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Obtusifoliol 14α-Demethylase (CYP51) Antisense *Arabidopsis* Shows Slow Growth and Long Life

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Obtusifoliol 14α-demethylase is a plant orthologue of sterol 14α-demethylase (CYP51) essential in sterol biosynthesis. We have prepared CYP51 antisense *Arabidopsis* in order to shed light on the sterol and steroid hormone biosynthesis in plants. *Arabidopsis* putative CYP51 cDNA (*AtCYP51*) was obtained from *Arabidopsis* expressed sequence tag (EST) library and its function was examined in a yeast lanosterol 14α-demethylase (Erg11) deficient mutant. A recombinant AtCYP51 protein fused with a yeast Erg11 signal-anchor peptide was able to complement the *erg11* mutation, which confirmed AtCYP51 to be a functional sterol 14α-demethylase. *AtCYP51* was then used to generate transgenic *Arabidopsis* by transforming with pBI vector harboring *AtCYP51* in the antisense direction under *CaMV35S* promoter. The resulting transgenic plants were decreased in accumulation of *AtCYP51* mRNA and increased in the amount of endogenous obtusifoliol. They showed a semidwarf phenotype in the early growth stage and a longer life span than control plants. This newly found phenotype is different from previously characterized brassinosteroid (BR)-deficient campesterol biosynthesis mutants.

Key Words: **sterol; sterol 14α-demethylase; CYP51; obtusifoliol 14α-demethylase; brassionosteroid; transgenic; *Arabidopsis*.**

Sterols are essential components of eukaryotic membranes and are also precursors of steroid hormones in higher plants and animals. Brassinosteroids (BRs) are plant hormones that have important roles in plant development processes, including cell elongation and division. The *Arabidopsis* dwarf mutant *det2* has a photomorphogenic phenotype that can be rescued by the administration of exogenous BRs (1). It was later determined that *DET2* is a gene involved in the biosynthesis of brassinolide (BL); one of the most important BRs. Since then, *det2*-like *Arabidopsis* dwarf mutants such as *dwf4* and *cpd* have been isolated and their roles in BL biosynthesis identified (2) (Fig. 1).

Recently, not only BR but also campestrol biosynthesis was found to be blocked in some of *det2*-like *Arabidopsis* dwarf mutants, including *dwf7*, *dwf5* and *dwf1* (3) (Fig. 1). In addition, *fackel*, a sterol 14-reductase mutant with a *det2*-like phenotype, was isolated very recently (4, 5). Since phytosterol biosynthesis is not a specific pathway for BR biosynthesis and the endogenous amount of phytosterol is much higher than those of BRs (6), it was surprising to see that campesterol biosynthesis mutants also exhibited BL-deficient phenotypes.

Therefore, we have been interested in how sterol biosynthesis is regulated in higher plants and how a plant phenotype will be modified when the upstream steps of campesterol biosynthesis are blocked. For this purpose, we focused our attention on obtusifoliol 14α-demethylase, which is involved in an early biosynthetic step for which no mutants are presently known.

Obtusifoliol 14α-demethylase is a sterol 14α-demethylase, which is classified as CYP51, a member of the cytochrome P450 monooxygenases superfamily (7). CYP51 is essential for sterol biosynthesis and it is assumed to be the only orthologous P450 family that exists in the fungi, mammal and plant kingdoms (8). Different CYP51 enzymes are reported to have different substrate specificities (Fig. 2) (9–11) and clones

The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank with Accession No. AB014459.

Abbreviations used: EST, expressed sequence tag; RACE-PCR, rapid amplification of cDNA end-polymerase chain reaction; ORF, open reading frame; BR, brassinosteroid; BL, brassinolide.

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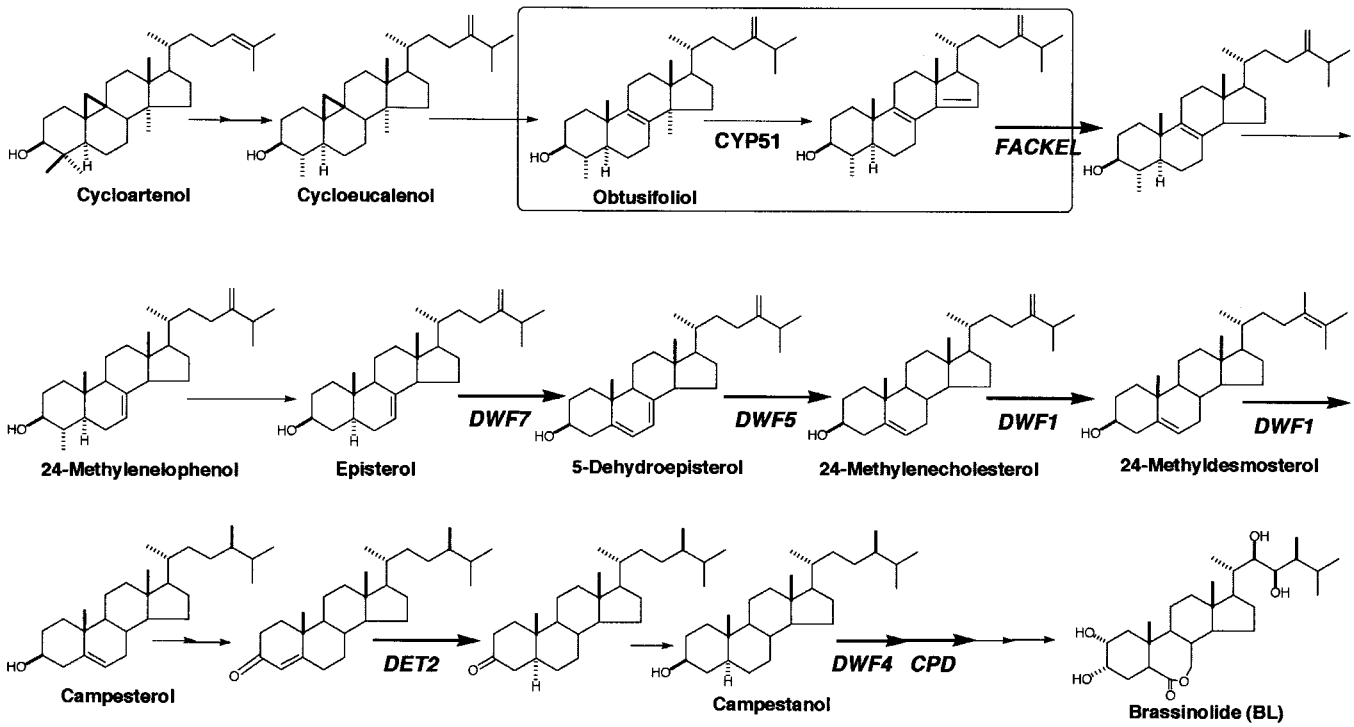

FIG. 1. Campesterol and brassinolide biosynthesis in *Arabidopsis. det2, dwf4, cpd, dwf7, dwf5, dwf1* and *fackel* are brassinosteroids (BRs)-deficient mutants. The reaction catalyzed by obtusifoliol 14α-demethylase (CYP51) is boxed.

from yeast, pathogenic fungi, rat, human and monocot plants have been identified to date (on DDBJ Database). In monocot plants, CYP51 activity has been reported in microsomal enzyme system from *Zea mays* (11) and *Sorghum bicolor* (12) and cDNA clones have recently been isolated from *Sorghum bicolor* (13) and *Triticum aestivum* (14). In dicot plants, however, CYP51 activity has yet to be detected, and no cDNA clones have been identified. Yet a dicot plant *Arabidopsis* has an advantage in molecular genetics, we decided to isolate *Arabidopsis CYP51* (*AtCYP51*) cDNA and analyze its role in sterol biosynthesis by constructing transgenic plants expressing antisense *AtCYP51* mRNA. According to *Arabidopsis* genome database,

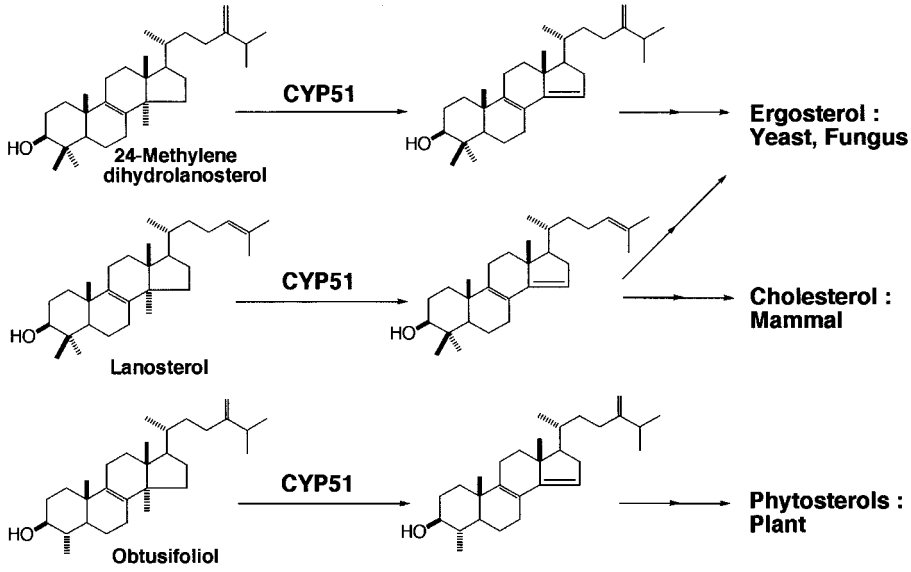

FIG. 2. Reactions catalyzed by sterol 14α-demethylases (CYP51s) in different organisms.

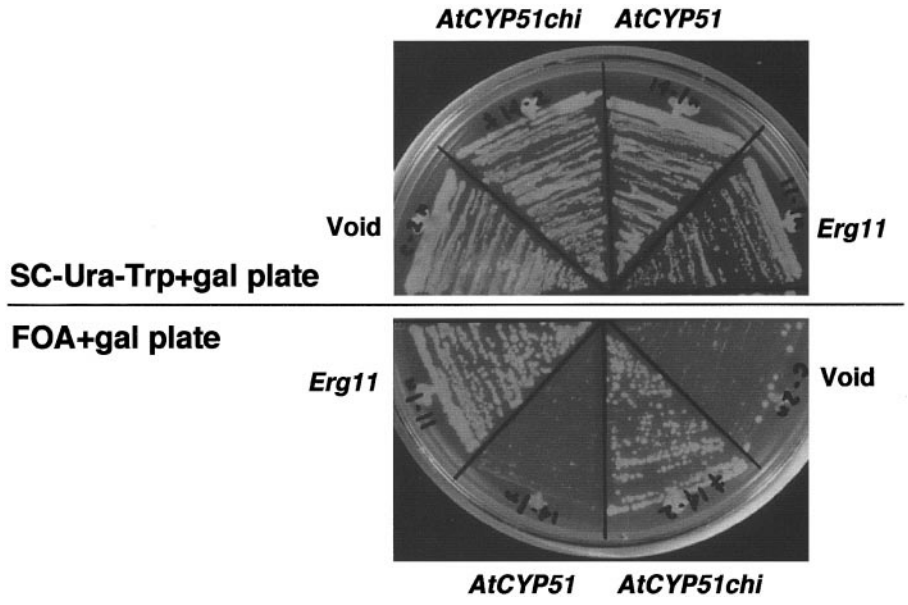

FIG. 3. Complementation of *erg11* yeast by *AtCYP51chi*. The host yeast strain was maintained by pYO326 carrying *ERG11* that complements the *erg11*, a lethal mutation. The host can survive only when the cDNA subcloned into pNMV4 complements the *erg11* mutation on FOA which selects against pYO326.

there are two putative *Arabidopsis* CYP51 genes. One is CYP51A1 on chromosome 2 (64% identical to Sorghum CYP51 with slightly modified heme binding region consensus sequence) and the other is CYP51A2 on chromosome 1 (76% identical to Sorghum CYP51 with conserved heme binding region consensus sequence). In this study, we used a clone CYP51A2 to construct antisense *Arabidopsis* and analyze its phenotype as well as chemotype.

MATERIALS AND METHODS

Plant material, plant cDNAs, yeast strains, vectors, and reagents. *Arabidopsis* Ecotype Columbia seeds were purchased (Lehle Seeds). An *Arabidopsis* Matchmaker cDNA library subcloned into pGAD10 was purchased from Clontech. *Arabidopsis* EST clone 225D4T7 was provided by the *Arabidopsis* Biological Resource Center. Oligonucleotide synthesis was done by Pharmacia. A yeast lanosterol 14α-demethylase deficient strain ANY200Δ*erg11* and a *URA* vector pYO326-ERG11 containing *ERG11* under *GAL1* promoter were kindly provided by Dr. K. Homma (15). A *TRP1* vector pNMV4 with *GAL1* promoter was a kind gift from Dr. N. Matsuda (16). pT7Blue T-Vector (Novagen) was used for subcloning and sequencing. *Agrobacterium* vector pBIX was made by a slight modification of pBI121 (Toyobo). Authentic obtusifoliol was extracted from *Oryza sativa*. Other reagents were purchased.

Amplification of the cDNA 5′-end. EST clone 225D4T7 was sequenced by the dideoxynucleotide termination method using a Thermo Sequenase Cycle Sequencing Kit (Aloka). Primers 4A, 5′-CACCACTCTTCCCCCATTTAGAGAAG-3′, and 3A, 5′-AGAACCGAAACTGCTCCTGACGAACAG-3′ (specific for 225D4T7), and primers GAD10F1, 5′-GGACGGACCAACTGCGTATAACGCG-3′, and GAD10F2, 5′-CGATGATGAAGATACCCCACCAAACCC-3′ (specific for the pGAD10 vector), were used for nested 5′-RACE-PCR with *Arabidopsis* cDNA library as template. The nested PCR was performed with GAD10F1 and 4A using a step program (0.5 min at 94°C, 0.5 min at 60°C, 2 min at 72°C, 25 cycles) for a first round, followed by a second round of PCR with the primer set of GAD10F2 and 3A. The nested PCR product (ca. 600 bp) was ligated to pT7Blue T-Vector with a DNA Ligation Kit Ver. 2 (Takara Biochemicals), propagated in *Escherichia coli* and sequenced.

Amplification of the cDNA ORF. The deduced *Arabidopsis* obtusifoliol 14α-demethylase (*AtCYP51*) ORF, yeast lanosterol 14α-demethylase (*ERG11*) and the chimeric *ERG11*::*AtCYP51* (*AtCYP51chi*) were obtained by PCR using primers containing *Eco*RI sites for subcloning to pNMV4 vector. PCR was performed using the same method as described for 5′-RACE-PCR except that the annealing temperature was 63°C. Primers 225D4T7-N (5′-CCAGAATTCAAAACAATGGAATTGGATTC-3′) and 225D4T7-C (5′-ATATATGAATTCTCTTTAAGAAAGCTGGCGCCTCTTGT-3′) were used for the amplification of *AtCYP51*. The 1467-bp PCR product was sequenced in both strands and subcloned into pNMV4 to construct the plasmid pNMV4-AtCYP51. pNMV4-ERG11 was constructed in the same manner using primers ERG11-5E (5′-GAGGAATTCAAGGATGTCTGCTACCAAGTC-3′) and ERG11-3E (5′-TTAGAATTCTTAGATCTTTTGTTCTGGATTTC-3′). For the construction of pNMV4-AtCYP51chi, a 108-bp PCR product amplified using primers ERG11-SE-S (5′-ATATATGTCGACGAATTCATGTCTGCTACCAAGTCAATCGTTGG-3′) and ERG11-N-A (5′-CACTAGAGGCGGCCGCTCCTTTCTCAAAGAATATAGTAATTGC-3′) was ligated with another 1359-bp PCR product obtained using primers 225D4T7-N-S (5′-GAAGCGGCGGCCGCCTACTCTTAAAGCTTGGCCTCCATTG-3′) and 225D4T7-C, and the ligation product was subcloned into pNMV4.

Expression of AtCYP51 in the yeast erg11 mutant. The yeast diploid strain ANY200Δ*erg11* was transformed with pYO326-ERG11 by the lithium acetate method (17). Transformants were sporulated and subjected to tetrad dissection to isolate a haploid ANY200Δ*erg11* (pYO326-ERG11). The resulting *erg11* mutant harboring pYO326-ERG11 was transformed with pNMV4-AtCYP51, pNMV4-ERG11 or pNMV4-AtCYP51chi. Transformants were selected on plates of synthetic complete media (SC) (17) lacking uracil and tryptophan and supplemented with galactose (SC-Ura-Trp+gal plate). These transformants were transferred to plates containing fluoro-orotic acid

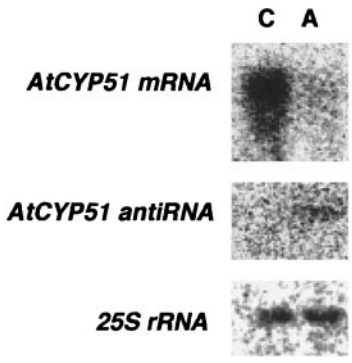

FIG. 4. *AtCYP51* mRNA expression in T3 progeny of the antisense transformants. Five micrograms of total RNAs, which were extracted from control and *AtCYP51* antisense transformant, were used for the Northern analysis. C, control *Arabidopsis* (transformed wih void pBIX vector). A, *AtCYP51* antisense *Arabidopsis.*

(FOA) and galactose (FOA+gal plate), and assayed for complementation of the *erg11* mutant.

Northern analysis. For Northern blotting, plant RNA was prepared from 1 g (fresh weight) of leaves by the ATA-phenol-SDS method (18). Both sense and antisense *AtCYP51* RNA fragments were labeled according to the instructions provided by the Riboprobe Combination System (Promega). Hybridization was done at 68°C, 0.1× SSPE, 0.1% SDS.

Plant transformation. To construct the *Agrobacterium* vector, the entire ORF of *AtCYP51* was amplified by PCR as described above. *Xho*I site was introduced upstream of the ATG codon and *Bam*HI site was placed downstream of the stop codon using primers 14DMA-5′X (5′-TCCAACTCGAGAAACAATGGAATTGGATTCGGAG-3′) and 14DMA-3′B (5′-TAAACGGATCCTTAAGAAAGCTGGCGCCTCTTG-3′). Triparental mating method was used to transform *Agrobacterium* GV3101 (pPM90) (19) with pBIX-AtCYP51a, which contained antisense *AtCYP51* under the control of *CaMV35S* promoter. *Arabidopsis* plants were transformed with *Agrobacterium* using the floral dip technique (20). Integration of the transgene was confirmed by PCR using the *35S* promoter primer (5′-GTGGAAAAAGAAGA-CGTTCCAACCACGTCTTCA-3′) and 225D4T7-N primer.

Analysis of endogenous sterols. Control and transgenic *Arabidopsis* seedlings were extracted with MeOH:$CHCl_3$ (4:1). [$^2H_7$]24-methylenecholesterol (500 ng), [$^2H_6$]campesterol (20 μg), and [$^2H_6$]campestanol (500 ng) were added to extracts (1 g fresh weight equivalent) as internal standards. Each extract was partitioned three times between $CHCl_3$ (20 mL) and water (40 mL). The $CHCl_3$-soluble fraction was purified using a silica gel cartridge (Sep-Pak Vac Silica, 2 g, Waters) and the eluate was subjected to ODS-HPLC

TABLE 1

Endogenous Sterol Levels of T3 Progeny

	C	A (μg/g fresh weight)
Obtusifoliol	0.45	7.40
24-Methylenecholesterol	1.46	1.47
Campesterol	18.0	21.7
Campestanol	0.21	0.26

Note. Amount of sterols (μg/g fresh weight) was quantified in C, control *Arabidopsis* (transformed with void vector), and A, *AtCYP51* antisense *Arabidopsis.*

FIG. 5. Slow growth of *AtCYP51* antisense transformants. *AtCYP51* antisense transformants and a control plant (transformed with void pBIX vector) were grown on soil under the continuous light for 50 days after sowing.

(Senshu Pak ODS 1151-D, 4.6 × 150 mm, Senshu Scientific Co., Ltd., Tokyo) at a flow rate of 1 mL min^{-1} with 100% MeOH. Fractions collected every 0.5 min (Rt, 9–18 min) were trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and analyzed by full-scan GC-MS as previously described (21). The endogenous levels of 24-methylenecholesterol, campesterol and campestanol were determined as the ratio of the peak areas of molecular ions for the endogenous sterol to the internal standard. Molecular ions of the internal standard and the endogenous sterol were as follows: 24-methylenecholesterol, *m/z* 477 and 470; campesterol, *m/z* 478 and 472; and campestanol, *m/z* 480 and 474. The endogenous level of obtusifoliol was estimated from the peak area of molecular ion (*m/z* 498) as compared to that of an authentic specimen.

RESULTS

Cloning of Arabidopsis obtusifoliol 14α-demethylase cDNA. Arabidopsis EST clone 225D4T7 was found to be highly homologous to *Sorghum CYP51.* Its deduced sequence appeared to contain the C-terminus of putative *Arabidopsis* obtusifoliol 14α-demethylase (AtCYP51). The full length sequence was obtained by 5′-RACE, whose deduced ORF, 1467 bp in length, encoded a 489-amino acid protein with 76% identity to *Sorghum* CYP51 and 36% identity to yeast lanosterol 14α-demethylase. The heme binding region consensus sequence XFXXGXRXCX was also highly conserved in

AtCYP51, as it is in all known eukaryotic P450s. The CYP51 family diverges from the consensus sequence with a histidine substitution at the highly conserved arginine, and has a conserved heme binding region with the sequence YXXFGXGRHRCXGEXFAYXQ (13). From the high level of sequence identity with *Sorghum* CYP51 and the conservation of unique sequence in CYP51, AtCYP51 seemed to be a possible *Arabidopsis* CYP51.

Putative AtCYP51 could complement the yeast Δerg11 strain. In order to identify the function of the putative *AtCYP51,* we examined whether this gene could complement the *ERG11*-deficient yeast strain. Since *ERG11* is essential for cell growth, we constructed the ANY200Δ*erg11* diploid strain containing pYO326-ERG11. After sporulation and tetrad dissection, a haploid Δ*erg11* strain containing pYO326-ERG11 was selected and used for complementation analysis. The 1467-bp *AtCYP51* ORF was amplified by PCR, sequenced and subcloned into pNMV4, to make pNMV4-AtCYP51. pNMV4-ERG11 was constructed as a positive control and pNMV4-AtCYP51chi was also constructed by the same strategy used for wheat *CYP51* (14). These three constructs were introduced into the haploid Δ*erg11*/pYO326-ERG11 strain and transformants were grown on fluoro-orotic acid plate containing galactose (FOA+gal plate) for 3 days. As shown in Fig. 3, cells carrying pNMV4-AtCYP51chi grew as well as the pNMV4-ERG11 positive control on FOA+gal plate. These results clearly indicate that AtCYP51 possessed actual sterol 14α-demethylase activity. Therefore, we concluded that we cloned a functional CYP51 from *Arabidopsis.*

Antisense AtCYP51 transgenic plants. Arabidopsis plants were transformed with antisense *AtCYP51* using *A. tumefaciens* GV3101. Twelve independent regenerates were transplanted to soil and grown to maturity in a culture room at 23°C with continuous light. Individual plants were self-crossed to yield T3 seeds which were plated on kanamycin-containing medium for segregation analysis.

The amounts of sense and antisense transcripts in transformants were analyzed by Northern hybridization using total RNA extracted from young leaves and the entire *AtCYP51* ORF as a probe. A homozygous line of antisense plants showed decreased levels of *AtCYP51* mRNA compared to control plants (transformed with void pBIX vector). Transformants also showed expression of antisense *AtCYP51* RNA (Fig. 4).

Endogenous sterols in the control and antisense plants were analyzed by GC-MS. Obtusifoliol in the antisense plants was accumulated to levels 16 times higher than that of control plants while campesterol and campestanol levels were unchanged (Table 1). Careful observation revealed the accumulation of other probable obtusifoliol isomers (m/z = 498, trimethylsilylated) in the antisense plants (data not shown), indicating that antisense transformants was indeed AtCYP51-deficient. The antisense plants showed semidwarfism during the first 30 days of development. Control *Arabidopsis* plants ordinarily finish their life cycle after going to seed after a month. Fifty days after germination, when the control plants had withered, antisense *AtCYP51* plants were still alive, and stem growth slowly continued until the height of the control plants was reached (Fig. 5). The observed phenotype is clearly different from known BR biosynthetic mutants such as *det2,* and also distinct from previously characterized campesterol biosynthetic mutants, *dwf5, dwf7, dwf1* and *fackel.*

DISCUSSION

AtCYP51 expression. Fungi, animals and plants differ in the structure of membrane sterols and their biosynthetic pathways; however, the most important structural features of sterol molecules have been conserved among all eukaryotes. Sterol-14α-demethylation is one of these features and sterol-14α-demethylase, also called CYP51, has been highly conserved throughout the evolution of eukaryotes. *CYP51* clones from fungi, animals and monocot plants have been characterized in the past decade; however, CYP51 has not been detected nor characterized in dicot plants. We used the sequence of *Arabidopsis* EST clone 225D4T7, which showed 76% identity to the C-terminus of *Sorghum* obtusifoliol 14α-demethylase, to isolate the full-length clone of a putative *Arabidopsis* obtusifoliol 14α-demethylase (*AtCYP51*).

Previously, data obtained with heterologously expressed CYP51s have clearly revealed the differences in the substrate specificities of animal, fungal and plant CYP51 enzymes (Fig. 2) (22). It has also been shown that the N-terminus of each CYP51 is an important region for both substrate recognition and catalytic activity (23). Therefore, we attempted to alter the substrate specificity of AtCYP51, so that it could metabolize lanosterol by substituting the 5′-terminus of *AtCYP51* with that of *ERG11* to make *AtCYP51chi.* This chimeric clone complemented the disruption of yeast sterol 14α-demethylation activity, proving that AtCYP51 is a functional sterol 14α-demethylase (Fig. 3). On the other hand, intact *AtCYP51* did not complement the *erg11* mutation, suggesting that AtCYP51 has strict substrate specificity, as is the case with CYP51s from monocot plants. The characterization of CYP51 in dicot plants will contribute to the analysis of the molecular evolution and substrate specificity of CYP51.

Dwarfism and slow growth phenotype of antisense AtCYP51 transformants. Antisense *AtCYP51 Arabidopsis* transgenics were generated and showed dwarfism during early development and slow growth during

maturation (Fig. 5). This phenotype is clearly different from that of known campesterol-deficient mutants such as *dwf5*, *dwf7*, *dwf1* and *fackel*, which are apparently dwarf during maturation. In fact, endogenous sterol analysis of antisense *Arabidopsis* showed that the accumulation levels of campesterol were as same as those of control plants. On the other hand, the amount of accumulated obtusifoliol was about 16 times higher in the antisense plants than that of control plants (Table 1). This high level of accumulation of obtusifoliol could be caused by the antisense effect, as the Northern analysis clearly showed decreased levels of *AtCYP51* mRNA in antisense plants (Fig. 4). In many cases of plant mutants which are deficient in plant hormone biosynthesis, accumulation of intermediate metabolites just before the disrupted step were observed. Therefore we successfully produced a mutant mimetic plant by the antisense effect. As mentioned above, *dwf5*, *dwf7*, *dwf1* and *fackel* all show dwarf phenotypes accompanied by campesterol and BR deficiencies. On the other hand, antisense *AtCYP51 Arabidopsis* showed dwarfism during early developmental stages but did not show campesterol deficiency. In addition, antisense seedlings grown in the dark had long and etiolated hypocotyls (data not shown), indicating that they are not BR-deficient, since all reported BR-deficient plants have photomorphogenic-like shortened hypocotyls when grown in the dark. These results indicate that CYP51 is the latest biosynthetic step whose blockage does not confer typical BR-deficient phenotype.

These observations suggest that dwarfism of transgenics is caused by something other than BR deficiency. One of the possible explanations for the dwarfism of our antisense *AtCYP51* transgenics might be the accumulation of obtusifoliol and its isomers. These compounds might be plant growth regulators as well as important membrane constituents. Although the structures of the obtusifoliol isomers have not yet been elucidated, these results indicate that there might be a branched sterol biosynthetic pathway. In yeast, the sterol biosynthetic pathway has been intensively studied and the enzyme functioning upstream of sterol 14α-demethylase was proven to be essential for viability (24). In plants, the existence of separate sterol biosynthetic pathways has not been completely elucidated. However, sterol 14α-demethylase may have a crucial role, since it accounts for about 20% of microsomal P450s and about 0.2% of microsomal protein in *Sorghum bicolor* (12). In the event of loss-of-function mutations in the biosynthetic genes upstream of CYP51, a bypass pathway may have developed in plants in order to ensure survival.

Sterol 14α-demethylase has been regarded as one of the key enzymes of sterol biosynthesis in all eukaryotes and is the target enzyme for the azole antifungals. These fungicides have been useful for investigating and manipulating plant sterol synthesis *in vivo*, and also been used as molecular probes for screening resistant plant sterol mutants. One such example is a selection of tobacco cell lines which have a modified profile of 14α-methyl-sterol (obtusifoliol and its derivatives) and/or phytosterol, using the resistance to the triazole CYP51 inhibitor LAB170250F (25). Endogenous sterol analysis revealed that most of these resistant cells accumulated 14α-methyl-sterols while campesterol levels were unaltered. This endogenous sterol profile is similar to that of current antisense *AtCYP51 Arabidopsis*. In this study, the accumulation of putative obtusifoliol isomers was observed in antisense *AtCYP51 Arabidopsis*. The identification of these compounds as well as the elucidation of their roles in normal plant growth would be a next important step towards understanding the physiological role of sterols in general.

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