Ajuga Δ24-Sterol Reductase Catalyzes the Direct Reductive Conversion of 24-Methylenecolesterol to Campesterol

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The nucleotide sequence reported in this paper has been submitted to the DNA Data Bank of Japan (DDBJ) with accession number LC070675 (ArDWF1).

The abbreviations used are: DWF1, Dwarf1; ArDWF1, Ajuga reptans var. atropurpurea Dwarf1; OsDWF1, Oryza sativa Dwarf1, EST, expression sequence tag; GC-MS, gas chromatography-mass spectrometry; EtOAc, ethyl acetate; MeOH, methanol; HPLC; high performance liquid chromatography.
Abstract

Dimunito/Dwarf1 (DWF1) is an oxidoreductase enzyme that is responsible for the conversion of C_{29} and C_{29-}Δ_{24(28)}-olefinic sterols to 24-methyl- and 24-ethylcholesterols. Generally the reaction proceeds in two steps via the Δ_{24(25)}-intermediate. In the present study, we characterized the ArDWF1 gene from an EST library of Ajuga reptans var. atropurpurea hairy roots. The gene was functionally expressed in the yeast T21 strain. The in vivo and in vitro study of the transformed yeast indicated that ArDWF1 catalyzes the conversion of 24-methylenecholesterol to campesterol. A labeling study followed by gas chromatography-mass spectrometry (GC-MS) analysis suggested that the reaction proceeded with retention of the C-25 hydrogen. The 25-H retention was established by the incubation of the enzyme with (23,23,25-3H_{2},28-13C)-24-methylenecholesterol followed by 13C NMR analysis of the resulting campesterol. Thus, it has been concluded that ArDWF1 directly reduces 24-methylenecholesterol to produce campesterol without passing through a Δ_{24(25)}-intermediate. This is the first characterization of such a unique DWF1 enzyme. For comparison purposes, Oryza sativa DWF1 (OsDWF1) was similarly expressed in yeast. An in vivo assay of OsDWF1 supported the generally accepted two-step mechanism because the C-25 hydrogen of 24-methylenecholesterol was eliminated during its conversion to 24-methylcholesterol. As expected, the 24-methylcholesterol produced by OsDWF1 was a mixture of campesterol and dihydrobrassicasterol. Furthermore, the 24-methylcholesterol contained in the Ajuga hairy roots was determined to be solely campesterol through its analysis using chiral GC-MS. Therefore, ArDWF1 has another unique property in that only campesterol is formed by the direct reduction catalyzed by the enzyme.

In vertebrates, cholesterol is the major sterol, whereas in higher plants, commonly occurring sterols are C_{29} sterols, sitosterol (1) and stigmasterol, and a C_{28} sterol, campesterol (2). Campesterol is frequently accompanied by dihydrobrassicasterol (3) (1,2). Latter steps in phytosterol biosynthesis have received considerable attention, because of their intriguing chemistry, biochemistry and biology. Recent studies through genetic mutations have uncovered an essential role of these phytosterols at the cellular level in hormone signaling, organized divisions and embryo patterning (3).

It is generally regarded that these typical plant sterols are biosynthesized from precursor Δ_{24(28)}-olefinic sterols, i.e., isofucosterol (4) and 24-methylenecholesterol (5) in two steps: a double bond isomerization from Δ_{24(28)} to Δ_{24(25)} (leading to 24-ethyldesmosterol (6) and 24-methyldesmosterol (7)) and a reduction of the Δ_{24(25)}-intermediates to the saturated C_{29} and C_{28} sterols (1 and 2/3). Interestingly, the reduction of 24-ethyl desmosterol affords only sitosterol, whereas the reduction of 24-methyldesmosterol yields a C-24 epimeric mixture of campesterol and dihydrobrassicasterol (1,2). The two-step mechanism has been proposed mainly based on the metabolic behavior of the C-25 hydrogen of the Δ_{24(28)}-olefinic sterol, which is eliminated during plant sterol biosynthesis (4-6). The C_{28}-Δ_{24(28)}-olefinic sterol (e.g., 24-methylenecholesterol) is produced by the action of sterol methyl transferase (SMT-1) to a Δ_{24(25)}-intermediate (cycloartenol is reportedly the most preferable substrate (7) and then followed by hydrogen migration from C-24 to C-25 and elimination of the C-28 hydrogen (8-10). The homologous C_{29}-Δ_{24(28)}-olefinic sterol (e.g., isofucosterol) is produced by the action of SMT-2 to a C_{28}-Δ_{24(25)}-olefinic intermediate (24-methyleneophenol is reportedly the most preferred substrate) followed by elimination of the C-28 hydrogen (Fig. 1).

The Dimunito/Dwarf1 (DWF1) gene, which encodes an oxidoreductase with an FAD binding domain, was first cloned from Arabidopsis thaliana (AtDWF1) (11), and it was subsequently indicated that this gene is involved in both the double bond isomerization and the reduction step (12). A. thaliana and Oryza sativa (13) each have a single DWF1 gene, whereas tomato and potato have two DWF1 ortholog genes (SSR1 and SSR2) (14). Therefore, it is highly likely that DWF1 of A. thaliana as well as O. sativa catalyzes the formation of sitosterol as well as campesterol and dihydrobrassicasterol. It has been recently reported that tomato SSR1 has a substrate specificity for 24-methylenecholesterol, affording 24-methylcholesterol, whereas SSR2 preferentially reduces a Δ_{24(25)}-sterol (cycloartenol/desmosterol) to produce cycloartenol/cholesterol (14).

It is reported that the double bond migration of the C_{29}-Δ_{24(28)}-olefinic sterol proceeds through a syn-S_{2} mechanism (15). Furthermore, the reduction of the C_{29}-Δ_{24(25)}-olefinic sterol proceeds in a trans-addition manner, wherein the hydrogen atoms are introduced from both the 24-Si, 25-Re and the 24-Re, 25-Si faces to give campesterol and dihydrobrassicasterol, respectively, in O. sativa cell culture and in Catharanthus roseus callus (16,17). It has also been indicated that 24-ethyl desmosterol is reduced to give...
sitosterol in a trans-addition manner, wherein the hydrogen atoms are introduced only from the 24-Si, 25-Re face (16,18).

In the study of phytoecdysteroid biosynthesis, we used the hairy roots of Ajuga reptans var. atropurpurea (Labiatae family) (19-22). Ajuga plants have unique sterol profiles in which clerosterol (8) and dehydroclerosterol (9) are the major sterols (20,23). These sterols are formed by the trans-methylation of a C28 = Δ24(28) intermediate followed by a 1,2-hydrogen shift of C-25 to C-24 and deprotonation at C-27 (24). Ajuga plants also contain C29-, C28- and C27-phytoecdysteroids, including cyasterone and 20-hydroxyecdysone. We reported that in Ajuga hairy roots, cholesterol is a precursor of 20-hydroxyecdysone (19), while cyasterone is biosynthesized from clerosterol (25). To accelerate our study on the biosyntheses of sterols and ecdysteroids in Ajuga plants, we recently generated an EST library of Ajuga hairy roots (26), from which a DWF1 ortholog gene (designated as ArDWF1) was identified.

In this paper, we report a unique reaction mechanism catalyzed by ArDWF1, which is distinct from the generally accepted two-step mechanism. O. sativa Dwarf1 (OsDWF1) was also studied as the basis for comparison.

EXPERIMENTAL PROCEDURES

Analytical Method—The 1H and 13C NMR spectra were recorded using a Bruker DRX-500 (500 MHz for 1H and 125 MHz for 13C) spectrometer in CDCl3 (99.8% atom enriched, Isotec) as the solvent. The 1H chemical shifts are reported in reference to the signal of tetramethylsilane (δH 0.00), while 13C chemical shifts are in reference to the solvent signals (δC 77.0 for CDCl3).

GC-MS Analysis—A mass spectrometer (JMS-AM SUN200; JEOL) was connected to a gas chromatograph (6890A; Agilent Technologies) with an electron ionization of 70 eV, a source temperature of 250 ºC, a DB-1ms column of 30 m × 0.25 mm, a film thickness of 0.25 µm (J&W Scientific), and an injection temperature of 250 ºC. The following column temperature program was used: 80 ºC for 1 min, an increase to 300 ºC at a rate of 20 ºC/min, and held at 300 ºC for 12 min. The interface temperature was 280 ºC, the carrier gas was He, and the flow rate was 1 ml/min. Splitless injections were performed.

GC-MS analysis was performed using a chiral column, HP-Chiral 20β (30 m × 0.25 mm, film thickness of 0.25 µm, Agilent) with the following column temperature program: held at 80 ºC for 1 min, raised to 250 ºC at a rate of 20 ºC/min, and held at 250 ºC for 71 min. Authentic campesterol and dihydrobrassicasterol were injected as references.

HPLC analysis—An LC-6A apparatus (Shimadzu) equipped with an SPD-6A UV detector (215 nm) and a Shim-Pack CLC-ODS column (15 cm × 6 mm i.d., Shimadzu) was used.

cDNA Cloning of ArDWF1—The details of the preparation of an EST library from the Ajuga hairy roots will be reported elsewhere (26). Keyword searches of the annotated ESTs identified only one DWF1 contig. The sequence data allowed the design of primers with the following sequences: 5'-AGTCCGATCCATGTCAATGAGGTCC CCC-3' and 5'-AGTGCATAGTGAAGGC AACATTCAATGGAAG3'. The open reading frame of ArDWF1 was amplified by PCR (initial denaturing for 2 min at 98 ºC; 35 cycles of 94 ºC for 10 s, 55 ºC for 5 s, and 72 ºC for 30 s; and a final extension reaction for 5 min at 72 ºC) using PrimeSTAR max with the template (a cDNA library that was constructed from Ajuga hairy roots) and the primers (described above). The PCR product was ligated into the galactose-inducible yeast expression vector pESC-LEU using the BamHI and Nhel sites included in the primers. The identity of the inserted cDNA was confirmed by sequencing.

Yeast transformation by ArDWF1 and OsDWF1—The yeast T21 strain that produces 24-methylenecolesterol, which was constructed by Sawai et al. (14), was transformed with the plasmids or the empty pESC-LEU vector as a control using the Frozen-EZ Transformation II kit (Zymo Research, California, USA). The transformants were selected using a synthetic complete medium (Takara) containing 2% (w/v) glucose supplemented with −His/−Leu or −Ura/−His DO supplement (Takara Bio) for 2 days at 30 ºC.

cDNA Cloning of OsDWF1—Seeds of the O. sativa L. cultivar Nipponbare (Nihon Nourken, Inc.) were sterilized with running water at 25 ºC for 12 h, and then vernalization was done at 4 ºC under dark conditions for 12 h. The seeds were germinated at 28 ºC using a photoperiodic cycle of 12 h light/12 h dark for 14 days. The total RNA of the seedlings was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer’s specifications.
The extract was treated with DNase using the RNase-Free DNase Set (Qiagen) and purified using the RNaseasy kit. First-strand cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) utilizing 1 μg of total RNA. A PCR fragment of OsDWF1 was amplified by PCR with the primers set 5'-ATGGCAGATCTGCAGGAGCC-3' and 5'-TTACGCCTCATCAGCTTAGGC-3' and template rice cDNA. The insert was cloned into the Gateway entry vector pENTR/D-TOPO (Life Technologies) and then into the yeast expression vector pYES-DEST52 (Life Technologies) with the Gateway LR clonase (Life Technologies) reaction. The obtained plasmid and empty vector were introduced into the yeast T21 strain.

**In Vivo Assay with ArDWF1 and OsDWF1 Expressing Yeasts**—The transformed yeast cells were inoculated into 5 ml of the same medium and cultured for 2 days at 30 °C. The cells were harvested by centrifugation and resuspended in 5 ml of new medium containing 2% (w/v) galactose instead of glucose. After incubation for another 2 days at 30 °C, the cells were disrupted by vortexing with glass beads (0.5 mm diameter) and extracted with ethyl acetate (EtOAc) (5 ml × 3). The organic phase of the extraction was dried over Na2SO4 and concentrated in vacuo. A part of the residue was trimethylsilylated with N-methyl-N-trimethylsilyl trifluoroacetamide (Sigma-Aldrich) at 80 °C for 30 min and analyzed by GC-MS.

**Determination of the C-24 Stereochemistry of 24-Methylcholesterol produced in vivo by 1H NMR spectroscopy**—The cultivation of ArDWF1 expressing yeast was carried out in 500 ml of synthetic complete medium containing 2% (w/v) glucose supplemented with the –His/–Leu DO supplement. The production cultures were inoculated with 5% (w/v) of the pre-culture with the same medium. After incubation of the culture for 2 days at 30 °C and 150 rpm, the cells were harvested and resuspended in the same volume of fresh medium containing 2% (w/v) galactose instead of glucose. Further incubation occurred for 2 days at 30 °C. Then, the harvested cells were lyophilized, ground with a mortar and pestle and extracted with EtOAc (10 ml × 3). The concentrated residue was chromatographed over silica gel with hexane-EtOAc (4:1) to separate the sterol fraction. The fraction was further separated by reversed-phase HPLC (solvent MeOH; flow rate 1.0 ml/min; retention time 16.4 min) to yield 24-methylcholesterol, which was analyzed by 1H NMR.

A large-scale incubation (1.5 l of the medium) of yeast expressing OsDWF1 was carried out in the same manner as for ArDWF1 expressing yeast. The harvested cells were treated as described above to give 24-methylcholesterol, which was analyzed by 1H NMR.

**Determination of the C-24 Stereochemistry of 24-Methylcholesterol contained in Ajuga hairy roots and O. sativa callus by Chiral GC-MS**—The hairy roots of Ajuga reptans var. atropurpurea were maintained as described previously (18). The hairy roots were transferred to a 100 ml Erlenmeyer flask containing 1 x MS medium (30 ml) supplemented with 3% (w/v) sucrose and were incubated at 25 °C on a rotary shaker (70 rpm) in the dark for 3 weeks. The hairy roots were harvested, washed with water and lyophilized (dry wt. 0.5 g). A portion of the dried hairy roots (50 mg) was ground and extracted with CHCl3-methanol (1:1, 2 ml). After the addition of Celite to the extract, the solvents were removed under reduced pressure. The desorbed sample was placed in a Sep-Pak Vac (silica, 500 mg/6 ml; Waters) and was eluted with hexane-EtOAc (2:1, 8 ml) and then CHCl3-methanol (1:1, 8 ml). The hexane-EtOAc eluent was concentrated, and the residue was saponified with 4 M KOH (1 ml) in ethanol for 1 h at 80 °C and then diluted with water (2 ml). The CHCl3-methanol eluent was concentrated, and the residue was hydrolyzed with 1 ml each of methanol and 4 M HCl for 1 h at 80 °C. Both reaction mixtures were extracted with hexane (2 ml × 2), and the hexane layers were combined and concentrated to dryness. A portion of the residue was trimethylsilylated and analyzed by GC-MS using a chiral column.

O. sativa cell culture was maintained as described previously (16). Cultured cells of O. sativa were transferred to a 100 ml Erlenmeyer flask containing N6 liquid medium (30 ml) supplemented with 2% (w/v) sucrose, N6 vitamin (0.3 ml), proline (2.8 g/l), casein hydrolysate (300 mg/l), 2,4-dichlorophenoxyacetic acid (2 mg/l), and myo-inositol (100 mg/l) and grown on a rotary shaker (70 rpm) at 25°C in the dark for 14 days. The cells harvested by filtration were processed as described for the Ajuga hairy roots, and the hexane extract was analyzed similarly.

**Administration of (23,23,25-2H3,28-13C)-24-methylenecholesterol to Ajuga hairy roots and O. sativa callus**—Ajuga hairy roots were cultured in a 100 ml Erlenmeyer flask for 2 weeks as described above. A sterilized solution of (23,23,25-2H3,28-13C)-24-methylenecholesterol (2.0 mg) and methyl-β-cyclodextrin (100 mg) in H2O (2.0 ml) was added to the liquid medium, and incubation was continued for another 2 weeks. The hairy roots were harvested and
treated as described for above, and the hexane extract was trimethylsilylated and analyzed by GC-MS.

Similarly, the labeled 24-methylencholesterol (2.0 mg) was added to O. sativa cells that were grown in a 100-ml Erlenmeyer flask for 2 weeks. The cell culture was incubated for another 2 weeks, and the hexane extract was obtained as described above. The residue was analyzed by GC-MS after trimethylsilylation.

**Incubation of Labeled Sterol Substrates with ArDWF1 Expressing Yeast Homogenate** — The transformant was cultured in synthetic complete medium (250 ml) containing 2% (w/v) glucose supplemented with the –His/–Leu DO supplement for 2 days at 30 °C. The cells were harvested and resuspended in new medium (250 ml) containing 2% (w/v) galactose instead of glucose. After incubation for one more day at 30 °C, the cells were harvested and resuspended in 100 mM Tris-HCl buffer (5 ml, pH 7.23) containing 0.1 mM dithiothreitol and 1 mM EDTA. The cells were lysed using a bead-beater (Wakenyaku) with glass beads (0.5-mm diameter, 5 ml for the cell amount of approximately 10 ml) for 30 s × 4 with 4 min cooling intervals. The resulting cell homogenate was centrifuged at 1,000 g for 5 min at 4 °C. The supernatant was used for an enzymatic assay (5 ml per substrate). The assay mixture contained 30 mM nicotinamide, 3.5 mM NADPH, 3.5 mM FAD, 0.5 mg/ml bovine serum albumin, 1.25% (w/v) methyl-β-cyclodextrin (Wako Pure Chemical Industries). The assay solution was incubated for 3 h at 25 °C for 24 h, the medium together with the cells was collected and mixed with EtOAc (2 mL). The mixture was vortexed and centrifuged. The supernatant was separated. The residue was extracted with EtOAc once more, and the combined EtOAc layer was concentrated. The residue was analyzed by GC-MS as the TMS ether.

**Incubation of Labeled Sterol Substrates with OsDWF1 Expressing Yeast Homogenate** — The yeast homogenate was prepared in the same manner as described for the homogenate of the ArDWF1 expressing yeast. (23,23,25,28-¹³C)-24-methylenecholesterol and (28,28,28-¹³C)-24-methylenecholesterol were also incubated with the homogenate for 24 h, as described for the ArDWF1 expressing yeast homogenate. The EtOAc extract was passed through a Sep-Pak Vac (C18, 500 mg/6 ml; Waters) using MeOH. The eluate was concentrated and purified by HPLC (solvent, MeOH; flow rate 1.0 ml) to obtain 24-methylenecholesterol, if any was present. This was analyzed by GC-MS as the TMS ether.

**Transfection of S2 Cells** — The cDNA of OsDWF1 was cloned into a pUAST vector using its EcoRI and NotI sites. An empty vector of pUAST was used as a negative control. Drosophila melanogaster S2 cells were cultured in Schneider’s Drosophila medium (Life Technologies) with 10% (v/v) heat-inactivated fetal calf serum and a penicillin-streptomycin solution (Wako). The S2 cells were transfected with the vector containing the OsDWF1 and Actin5c-GAL4 constructs (a gift from R. Niwa) in 60 mm dishes using the Effectene transfection reagent (Qiagen) as described previously (27).

**Incubation of 24-Methylenecholesterol with Transfected S2 Cells** — Forty eight hours after the transfection of S2 cells, the medium was replaced with fresh medium (2 ml) containing the labeled 24-methylenecholesterol (2.0 mg) and methyl-β-cyclodextrin (0.5% w/v). After incubation at 25 °C for 24 h, the medium together with the cells was collected and mixed with EtOAc (2 mL). The mixture was vortexed and centrifuged. The supernatant was separated. The residue was extracted with EtOAc once more, and the combined EtOAc layer was concentrated. The residue was analyzed by GC-MS as the TMS ether.

**Steroidal Compounds** — 24-Methylenecholesterol was prepared as previously reported (28). (23,23,25,28-¹³C)-24-methylenecholesterol was prepared using a slight modification of the synthesis of (23,23,25-²H₁)-24-methylenecholesterol (28), in which ¹³C-methyltriphenylphosphonium iodide (Tokyo Chemical Industry) was used in place of its non-labeled counterpart. (28,28,28-²H₁,28-¹³C)-24-methylcastasterone was prepared from 24-oxocholesterol 3-O-t-butyldimethylsilyl ether (29) in three steps: a Grignard addition of ¹³CD₂₃-methylmagnesium iodide prepared from ¹³CD₂₃ (Cambridge Isotope Laboratories), a dehydration of the resulting tertiary alcohol with
POCl₃ in pyridine, and a desilylation step with tetrabutylammonium fluoride/tetrahydrofuran (1.0 M solution). HPLC was used for the final separation (solvent MeOH; flow rate, 1.2 ml/min; tR 15.6 min) of the ∆24(25)-olefinic isomer from ∆23(24)- and ∆24(28)-olefinic isomers. The compound was obtained as white needles, mp. 141-142 ºC (crystallized from MeOH), 1H-NMR (500 MHz, CDCl₃) intense signal at δ 11.9 (C-18), 17.6 (heptet, J=18.8 Hz, C-28), 18.8 (C-21), 19.4 (C-19), 20.0 (d, J=3.8 Hz, C-26), 20.5 (d, J=5.0 Hz, C-27), 21.1 (C-11), 23.3 (C-15), 28.2 (C-16), 31.0 (d, J=3.8 Hz, C-23), 31.7 (C-2), 31.9 (C-7, C-8), 34.3(C-22), 36.0 (C-20), 36.5 (C-5), 37.3 (C-1), 39.8 (C-1), 42.3 (C-4, C-13), 121.7 (C-6), 123.3 (C-25), 128.3 (d, J=43.8 Hz, C-25), 140.8 (C-8) (30).

Phytosterols and dihydrobrassicasterol were prepared as previously reported (31).

**RESULTS**

**Identification of ArDWF1 Gene** — The protein sequence of ArDWF1 and comparison with those of known DWF1 genes are shown in Figs. 2 and 3. ArDWF1 and OsDWF1 were found to have an FAD binding domain and a transmembrane region, as these are also found in AtDWF1 (12).

*Fig. 2*

*Fig. 3*

**ArDWF1 and OsDWF1 Expressing Yeast T21 Cells Produce 24-Methylcholesterol In Vivo** — It is expected that DWF1 catalyzes the conversion of the endogenous 24-methylenecholesterol to 24-methylcholesterol if the yeast T21 strain is further transformed with the DWF1 gene. We used this particular yeast transformant in the present study, since exogenously added 24-methylenecholesterol would have difficulty entering the yeast cells. As expected, GC-MS analysis clearly indicated that ArDWF1 expressing yeast produced 24-methylcholesterol in vivo, whereas the vector control showed a sole peak of 24-methylenecholesterol (Fig. 4). OsDWF1 expressing yeast produced 24-methylcholesterol as well but with a 3-fold less efficiency (estimated by comparing peak integration) than ArDWF1 expressing yeast.

*Fig. 4*

*The C-24 Configuration of 24-Methylcholesterol* — It is known that campesterol and dihydrobrassicasterol can be distinguished by high-resolution ¹H-NMR spectroscopy. The ¹H-NMR analyses of the HPLC-purified 24-methylcholesterols, which were obtained in a large-scale incubation, unambiguously indicated that ArDWF1 produced campesterol (2) as a single product, whereas OsDWF1 produced an approx. 2:1 mixture of campesterol (2) and dihydrobrassicasterol (3) (Fig. 5). Because the formation of campesterol as a single stereoisomer by ArDWF1 was rather unexpected, the C-24 stereochemistry of the 24-methylcholesterol of *Ajuga* hairy roots and *O. sativa* callus was further investigated.

*Fig. 5*

Our preliminary GC-MS study indicated that 24-methylcholesterol consisted of only approx. 0.5–1% of the sterol fraction of *Ajuga* hairy roots. Thus, we sought a method that could be applied in the present case and found that GC-MS analysis using a chiral capillary column was applicable. The analysis indicated that *Ajuga* hairy roots contained only campesterol as its 24-methylcholesterol, whereas *O. sativa* callus contained an approximately 1:1 mixture of campesterol and dihydrobrassicasterol as shown in Fig. 6. The results qualitatively agreed with those of the in vivo experiments. Therefore, it is conceivable that ArDWF1 functions as the sole enzyme responsible for the biosynthesis of 24-methylcholesterol in *Ajuga* plant.

*Fig. 6*

**Conversion of (23,23,25-2H₃,28-13C)-24-Methylenecholesterol to 24-Methylcholesterol in Ajuga Hairy Roots and *O. sativa* Callus** — The feeding experiments of the labeled substrate were carried out to provide insight into the reaction mechanism of the reductive conversion, focusing on the metabolic fate of H-25. GC-MS analysis (Fig. 7) of the biosynthesized 24-methylsterol (campesterol) fed to *Ajuga* hairy roots displayed its molecular ion at m/z 476 [M]+. Non-labeled campesterol, which was originally present in the homogenate, showed the molecular ion at m/z 472 [M]+. This result indicated that during the reductive conversion, the hydrogen atom at C-25 was retained, most likely at the original C-25 position. In contrast, the 24-methylcholesterol obtained from *O. sativa callus* showed its molecular ion at m/z 475 [M]+, which indicated a loss of one of the deuterium atoms, presumably 25-2H, during the conversion. The finding agreed with the generally accepted two-step mechanism.
involving the isomerization of $\Delta^{24(28)}$ to $\Delta^{24(25)}$, which requires elimination of H-25.

<Fig. 7>

In Vitro Assay of ArDWF1 Using Transformed Yeast Homogenate — The incubation of (23,23,25-2H$_3$,28-13C)-24-methylenecholesterol with a cell free homogenate (1500 g supernatant, supplemented with NADPH and FAD) of ArDWF1 expressing yeast followed by GC-MS analysis of the product indicated that the substrate was converted into 24-methylcholesterol with good efficiency (Fig. 8). Furthermore, the MS spectrum of the product clearly showed that three deuterium atoms were retained in the molecule. The retention of 25-2H was inconsistent with the two-step mechanism. (28,28,28-2H$_3$,28-13C)-24-Methylcholesterol was not converted to 24-methylcholesterol using the same homogenate (data not shown).

<Fig. 8>

Mechanism of the Conversion of 24-Methylenecholesterol to 24-Methylcholesterol with ArDWF1 — It was found that H-25 of 24-methylenecholesterol was retained during its conversion to 24-methylcholesterol with ArDWF1, whereas it was eliminated when the reaction was done by OsDWF1. Based on the above results, we proposed a direct reduction mechanism without involving the double bond isomerization for ArDWF1. However, one may question the possibility of the double bond isomerization concomitant with 1,3-hydrogen migration from C-25 to C-28. In this case, the observed retention of H-25 cannot necessarily eliminate the $\Delta^{24(25)}$ intermediate because the hydrogen atom originally located at C-25 can reside at C-28 after the reaction. The 2H-decoupled 13C NMR spectrum of 24-methylcholesterol (campesterol) derived from (23,23,25-2H$_3$,28-13C)-24-methylenecholesterol exhibited an enriched C-28 signal at $\delta$ 15.30 ppm (Fig. 9). The data unambiguously indicated that C-28 was not substituted with any deuterium atom. If the deuterium atom migrated from C-25 to C-28, the C-28 signal should resonate at around $\delta$ 15.00 because of one-bond deuterium isotope shift. The very small upfield shift of C-28 (0.06 ppm from non-labeled C-28) could be attributed to the three-bond deuterium isotope shift of 23,23,25-2H$_3$. We concluded that the conversion of 24-methylenecholesterol to campesterol by ArDWF1 did not pass through a $\Delta^{24(25)}$-intermediate.

<Fig. 9>

In Vitro Assay of OsDWF1 Using Transformed Yeast Homogenate — (23,23,25-2H$_3$,28-13C)-24-methylenecholesterol was similarly incubated with a cell-free homogenate prepared from OsDWF1 transformed yeast. GC-MS analysis of the incubation product indicated no formation of 24-methylcholesterol (data not shown). The result was markedly different from that with ArDWF1 transformed yeast. In contrast, (28,28,28-2H$_3$,28-13C)-24-methylcholesterol was converted to 24-methylcholesterol in a few % yield. The mass chromatogram tracing the m/z 426 ion (Fig. 10) clearly showed this enzymatic conversion. The GC-MS data indicated that the three deuterium atoms at C-28 were retained during the reduction. It is conceivable that the double bond isomerization step is disturbed for some reason in the cell-free homogenate, which led to the failure of the in vitro conversion of 24-methylencholesterol to 24-methylcholesterol. In this context, it has been reported that AtDWF1 is a Ca$^{2+}$/calmodulin-binding protein and the binding is critical for the catalysis of DWF1 (32).

<Fig. 10>

Conversion of 24-Methylenecholesterol to 24-Methylcholesterol Using Transfected S2 Cells—It has been reported that the in vitro expression of Arabidopsis thaliana DWF1 in yeast and Escherichia coli was not successful (11). The present failure of in vitro expression of OsDWF1 agreed with these previous results. Thus, we tried to express the OsDWF1 gene using an alternative system. S2 cells were transfected with OsDWF1 and the cells were incubated with (23,23,25-2H$_3$,28-13C)-24-methylenecholesterol. GC-MS analysis, shown in Fig. 11, indicated that the exogenously added labeled substrate was converted to 24-methylcholesterol with a good yield. The mass spectrum of the resulting 24-methylcholesterol showed a molecular ion at m/z 475, which implies the loss of one deuterium atom, presumably from C-25, during the conversion. The data were consistent with that found for yeast in vivo. Therefore, it was concluded that OsDWF1 converts 24-methylenecholesterol to 24-methylcholesterol in the two-step mechanism.

<Fig. 11>

DISCUSSION
The present paper focused on a unique feature of ArDWF1. ArDWF1 was expressed in the yeast T21 strain. An in vivo assay indicated that ArDWF1 was able to convert endogenous 24-methylenecholesterol to 24-methylcholesterol. In agreement with the in vivo data, 24-methylenecholesterol was converted to 24-methylcholesterol in the in vitro assay. It was subsequently established that the 24-methylcholesterol produced consisted only of campesterol.

The mechanism of the ArDWF1 catalyzed reaction was investigated through labeling experiments. The C-25 hydrogen of 24-methylenecholesterol was retained during its conversion to campesterol in vivo and in vitro. These results were markedly different from those of typical biosyntheses of plant sterols, in which the C-25 hydrogen was eliminated during the process. Indeed, a parallel experiment expressing OsDWF1 in yeast in vivo showed that the C-25 hydrogen was lost in the conversion of 24-methylenecholesterol to 24-methylcholesterol. The results presented herein indicated that ArDWF1 catalyzes the direct reductive conversion of 24-methylenecholesterol to campesterol without passing through the Δ5(25)-olefinic intermediate. This is the first report describing the direct reductive conversion of 24-methylenecholesterol to 24-methylcholesterol in higher plants, as opposed to the two-step mechanism. 24-Methyldesmosterol was not converted to campesterol in the ArDWF1 expressing yeast in vitro, and this is understandable because the compound is not an obligatory intermediate in Ajuga plants.

Another interesting feature of ArDWF1 is that the formed 24-methylcholesterol consisted of solely campesterol. This is in contrast to the general view that 24-methylcholesterol is a C-24 epimeric mixture (campesterol and dihydrobrassicasterol) in higher plants. It has been reported that the 24-methylcholesterol of Glycine max (33), Zea mays (33), O. sativa (16), C. roseus (16) and A. thaliana (data not shown) consists of a mixture of campesterol and dihydrobrassicasterol. The composition of 24-methylcholesterol in the sterol fraction of Ajuga hairy roots was examined and determined to contain only campesterol, as revealed by chiral GC-MS analysis.

For comparative purposes, OsDWF1 was similarly expressed in yeast. 24-Methylenecholesterol was converted to 24-methylcholesterol (a mixture of campesterol and dihydrobrassicasterol) in an in vitro assay. The in vitro assay failed to reproduce the conversion, in contrast to the case of ArDWF1. However, OsDWF1 had a weak activity for the conversion of 24-methylenecholesterol to 24-methylcholesterol in vitro. The reason for its failure to convert 24-methylenecholesterol to 24-methylcholesterol remains unclear.

OsDWF1 transfected S2 cells were able to convert exogenously added 24-methylenecholesterol to 24-methylcholesterol with good efficiency. This finding suggests that S2 cells are an alternative host for DWF1 gene expression.

One may question as to whether a homolog of 24-methylenecholesterol, isofucosterol, can be a substrate of ArDWF1 and OsDWF1. Our preliminary results showed that isofucosterol was not converted to sitosterol with ArDWF1 and OsDWF1 expressing yeast T21 cells. Yeast T21 cells expressing the SSR2 genes of tomato and potato were reported to catalyze the reduction of desmosterol to cholesterol in vivo. Cholesterol is a minor sterol (approx. 5% of the total sterol of the Ajuga hairy roots). Therefore, it would be interesting to examine whether ArDWF1 converts desmosterol to cholesterol. The enzyme activities of ArDWF1 could be affected by the steroid nuclei structures of the substrates. A further systematic study including the incubation of various olefinic sterols having different nuclei structures are required to fully understand the substrate specificity of ArDWF1.

Historically, 24-methylcholesterol has been analyzed by GC and reported without defining the C-24 stereochemistry except for a few cases. At the present time, the C-24 stereochemistry can be determined by high resolution 1H NMR and chiral GC. The stereochemistry of C-24 will be a subject of further study to accumulate information on plants that contain solely campesterol as their 24-methylcholesterol. This will help answer how widely this unique DWF1 is distributed among higher plants. It remains a possibility that the unique sterol profile of Ajuga plants, which is devoid of sitosterol, is associated with the occurrence of this unique DWF1. Campesterol produced by ArDWF1, though a very minor sterol of Ajuga plants, may serve as a precursor of the plant hormone, brassinolide (34).

The amino acid sequence of ArDWF1 showed a high similarity to those of known DWF1s, e.g., OsDWF1 (81% identity), Arabidopsis thaliana DWF1 (79% identity), and Glycine max DWF1 (88% identity) (Fig. 2). Comparison of the amino acid sequence of ArDWF1 with those of known DWF1s revealed that there are more than ten characteristic differences including 460Ile-463Cys, as shown in Fig. 2. To gain insight into which amino acid difference(s) is associated with the unique features of ArDWF1, site-directed mutagenesis studies are awaited.

Δ24-Sterol reductases have been classified into two groups, Δ24(25)-sterol reductase (EC 1.3.1.72) and Δ24(28)-sterol reductase (EC 1.3.1.71). DHCR24
(Seladin-1) of human (35) and typical DWF1s are included in the former group, while the latter group includes ERG4 of yeast (Δ24(24(1))-sterol reductase) (36). ArDWF1 can be assigned as a member of Δ24(28)-sterol reductase family on the basis of its substrate specificity and the reduction mechanism.

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The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: Y. T., K. O., and Y. F. designed the study and wrote paper. Y. T. and K. O. characterized functions of DWF1s. H. Seki., T. A., T. M., H. Suzuki designed and constructed vectors for yeast expression. Y. F. synthesized steroidal compounds. All authors analyzed the results and approved the final version of the manuscript.

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Figure legends:

**Figure 1.** Biosynthetic pathway of 24-methyl and 24-ethyl sterols in higher plants. The present study determined that ArDWF1 catalyzes the direct reduction of a Δ24(28) sterol (indicated by the red arrow) as opposed to the typical two-step mechanism that includes double bond migration. To simplify the discussion, all structures are depicted as having 3β-hydroxy-Δ5 nuclei. However, this does not necessarily mean that the reactions proceed as the nuclei structure.

**Figure 2.** Alignment of the amino acid sequences of ArDWF1, OsDWF-1 and AtDWF1. The red bar represents the transmembrane region, while the blue bar indicates the FAD binding domain (deduced by SOSUI and PROSITE, respectively). The Ca2+/calmodulin binding region is shown in the blue square. The red square indicates the amino acid that is peculiar to ArDWF1. ArDWF1, *Ajuga reptans* var. atropurpurea DWF1; GmDWF1, *Glycine max* DWF1; AtDWF1, *Arabidopsis thaliana* DWF1; OsDWF1, *Oryza sativa* DWF1; ZmDWF1, *Zea mays* DWF1.

**Figure 3.** Phylogenetic tree showing the relationship of various plant DWF1s. The phylogenetic tree was generated based on the entire amino acid sequences of the proteins using the ClustalW program (www.ddbj.nig.ac.jp/E-mail/clustalw-j.html).

**Figure 4.** *In vivo* expression of ArDWF1 and OsDWF1 in yeast. GC-MS analysis (total ion chromatogram (TIC)) of sterols produced by ArDWF1 (left) and OsDWF1 (right) expressing yeast T21 cells. The peak at *t*<sub>R</sub>=17.24 min was identified as 24-methylcholesterol by direct comparison with campesterol in terms of retention time and mass spectrum. 24-Methylcholesterol produced by ArDWF1 was subsequently found to be campesterol, whereas that obtained by OsDWF1 was determined to be a mixture of campesterol and dihydrobrassicasterol (see text).

**Figure 5.** Determination of the C-24 configuration of 24-methylcholesterol obtained from yeast transformed with ArDWF1 (upper) and OsDWF1 (lower) by 1H NMR. Upfield regions of the spectra are shown. The doublet signals indicated by blue and red correspond to those of campesterol and dihydrobrassicasterol, respectively.

**Figure 6.** The C-24 configuration of 24-methylcholesterol as determined by GC-MS (TIC) using a chiral capillary column. A: From *Ajuga* hairy roots. B: From *Oryza sativa* callus. C: Authentic campesterol. D: A 1:1 mixture of authentic dihydrobrassicasterol and campesterol.

**Figure 7.** GC-MS analysis of the 24-methylcholesterol obtained upon feeding (23,23,25-2H3,28-13C)-24-methylencholesterol to *Ajuga* hairy roots (A) and *Oryza sativa* callus (B). The mass spectra were recorded at the peak top of the labeled 24-methylcholesterol indicated by the arrow. The molecular ion peak at *m/z* 476 for the ArDWF1 product indicated that all three deuterium atoms were retained during the conversion, whereas the molecular ion peak at *m/z* 475 for the OsDWF1 product implies that one of the deuterium atoms, most likely 25-2H, was lost during the transformation.

**Figure 8.** GC-MS analysis (mass chromatograms of *m/z* 476 ion) of the reaction product obtained upon incubation of (23,23,25-2H3)-24-methylencholesterol with *ArDWF1* expressing yeast homogenate. The mass spectrum was recorded at the peak top (*t*<sub>R</sub>=17.70) of the labeled product. The molecular ion peak at *m/z* 476 for the ArDWF1 product indicated that all the three deuterium atoms were retained during the conversion.

**Figure 9.** 13C NMR analysis of the campesterol obtained upon incubation of (23,23,25-2H3,28-13C)-24-methylenessterol with *ArDWF1* expressing yeast homogenate. The C-28 signal that originated from the substrate is exhibited at δ 15.30 as an intense peak.

**Figure 10.** GC-MS analysis (mass chromatograms of *m/z* 476 ion) of the reaction product obtained upon incubation of (28,28,28-2H3,28-13C)-24-methyldesmosterol with OsDWF1 expressing yeast (upper) and vector control (lower). The mass spectrum was recorded at the peak top of the product. The peak at *t*<sub>R</sub>=17.74
min corresponds to the substrate. The data indicated that OsDWF1 was able to reduce the substrate to 24-methylcholesterol, although the enzyme activity was very poor.

Figure 11. GC-MS analysis of the reaction product obtained upon incubation of (28,28,28-2H$_3$,28-13C)-24-methylenecholesterol with OsDWF1 transfected S2 cells (upper). Vector control (lower). MS spectrum was recorded at the peak top of the product, which clearly indicated one of the deuterium atoms, presumably 25-2H, was eliminated during the conversion.
Figure 1
Figure 2
Figure 3

Figure 4
Figure 5

Figure 6
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Figure 8

Figure 9
Figure 10

Figure 11
Ajuga Δ24-Sterol Reductase Catalyzes the Direct Reductive Conversion of 24-Methylenecholesterol to Campesterol
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