEAFQLTAQYTIIPKGTMVFPSVYESSFQGFQD--ADAFDPDRFFSEARREDVV 443
Se 710A11   374:TMVPHIAGEEFRLTEDYIVIPKGTIVFPSVFDSSFQGFPE--PEKFEPDRFMEE-RQEERV 430
Cr 710B     389:PMVPMRAKAPFKLTETYTAPKGALIVPSLVAACKQGYSN--PDSFDPDRFSPERA-EDIK 445
Sc 61       404:IMVRYVVKKNEPVSPNYTAPKGAMLIIPTLYPALHDPVVFPHPQVFIPERWVEGSKASEAK 463
Dicty 524   340:VMVPHENIEDIVIGDNVHVPKGTMILFSIWSAHFQEGGYSDEHYKFDPQRFDSVRKEDVTC 399

At 710A1    420:FKRNFLAFGWGVCVGQRYALNHIVLFIAMFSSLLDFKRLRSDGCDEIVMYCPTI-SPKD 478
Os 710A5    444:YKRNFIAFGAGPHACVGQRYAINHIMLAVFVSLVDFRRERTEGCDVPVVMPTM-VRPD 502
Se 710A11   431:YKKNFLAXGAGPHACVGQKYAINHIMLXIAMFTALIDFKPHKTDGCDDIIVIIPTI-APKD 489
Cr 710B     446:YASNFLMFGHGPHYCVGKEYAMNHLTVFLALLATSLDFPRIRSKVSDDILMLPTL-YPGD 504
Sc 61       464:K-NWLV-FGGGPHVCLGQTYVMITFAALLGKEFALYTDEHHTVTPLSEKIKVFATAI-FPKD 523
Dicty 524   400:AKNSLV-FGAGPHFCIGKELAKNNQIEVEITKLAMSTEWTHNKTPNGDEJII-FGPTIFPKD 457

At 710A1    479:---------GCTVFLSRRVAKYPNFS------------- 495
Os 710A5    503:---------GCVVYLKQR-------------------- 511
Se 710A11   490:---------DCKVFLAHRCTR-------------- 501
Cr 710B     505:SIFDLSWSAKK------------------------ 515
Sc 61       521:DLL--------------------LTFKKRDPITGEVFE 538
Dicty 524   458:GCN--------------------------ITIKARN--------- 467
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Figure 2. Alignment of Amino Acid Sequences of CYP710 Proteins.

The deduced amino acid sequences of CYP710A1 (At 710A1) and CYP710A11 from tomato (Se 710A11) proteins were aligned with CYP710A5 (Os 710A5) from *O. sativa*, CYP710B (Cr 710B) from *C. reinhardtii*, CYP61 (Sc 61) from *S. cerevisiae*, and CYP524 (Dicty 524) from *D. discoideum*. The double-headed arrow above the sequences indicates the conserved amino acids in the putative substrate recognition site (SRS4) of P450s (Gotoh, 1992). The conserved Ala-299 residue (numbered as in CYP710A1) is boxed in green. The arrowhead points to the heme ligand Cys residues. The conserved residues are boxed in red.

RESULTS

CYP710A Sequences

Arabidopsis contains four genes encoding putative P450 proteins belonging to the CYP710A subfamily (The Arabidopsis Information Resource, http://www.arabidopsis.org/): *CYP710A1*, *CYP710A2*, *CYP710A3*, and *CYP710A4*. No introns were found

in these *CYP710A* genes in *Arabidopsis*, and we amplified by PCR the putative coding sequences of *CYP710A1* and *CYP710A2* genes encoding polypeptides of 495 and 499 amino acids, respectively, with the calculated molecular masses of 55,723 and 56,344 D, respectively. The predicted amino acid sequence of CYP710A1 is 81.9, 77.7, and 76.1% identical to those of CYP710A2, CYP710A3, and CYP710A4, respectively. The CYP710A3 protein sequence is 73.9 and 93.9% identical to those of CYP710A2 and CYP710A4 proteins, respectively. Using the amino acid sequence of CYP710A1 as the bait for a tBLASTn search at The Institute for Genomic Research (TIGR) Gene Indices (http://tigrblast.tigr.org/tgi/), we identified a tomato (*Lycopersicon esculentum*) EST clone containing a putative entire coding region for tomato CYP710A11. The deduced primary structure consists of 501 amino acids with a calculated molecular mass of 57,511 D, and the amino acid sequence is 59.0% identical to that of CYP710A1. Both the entire coding sequences of *CYP710A1* and *CYP710A2* and the full-length EST clone of *CYP710A11* were analyzed for further experiments.

Insect Cell Expression of *CYP710A1*, *CYP710A2*, and *CYP710A11*

To clarify the C-22 desaturation activity, we characterized the enzymatic properties of CYP710A1, CYP710A2, and CYP710A11 proteins. The entire coding sequences of the *Arabidopsis CYP710A* genes (*CYP710A1* and *CYP710A2*) and the tomato *CYP710A11* cDNA were expressed in insect cells using a baculovirus expression vector system. SDS-PAGE analysis (see Supplemental Figure 1 online) demonstrated that protein bands of ~55 kD appeared in the microsomal fractions in the insect cells infected with the recombinant viruses carrying the expression cassettes of *CYP710A1*, *CYP710A2*, and *CYP710A11*, respectively. The apparent molecular masses of these expressed proteins were in good agreement with those calculated from the deduced primary structures of CYP710A1, CYP710A2, and CYP710A11 proteins. These recombinant proteins were recovered in the microsomal membrane fractions (100,000*g* pellet) and used for spectrophotometric determination of P450 (Figure 3). The microsomal fractions from insect cells expressing the recombinant CYP710A proteins showed the expected reduced CO difference spectrum with the absorption maximum at 449 nm. The specific contents of the recombinant CYP710A1, CYP710A2, and CYP710A11 P450 proteins were 100, 71, and 230 pmoles P450/mg microsomal protein, respectively. For the expression of CYP710A1 and CYP710A2 proteins in insect cells, we used the PCR-amplified genomic DNA fragments containing the putative coding sequences of *CYP710A1* and *CYP710A2* genes. The successful accumulation of P450 proteins with the CO difference spectra (Figure 3) indicated that these *Arabidopsis CYP710A* genes contain no intron as predicted. No significant P450 accumulation could be detected in the mock-infected insect cells under the same experimental conditions (data not shown).

Enzyme Assay

The microsomal fractions from the insect cells expressing the recombinant CYP710A proteins were used for P450 functional
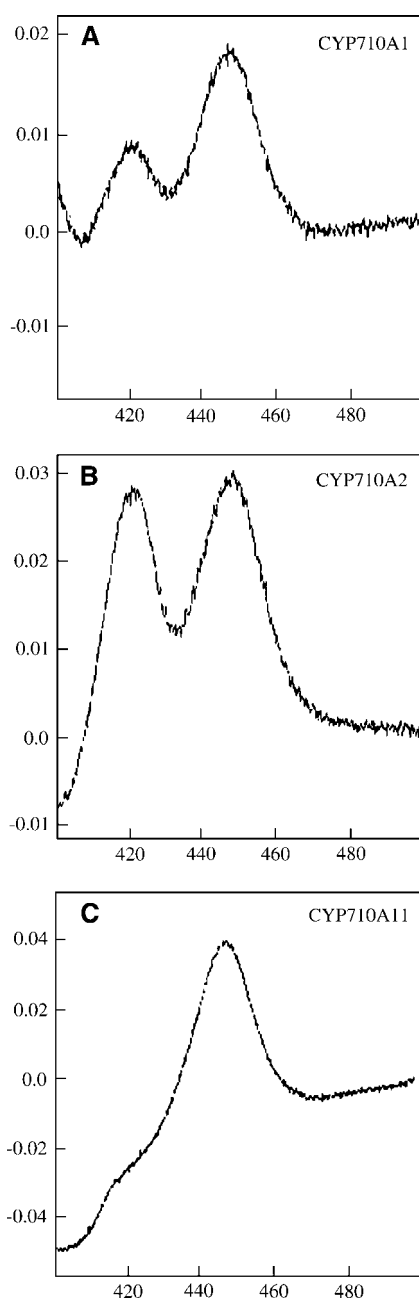
Figure 3. Heterologous Expression of Recombinant CYP710A Proteins in Insect Cells.

Reduced CO difference spectra of recombinant CYP710A1, CYP710A2, and CYP710A11. The recombinant P450 samples of 2 mg microsomal protein/mL of CYP710A1 **(A)** and CYP710A11 **(C)** and 4.6 mg microsomal protein/mL of CYP710A2 **(B)** were used for the spectrophotometric analyses.

characterization using the potential substrates (β-sitosterol, campesterol, and 24-*epi*-campesterol) for the C-22 desaturation reactions (Figure 1). The gas chromatography analyses of the reaction products from the *Arabidopsis* CYP710A1 and tomato CYP710A11 microsomes with β-sitosterol as the substrate

showed specific peaks at the same retention time (18.5 min, indicated by the arrows in Figure 4A) as that of stigmasterol. A very weak level of stigmasterol production was also detected from the *Arabidopsis* CYP710A2 assay (Figure 4B). The gas chromatography–mass spectrometry (GC-MS) fragmentation patterns obtained from the peaks in the selective ion mode (SIM) (Figure 4B) demonstrated that the putative reaction products were actually stigmasterol, indicating that the CYP710A1, CYP710A2, and CYP710A11 proteins catalyzed the C-22 desaturase reaction to produce stigmasterol from β-sitosterol in vitro.

It has been known that *Brassicaceae* family plants contain 24-methyl-Δ^{22}-sterols as a mixture of brassicasterol (24*R*-epimer) and crinosterol (24*S*-epimer), which are derived from 24-*epi*-campesterol and campesterol, respectively (Matsumoto et al., 1983; Benveniste, 2004). When campesterol was used as the substrate for the CYP710A1 and CYP710A2 assays, no crinosterol production was detected (Figure 4B). Then, we synthesized 24-*epi*-campesterol to clarify whether the stereoconfigurations of the 24-methyl group might be distinguished by the *Arabidopsis* CYP710A proteins. When the CYP710A2 microsomes were assayed with the synthesized 24-*epi*-campesterol (containing 30% campesterol as impurity), a new product peak appeared at the same retention time (Figure 4A, 16.4 min, indicated by the arrowhead) as that of brassicasterol (Figures 4A and 4B). The fragmentation pattern perfectly matched that of brassicasterol. Interestingly, 24-*epi*-campesterol could not be the substrate of CYP710A1, and no corresponding Δ^{22}-sterol was produced (Figure 4B). These results indicated that *Arabidopsis* CYP710A2 was the desaturase responsible for the production of brassicasterol from 24-*epi*-campesterol (Figure 1). A weak activity of crinosterol production was detected from the CYP710A11 assay with campesterol as the substrate (see Supplemental Figure 2 online), suggesting that tomato plants might produce crinosterol. No reaction products were detected when 24-*epi*-campesterol was added for the CYP710A11 assay (see Supplemental Figure 2 online).

These recombinant CYP710A reactions were completely dependent on the addition of NADPH, and NADH was not a substitute for NADPH (data not shown). The microsomal assays for the desaturase reactions proceeded even without adding recombinant NADPH-P450 reductase (Mizutani and Ohta, 1998), indicating that the endogenous NADPH-P450 reductase in insect cells could support the desaturase reactions of these recombinant CYP710A proteins to some extent. These results were consistent with our previous results from microsomal assays of *Arabidopsis* CYP707A proteins that are abscisic acid 8′-hydroxylases (Saito et al., 2004). When the CYP710A1, CYP710A2, and CYP710A11 proteins were assayed with cholesterol and fucosterol, no reaction products were detected under our experimental conditions (data not shown), and no oxidation products (Figure 4A) were detected from the insect cells expressing CYP73A5, which is a cinnamate 4-hydroxylase (Mizutani et al., 1997). In addition, *CYP710A1* failed to functionally complement the sterol C-22 desaturation activity with ergosta-5,7,24(28)-ergostatrienol as the substrate in a yeast strain of *CYP61* (*ERG5*) gene disruption (T. Morikawa, unpublished results). These results indicated that the substrate specificities of *Arabidopsis* CYP710A1 and tomato CYP710A11 proteins were rather strict
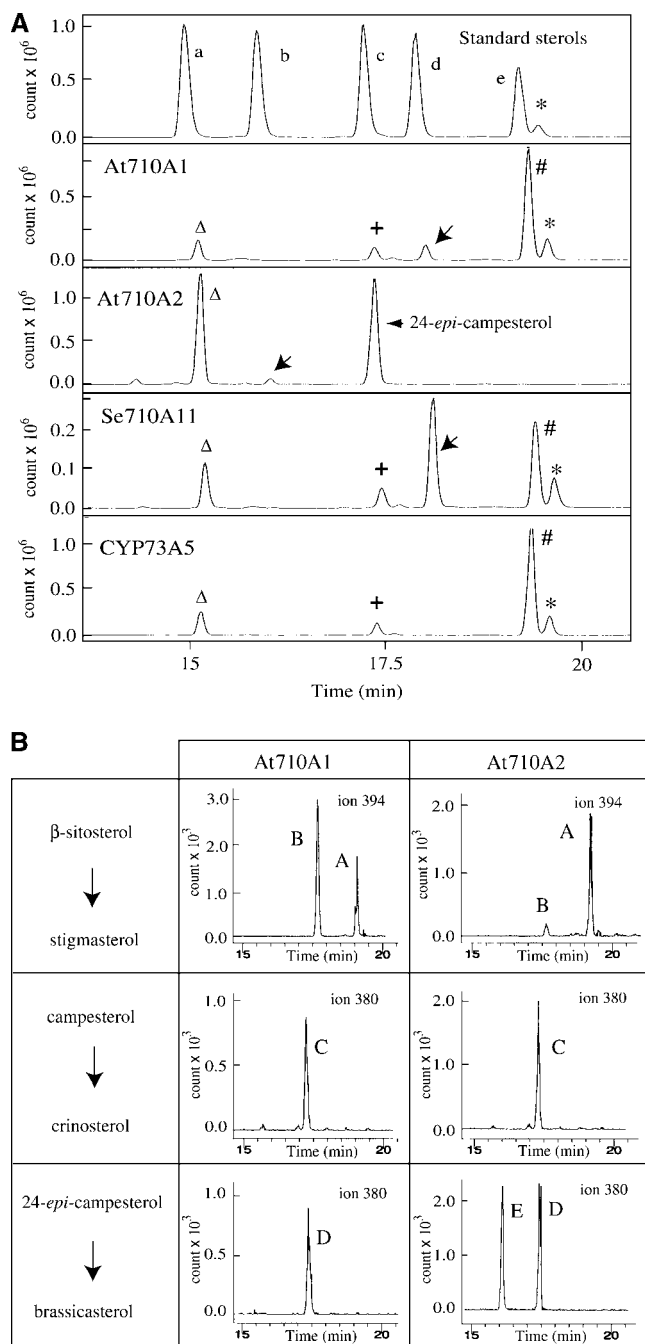
Figure 4. GC-MS Analysis of the Enzyme Reaction.

(A) Reaction products from the recombinant CYP710A assays were analyzed in GC-MS total ion chromatograms. The top panel represents the total ion chromatogram of standard sterols at specific retention times (RTs): a, cholesterol-trimethylsilyl (TMS) (RT = 15.5 min; mass-to-charge value [m/z] = 458); b, brassicasterol-TMS (RT = 16.4 min; m/z = 380); c, campesterol-TMS (RT = 17.4 min; m/z = 383); d, stigmasterol-TMS (RT = 18.5; m/z = 394); e, β-sitosterol (RT = 19.1; m/z = 396). The assays of the At 710A1 (with 10 pmoles of CYP710A1 P450/mL) and the 710A11 (with 20 pmoles of CYP710A11 P450/mL) proteins were done with β-sitosterol as the substrate (60-min reactions), and the At 710A2 assay (90-min reaction with 100 pmoles of CYP710A2 P450/mL) was per-

toward β-sitosterol and that *Arabidopsis* CYP710A2 was capable of producing brassicasterol and stigmasterol from 24-*epi*-campesterol and β-sitosterol, respectively.

Enzymatic Properties of CYP710A1, CYP710A2, and CYP710A11

The C-22 desaturase reactions were further investigated using the CYP710A1, CYP710A2, and CYP710A11 microsomes (Table 1). The K_m values for β-sitosterol of CYP710A1 and CYP710A11 were estimated to be 1.0 and 3.7 μM, respectively, and the kinetic constant (k_{cat}) values were calculated as 0.53 and 10 min^{-1}, respectively. The K_m values indicated that both CYP710A1 and CYP710A11 have high affinity toward β-sitosterol as the substrate. The CYP710A2 activity (Figure 4) was not high enough for reproducible kinetic studies to determine the K_m values for 24-*epi*-campesterol and β-sitosterol. The prominent peak at 420 nm observed with the reduced CO spectrum (Figure 3B) implicated the possibility that processes of correct folding and appropriate heme integration might not have efficiently proceeded with the CYP710A2 in the insect cells. This might be the case for CYP710A1 as well (Figure 3A). Furthermore, the synthesized 24-*epi*-campesterol contained 30% campesterol as impurity, and the correct K_m value for 24-*epi*-campesterol could not be determined. Relative activities were thus obtained to compare the substrate specificities of CYP710A2. The activities of brassicasterol production and stigmasterol production were 0.028 and 0.0027 nmoles/nmol P450/min, respectively, at 10 μM of the substrate concentrations. No reaction product was detected from the CYP710A2 assay when campesterol alone was used as the substrate (Figure 4B). The brassicasterol production activity level determined with CYP710A2 was <5% of the stigmasterol production activity of CYP710A1 under the same experimental conditions. These enzyme assay results indicated that the substrate specificities of CYP710A proteins were fairly strict in terms of the sterol side chain structures, whereas the catalytic efficiencies were not notably higher than other recombinantly expressed P450s (Mizutani et al., 1997; Saito et al., 2004).

formed using the synthesized 24-*epi*-campesterol (containing 30% campesterol as impurity) as the substrate (indicated by the arrowhead). The positions of the reaction products are indicated by the arrows. Sterol structures were identified by reference to relative RT and mass spectra. The pattern of fragment ions with m/z values of 484, 394, 255, and 129 were attributed to stigmasterol, and the fragment ions (m/z = 470, 380, 365, and 129) were used to identify brassicasterol/crinosterol. The 24-epimers were not separately analyzed under our experimental conditions. Campesterol and an unknown compound, both contaminants in β-sitosterol (substrate), are shown by a plus sign and an asterisk, respectively. Cholesterol (from the insect cell microsomes) and β-sitosterol (substrate) are indicated by open triangles (RT = 15.1 min) and pound signs, respectively. Recombinant CYP73A5 microsomes were used for the assay with β-sitosterol (bottom panel).

(B) GC-MS in SIM. A, β-sitosterol; B, stigmasterol; C, campesterol; D, 24-*epi*-campesterol (containing 30% campesterol as impurity); E, brassicasterol. The m/z value of 394 was used for SIM analysis of stigmasterol production, and m/z of 380 was used for the detection of brassicasterol/crinosterol.

Table 1. Enzymatic Properties of Recombinant P450s of *Arabidopsis* CYP710A1 and Tomato CYP710A11

| Enzyme	Activity		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
CYP710A1	1.0 ± 0.090	0.53 ± 0.021	0.53 ± 0.026
CYP710A11	3.7 ± 0.11	10 ± 0.22	2.8 ± 0.023

C-22 Desaturase Activities in Plants

Functions of CYP710 proteins in plants were investigated by transgenic approaches. The full-length coding sequences of the *Arabidopsis CYP710A* genes (*CYP710A1* and *CYP710A2*) and the tomato *CYP710A11* cDNA were put under the control of the cauliflower mosaic virus 35S promoter on a pBIN-derived binary vector, and the constructs (pBINCYP710A1, pBINCYP710A2, and pBINCYP710A11) were then subjected to *Agrobacterium tumefaciens*–mediated transformation of *Arabidopsis* plants using the floral dip method (Clough and Bent, 1998). Based on the screening for kanamycin resistance, we identified 15, 7, and 22 independent T1 lines from the transformation experiments of *CYP710A1*, *CYP710A2*, and *CYP710A11*, respectively. T-DNA integration events were confirmed through PCR using genomic DNA (data not shown). We obtained three, three, and five independent transgenic T2 lines as the overexpressors of *35S:CYP710A1* (At11, At12, and At13), *35S:CYP710A2* (At21, At22, and At23), and *35S:CYP710A11* (Se1, Se2, Se3, Se4, and Se5), respectively.

The correlation between P450 transgene expression levels (Figure 5) and sterol compositions (Table 2) were analyzed in these transgenic plants. It should be noted that the 24-epimers were not separately analyzed in this study. In the *35S:CYP710A1*-, *35S:CYP710A2*-, and *35S:CYP710A11*-transgenic lines, the large accumulations of the transgene transcripts were evident (Figure 5). No significant differences in the endogenous *CYP710A1* and *CYP710A2* expression levels were observed between wild-type plants and transgenic lines. All the *35S:CYP710A1* lines contained stigmasterol at higher levels ranging from 21 to 110 μg/g fresh weight, corresponding to an ∼6- to 32-fold increase compared with that in the wild-type plants (Table 2). Six- to 28-fold increases in the stigmasterol levels (22 to 98 μg/g fresh weight) were also demonstrated in the *35S:CYP710A11* lines (Table 2). No significant changes were observed in the brassicasterol/crinosterol (24-methyl-Δ^{22}-sterols) and 24-*epi*-campesterol/campesterol (24-methyl-sterols) levels in both the *35S:CYP710A1* and *35S:CYP710A11* lines (Table 2). The weak activity of tomato CYP710A11 toward campesterol detected in the enzyme assay (see Supplemental Figure 2 online) was not reflected in the crinosterol production in *Arabidopsis* plants (Table 2). In these transformants, β-sitosterol levels decreased, while the ratio of 24-methyl sterols to 24-ethyl sterols remained largely unchanged. In the *35S:CYP710A2* lines, brassicasterol/crinosterol levels increased by ∼18 times from that in wild-type plants (Table 2). The elevated levels of brassicasterol/crinosterol were accompanied by the decreases in 24-*epi*-campesterol/

campesterol levels (Table 2), supporting in planta that *Arabidopsis* CYP710A2 was involved in the desaturase reaction to produce brassicasterol/crinosterol from 24-*epi*-campesterol/campesterol. Stigmasterol levels also dramatically increased, and this increase was associated with the decrease in β-sitosterol levels (Table 2), indicating that CYP710A2 was also implicated in the stigmasterol production from β-sitosterol (Figure 4). The accumulation levels of the Δ^{22}-sterols in the *35S:CYP710A2* lines were comparable to those in the *35S:CYP710A1* and *35S:CYP710A11* lines (Table 2), implicating the possibility that the activity of CYP710A2 (Figure 3) might not be fully expressed in the enzyme assay.

In addition to the overexpression studies, the brassicasterol/crinosterol production activities of CYP710A2 were further investigated using plants carrying a T-DNA insertion within *CYP710A2* (Figure 6A, *cyp710a2* line). Clear phenotypic alterations were not observed in the *cyp710a2* plants grown in soil under continuous light at 22°C. The T-DNA insertion event was in the coding sequence of *CYP710A2* (Figure 6A), and no apparent *CYP710A2* transcript accumulation was detected (Figure 6B). In the *cyp710a2* plants, the brassicasterol/crinosterol production activity was almost completely abolished (Table 2). On the other hand, the stigmasterol content in the *cyp710a2* plants was maintained at the same level as that of wild-type plants, which
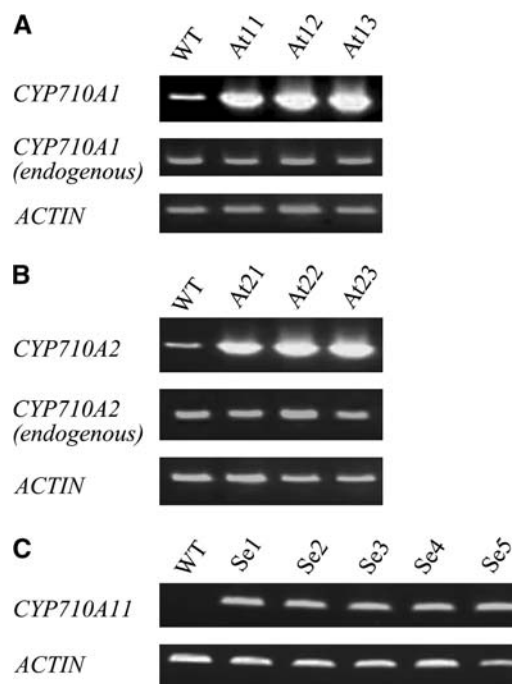
Figure 5. Overexpression of *CYP710A* Genes in *Arabidopsis* Transgenic Lines.

(A) RT-PCR analyses of the expression levels of the *CYP710A1* transgene in the *35S:CYP710A1* lines (At11 to At13).
(B) RT-PCR analyses of the expression levels of the *CYP710A2* transgene in the *35S:CYP710A2* lines (At21 to At23).
(C) RT-PCR analyses of the expression levels of the *CYP710A11* transgene in the *35S:CYP710A11* lines (Se1 to Se5).

Table 2. Sterol Compositions in *35S:CYP710A1-*, *35S:CYP710A2-*, and *35S:CYP710A11*-Transgenic *Arabidopsis* Lines

Sample	24-Methyl-Δ²²-Sterols[a]	24-Methyl-Sterols[a]	Stigmasterol	β-Sitosterol
Wild type	0.85 ± 0.062	23 ± 2.4	3.4 ± 0.52	250 ± 20
At11	1.9 ± 0.62	19 ± 1.5	110 ± 9.9	81 ± 3.7
At12	0.22 ± 0.052	19 ± 2.1	21 ± 4.5	160 ± 6.9
At13	0.50 ± 0.010	21 ± 3.2	86 ± 16	140 ± 9.1
At21	15 ± 1.4	4.5 ± 0.33	81 ± 7.2	170 ± 3.8
At22	16 ± 0.22	2.5 ± 0.20	83 ± 9.1	110 ± 13
At23	14 ± 1.8	4.1 ± 0.44	50 ± 7.7	120 ± 5.8
Se1	0.66 ± 0.16	17 ± 0.43	27 ± 5.8	190 ± 27
Se2	0.63 ± 0.10	20 ± 2.6	27 ± 3.6	190 ± 27
Se3	1.20 ± 0.20	26 ± 0.28	98 ± 29	190 ± 61
Se4	0.96 ± 0.067	20 ± 3.5	46 ± 2.5	170 ± 33
Se5	0.60 ± 0.053	16 ± 1.2	22 ± 4.9	140 ± 20
cyp710a2	n.d.[b]	20 ± 1.1	3.7 ± 0.72	170 ± 14

The same plant samples were divided into two parts for the sterol analyses and the RT-PCR to monitor the transgene expression levels (Figure 5). Data are the means ± SD of triplicate determinations. Values are given in μg/g fresh weight.

[a] The C24-epimers were not separately analyzed. 24-Methyl-Δ²²-sterols are a mixture of brassicasterol and crinosterol, and 24-methyl-sterols consist of 24-*epi*-campesterol and campesterol.

[b] n.d., not detected.

was in agreement with the finding that CYP710A1, as well as CYP710A2, was responsible for the stigmasterol production. Thus, the activities of CYP710A1 and CYP710A2 proteins were redundant in terms of the production of stigmasterol from β-sitosterol. The enzyme assay results showed that CYP710A2 was highly specific for 24-*epi*-campesterol for producing brassicasterol (Figure 4), and campesterol was not accepted as the substrate. In addition, the *cyp710a2* plants contained no 24-methyl-Δ²²-sterols (Table 2). These results suggested that *Arabidopsis* plants might produce brassicasterol from 24-*epi*-campesterol as the major 24-methyl-Δ²²-sterol through the enzyme activity of CYP710A2. No significant difference was observed in the 24-methyl-sterol levels (Table 2) between wild-type plants and the *cyp710a2* plants. The first committed step in the BR biosynthesis is the enzymatic conversion of campesterol/24-*epi*-campesterol into campestanol via the formation of 24-methyl-cholest-4-en-3β-ol and 24-methyl-cholest-4-en-3-on (Noguchi et al., 1999), and brassicasterol/crinosterol are not involved in the BR biosynthetic pathway (Figure 1). In the *cyp710a2* plants, campesterol/24-*epi*-campesterol accumulated at the same level as that in the wild type (Table 2), while brassicasterol/crinosterol was depleted. Thus, BR biosynthesis should not be affected in this T-DNA mutant line.

Expression Profiles of *CYP710A* Family Genes in *Arabidopsis*

We determined tissue-specific expression profiles of the *CYP710A* genes in *Arabidopsis* by RT-PCR (Figure 7). Transcript accumulation from *CYP710A1* expression was detected at higher levels in various organs, including roots, leaves, and flowers,

while the levels in stems and mature siliques were found to be very low. The expression of *CYP710A2* was ubiquitous except for a weak signal in mature siliques. *CYP710A3* expression was specific for the stem, and very weak expression of *CYP710A4* was detected in roots. These results indicated specific regulation mechanisms for the expression of *CYP710A* genes in different tissues and organs.

We generated transgenic *Arabidopsis* lines of promoter:β-glucuronidase (GUS) fusions to monitor the expression profiles of *CYP710A1* (*A1P:GUS*), *CYP710A2* (*A2P:GUS*), *CYP710A3*
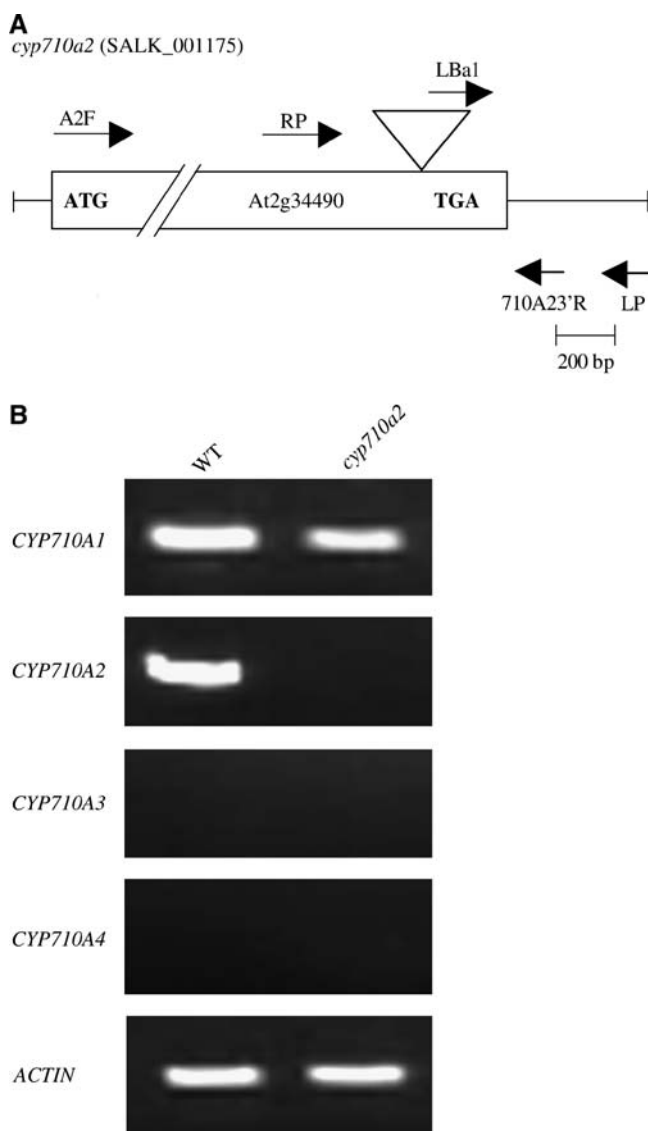

Figure 6. T-DNA Insertion Mutagenesis.

(A) Schematic diagram of the T-DNA insertion event within *CYP710A2* in the *cyp710a2* line (SALK_001175). The primers (Table 3) used for the analysis of the T-DNA insertion are shown.

(B) Expression levels of *CYP710A* genes (*CYP710A1*, *CYP710A2*, *CYP710A3*, and *CYP710A4*) in the *cyp710a2* line.
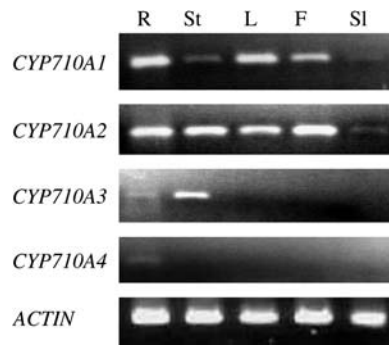
Figure 7. Expression Profiles of *CYP710A* Genes in *Arabidopsis*.

Endogenous expression levels of *CYP710A1*, *CYP710A2*, *CYP710A3*, and *CYP710A4* genes were analyzed in wild-type *Arabidopsis* plants. R, root; St, stem; L, leaf; F, inflorescence stem; Sl, mature silique.

(*A3P*:*GUS*), and *CYP710A4* (*A4P*:*GUS*). In the *A1P*:*GUS* plants (Figure 8), the reporter expression was restricted to the vascular tissues in both shoots and roots. The GUS staining was seen in sepals, and both primary and lateral root tips were also GUS stained. It should be noted that only older inflorescences were consistently stained, while no staining was observed in younger tissues. The staining in the *A2P*:*GUS* plants was observed in both root vascular tissues and leaf tissues with higher levels in younger leaves. The staining was detected in inflorescence stems, together with carpels and seeds as well. The GUS staining also suggested significant levels of *CYP710A2* expression in siliques, whereas only a lower level of mRNA accumulation was detected by semiquantitative PCR analysis (Figure 7). On the other hand, it was suggested that the *CYP710A3* expression was restricted to the primary root caps and immature petals, and no GUS staining was detected in other tissues. In the *A4P*:*GUS* plants, the GUS staining was only seen in the roots, including root hair, but no staining was observed in the root tips. These results suggest that *CYP710A* genes in *Arabidopsis* may be under strict regulations for tissue and developmental stage-specific expression.

DISCUSSION

CYP710 Family P450 Genes

We have demonstrated both in vitro and in planta that the P450 enzymes, CYP710A1 from *Arabidopsis* and CYP710A11 from tomato, were responsible for the C-22 desaturation reaction with β-sitosterol as the substrate, yielding stigmasterol as the product (Figure 1), and that CYP710A2 from *Arabidopsis* was responsible for the production of brassicasterol and stigmasterol from 24-*epi*-campesterol and β-sitosterol (Figure 1), respectively. The T-DNA mutagenesis study demonstrated that *CYP710A2* was essential for the brassicasterol/crinosterol production in *Arabidopsis*, indicating that unidentified desaturases were not able to compensate for the C-22 desaturase reaction catalyzed by CYP710A2. While we presented evidence for the C-22 desa-

turases only from *Arabidopsis* and tomato, it is possible that the desaturation reactions in other plant species may be also catalyzed by the CYP710A family members.

CYP710A genes are present in diverse plant species from gymnosperms to monocots and dicots (Nelson et al., 2004), and CYP710 family sequences are also found as CYP710B in lower photosynthetic unicellular eukaryotes, including red algae (*Cyanidioschyzon merolae*) and green algae (*Chlamydomonas reinhardtii*). The deduced amino acid sequence of *Arabidopsis* CYP710A1 is 40.4 and 37.9% identical to that of CYP710B from the green alga, *C. reinhardtii*. These CYP710 proteins from plants and lower eukaryotes contained the functional domains, such as the N-terminal hydrophobic membrane-anchoring region of 30 to 50 amino acids and a conserved Cys residue in the C-terminal portion involved in binding the heme iron in the fifth coordination site (Schuler and Werck-Reichhart, 2003). The sequence conservation of F(L/M)FA(A/S)QDA(S/T)(S/T)S at the substrate recognition site (SRS4) on the putative I helix region (Gotoh, 1992) is characteristic of the CYP710 proteins and is thought to be crucial for C-22 desaturation activity.

Figure 2 shows the alignment of the deduced primary structures of *Arabidopsis* CYP710A1 and tomato CYP710A11 proteins with those of CYP710A5 from *Oryza sativa*, CYP710B from *C. reinhardtii*, CYP524 from *Dictyostelium discoideum*, and CYP61 from *Saccharomyces cerevisiae*. It has been reported that *C. reinhardtii* produces ergosterol and 7-dehydroporiferasterol as the major end sterols (Salimova et al., 1999), suggesting that CYP710B may be the C-22 desaturase in this algae. The CYP710 family is one of four P450 families (CYP51, CYP97, CYP710, and CYP711) conserved between higher plants and algae, implying that the sterol C-22 desaturation reaction may be essential in all plant taxa. A partial CYP710 sequence was also identified from *Physcomitrella patens* (contig629 at PHYSCObase, http://moss.nibb.ac.jp/), suggesting that the C-22 desaturase may also function in bryophytes, the ancestors of vascular plants.

A phylogenetic tree (Figure 9; see Supplemental Figure 3 online) was obtained with CYP710A proteins from higher plants, CYP710B proteins from *C. reinhardtii* and *C. merolae*, and fungal CYP61 sequences including *S. cerevisiae* and *S. pombe*. The higher plant CYP710 proteins formed a cluster distinct from the fungal CYP61 cluster. These P450 sequences of CYP710 proteins and CYP61 proteins were distant from those of another P450 monooxygenase, CYP51, involved in the sterol biosynthetic pathway catalyzing the sterol 14-demethylase reaction (Benveniste, 2004). It should be noted that CYP524 from a cellular slime mold (*D. discoideum*), containing the sequence conservation in the substrate recognition site (Figure 2), was located on the same branch of CYP710 proteins in the phylogenetic tree (Figure 9). *D. discoideum* produces 4α-methylergostanol, 24β-ethycholesta-8,22-enol, and dictyosterol as Δ^{22}-sterols (Nes et al., 1990), suggesting the possibility that these different subfamily P450s may be involved in the same catalytic function. Sterol analysis indicated that the slime mold evolved from algal rather than from fungal ancestors (Nes et al., 1990), which is consistent with the close location of CYP524 and CYP710B in the phylogenetic tree (Figure 8).

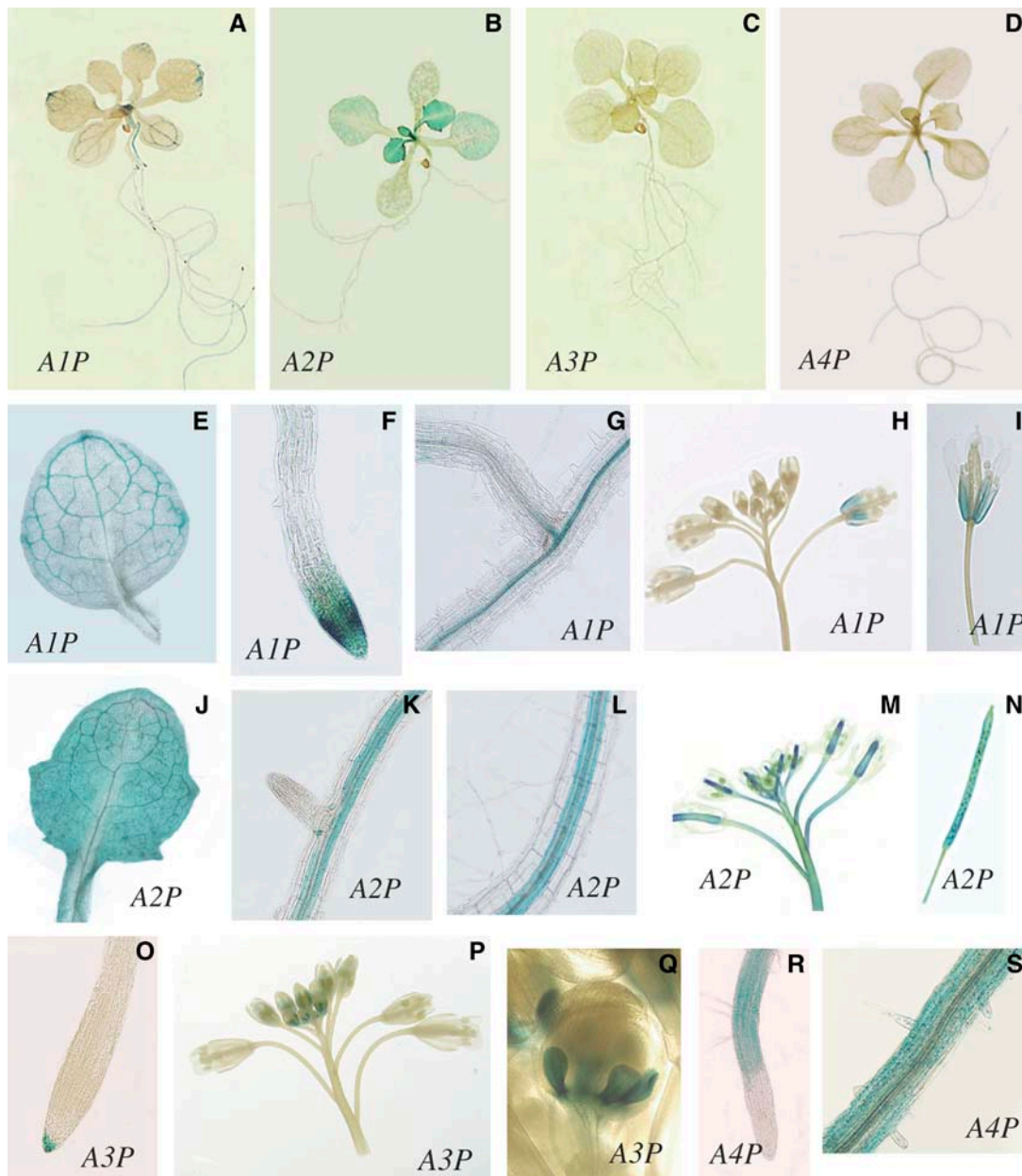**Figure 8.** Promoter:GUS Staining.

Promoter:GUS fusion analyses for differential expression patterns of *CYP710A1* **(A)**, *CYP710A2* **(B)**, *CYP710A3* **(C)**, and *CYP710A4* **(D)**. Tissue-specific expression patterns are shown for *CYP710A1* (**[E]** to **[I]**), *CYP710A2* (**[J]** to **[N]**), *CYP710A3* (**[O]** to **[Q]**), and *CYP710A4* (**[R]** and **[S]**).

Δ²²-Sterols

The *Arabidopsis* CYP710A1 and tomato CYP710A11 proteins catalyzed the C-22 desaturation reaction with β-sitosterol as the primary substrate. In the *35S*:*CYP710A1*- and *35S*:*CYP710A11*-transgenic lines, the stigmasterol levels increased by 6- to 32-fold and 6- to 28-fold compared with those of wild-type plants, respectively. These results substantiated the fact that the C-22 desaturation activity of the CYP710A1 and CYP710A11

proteins were also functioning in plants to produce stigmasterol from β-sitosterol.

CYP710A2 was able to act on 24-*epi*-campesterol and β-sitosterol to yield brassicasterol and stigmasterol, respectively. These enzyme activities were confirmed using the *35S*:*CYP710A2*-transgenic plants and the *cyp710a2* line containing the T-DNA insertion within *CYP710A2* (Table 2). In the enzyme assay experiments, CYP710A2 was specific for 24-*epi*-campesterol, indicating that brassicasterol should be the major form of
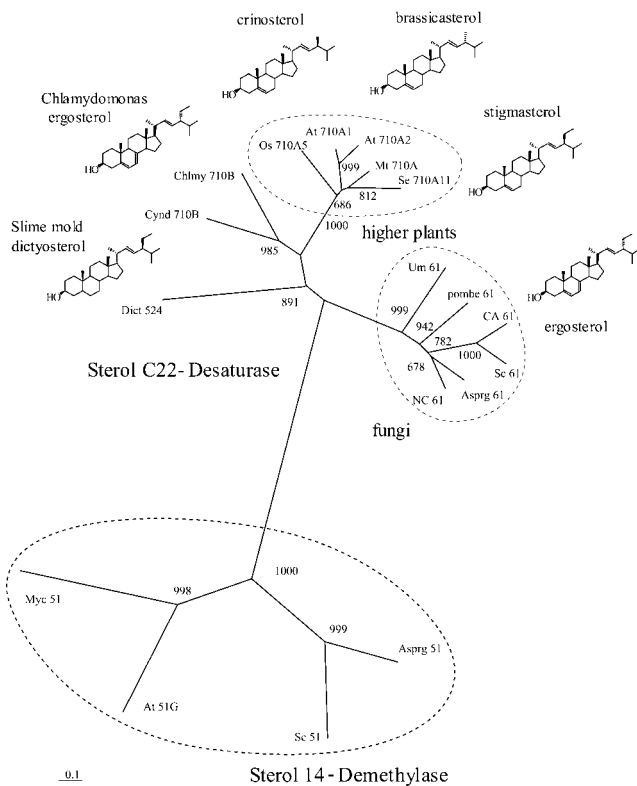
Figure 9. Phylogenetic Tree for CYP710 Proteins and Major Δ²²-Sterols.

Phylogenetic tree of CYP710-related sequences were obtained with CYP710A1 (At 710A1) and CYP710A2 (At 710A2) from *Arabidopsis*, CYP710A11 (Se 710A11) from tomato, CYP710A (Mt 710A) from *M. truncatula*, CYP710A5 (Os 710A5) from *O. sativa*, CYP710B (Chlmy 710B) from *C. reinhardtii*, CYP710B (Cynd 710B) from *C. merolae*, CYP524 (Dict 524) from *D. discoideum*, CYP61 (pombe 61) from *S. pombe*, CYP61 (NC 61) from *N. crassa*, CYP61 (CA 61) from *C. albicans*, CYP61 (Sc 61) from *S. cerevisiae*, CYP61 (Asprg 61) from *A. fumigatus*, CYP61 (Um 61) from *U. maydis*, CYP51F1 (Asprg 51) from *A. nidulans*, CYP51F1 (Sc 51) from *S. cerevisiae*, CYP51G1 (At 51G) from *Arabidopsis*, and CYP51B1 (Myc 51) from *M. tuberculosis*. Major Δ²²-sterols from higher plants (stigmasterol, brassicasterol, and crinosterol), *C. reinhardtii* (ergosterol), and *D. discoideum* (dictyosterol) are shown next to the corresponding genes. Values at branch nodes indicate numbers of bootstrap trials out of 1000 that produced each node.

could not be functionally expressed in our system (data not shown), also should be elucidated for complete understanding of the biosynthesis of the Δ²²-sterols in *Arabidopsis*.

It has been reported that proper ratios of β-sitosterol to campesterol are imperative for normal plant development and sexual reproduction (Schaller, 2003; Benveniste, 2004). While the *CYP710A1*, *CYP710A2*, and *CYP710A11* overexpressors accumulated stigmasterol at ∼6- to 32-fold higher levels, with a concurrent decrease in the β-sitosterol level, of that in wild-type plants, there were no significant changes in the ratio of 24-ethyl sterols (β-sitosterol and stigmasterol) to 24-methyl sterols (campesterol and brassicasterol). The *35S:CYP710A1*, *35S:CYP710A2*, and *35S:CYP710A11* lines displayed no drastic phenotypic alterations, such as dwarfism or developmental abnormalities, indicating that the accumulation of the Δ²²-sterols at the observed levels was not influencing BR biosynthesis and other developmental processes revealed by studies of mutants such as *smt1/orc/cph*, *hyd1*, *hyd2/fk*, *smt1*, and *smt2/cvp1* (Clouse, 2000; Lindsey et al., 2003).

The *cyp710a2* plants in which *CYP710A2* function was abolished by the T-DNA insertion (Figure 6, Table 2) did not exhibit clear phenotypic alterations. The enzyme assay results indicated that *Arabidopsis* CYP710A2 was involved in the production of both Δ²²-methyl-sterols (brassicasterol/crinosterol) and Δ²²-ethyl-sterol (stigmasterol). The *cyp710a2* plants did not accumulate brassicasterol/crinosterol, while stigmasterol was produced at a level comparable to that in wild-type plants (Table 2). Thus, *Arabidopsis* CYP710A2 is thought to be the crucial enzyme for the production of brassicasterol/crinosterol in *Arabidopsis*, and

the 24-methyl-Δ²²-sterol in *Arabidopsis*. This is consistent with the finding that the seeds of *Brassica* plants (rape [*Brassica napus*] and mustard [*Brassica juncea*]) contained the C-22 unsaturated forms of the 24-methyl sterols as a mixture of brassicasterol and crinosterol (10 to 30%) as the minor component (Matsumoto et al., 1983). In this study, the stereoconfigurations of the 24-methyl group were not distinguished, and we cannot rule out the possibility that campesterol may be a very weak substrate for the CYP710A2 reaction to accumulate crinosterol in planta. The ratio of brassicasterol to crinosterol should be clarified in *Arabidopsis* plants. The enzyme reactions catalyzed by CYP710A3 and CYP710A4, of which recombinant enzymes

Table 3. Primer Sequences

Primer	Primer Sequence (5′–3′)
A1F	CCGTCTAGAGAATTCATGGTTTTCTCTGTTTCTAT
A1R	CCGGTCGACCTCGAGTTAGGAAAAGTTGGGATACTT
A2F	CGCGGATCCATGGTTTTCTCAGTTTCCAT
A2R	CCGCTCGAGTCAGAGGTTCGGATACGTT
710A1QF	AGGCGCGTCGCAAAGTATCCCAACTTTTCC
710A13′R	CTCGTGGGCCACTCACTTCGAGAGA
710A2QF	ATCGTAACGTATCCGAACCTCTGA
710A23′R	CACGATCACGAACAACAATAATAGT
710A34QF	CCGCCTCGTTACCTCTCCTTGA
710A3QR	CAACACATATATTAGGTGAA
710A4QR	AAGAACACGAATTAAATATG
710ToF	ATGGCATCCATTTGGGGTTTGTTATCT
710ToR	TCATCGTGTGCACCTGTGTGCAAGGAAA
Act-F	ATGGCTGATGGTGAAGACATTC
Act-R	TCAGAAGCACTTCCTGTGAAC
A1pF	TGCTCTAGATGGAAGTCTTCGAGACTGAA
A1pR	TGCTCTAGAGTTTCTTTGTTTCTAGCTTG
A2pF	CCCAAGCTTAAGACTTTTGAACCATGTTA
A2pR	CCCAAGCTTCCTTTTTTTTTCCTCTTCT
A3pF	TGCTCTAGAACCACATGATATCTAATGGA
A3pR	TGCTCTAGATGCTCTTTGTTTTCTATTA
A4pF	CGCGGATCCTTTGCATTCATATCCGAAAT
A4pR	CGCGGATCCTGCTCTTTGTTTTCTATT
LP	CGGTTTTGGCTTTCTGTCGTT
RP	GGCGGTTTGCTCTTCGATTTT
LBa1	TGGTTCACGTAGTGGGCCATG

enzymes other than CYP710A2 also should be involved in stigmasterol production. This is consistent with the findings that both CYP710A1 and CYP710A2 enzymes catalyzed the C-22 desaturation reaction to produce stigmasterol from β-sitosterol in *Arabidopsis*. In the *cyp710a2* plants, campesterol/24-*epi*-campesterol, the direct precursors of BR biosynthesis (Figure 1), accumulated at the same level as that in wild-type plants, indicating that BR biosynthesis should not be affected. There are different possibilities for the lack of a phenotype in the *cyp710a2* line. Brassicasterol and crinosterol specifically occur in the *Brassicaceae* (Benveniste, 2004), but their physiological functions are not known. It is possible that the loss of brassicasterol/crinosterol production activity may not be detrimental to plants. It is also possible that the physiological roles of the Δ^{22}-sterols may be redundant. If this is the case, the defect in brassicasterol/crinosterol production activity should not be a critical factor for the growth of *Arabidopsis* containing stigmasterol at a normal level.

The tissue specificity of the Δ^{22}-sterol production activities remains to be clarified. The expression of *Arabidopsis CYP710A* genes was suggested to be under strict regulation in different tissues and organs (Figure 8), suggesting the possibility that phenotypic alterations may be observed in definite tissues at specific developmental stages. It has been reported that another P450 gene involved in sterol biosynthesis, *CYP51G1*, was strongly expressed in leaf vascular tissues (Kim et al., 2005), which was closely similar to the expression pattern of *CYP710A1* involved in stigmasterol production (Figure 8). *CYP51G1* (At1g11680) is the single functional gene (Kim et al., 2005) encoding obtusifoliol 14α-demethylase protein in *Arabidopsis* (Figure 1). The promoter:GUS experiments suggested that the brassicasterol/crinosterol production activities of CYP710A2 might be higher in younger leaves (Figure 8), implicating greater demands for brassicasterol/crinosterol in younger leaves. Sterol analyses of specific samples, such as mesophyll protoplasts, vascular tissues, and root tips, may provide information to link phenotypic alterations, if any, with differential distributions of the Δ^{22}-sterols in *Arabidopsis*.

The physiological roles of Δ^{22}-sterols are not well known. Studies from sterol mutants indicated that correct membrane properties are assured through the maintenance of proper sterol compositions, affecting cell functions such as cell polarity, auxin efflux, and ethylene signaling (Clouse, 2000; Lindsey et al., 2003). For example, the membrane localization of PIN1 and PIN3 proteins is disturbed in the sterol mutant *smt1^{orc}* (Willemsen et al., 2003), indicating that sterol profiles are important determinants of protein targeting in plants. Furthermore, *fk* and *hyd1* as well as *smt1/cph* mutants are characterized by altered sterol profiles and accumulation of abnormal sterol intermediates, exhibiting incomplete cell walls and aberrant cell wall thickenings in embryonic and postembryonic tissues together with ectopic callose and lignin deposits (Schrick et al., 2004). In animals and yeasts, lipid microdomains or rafts are defined as specialized detergent-resistant membrane regions enriched in sphingolipids and cholesterol, harboring proteins involved in crucial physiological processes, such as signal transduction for cellular proliferation and differentiation, vesicular trafficking, and cytoskeleton organization (Harder and Simons, 1997; Hakomori et al., 1998).

Recently, it has been reported that plant cells also contained lipid microdomains/rafts enriched in β-sitosterol, stigmasterol, and 24-methylcholesterol as well as cholesterol and sphingolipids (Mongrand et al., 2004; Borner et al., 2005). Involvement of Δ^{22}-sterols in the formation and functional properties of lipid rafts/microdomains remains to be clarified.

In this study, we show that CYP710A1 and CYP710A2 from *Arabidopsis* and CYP710A11 from tomato are the sterol C-22 desaturases. The identification of the sterol C-22 desaturase genes in plants enables the manipulation of the expression levels of *CYP710A* genes, leading to the direct modification of sterol composition in crop plants. Furthermore, generating transgenic plants lacking either specific Δ^{22}-sterol or all of the Δ^{22}-sterol species by means of gene knockout and RNA interference should shed light on the crucial roles of phytosterols underlying BR-independent processes of embryonic and postembryonic development.

METHODS

Materials

Arabidopsis thaliana ecotype Columbia (Col-0) (Lehle Seeds) seedlings were grown at 22°C under continuous light as described previously (Mizutani and Ohta, 1998). For sterol analysis, RNA isolation, and GUS staining, *Arabidopsis* seedlings were grown under a sterile condition on 0.8% (w/v) agar plates containing germination medium (GM) supplemented with 1× Murashige and Skoog salts and 1% (w/v) sucrose (Valvekens et al., 1998).

Isolation of CYP710 Coding Sequences from *Arabidopsis* and Tomato

The entire coding sequences of *CYP710A1* and *CYP710A2* genes in *Arabidopsis* contain no intron and were thus amplified by PCR using genomic DNA as the template. Genomic DNA was prepared from 2-week-old *Arabidopsis* seedlings as described (Sambrook et al., 1989). A primer set of A1F and A1R (Table 3) was used for the amplification of the *CYP710A1* coding sequence, and A2F and A2R (Table 3) was used for the amplification of the *CYP710A2* coding sequence. The PCR products for the coding sequences of *CYP710A1* and *CYP710A2* were cloned into a pDrive cloning vector (Qiagen) to give pDCYP710A1 and pDCYP710A2, respectively. A tomato (*Lycopersicon esculentum*) cDNA clone coding for a putative CYP710A protein was identified through a tBLASTn search at the TIGR Gene Indices (http://tigrblast.tigr.org/tgi/) against the tomato EST database using the amino acid sequence of CYP710A1. Among candidate sequences identified, we obtained an EST of cLEX15L1 from Clemson University Genomics Institute. The cLEX15L1 insert was completely sequenced and shown to contain the putative entire coding region of a tomato *CYP710A*. The tomato P450 sequence was designated as CYP710A11 (David Nelson, http://drnelson.utmem.edu/CytochromeP450.html).

Heterologous Expression in Insect Cells

Recombinant CYP710A proteins were prepared by expressing the full lengths of the *Arabidopsis CYP710A1* and *CYP710A2* coding sequences and the tomato *CYP710A11* cDNA in a baculovirus expression vector system as described previously (Mizutani et al., 1997), using the Bac-to-Bac baculovirus expression system (Invitrogen) and *Spodoptera furugiperda* cells (Sf9; Invitrogen). Briefly, pDCYP710A1 and pDCYP710A2 were double-digested with *Eco*RI-*Xho*I and *Bam*HI-*Xho*I, respectively, and the tomato *CYP710A11* cDNA insert of cLEX15L1 in pBluescript

SK− was isolated by digesting with *Bam*HI and *Xho*I. These CYP710A coding sequences were cloned into a pFastBac1 plasmid digested with appropriate restriction enzymes. The pFastBac1 constructs were then used for the preparation of recombinant bacmid DNAs by transformation of *Escherichia coli* strain DH10Bac (Invitrogen), and transfection of the insect cells was done according to the manufacturer's instructions (Invitrogen). For expression of the recombinant P450 proteins, Sf9 cells were maintained in Sf900II serum-free medium (Invitrogen) supplemented with 200 μM 5-aminolevulinic acid and 200 μM ferrous citrate to increase the low heme synthetic capacity of the insect cells (Saito et al., 2004).

Enzyme Assays

For preparation of microsomal fractions, the infected cells (60 mL of suspension-cultured cells) were washed with PBS and suspended in buffer A consisting of 20 mM potassium phosphate, pH 7.25, 20% (v/v) glycerol, 1 mM EDTA, and 1 mM DTT. The cells were sonicated, and cell debris was removed by centrifugation at 10,000g for 15 min. The supernatant was further centrifuged at 100,000g for 1 h, and the pellet was homogenized with buffer A as the microsomal fraction. The microsomal fractions were stored at −80°C until use. The complete reaction mixture (0.5 mL) consisted of 50 mM potassium phosphate, pH 7.25, recombinant CYP710A microsomes (50 to 500 μg protein/mL), 100 μM NADPH, and sterol substrates at different concentrations ranging from 1 to 100 μM. To monitor full P450 activities, microsome assays were supplemented with 0.1 unit/mL of a purified recombinant *Arabidopsis* NADPH-P450 reductase preparation (Mizutani and Ohta, 1998). After 5 to 90 min at 30°C, the reactions were stopped by adding 50 μL of 1 N HCl, and the reaction products were extracted four times with an equal volume of ethyl acetate. The ethyl acetate extracts were evaporated to dryness, and TMS ether derivatives for GC-MS analysis were prepared in a 1:1 mixture (40 μL) of pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide (Wako Pure Chemical Industries) containing 1% (v/v) trimethylchlorosilane (Nacalai Tesque) at 90°C for 1 h. For calculation of enzyme kinetic parameters, linear regression was used in double-reciprocal analyses of activity data (Saito et al., 2004).

Semiquantitative RT-PCR Analysis

RT-PCR analyses were done for detailed comparison of the expression levels of *CYP710A1*, *CYP710A2*, *CYP710A3*, and *CYP710A4* genes in *Arabidopsis*. Total RNA was isolated using an RNeasy plant mini kit (Qiagen), and genomic DNA contamination was eliminated using an RNase free DNase set (Qiagen). First-strand cDNA was synthesized using the Takara RNA PCR kit (AMV) version 3.0 in a 10-μL reaction mixture containing 300 ng of total RNA using an oligo(dT)$_{16}$ as the reverse primer. The reverse transcription reactions were performed at 30°C for 10 min, 50°C for 30 min, and 95°C for 5 min and then chilled to 5°C for 5 min. The PCR was done using 2 μL of the reverse transcription products as the template in 10 μL of reaction mixture containing 1 mM MgCl$_2$, 0.2 mM deoxynucleotide triphosphate mixture, 0.025 unit/μL Takara Ex Taq HS, and 0.2 μM of primers using the Takara RNA PCR kit (AMV) version 3.0. The PCR was programmed in 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min followed by an extension period of 10 min at 72°C. The gene-specific primer set for *CYP710A1* was a pair of 710A1QF and 710A13′R (Table 3). A set of 710A2QF and 710A23′R (Table 3) was used for the analysis of *CYP710A2* gene expression. For the *CYP710A3* expression analysis, a set of 710A34QF and 710A3QR (Table 3) was prepared, and a set of 710A34QF and 710A4QR (Table 3) was used for *CYP710A4* analysis. *Arabidopsis ACTIN* (Nairn et al., 1988) as the internal control was amplified under the same PCR conditions using the primer pair Act-F and Act-R (Table 3).

Overexpression of CYP710A

The entire coding sequences of *CYP710A1* and *CYP710A2* were double digested with *Xba*I-*Sal*I and *Bam*HI-*Xho*I from pDCY710A1 and pDCY-P710A2, respectively, and cloned into an *Xba*I-*Sal*I and a *Bam*HI-*Xho*I double-digested pBIN-based vector (Clontech) to yield pBINCYP710A1 and pBINCYP710A2, respectively. From cLEX15L1, *CYP710A11* cDNA was excised with *Bam*HI and *Xho*I and cloned into a *Bam*HI-*Xho*I double-digested pBIN vector (pBINCYP710A11). These plasmids were electroporated into *Agrobacterium tumefaciens* strain EHA105 and transformed into *Arabidopsis* using the floral dip method (Clough and Bent, 1998). T1 seeds were screened on GM agar plates containing 25 μg/mL kanamycin, and resistant seedlings were transferred to soil and allowed to set seed. *35S*:*CYP710A1*, *35S*:*CYP710A2*, and *35S*:*CYP710A11* homozygous lines were selected by examining the kanamycin resistance of T3 seedlings.

Transgene expression levels in *35S*:*CYP710A1*, *35S*:*CYP710A2*, and *35S*:*CYP710A11* plants were analyzed by RT-PCR. Total RNA was extracted from rosette leaves of T2 plants using an RNeasy plant mini kit. The same plant samples were also used for the sterol composition analyses. RT-PCR was done using the Takara RNA PCR kit (AMV) version 3.0. The primer set of A1F and A1R (Table 3) was used to check the expression levels of both the *CYP710A1* transgene and endogenous *CYP710A1*, and the pair of A2F and A2R was used to monitor the transcripts from both the *CYP710A2* transgene and endogenous *CYP710A2*. *CYP710A11* expression levels were studied using a set of 710ToF and 710ToR (Table 3). Endogenous expression levels of *CYP710A1* and *CYP710A2* genes in the transformants were studied using a set of primers of A1F and 710A13′R and a set of A2F and 710A23′R, respectively (Table 3). The primer sequences of 710A13′R and 710A23′R were derived from the 3′-noncoding regions of *CYP710A1* and *CYP710A2* genes, respectively, and the PCR products thus obtained were ascribed to the transcripts from the expression of *CYP710A1* and *CYP710A2* genes driven by their own promoters. *Arabidopsis ACTIN* (Nairn et al., 1988) as the internal control was amplified under the same PCR conditions.

T-DNA Insertion Lines

A T-DNA insertion event within the *CYP710A2* coding sequence, *cyp710a2* (SALK_001175), was found by the database search with the SIGnAL Arabidopsis Gene Mapping Tool (Alonso et al., 2003; http://signal.salk.edu/cgi-bin/tdnaexpress), and the seeds were obtained from ABRC (Columbus, OH). A gene-specific primer set of LP and RP and a T-DNA border primer LBa1 (Table 3) were used to clarify the T-DNA insertion event. Individual plants homozygous for a T-DNA insertion in *CYP710A2* were identified by PCR screening and segregation analysis. The primer pairs of 710A1QF and 710A13′R, A2F and 710A23′R, 710A34QF and 710A3QR, and 710A34QF and 710A4QR (Table 3) were used to check the expression levels of *CYP710A1*, *CYP710A2*, *CYP710A3*, and *CYP710A4* genes, respectively. *Arabidopsis ACTIN* (Nairn et al., 1988) as the internal control was amplified under the same PCR conditions using a primer pair of Act-F and Act-R (Table 3). Other Salk T-DNA insertions related to *CYP710A2* were found outside the coding sequence and excluded from the analysis.

Promoter:GUS

For promoter:GUS analysis, promoter regions of *Arabidopsis CYP710A* genes including 5′-upstream portions were amplified by PCR from genomic DNA as the template. A 2012-bp fragment (*A1P*) for *CYP710A1* was amplified using a primer pair of A1pF and A1pR (Table 3), and a 2008-bp fragment for *CYP710A2* (*A2P*) was obtained using A2pF and A2pR (Table 3). As the *CYP710A3* promoter (*A3P*), a 2002-bp fragment was amplified using A3pF and A3pR (Table 3). A 2060-bp

fragment as for *CYP710A4* (*A4P*) was obtained using a primer set of A4pF and A4pR (Table 3). The PCR products were subcloned into a pDrive cloning vector (Qiagen), and the inserts were completely sequenced. The restriction fragments were excised and subcloned into a plant transformation vector pBI101.1 (Clontech) to give pBI101-A1P, pBI101-A2P, pBI101-A3P, and pBI101-A4P. These plasmids were electroporated into *A. tumefaciens* strain GV3101 and transformed into *Arabidopsis* using the floral dip method (Clough and Bent, 1998). T1 seeds were screened on medium containing 25 µg/mL kanamycin, and resistant seedlings were transferred to soil and allowed to set to seed. Homozygous lines were selected by examining the kanamycin resistance of T3 seedlings.

Staining of Promoter:GUS Transgenic Plants

After 3 weeks of growth on GM plates, the promoter:GUS transgenic plants (*A1P*:*GUS*, *A2P*:*GUS*, *A3P*:*GUS*, and *A4P*:*GUS*) were first treated with 90% (v/v) ice-cold acetone for 1 h and submerged in GUS staining buffer containing 50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% (w/v) Triton X-100, and 0.5 mg/L 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Nacalai Tesque). The tissues were then infiltrated with staining buffer under vacuum and incubated overnight at 37°C (Jefferson, 1987). After rinsing with water, tissues were cleared with ethanol:acetic acid (9:1, v/v), which was followed with washes using 90, 70, and 50% (v/v) diluted ethanol. The tissues were then left in 50% glycerol for later photography.

Sterol Analysis

Samples (50 mg) were frozen and homogenized in liquid N_2, and total sterol fractions were extracted for 30 min at room temperature in 5 mL chloroform/methanol (1:2, v/v). By adding 2 mL of 1% (w/v) KCl and 1 mL of chloroform, sterols were recovered in the organic phase. Two milliliters of methanol/water (10:9, v/v) was then added to the organic phase and evaporated to dryness under a N_2 stream. The saponification was done in 2.5 mL of 1 M KOH in methanol at 90°C for 1 h, and 2 mL chloroform and 2.5 mL water were added to recover the organic phase. After adding 1.25 mL of 0.5 M KOH and 6 mL water, the organic phase was evaporated to dryness in vacuo and used for the TMS derivatization. Sterols were analyzed by GC-MS on a Saturn 2200 GC-MS system (Varian) with a CP-SIL5 CB LOW BLEED/MS column (30 m × 0.25 mm) (Varian). The port temperature for split injections (a split ratio of 50%) was 270°C, and helium was used as the carrier gas at a flow rate of 1.5 mL/min. The temperature program was started with a slow rise from 230 to 285°C (2°C/min), from 285 to 320°C (10°C/min), and finally 10 min at 320°C. Standards consisting of cholesterol, brassicasterol, campesterol, stigmasterol, and β-sitosterol were used for quantification and identification. The peak areas were automatically calculated using the 2200 Workstation (Varian), and sterol amounts were determined from the ratio of the peak area of each sterol to that of 5-α-cholestane as an internal standard (Dyer et al., 1995). Sterol structures were identified by reference to relative retention time and mass spectra. The pattern of fragment ions with *m/z* values of 484, 394, 255, and 129 were attributed to stigmasterol, and the fragment ions (*m/z* = 470, 380, 365, and 129) were used to identify brassicasterol/crinosterol. The 24-epimers were not separately analyzed under our experimental conditions.

DNA Sequence Analysis

DNA sequences were determined from both strands using CEQTM DTCA-Quick Start kits (Beckman Coulter) and a DNA sequencer, CEQTM2000XL DNA analysis system (Beckman Coulter). DNA and amino acid sequences were analyzed using GENETYX software (Software Development).

Phylogenetic Analysis

Amino acid sequences were aligned at DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) using the ClustalW program (Thompson et al., 1994), and phylogenetic trees were determined using the neighbor-joining method (Saitou and Nei, 1987) with bootstrap analysis (1000 replicates) and Kimura's correction for protein distances (Kimura, 1983). The tree was visualized using TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The following sequences were analyzed: CYP710A1 and CYP710A2 from *Arabidopsis*, CYP710A11 from tomato, CYP710A from *Medicago truncatula*, CYP710A5 from *Oryza sativa*, CYP710B from *Chlamydomonas reinhardtii*, CYP710B from *Cyanidioschyzon merolae*, CYP524 from *Dictyostelium discoideum*, CYP61 from *Schizosaccharomyces pombe*, CYP61 from *Neurospora crassa*, CYP61 from *Candida albicans*, CYP61 from *Saccharomyces cerevisiae*, CYP61 from *Aspergillus fumigatus*, CYP61 from *Ustilago maydis*, CYP51F1 from *Aspergillus nidulans*, CYP51F1 from *S. cerevisiae*, CYP51G1 from *Arabidopsis*, and CYP51B1 from *Mycobacterium tuberculosis*.

Chemical Synthesis

Crinosterol was synthesized for identification of reaction products in the enzyme assay experiments. A mixture of (22*E*,24*S*)-6β-methoxy-3α,5-cyclo-5α-ergost-22-ene (340 mg, 0.82 mmoles) and a catalytic amount of *p*-toluenesulfonic acid in an 80% aqueous tetrahydrofuran solution (10 mL) was refluxed for 3 h. The reaction mixture was cooled to room temperature and poured into a saturated aqueous sodium hydrogencarbonate solution (30 mL) followed by extraction with ethyl acetate (1 × 20 mL, 4 × 10 mL). The combined organic layer was washed with brine (15 mL), dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The crude product was purified by flash column chromatography (hexane:ethyl acetate = 4:1) to yield crinosterol (258 mg, 79% yield) as a colorless solid. NMR δ_H (300 MHz, CDCl$_3$): 0.69 (3H, s), 0.82 (3H, d, *J* = 6.9), 0.84 (3H, d, *J* = 6.9), 0.91 (3H, d, *J* = 6.6), 1.00 (3H, d, *J* = 5.7), 1.01 (3H, s), 3.52 (1H, m), 5.16 (2H, m), and 5.35 (1H, m). All physicochemical properties were identical to those reported previously (Lang and Djerassi, 1982; Anastasia et al., 1983).

The 24-*epi*-campesterol was synthesized as the substrate for the enzyme assay of recombinant CYP710A enzymes. A mixture of (22*E*,24*R*)-6β-methoxy-3α,5-cyclo-5α-ergost-22-ene (305 mg, 0.74 mmoles) and platinum(II) oxide (50 mg) in ethyl acetate (25 mL) was stirred overnight under hydrogen atmosphere. The catalyst was removed by decantation, and the solvent was removed in vacuo to give (24*S*)-6β-methoxy-3α,5-cyclo-5α-ergostan (301 mg). This crude product was purified by flash column chromatography (hexane:ethyl acetate = 4:1) to give 24-*epi*-campesterol (208 mg, 70% yield for two steps) as a colorless solid. NMR δ_H (500 MHz, CDCl$_3$): 0.68 (3H, s), 0.776 (3H, d, *J* = 6.8), 0.783 (3H, d, *J* = 6.8), 0.86 (3H, d, *J* = 6.8), 0.92 (3H, d, *J* = 6.5), 1.01 (3H, s), 3.52 (1H, tt, *J* = 11.0, 4.7), and 5.35 (1H, m). NMR δ_C (125 MHz, CDCl$_3$): 11.84, 15.43, 17.58, 18.88, 19.38, 20.51, 21.07, 24.28, 28.18, 30.56, 31.44, 31.65, 31.89 (×2), 33.70, 36.17, 36.49, 37.24, 39.05, 39.75, 42.29 (×2), 50.12, 55.98, 56.74, 71.78, 121.70, and 140.75. NMR analysis showed that our 24-*epi*-campesterol preparation was contaminated by campesterol (~30% of the total material), a 24-epimer of the desired product. Although they could not be separated, the structures were established by comparing ^{1}H and ^{13}C NMR spectra with those of authentic campesterol. Khripach et al. (2002) reported inversion of stereochemistry at C-24 during the course of hydrogenation of C-24 substituted Δ^{22}-steroids. The same kind of isomerization might have occurred in our hydrogenation step of (22*E*,24*R*)-6β-methoxy-3α,5-cyclo-5α-ergost-22-ene.

NMR spectra were recorded on either the Bruker ARX-500 or the JEOL JNM-AL300. Chemical shifts have been reported in the δ value by ppm unit relative to tetramethylsilane (δ 0 ppm for ^{1}H) and the solvent

(δ 77.0 ppm for ^{13}C) as an internal standard in deuteriochloroform (CDCl$_3$) solution. All *J*-values are given in hertz.

The (22*E*,24*S*) and (22*E*,24*R*)-6β-methoxy-3α,5-cyclo-5α-ergost-22-ene were prepared from stigmasterol as described (Takatsuto et al., 1997). Flash column chromatography was done using Kieselgel 60 (Merck) as the adsorbent.

Assay Methods

All spectrophotometric determinations were done at room temperature using a Cary 300 spectrophotometer (Varian). P450 was estimated from the CO difference spectrum using an extinction coefficient ($\Sigma = 91$ mM^{-1} cm^{-1}). NADPH-P450 reductase was assayed by measuring its NADPH-cytochrome *c* reductase activity, and the rate of cytochrome *c* reduction was calculated from the A_{550} change using an extinction coefficient ($\Sigma = 21$ mM^{-1} cm^{-1}). Protein was determined using the Coomassie protein assay reagent (Bio-Rad Laboratories).

Accession Numbers

Sequence data from this article can be found in the GenBank database under the following accession numbers: *CYP710A1* (AB219423), *CYP710A2* (AB233425), *CYP710A3* (At2g28850), *CYP710A4* (At2g28860), cLEX15L1 (AW622568), *CYP710A11* (AB223043), *M. truncatula* CYP710A (TC101450, http://tigrblast.tigr.org/tgi/), *O. sativa* CYP710A5 (NM_188395), *S. pombe* CYP61 (CAB11640), *N. crassa* CYP61 (EAA32679), *C. albicans* CYP61 (EAK98020), *S. cerevisiae* CYP61 (CAA89116), *A. fumigatus* CYP61 (EAL88107), *U. maydis* CYP61 (EAK81168), *A. nidulans* CYP51F1 (EAA59021), *S. cerevisiae* CYP51F1 (AAB68433), *Arabidopsis* CYP51G1 (NP_172633), and *M. tuberculosis* CYP51B1 (NP_215278). *C. reinhardtii* CYP710B (ChlamyDB, http://www.chlamy.org/chlamydb.html), *C. merolae* CYP710B (*Cyanidioschyzon merolae* Genome Project, http://merolae.biol.s.u-tokyo.ac.jp/), and *D. discoideum* CYP524 (dictyBase, http://dictybase.org/) sequences were retrieved from the database indicated.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Heterologous Expression of Recombinant CYP710A Proteins in Insect Cells.

Supplemental Figure 2. GC-MS in the Selected Ion Monitoring Mode for Tomato CYP710A11 Assay.

Supplemental Figure 3. Multiple Sequence Alignment Used for Phylogenetic Analysis Visualized Using CINEMA5 (Parry-Smith et al., 1998, http://umber.sbs.man.ac.uk/dbbrowser/CINEMA2.1/).

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